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TITLE: Progesterone Receptor Scaffolding Function in Breast Cancer

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Progesterone receptors (PR) are critical mediators of mammary gland development and contribute to breast cancer progression. Progestin-induced rapid activation of cytoplasmic protein kinases leads to selective regulation of growth-promoting genes by phospho-PR species. We have shown that phosphorylation of PR Ser81 is ck2-dependent, progestin-regulated and cell cycle-dependent in intact cells. Mutation of the CD domain in PR (mCD PR) abrogates phosphorylation on Ser81, indicating that the CD domain is necessary to facilitate phosphorylation at this site (Ser81). Additionally, we showed that an interaction between PR and MKP3, a regulator of the ERK family, is dependent on the CD domain. Regulation of selected genes by PR-B also required the CD domain for basal and/or progestin-regulated (STAT5A, Wnt1, MKP3) expression/repression. We conclude that the CD domain of PR facilitates protein interactions that are critical to PR-dependent transcription of genes involved in proliferation and mammary stem cell maintenance. Experiments to determine how MKP3 binding mechanistically regulates transcription of these genes, as well as phosphorylation at Ser81 (by ck2) are currently underway. Understanding how mitogenic protein kinases, such as ck2, alter PR phosphorylation and function is critical to fully understanding breast tumor etiology and developing better targeted therapies. Recent clinical data linking the progesterone component of hormone-replacement therapy regimens with the development of breast cancer underscores the importance of understanding how PR works in the context of breast cancer and high kinase environments. Due to the ubiquitous nature of ck2 and its prevalence in many different types of cancer, there has been much interest in the development of ck2 inhibitors as anti-cancer agents. Clinical ck2 inhibitors, in combination with more specific anti-progestins (new classes of selective progesterone receptor modulators or SPRMs), could provide an effective combination of targeted therapy for breast cancer treatment.
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INTRODUCTION

Progesterone receptors (PR) are critical for massive breast epithelial cell expansion during mammary gland development and contribute to breast cancer progression. Nuclear PR activates transcription of PR-target genes, either directly through binding to progesterone response elements (PREs), or indirectly through tethering interactions with other transcription factors (AP1, SP1, STATs). PR is highly post-translationally modified, primarily on N-terminal serine (phosphorylation) and lysine (ubiquitination and sumoylation) residues [1-3]. These modifications significantly alter receptor stability, localization, transcriptional activity and promoter selectivity [4]. In addition to MAPK and cdk2, casein kinase II (ck2), a kinase often overexpressed in breast cancer, has been shown in vitro to phosphorylate PR Ser81 [5-7]. Finally, recent clinical data has shown that women taking hormone-replacement therapy whose regimens included estrogen and progesterone, but not estrogen alone, had an increase in breast tumor number and size [8, 9]. In light of these data, understanding how mitogenic protein kinases alter PR is critical to understanding breast tumor etiology and developing better treatments. Progestin-bound PRs induce rapid activation of cytoplasmic protein kinases, leading to regulation of growth-promoting genes by transcription complexes that include phospho-PR species. We propose that hormonal and growth factor signals converge at the level of PR-target gene promoter selection. We identified a putative common docking (CD) domain in the N-terminal B-upstream segment (BUS) of PR-B. [10]. CD domains are regions through which MAPKs (i.e. ERK) interact with their activators, MAPK kinases (MKKs; i.e. MEK1) and inactivators, MAPK-phosphatases (MKPs) [10, 11]. Another nuclear receptor, PPARγ, has also been shown to interact with MEK1 through a similar domain [12]. The PR CD domain, DPSDE, is an exact match to the CD domain of ERK2, suggestive of PR direct binding with MEK1 and/or MKPs. We created a CD domain mutant (mCD PR) that is differentially post-translationally modified following treatment with synthetic progesterone (R5020), as indicated by its lack of phosphorylation-dependent gel retardation, or “up-shift”, when analyzed by Western blotting. These data suggest that mutation of the CD domain disrupts interactions with kinases that are responsible for direct phosphorylation of PR. Because mCD PR fails to up-shift upon ligand-binding, we screened for protein kinases whose target sequences are within close proximity of PR’s CD domain; PR Ser81 is a known ck2 site in the PR N-terminus. ck2 is a ubiquitously expressed, constitutively active kinase that is overexpressed in every cancer examined thus far, including breast cancer [5, 6]. Interestingly, in breast cancer cells treated with highly specific ck2 inhibitors, TBB and DMAT, we observed a loss of the progesterone-dependent PR up-shift, similar to the behavior of the mCD PR mutant. This affect on PR was specific to inhibition of ck2, as treatment with other kinase inhibitors did not affect PR gel mobility following treatment with R5020. These data suggest that ck2 may contribute to protein interactions and/or PR activity via direct phosphorylation of PR. Additionally, these data suggest that protein interactions mediated through the CD domain may affect PR Ser81 phosphorylation. We hypothesize that the PR CD domain mediates direct interactions with mitogenic protein kinases (MEKs, ck2) that phosphorylate PR, thereby dictating downstream signaling and target-gene specificity. In the context of breast cancer where protein kinases are inappropriately activated, hyperactive PR may lead to reprogramming of breast cancer cells, altering their hormone sensitivity and driving breast cancer progression.
MAJOR RESEARCH TASKS:

**Task 1: Analysis of the signaling molecules that require the CD domain for PR docking (Months 1-12):**

Human PR exists in two primary isoforms: PR-B and PR-A. The full-length receptor, PR-B (116 kDa), contains a unique N-terminal segment, termed the B-upstream segment (BUS), that is not present in the truncated isoform, PR-A (94 kDa). As reported above, we have created a CD domain mutant PR (mCD PR) of PR-B. To identify possible protein interactions that may be disrupted upon mutation of this domain, we used co-immunoprecipitation (Co-IP) assays to screen for putative interacting proteins. We tested the ability of mCD PR to interact with MKP3, a protein previously shown to interact with ERK2 through an identical CD domain [11]. Using COS cells that had been transiently transfected with wt PR-B (hereby referred to as wt PR) or mCD PR, as well as myc-tagged MKP3, we showed that while wt PR interacts with MKP3 both in the presence and absence of ligand, mCD PR failed to interact with MKP3 (Fig. 1). Subsequent biochemical experiments using various PR mutants showed how critical the CD domain is in facilitating the PR-MKP3 interaction. The CD domain is located in the BUS region that is unique to PR-B, therefore, PR-A lacks the CD domain. Moreover, we hypothesized that PR-A would not participate in protein complexes whose formation was dependent on the presence of the CD domain. Using COS cells transiently transfected with PR-A (lacking the CD domain), we showed that the CD domain is critical for PR-MKP3 protein complex formation (Fig. 2). The interaction between PR-A (lacking the CD domain) and MKP3 is significantly compromised when compared to wt PR (PR-B) (compare lanes 2 and 5). When an artificial CD domain is added onto the N-terminus of PR-A (creating CD-PR-A; lane 6), this artificial PR construct regains the ability to interact with MKP3; an effect that is reversed upon mutation of the artificial CD domain (mCD-PR-A; lane 7). These experiments strongly suggest that the CD domain is critical to facilitating the interaction between wt PR and MKP3.

Co-IP experiments studying a putative interaction between PR and ck2 have thus far been unsuccessful due to limitations in the ability to overexpress ck2. We continue to troubleshoot these experiments, however, the CD domain does not contain sequences known to facilitate interactions between ck2 and its respective substrates, suggesting that a putative interaction between PR and ck2 may be indirect. Co-IPs between PR and other members of the MKP or MEK family have not been tested. These data indicate that PR interacts with MKP3 in a CD domain-dependent manner.

**Figure 1. mCD PR fails to interact with MKP3.**
COS cells were co-transfected with wt or mCD PR, myc-MKP3 or respective vector controls. Following a 24 hr incubation in serum-free media, cells were treated with EtOH or 10nM R5020 for 60 min. Cell lysates were immunoprecipitated with a PR antibody, and the resulting co-immunoprecipitated protein complexes were analyzed by Western blotting (top two panels). Bottom two panels represent total cell lysates.
**Task 2: Analysis of PR phosphorylation sites that are altered by CD domain interactions (Months 1-12):**

The phosphorylation status of mCD PR in response to ligand was analyzed using phospho-specific PR antibodies. HeLa cells were transiently transfected with wt or mCD PR, and PR phosphorylation in response to ligand was analyzed by Western blotting using antibodies directed to PR Ser294, 345 and 400 (Fig. 3). Interestingly, mCD PR appears to be phosphorylated on an earlier time course as compared to wt PR, with R5020-induced phosphorylation occurring earlier in cells transfected with mCD PR. In contrast, when measuring levels of Ser81 phosphorylation, mCD PR is not phosphorylated on this site in response to ligand in transiently transfected HeLa cells (Fig. 4 - left) or breast cancer cells (T47D) stably expressing wt or mCD PR (Fig. 4 - right). PR is an ER target gene. Thus, estradiol is generally required for robust PR expression in steroid hormone receptor-positive breast cancer models, complicating experimental isolation of PR action (i.e. independent of estrogen) [13]. To bypass the need for added estrogen, a naturally occurring PR-negative variant of the T47Dco human breast cancer cell line, termed T47D-Y, was used to create stable cell lines constitutively expressing wt PR-B (T47D-YB), PR-A (T47D-YA; used below) or mCD PR (T47D-mCD PR) [14]. A time course of Ser81 phosphorylation in response to R5020 treatment verified that mCD PR is phosphorylated (Fig. 5) at dramatically reduced levels as compared to wt PR (Fig. 5). These data indicate that the kinetics of Ser81 phosphorylation of mCD PR are not altered, but rather the absolute levels are diminished. Cumulatively, these data suggest that mutation of the CD domain differentially affects PR phosphorylation in a site-specific manner: some sites show hyper-phosphorylation, whereas other newly characterized PR phosphorylation sites (Ser81; see Appendix C) show decreased phosphorylation in response to ligand, indicating an impaired interaction with a PR-modifying kinase, like ck2 (the kinase shown in vitro and in vivo to phosphorylate PR on Ser81) [7, 15].

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**Figure 2. PR CD domain mediates interaction with MKP3.**
COS cells were co-transfected with myc-MKP3 (or vector control) and one of the following PR constructs: vector (lane 1), wt PR-B (lanes 2-3), mCD PR (lane 4), PR-A (lane 5), CD-PR-A (PR-A with a CD domain attached to the N-terminus; lane 6), or mCD-PR-A (PR-A with a mutant CD domain attached to the N-terminus; lane 7). Lysates were isolated following a 24 hr incubation in serum-free media. Cell lysates were immunoprecipitated with a PR antibody, and the resulting co-immunoprecipitated protein complexes were analyzed by Western blotting (top two panels). Bottom two panels represent total cell lysates.

**Figure 3. Earlier time-course for progesterone-induced phosphorylation of mCD PR as compared to wt PR.**
HeLa cells were transfected with either wt or mCD PR. Following transfection, cells were starved for 24 hr in serum-free media and then treated with 10nM R5020 for 0-60 min. Total cell lysates were analyzed by Western blotting.
The lack of mCD PR Ser81 phosphorylation suggests that the CD domain unique to PR-B may facilitate specific interaction(s) between PR-B and one or more factors that are required for ck2-dependent PR Ser81 phosphorylation. Notably, an interaction between ck2 and MAP kinase phosphatase 3 (MKP3) has previously been reported [16]. MKPs use “D” domains to interact with CD domains in their respective substrates (MAPKs). For example, MKP3 interacts with and inhibits Erk2 phosphorylation by binding to the CD domain in Erk2. Similarly, we hypothesized that MKP3 may interact with PR through its CD domain, thus facilitating complex formation (i.e. recruitment of ck2) needed to for robust PR Ser81 phosphorylation (Figs. 4 and 5).

Interaction between PR and MKP3 is mediated through the CD domain of PR (Figs. 1 and 2) and this domain is required for PR Ser81 phosphorylation (Figs. 4 and 5). We previously identified PR Ser81 as a ck2-dependent site regulated in response to treatment of breast cancer cells with progestin and phosphorylated during S-phase in the absence of progestin [15]. If MKP3 primarily functions to recruit ck2 for PR Ser81 phosphorylation, then abrogation of this interaction should block this phosphorylation event. Specific siRNA was used to knock-down MKP3 protein expression in breast cancer cells. We knocked down MKP3 in T47D-YB cells, and analyzed progestin-induced PR Ser81 phosphorylation. Although MPK3 knockdown efficiency was weak (~50% as determined by densitometry), cells transfected with MKP3 siRNA exhibited decreased PR Ser81 phosphorylation (Fig. 6 - left) relative to cells transfected with non-silencing control siRNA; 50% knockdown in MKP3 translated to a 75% reduction in PR Ser81 phosphorylation (Fig. 6 – right).
MKP3 is a negative regulator of Erk1/2 phosphorylation and activity. As a control for functional MKP3 knock-down, we measured Erk1/2 phosphorylation under similar conditions (Fig. 7 - left). As expected, T47D-YB cells treated with MKP3 siRNA contained activated MAPK activity relative to controls, indicating effective MKP3 knock-down (i.e. an indirect measure of phosphatase activity). To confirm these results, we chemically modulated MKP3 activity. Reactive oxygen species, produced as a result of treatment of cells with agents such as H2O2, block MKP phosphatase activity [17, 18], thereby resulting in higher levels of Erk1/2 phosphorylation (schematic, Fig. 7 - middle). Therefore, to determine if MKP3 phosphatase activity (as compared to protein presence) is required for PR Ser81 phosphorylation, we treated cells with H2O2 to block MKP3 activity without altering protein levels. T47D-YB cells treated with either 1mM H2O2 or vehicle alone followed by R5020 showed similar levels of Ser81 phosphorylation (Fig. 7 - right) despite MKP3 phosphatase inhibition, as measured by increased phospho-Erk1/2. These data suggest that MKP3 enzyme activity is not required for Ser81 phosphorylation, as Ser81 levels remain unchanged even under conditions where MKP3 phosphatase activity is greatly diminished (Fig. 7 - right). Cumulatively, these data suggest that MKP3 protein, but not phosphatase activity, is required for efficient PR Ser81 phosphorylation, indicating that MKP3 serves a scaffolding function to support ck2-dependent PR Ser81 phosphorylation.

**Figure 6. MKP3 is necessary for PR Ser81 phosphorylation.** Left: T47D cells stably expressing wt PR-B (T47D-YB) were transfected with 50nM non-silencing (NS) or MKP3 siRNA. 72hr following transfection, cells were treated with 10nM R5020 (or EtOH) for 60min. Lysates were analyzed via Western blotting using p-S81, PR and MKP3 antibodies. Right: The ratio of Ser81 phosphorylated PR bands (in R5020-treated cells) to total PR bands was determined using densitometry, and compared between cells transfected with NS or MKP3 siRNA.

**Figure 7. MKP3 activity is not necessary to support PR Ser81 phosphorylation.** Left: T47D-YB cells were transfected and treated (as described in Fig. 6), and lysates were analyzed via Western blotting using p-Erk1/2 and Erk1/2 antibodies. Middle: Schematic showing how ROS production (increased through treatment with H2O2) decreases MKP3 phosphatase activity, which subsequently leads to an increase in Erk1/2 activity and phosphorylation. Right: T47D-YB cells were pre-treated with 1mM H2O2 for 20min, followed by 10nM R5020 (or EtOH) for 30 min. Lysates were analyzed via Western blotting using p-S81, PR, p-Erk1/2 and Erk1/2 antibodies.
To determine if Ser81 phosphorylation is dependent on phosphorylation at other sites within PR (i.e. pre-requisite phosphorylation needed to subsequently obtain Ser81 phosphorylation), we analyzed S81 phosphorylation of previously characterized PR-phosphorylation mutants (S294A, S345A and S400A). Ser81 phosphorylation was measured in HeLa cells that were transiently transfected with various PR phosphorylation mutants (alanine mutants for serine phosphorylation at Sers 294, 345 and 400). Each of these PR phosphorylation mutants retained the ability to get phosphorylation on Ser81, indicating that phosphorylation at Sers 294, 345 or 400 is not required to obtain Ser81 phosphorylation (Fig. 8).

To characterize PR phosphorylation by ck2, the kinase previously shown in vitro to phosphorylate PR on Ser81 [7], we analyzed ligand-activated PR phosphorylation in the presence of two highly-specific, synthetic ck2 kinase inhibitors, TBB and DMAT. Data from two different cell lines stably expressing wt PR, HeLa-PR and T47D-YB, showed that treatment with both inhibitors significantly decreased phosphorylation of Ser81 in response to ligand (Appendix C; Fig. 2A-C). In addition to ligand, we found that Ser81 phosphorylation was differentially activated during specific phases of the cell cycle, independent of ligand. Ser81 was phosphorylated in the G1/S phase of the cell cycle (in the absence of ligand); an effect shown to be dependent on ck2 (Appendix C; Fig. 2D). We have not yet analyzed the effect of ck2 knockdown (using si/shRNA technology) on Ser81 phosphorylation, but predict that the outcome will be similar to using synthetic kinase inhibitors. These data indicate that PR phosphorylation on Ser81 is regulated by ck2, both in response to ligand and in a cell cycle-dependent manner [15].

**Task 3: Analysis of CD domain-dependent PR transcriptional activity (Months 6-18):**

Although we have been technically unsuccessful in measuring PR transcriptional activity via PRE-luciferase assays in the presence of ck2 inhibitors (long term inhibition of ck2, as is necessary to measure PR transcriptional products by luciferase, proved to be toxic to both HeLa-PR and T47D-YB cells), we have focused on studying the downstream consequence of ck2 kinase action: phosphorylation on PR Ser81 (thoroughly characterized in Appendix C; [15]). To study the functional significance of PR phosphorylation at this site, we created a PR mutant that cannot get phosphorylated by ck2 by mutating Ser81 to alanine (S79/81A PR). Point mutation of phosphorylated residues within phospho-proteins can shift specificity to adjacent or very nearby phospho-acceptor sites that are not detected using mass spectrometry of the wt protein [19]. Thus, both PR residues (Ser79 and Ser81) were mutated to ensure that nearby Ser79 is not weakly targeted by highly active kinases (in vivo) when Ser81 is mutated. Phospho-Ser81 PR antibody specificity was verified using the double phospho-mutant receptor (S79/81A PR). The S79/81A PR mutant does not get phosphorylated on Ser81, but retains functional transcriptional activity as measured by PRE-luciferase (Appendix C, Fig. 3B). Stable cell lines were created using this mutant and were used for subsequent experiments (Appendix C, Fig. 4).

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Figure 8. Ser81 phosphorylation independent of other PR phosphorylation.
HeLa cells were transfected with the respective PR construct as labeled. Following transfection, cells were starved for 24 hr in serum-free media and then treated with vehicle (EtOH) or 10nM R5020 for 60 min. Total cell lysates were analyzed by Western blotting.
Specifically, T47D-S79/81A PR cells were used to measure transcription of endogenous PR target genes. We found that Ser81 PR phosphorylation regulated transcription in a ck2-dependent manner of a subset of PR target genes known to be involved in cell growth and prevention of apoptosis, including BIRC3, HSD11β2 and HbEGF (Appendix C, Figs. 5-7). ChIP and re-ChIP experiments (Appendix C, Fig. 8) showed that Ser81 phosphorylation was required for PR recruitment to these genes, both basally (BIRC3, HSD11B2) and in response to ligand (HbEGF).

Analysis of PR target gene transcription in cells stably expressing mCD PR yielded results suggesting that the CD domain regulates known PR-target genes critical to cell growth and mammary stem cell maintenance (STAT5A and Wnt1; Fig. 9). T47D cells stably transfected with mCD PR showed significant defects in activating transcription of STAT5A and Wnt1 in response to ligand as compared to wt PR (Fig. 9). Activation of these target genes in mCD PR-expressing cells mimicked what was seen in cells stably expressing S79/81A PR, reinforcing the link between the CD domain and Ser81 phosphorylation. Interestingly, basal levels of STAT5A were also affected similarly by mutation of the CD domain and disruption of Ser81 phosphorylation. Both mCD PR and S79/81A PR-expressing cells showed decreased basal (in the absence of ligand) levels of STAT5A transcription (Fig. 10), indicating that phosphorylation at Ser81 (facilitated by the CD domain) is required to maintain PR-dependent transcription of STAT5A.

To investigate the mechanism through which wt PR (via Ser81 phosphorylation) regulates transcription of these genes, we analyzed transcription in the presence of a specific JAK-STAT inhibitor, AG490 [20, 21]. STAT5A has previously been shown to be regulated in a PR- and JAK/STAT-dependent manner; transcription was decreased in the presence of the inhibitor (AG490) [22]. Because PR regulation of STAT5A is Ser81 phosphorylation-dependent (via PR’s CD domain; see Fig. 9), we hypothesized that other Ser81-regulated genes (i.e. Wnt1) were also similarly regulated by STATs. Cells expressing wt PR were pre-treated with 50µM AG490 (or vehicle; DMSO) for 1hr, followed by 10nM R5020 or EtOH (6hr). Under these conditions,
treatment with AG490 dramatically reduced transcription of STAT5A (previously shown in [22]), as expected (Fig. 11 – middle). Interestingly, treatment with AG490 significantly diminished Wnt1 regulation by progestin (Fig. 11 – left), suggesting that STAT5 activity is required for PR-dependent transcription of Wnt1. Other PR-target genes previously shown to be phospho-Ser81 PR-dependent [15], such as HSD11β2 and BIRC3, were also similarly regulated in a STAT-dependent fashion (data not shown) [22]. c-myc, a PR-target gene whose regulation is PR Ser81- and STAT-independent was not affected by treatment with the JAK/STAT pathway inhibitor (Fig. 11 – right) [15, 22]. These data suggest that regulation of a subset of PR-target genes, those that are regulated by PR Ser81-phosphorylation, is dependent on STAT pathway activation. Of note, Ser81 phosphorylation was unaffected by the STAT inhibitor (data not shown). Moreover, these data provide insight into the previously unknown regulation of PR-dependent Wnt1 transcription, a gene known to be critically involved in mammary stem cell maintenance and proliferation.

To further understand how phospho-Ser81 PR regulates transcription of Wnt1 in a STAT-dependent fashion, we performed an in silico analysis of the Wnt1 promoter and enhancer regions to reveal any transcriptional regulatory regions. We identified four putative full-length PRE binding regions (Fig. 12; triangles), including a site located in the proximal promoter region (~1kb upstream from the transcriptional start site [TSS]; PRE1), as well as three PREs located downstream in transcriptional enhancer sites (+44, 65 and 66kb from the TSS; PREs 2-4, respectively). Interestingly, we also identified four putative STAT5 binding sites (interferon gamma activation sites [GAS]) in the same region (Fig. 12; rectangles). To determine if PR and STAT5 coordinate to regulate transcription of Wnt1, we performed chromatin immunoprecipitation (ChIP) assays to detect the presence of PR and/or STAT5 on sites within the Wnt1 promoter/enhancer region. ChIP analysis was performed on lysates from EtOH- or R5020-treated T47D cells stably expressing wt PR, or from PR-null cells (negative control), using PR and STAT5-specific antibodies. In the presence of ligand, we detected robust recruitment (~11-fold) of wt PR to PRE1 (located just upstream of the Wnt1 TSS). Moderate levels of PR recruitment (~4-fold with ligand) were detected at two other PREs located downstream of the Wnt1 TSS (PREs 2 and 4; Fig. 13 - left). Statistically significant levels of ligand-induced recruitment were not seen at PRE3, although PR is present at this site in wt PR-expressing cells (ligand-independent; as compared to IgG controls). Notably, PR was also detected at the four putative GAS sites (GAS1-4) identified in the Wnt1 promoter/enhancer region; levels of PR at the GAS sites were unchanged in response to ligand (Fig. 13 - left). These data indicate that PR is recruited to regulatory regions in the Wnt1 promoter/enhancer in response to ligand, and that PR is present in a ligand-independent fashion at GAS sites within those same areas. Immuno-precipitation of lysates from T47D cells stably expressing wt PR, or PR-null cells (not shown), using a STAT5-specific antibody showed complex patterns of STAT5 binding. STAT5 was present (unchanged with ligand) at two of the four GAS sites (GAS 2 and 3), present (unchanged with ligand) at three of the four PREs (PREs 1, 2 and 4), and not present at the other sites (GAS1 and 4, PRE3) (Fig. 13 - left). Finally, we performed ChIP analysis on lysates from EtOH- or R5020-treated T47D cells stably expressing wt PR, or from PR-null cells (negative control; not shown), using ck2 and MKP3-specific antibodies, proteins previously shown to be critical for PR Ser81-phosphorylation, and
therefore, regulation of these genes (see Fig. 6 and [15]). In cells expressing wt PR, MKP3 and ck2 were both recruited to PRE1 in a ligand-dependent manner (Fig. 13 - right). Cumulatively (summarized in table in Fig. 13), these data suggest that regulation of the Wnt1 promoter is a complex process involving PR, STAT5, MKP3 and ck2.

**Figure 12. Schematic of Wnt1 promoter/enhancer.**
A schematic of the Wnt1 promoter/enhancer regions. PRE sites (triangles) and STAT5-GAS sites (rectangles) are located up- and down-stream of the Wnt1 transcription start site (TSS; denoted with an arrow). Distance of the regulatory regions from the TSS are listed in kilobases (kb).

**Figure 13. PR, STAT5, MKP3 and ck2 regulate PR-dependent Wnt1 transcription.**
Left - top: T47D-Y cells stably expressing wt PR-B or unmodified cells (PR-null; not shown) were serum-starved for 18hr. Cells were then treated with EtOH or 10nM R5020 for 60min. Fixed lysates were subjected to ChIP with antibodies against PR-B or STAT5 (or species-specific IgG as controls), and qPCR was performed on the isolated DNA using primers designed to amplify the respective regulatory region (PRE1-4 and GAS1-4). Fold recruitment of PR or STAT5 in R5020 condition over EtOH is shown. The lack of a bar indicates that STAT5 is not present (not over IgG) at that site. Error bars represent ±STD of triplicate experiments (left – top). Asterisks represent PR recruitment that achieves statistical significance (p<0.05) when compared to a site where PR (GAS1) or STAT5 (PRE2) is present, but not recruited with ligand. Left – bottom: the table below represents a summary of the data presented in the above graph. Right: T47D-Y cells stably expressing wt PR-B or unmodified cells (PR-null; not shown) were serum-starved for 18hr. Cells were then treated with EtOH or 10nM R5020 for 60min. Fixed lysates were subjected to ChIP with antibodies against MKP3 or ck2a (or species-specific IgG as controls, not shown), and qPCR was performed on the isolated DNA using primers designed to amplify PRE1. Fold recruitment of MKP3 or ck2α in R5020 condition over EtOH is shown.
Finally, transcription of MKP3, both basally and in response to ligand, appears to be altered in mCD PR-expressing cells. In the absence of ligand, MKP3 mRNA (Fig. 14 – left) and protein (not shown) levels are significantly elevated in mCD PR cells as compared to cells expressing wt PR. Moreover, in response to ligand, MKP3 levels are repressed in wt PR-expressing cells, an effect that is impaired in cells expressing mCD PR (Fig. 14 - right; 0-18hr). Interestingly, this phenotype (altered basal and ligand-dependent transcription in mCD PR cells) is not shared by the S79/81A expressing cells, which behave similarly to wt PR-expressing cells. These data indicate that the transcriptional control of MKP3 is independent of Ser81 phosphorylation, and is regulated by the CD domain through a mechanism that has yet to be defined. Experiments are underway to further characterize this phenotype, and to determine the connection between MKP3 protein binding through the CD domain (Figs. 1-2) and subsequent regulation of MKP3 mRNA/protein levels (Fig. 14). This is an interesting experimental result that we are eager to analyze.

Task 4: Analysis of CD domain-dependent rapid signaling events (Months 6-12):

Following treatment with ligand, PR has been shown to rapidly activate (within 15 min) protein kinases, such as MAPK (Erk1/2), c-Src and Akt. To determine if the CD domain of PR is necessary for this function, we transfected HeLa cells with wt and mCD PR constructs and measured MAPK phosphorylation following short treatments with R5020 (Fig. 15). Preliminary experiments suggest that wt and mCD PR similarly activate phosphorylation of Erk1/2, suggesting that the CD domain is not required for this effect. Rapid activation of c-Src and Akt have not been tested. Experiments to test the ability of mutant S79/81A PR to rapidly activate cellular kinases have not yet been initiated, but we would expect the results to be similar to those obtained with mCD PR.
**Task 5: Analysis of the effect of PR’s CD domain on cell proliferation (Months 12-30):**

To determine if mCD PR expressing cells exhibit proliferation defects, we analyzed their cell cycle profile in response to progestin-treatment. It is well documented that progestins induce S-phase entry in cells expressing wt PR-B [23-25]. This ligand-dependent effect fails to occur in cells expressing only PR-A [26]. We analyzed progestin-induced S phase entry in T47D breast cancer cells stably expressing wt PR-B, PR-A or mCD PR. Indeed, when we treated wt PR-B expressing cells with R5020 for 18hrs, we observed a predictable increase in S-phase entry that was not observed in cells expressing wt PR-A (Fig. 16). Interestingly, when mCD PR expressing cells were treated with R5020, they exhibited the same defect in S-phase entry as PR-A expressing cells (Fig. 16). These data suggest that the PR-B CD domain is essential for proliferative signaling, as measured by S-phase entry, in progestin-treated breast cancer cells.

**Task 6: Analysis of the effect of PR’s CD domain on anchorage-independent growth (Months 24-36):**

The ability of mCD PR cells to grown in an anchorage-independent manner has not yet been analyzed. However, these experiments have been conducted with regards to S79/81A PR-expressing cells. Interestingly, cells expressing mutant S79/81A PR, while retaining their ability to grown soft-agar colonies in response to ligand, formed significantly fewer colonies in the ligand-independent condition as compared to cells expressing wt PR (Appendix C, Fig. 4C). These data indicate that phosphorylation on Ser81, in the absence of ligand, contributes to cellular survival as measured by anchorage-independent growth.
KEY RESEARCH ACCOMPLISHMENTS

- Task 1 Milestone: MKP3 was identified as a protein that interacts with PR through the CD domain. Mutational studies revealed the critical contribution of the CD domain to facilitating the interaction between PR and MKP3.
- Task 2 Milestone: Ser81 is differentially phosphorylated due to mutation of the PR CD domain; mCD PR lacks phosphorylation at Ser81. Other PR phosphorylation sites studied appear to be hyper-phosphorylated on mCD PR as compared to wt PR.
- Task 2 Milestone: MKP3 protein, not phosphatase activity, is necessary to facilitate PR Ser81 phosphorylation.
- Task 2 Milestone: Ser81 phosphorylation occurs independently of other PR site phosphorylation.
- Task 2 Milestone: ck2 is the kinase responsible for phosphorylation of PR on Ser81.
- Task 2 Milestone: Ser81 phosphorylation is regulated basally, in response to ligand, and in a cell cycle-dependent manner; all PR Ser81 phosphorylation is ck2-dependent.
- Task 3 Milestone: A subset of endogenous PR target genes was identified that is regulated by phosphorylation at PR Ser81. This subset contains genes known to regulate cellular proliferation and/or survival.
- Task 3 Milestone: ChIP and re-ChIP experiments confirmed that Ser81 is required for PR recruitment to the aforementioned subset of Ser81-dependent target genes.
- Task 3 Milestone: STAT5A and Wnt1, genes known to be involved in breast cancer cell proliferation and mammary stem cell maintenance, are regulated by PR in a CD domain-dependent manner.
- Task 3 Milestone: Transcription of PR Ser81-dependent genes is STAT-dependent.
- Task 3 Milestone: Wnt1 promoter/enhancer regulation is a complex process involving PR, STAT5, MKP3 and ck2.
- Task 3 Milestone: Mutation of the CD domain in PR disrupts the transcriptional regulation of MKP3, a protein previously shown to interact with PR through the CD domain; mCD cells have higher levels of MKP3 and are no longer able to transcriptionally repress MKP3 in a PR-dependent manner.
- Task 4 Milestone: The CD domain of PR is not required for PR-dependent rapid activation of MAPK in response to ligand.
- Task 5 Milestone: Cellular proliferation rates are affected by mutations in the CD domain, as mCD PR-expressing cells lack progestin-dependent S-phase entry.
- Task 6 Milestone: Phosphorylation at Ser81 regulates the ability of PR-expressing cells to survive in an anchorage-independent manner in the absence of ligand.
REPORTABLE OUTCOMES

- Manuscripts:

  **Hagan C.R.** and Lange, C.A. Progesterone receptor regulation of Wnt1 in breast cancer cells is STAT-dependent. In submission. (Appendix A)


- Invited Presentations:


- Abstracts presented/meetings attended:


- Grants accepted:
K99/R00 Pathway to Independence Award (NIH/NCI). Title: CK2-dependent Phosphorylation of Progesterone Receptors Mediates Proliferative Signaling in Breast Cancer. Funding period: 2012-2017
CONCLUSION

Progesterone receptors (PR) are critical mediators of mammary gland development and contribute to breast cancer progression. Progestin-induced rapid activation of cytoplasmic protein kinases leads to selective regulation of growth-promoting genes by phospho-PR species. We have shown that phosphorylation of PR Ser81 is ck2-dependent, progestin-regulated and cell cycle-dependent in intact cells. Mutation of the CD domain in PR (mCD PR) abrogates phosphorylation on Ser81, indicating that the CD domain is necessary to facilitate phosphorylation at this site (Ser81). mCD PR expressing cells exhibit a defect in progestin-induced S-phase entry, indicating that they have a proliferation defect in comparison to wt PR expressing cells. Additionally, we showed that an interaction between PR and MKP3, a regulator of the ERK family, is dependent on the CD domain. This interaction is necessary to achieve PR Ser81 phosphorylation. Regulation of selected genes by PR-B also required the CD domain for basal and/or progestin-regulated (STAT5A, Wnt1, MKP3) expression/repression. We have shown that PR-regulation of Wnt1 is not only Ser81-dependent, but also requires STAT activation. Our data show that regulation of PR-dependent Wnt1 transcription is a complex process involving PR, STAT5, MKP3 and ck2. We conclude that the CD domain of PR facilitates protein interactions that are critical to PR-dependent transcription of genes involved in proliferation and mammary stem cell maintenance.

Understanding how mitogenic protein kinases, such as ck2, alter PR phosphorylation and function is critical to fully understanding breast tumor etiology and developing better targeted therapies. Recent clinical data linking the progesterone component of hormone-replacement therapy regimens with the development of breast cancer underscores the importance of understanding how PR works in the context of breast cancer and high kinase environments. Due to the ubiquitous nature of ck2 and its prevalence in many different types of cancer, there has been much interest in the development of ck2 inhibitors as anti-cancer agents. Clinical ck2 inhibitors, in combination with more specific anti-progestins (new classes of selective progesterone receptor modulators or SPRMs), could provide an effective combination of targeted therapy for breast cancer treatment.
References


15. Hagan, C.R., et al., ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B Isoform-antiprogestin-occupied B-receptors are switched to transcriptional agonists by cAMP.


Hagan C.R. and Lange, C.A. Progesterone receptor regulation of Wnt1 in breast cancer cells is STAT-dependent. In submission. (Appendix A)
Introduction

Progestrone is an ovarian steroid hormone essential for breast development. The progesterone receptor (PR) exists primarily in two co-expressed isoforms, PR-A and PR-B [1, 2], encoded for by the same gene with distinct promoters [3]. PR-B, the full-length receptor contains 164 amino acids at the N-terminus, a region unique to PR-B termed the B-upstream segment (BUS) [4]. Both receptors contain the same DNA binding domain (DBD), a hinge region (H) and two activator function (AF) domains; PR-B contains a third AF domain in the BUS [5]. Unliganded PR rapidly shuttles between the cytoplasm and the nucleus. Following ligand binding, PR undergoes dimerization (A:A, B:B, or A:B) and translocation to the nucleus. Nuclear PR activates or represses transcription of PR-target genes, either directly through binding to progesterone response elements (PREs), or indirectly through tethering interactions with other transcription factors (AP1, SP1, STATs) [6-8]. Initiation of transcription by PR involves recruitment of co-activators, inhibition of co-repressors, and reliance on basic transcriptional machinery (reviewed in [9]).

Recruitment of transcription factors and co-regulators occurs at gene promoter and enhancer regions that until recently were thought to be within a few kilobases (kb) of the respective transcriptional start site (TSS). Recently, however, whole genome binding site studies have revealed that nuclear receptors (and their corresponding transcriptional co-regulators) can bind to and enhance/repress transcription of genes over 50kb away from the TSS, likely through complex models of chromatin looping. No correlation exists between the distance of functional enhancers from TSS and the strength of transcriptional enhancement [10]. Additionally, these whole genome (cistrome) analyses have identified “pioneer factors” for nuclear receptors. These pioneer factors are specialized subsets of transcription factors that bind to and activate transcriptional enhancers, making them competent for subsequent transcriptional activation by downstream transcription factors (reviewed in [11, 12]). This activity has been reported for both FOXA1 and PBX1-dependent modulation of estrogen receptor binding, and similar pioneer factors have been identified for other nuclear receptors as well [11, 13, 14]. Importantly, pioneer factors bind DNA before the activation of transcription, thereby “marking” enhancer regions for sites of future active transcription.
PR is highly post-translationally modified, primarily on N-terminal serine (phosphorylation) and lysine (ubiquitination, sumoylation and acetylation) residues [15-19]. These modifications significantly alter receptor stability, localization, transcriptional activity and promoter selectivity. In addition to MAPK and cdk2, ck2 has recently been shown to phosphorylate PR on Ser81 [20-23]. ck2 is a ubiquitously expressed, constitutively active kinase that is overexpressed in every cancer examined thus far, including breast cancer [20, 24]. ck2-dependent phosphorylation of Ser81 (unique to PR-B) has been shown to regulate a specific subset of PR-target genes involved in breast cancer cell growth. In addition, ck2 was shown to be recruited along with Ser81-phosphorylated PR-B to enhancer sites of a subset of PR-target genes [23]. Notably, this work showed that ck2-dependent phosphorylation of PR Ser81 is a primary determinant of PR isoform-specific activity. However, the molecular interactions necessary to support Ser81 phosphorylation have yet to be understood.

Post-translational modifications also alter PR interactions with other proteins. There are a number of protein interaction domains in PR that have previously been characterized [25]. These domains include the estrogen receptor interaction domains (ERID; ER interaction [26]) and a proline-rich domain (c-Src interaction [27]). PR interacts with many other proteins whose interaction domains in PR have yet to be identified (i.e. MEK1, FGFR2, STAT5, JDP2, etc) [25, 28-30]. We previously identified a common docking (CD) domain in PR commonly found in members of the MAPK family [25]. These domains are used for interactions between MAPKs and their upstream activators (MEKs), negative regulators (MKPs), and downstream targets [31, 32]. CD domains are characterized by clusters of negatively charged amino acids (Aspartic or Glutamic acid) that form electrostatic interactions with a positively-charged “D domain” in the respective binding partner (MKKs, MKPs, MAPK targets; reviewed in [33]). The CD domain we identified in PR is an identical match to the CD domain in Erk2. This is the first identification of such a highly conserved domain in a nuclear receptor, a protein superfamily that would not be predicted to contain such a domain, however, the function of this domain in PR has not yet been determined.

Many cancers have upregulated protein kinases, such as MAPK, c-Src, cdk2 and ck2, which modify and/or activate PR [21, 34-36]. Additionally, recent clinical data has shown that women taking hormone-replacement therapy whose regimens included estrogen and progesterone, but
not estrogen alone, had an increase in breast tumor number and size [37, 38]. Notably, we recently identified a phospho-PR gene signature associated with decreased survival in women with luminal breast cancer who failed tamoxifen therapy [18]. In light of these data, understanding how mitogenic protein kinases (and proteins that regulate them) alter and interact with PR is critical to understanding breast tumor etiology and developing better treatments. Herein, we sought to identify proteins that interact with PR through a novel CD domain in PR. Moreover, we wanted to understand how interactions through this domain alter PR function and transcriptional profile.
Results

PR CD domain is required for progestin-induced Ser81 phosphorylation

We previously published an interaction between PR-B and MEK1, an upstream activator of the MAPK pathway. An *in silico* analysis of the PR amino acid sequence aimed at identifying protein interaction domains revealed the presence of a putative common docking (CD) domain in the N-terminus of PR-B (Fig 1A)[25]. PR’s CD domain is located in the N-terminal region of full-length PR-B known as the B-upstream segment (BUS), a region that is unique to PR-B, and, therefore, not found in PR-A (Fig 1B). To study the importance of this newly identified CD domain in modulating PR-B-specific functions, we mutated the critical negatively-charged amino acids (D-aspartic acid, E-glutamic acid) to alanines (A), creating the mCD PR mutant (Fig 1B). Similar mutational strategies have been used to study the CD domain in Erks and other MAPKs [31, 39].

To determine if mCD PR is able to bind DNA and activate transcription, we used PRE-luciferase reporter gene assays to compare transcription between wt and mCD PR-B. In transiently transfected HeLa cells treated with EtOH or 10nM R5020 (synthetic progesterone), wt and mCD PR activated similar fold levels of PRE-luciferase transcription (~15 fold R5020/EtOH; Fig 2A). Thus, mutation of the CD domain does not abrogate the transcriptional capacity of mCD PR (on a PRE-luciferase construct). A hallmark of wt PR-B activity is the ability to rapidly activate MAPK (Erk1/2) following short treatment with ligand (R5020; 10min), an effect that has been mapped to proline-rich and ERID interaction domains in the BUS region [26, 27, 40, 41]. HeLa cells transiently transfected with wt or mCD PR were treated with 10nM R5020 or vehicle (EtOH) for 0-10min. Basal p42 MAPK activity was slightly higher in cells expressing mCD PR, however similar levels of MAPK activation, as measured by Erk1/2 phosphorylation (10min), were achieved in cells transfected with either wt or mCD PR, indicating that the CD domain is not necessary for the rapid signaling actions of PR (Fig 2B). Interestingly, although CD domains in MAPKs interact with MEKs [25], mCD PR retained the ability to interact with MEK (as measured by co-immunoprecipitation assays (Co-IPs; data not shown), indicating another domain(s) is responsible for this interaction. These data suggest that mCD PR may act to suppress basal MAPK activity perhaps via other protein complexes that form through interaction with this domain (see below).
To determine if mCD PR expressing cells exhibit proliferation defects, we analyzed their cell cycle profiles in response to progestin. It is well documented that progestins induce S-phase entry in cells expressing wt PR-B [42-44]. This ligand-dependent effect fails to occur in cells expressing only PR-A [45]. PR is an ER target gene. Thus, estradiol is generally required for robust PR expression in steroid hormone receptor-positive breast cancer models, complicating experimental isolation of PR action (i.e. independent of estrogen) [46]. To bypass the need for added estrogen, a naturally occurring PR-negative variant of the T47Dco human breast cancer cell line, termed T47D-Y, was used to create stable cell lines constitutively expressing wt PR-B (T47D-YB), PR-A (T47D-YA) or mCD PR (T47D-mCD PR) [47]. We analyzed progestin-induced S phase entry in T47D breast cancer cells stably expressing wt PR-B, PR-A or mCD PR. Indeed, when we treated wt PR-B expressing cells with R5020 for 18hrs, we observed a predictable increase in S-phase entry that was not observed in cells expressing wt PR-A (Fig 2C). Interestingly, when mCD PR expressing cells were treated with R5020, they exhibited the same defect in S-phase entry as PR-A expressing cells (Fig 2C). These data suggest that the PR-B CD domain is essential for proliferative signaling, as measured by S-phase entry, in progestin-treated breast cancer cells.

PR phosphorylation has been characterized on multiple sites; phosphorylation is known to be a critical determinant of PR localization and receptor stability, transcriptional profile, tethering interactions, and promoter selectivity (reviewed in [48]). We analyzed PR phosphorylation on known ligand-induced, MAPK- or cdk2-regulated sites (Serines 294, 345 and 400) in HeLa cells transiently transfected with wt or mCD PR. Following treatment with R5020 (0-60min), cells transfected with mCD PR showed higher levels of phosphorylation on these proline-directed sites as compared to wt PR (Fig 3A). Additionally, the time course of phosphorylation appeared to occur with faster kinetics in cells expressing mCD PR compared to wt PR. Phosphorylation of mCD PR appeared as early as 10min, compared to wt PR phosphorylation starting at 30-60min. Phosphorylation at basally regulated sites (Ser190) was unchanged when comparing the two PR constructs (data not shown). These data indicate that on select sites, mCD PR phosphorylation is more rapid and robust compared to wt PR. In contrast, Ser81, a basally phosphorylated site recently characterized by our group to be regulated by ck2 in response to PR ligand-binding [23], fails to undergo phosphorylation in mCD PR (Fig 3B), an effect that was
seen in HeLa cells transiently transfected with the mCD PR mutant (Fig 3B – left), or in T47D cells stably expressing the mCD PR mutant (Fig 3B – right). PR Ser81 phosphorylation following 60min of progestin treatment was greatly diminished in T47D cells stably expressing mCD PR relative to cells expressing wt PR, however, it was not completely abrogated (Fig 3B right – dark exposure). A time course of Ser81 phosphorylation in response to R5020 treatment verified that mCD PR is phosphorylated (Fig 3C – top) at dramatically reduced levels as compared to wt PR (Fig 3C – bottom). These data indicate that the kinetics of Ser81 phosphorylation of mCD PR are not altered, but rather the absolute levels are diminished. Unlike the previously characterized ligand-dependent sites (S294, 345 and 400) whose regulation is almost entirely MAPK- or cdk2-dependent, Ser81 phosphorylation is dependent on the serine-threonine kinase, ck2. Cumulatively, these data suggest that mutation of PR’s CD domain alters the program for normal PR phosphorylation in response to ligand: some sites exhibit hyper-phosphorylation, while other sites fail to be phosphorylated. Moreover, these data indicate that the CD domain is necessary for efficient PR Ser81 phosphorylation in response to ligand.

MKP3 scaffolding, but not enzyme activity, through the CD domain is required for Ser81 phosphorylation

The lack of mCD PR Ser81 phosphorylation suggests that the CD domain unique to PR-B may facilitate specific interaction(s) between PR-B and one or more factors that are required for ck2-dependent PR Ser81 phosphorylation. Notably, interaction between ck2 and MAP kinase phosphatase 3 (MKP3) has previously been reported [49]. MKPs use “D” domains to interact with CD domains in their respective substrates (MAPKs). For example, MKP3 interacts with and inhibits Erk2 phosphorylation by binding to the CD domain in Erk2. Similarly, we hypothesized that MKP3 may interact with PR through its CD domain, thus facilitating complex formation (i.e. recruitment of ck2) needed to for robust PR Ser81 phosphorylation (Fig. 3). To test for PR and MKP3 interaction, COS cells were transiently co-transfected with constructs expressing either wt or mCD PR, MKP3 (myc-tagged) or vector only controls. In cells transfected with wt PR and MKP3, myc-tagged MKP3 co-immunoprecipitated with wt PR (Fig 4A; lanes 3-4). Myc-tagged MKP3 appears as a dimer by Western blotting, likely due to alternate translational start sites [50, 51]. In COS cells, the PR interaction with MKP3 was largely ligand-independent, but appeared slightly increased in response to progestin. In contrast, MKP3 failed to co-purify with mCD PR under the same experimental conditions (lanes 6-7). A long exposure of the film revealed very faint levels of co-immunoprecipitated MKP3 with mCD PR (data not shown),

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indicating that other domains in PR may weakly support interaction with MKP3, or that the interaction mediated by the CD domain was not completely abrogated with our mutational design. Regardless, these data indicate that the interaction between PR and MKP3 occurs primarily through the PR-B CD domain.

To determine the specificity of the PR-MKP3 interaction, we utilized various PR isoform-specific mutants (Fig 4B). Wt PR-B has an intact CD domain; mCD PR-B contains D/E to A mutations at three critical residues in the CD domain (see Figure 1B). PR-A lacks the BUS where the CD domain is located, and therefore lacks a CD domain. We therefore engineered artificial PR-A constructs containing either complete wt or mutant PR CD domains fused to the N-terminus (termed CD-PR-A and mCD-PR-A, respectively). COS cells were transiently transfected with control (wt) and mutant PR constructs, as well as myc-tagged MKP3. Reproducibly, wt PR-B and MKP3 exhibited a robust interaction; mCD PR-B displayed greatly reduced interaction with MKP3 (Fig 4C – compare lanes 2 and 4). PR-A co-immunoprecipitated a low or background level of myc-tagged MKP3, similar to that observed with mCD PR-B (lane 5). Transient expression of CD-PR-A and mCD-PR-A fusion proteins was poor relative to wt PR-A (see Input lysates blot, Fig 4C lanes 6-7). However, wt PR-A and both PR-A fusion proteins were visible in Western blots of immunoprecipitates (IP:PR blot, lanes 5-7). Despite the poor expression, the CD-PR-A fusion protein co-immunoprecipitated with myc-tagged MKP3 to equal or slightly greater levels than the background levels seen with wt PR-A. Mutation of CD in the PR-A fusion protein (mCD-PR-A) reversed this, returning MKP3 co-immunoprecipitation levels (lane 7) to approximately those seen with wt PR-A alone (lacking a CD domain) and mCD PR-B. These data indicate that the PR-B CD domain is the primary domain responsible for mediating the interaction between wt PR and MKP3. Other regions of both PR-B and PR-A (i.e. outside the BUS) are capable of weak interaction with MKP3 (lanes 5 and 7).

Interaction between PR and MKP3 is mediated through the CD domain of PR (Fig. 4) and this domain is required for PR Ser81 phosphorylation (Fig. 3). We previously identified PR Ser81 as a ck2-dependent site regulated in response to treatment of breast cancer cells with progestin and phosphorylated during S-phase in the absence of progestin [23]. If MKP3 primarily functions to recruit ck2 for PR Ser81 phosphorylation, then abrogation of this interaction should block this phosphorylation event. Specific siRNA was used to knock-down MKP3 protein expression in
breast cancer cells. We knocked down MKP3 in T47D-YB cells, and analyzed progestin-induced PR Ser81 phosphorylation. Although MPK3 knockdown efficiency was weak (~50% as determined by densitometry), cells transfected with MPK3 siRNA exhibited decreased PR Ser81 phosphorylation (Fig 5A - top) relative to cells transfected with non-silencing control siRNA; 50% knockdown in MKP3 translated to a 75% reduction in PR Ser81 phosphorylation (Fig 5A – bottom). MKP3 is a negative regulator of Erk1/2 phosphorylation and activity. As a control for functional MKP3 knock-down, we measured Erk1/2 phosphorylation under similar conditions (Fig 5B). As expected, T47D-YB cells treated with MPK3 siRNA contained activated MAPK activity relative to controls, indicating effective MKP3 knock-down (i.e. an indirect measure of phosphatase activity). To confirm these results, we chemically modulated MKP3 activity. Reactive oxygen species, produced as a result of treatment of cells with agents such as H2O2, block MKP phosphatase activity [52, 53], thereby resulting in higher levels of Erk1/2 phosphorylation (Fig 5C – top). Therefore, to determine if MPK3 phosphatase activity (as compared to protein presence) is required for PR Ser81 phosphorylation, we treated cells with H2O2 to block MPK3 activity without altering protein levels. T47D-YB cells treated with either 1mM H2O2 or vehicle alone followed by R5020 showed similar levels of Ser81 phosphorylation (Fig 5C – bottom) despite MKP3 phosphatase inhibition, as measured by increased phospho-Erk1/2. These data suggest that MKP3 enzyme activity is not required for Ser81 phosphorylation, as Ser81 levels remain unchanged even under conditions where MKP3 phosphatase activity is greatly diminished (Fig 5C). Cumulatively, these data suggest that MKP3 protein, but not phosphatase activity, is required for efficient PR Ser81 phosphorylation, indicating that MKP3 serves a scaffolding function to support ck2-dependent PR Ser81 phosphorylation.

PR Ser81 phosphorylation is required for select STAT-dependent progestin-induced transcriptional responses

It is well documented that PR transcriptional activity on endogenous gene promoters is a more accurate and sensitive read-out for PR action (as compared to PRE-luciferase assays). Post-translational modifications are known to alter PR promoter selectivity, directing PR to different subsets of promoters [54-56]. To determine how mutation of the CD domain in PR-B affects endogenous PR-dependent gene transcription, we analyzed mRNA expression of known PR-target genes in T47D cells expressing wt PR-B, mCD PR and a previously characterized PR mutant that cannot be phosphorylated on Ser81 (S79/81A PR) [23]. Because mCD PR shows
an intermediate Ser81 phosphorylation phenotype, we included the S79/81A PR mutant to differentiate effects that could be solely attributed to Ser81 phosphorylation. Interestingly, although wt PR and two PR mutants (mCD and S79/81A) exhibited equivalent transcriptional responses to ligand on a PRE-reporter gene construct (Fig 2A and [23]), transcription on endogenous promoters appears to be differentially regulated on a subset of PR-target genes. Following treatment with ligand (0-6hrs), cells expressing wt PR robustly activated two known PR-target genes, STAT5A (Signal transducer and activator of transcription 5A) [29, 57] and Wnt1 (wingless-type MMTV integration site family, member 1) [58-61], an effect not seen in PR-null cells (T47D-Y; null) (Fig 6A – top and middle; PR-null and wt PR-B). Cells expressing a PR mutant lacking Ser81 phosphorylation (S79/81A PR) showed dramatic deficiencies in the ability to activate STAT5A or Wnt1 transcription compared to wt PR expressing cells (Fig 6A – top and middle; wt PR-B and S79/81A). STAT5B, a weakly-activated PR-target gene, was unaffected by the PR mutations discussed herein (data not shown). Notably, S79/81A (phospho-mutant) PR-B was phenotypically identical to wt PR-A (not shown) in its inability to transcribe these genes. Interestingly, cells expressing mCD PR (a mutant shown to have weak Ser81 phosphorylation; Figs 3B and C) displayed an intermediate phenotype, further implicating dependence of Ser81 phosphorylation for PR-regulation of STAT5A and Wnt1. Finally, transcription of a Ser81-independent PR-target gene, Tissue Factor (TF) [23, 62], was unaltered in cells expressing wt or S79/81A PR (Fig 6A – bottom). Cells expressing mCD PR showed higher levels of TF transcription, perhaps due to hyper-phosphorylation observed on sites (Sers 294, 345 and 400; Fig 3A) in mCD PR previously identified to be critical for TF transcription [63]. Collectively, these data indicate that the CD domain in PR is responsible, through its ability to scaffold protein-interactions necessary for Ser81 phosphorylation, for transcription of Ser81-dependent PR-target genes known to be critical mediators of progestin-dependent growth, mammary stem cell maintenance and proliferation, and mammary gland development (STAT5A and Wnt1; see Discussion) [29, 57-61, 64, 65].

To investigate the mechanism through which wt PR (via Ser81 phosphorylation) regulates transcription of these genes, we analyzed transcription in the presence of a specific JAK-STAT inhibitor, AG490 [66, 67]. STAT5A has previously been shown to be regulated in a PR- and JAK/STAT-dependent manner; transcription was decreased in the presence of the inhibitor (AG490) [68]. Because PR regulation of STAT5A is Ser81 phosphorylation-dependent (via PR’s CD domain; see Fig 6A), we hypothesized that other Ser81-regulated genes (i.e. Wnt1) were also similarly regulated by STAT. Cells expressing wt PR were pre-treated with 50µM AG490 (or
vehicle; DMSO) for 1hr, followed by 10nM R5020 or EtOH (6hr). Under these conditions, treatment with AG490 dramatically reduced transcription of STAT5A (previously shown in [68]), as expected (Fig 6B – middle). Interestingly, treatment with AG490 significantly diminished Wnt1 regulation by progestin (Fig 6B – top), suggesting that STAT5 activity is required for PR-dependent transcription of Wnt1. Other PR-target genes previously shown to be phospho-Ser81 PR-dependent [23], such as HSD11β2 and BIRC3, were also similarly regulated in a STAT-dependent fashion (data not shown) [68]. c-myc, a PR-target gene whose regulation is PR Ser81- and STAT-independent was not affected by treatment with the JAK/STAT pathway inhibitor (Fig 6B – bottom) [23, 68]. These data suggest that regulation of a subset of PR-target genes, those that are regulated by PR Ser81-phosphorylation, is dependent on STAT pathway activation. Of note, Ser81 phosphorylation was unaffected by the STAT inhibitor (data not shown). Moreover, these data provide insight into the previously unknown regulation of PR-dependent Wnt1 transcription, a gene known to be critically involved in mammary stem cell maintenance and proliferation (see Discussion).

To further understand how phospho-Ser81 PR regulates transcription of Wnt1 in a STAT-dependent fashion, we performed an in silico analysis of the Wnt1 promoter and enhancer regions to reveal any transcriptional regulatory regions. We identified four putative full-length PRE binding regions (Fig 7A; triangles), including a site located in the proximal promoter region (~1kb upstream from the transcriptional start site [TSS]; PRE1), as well as three PREs located downstream in transcriptional enhancer sites (+44, 65 and 66kb from the TSS; PREs 2-4, respectively). Interestingly, we also identified four putative STAT5 binding sites (interferon gamma activation sites [GAS]) in the same region (Fig 7A; rectangles). To determine if PR and STAT5 coordinate to regulate transcription of Wnt1, we performed chromatin immunoprecipitation (ChIP) assays to detect the presence of PR and/or STAT5 on sites within the Wnt1 promoter/enhancer region. ChIP analysis was performed on lysates from EtOH- or R5020-treated T47D cells stably expressing wt PR, or from PR-null cells (negative control), using PR and STAT5-specific antibodies. In the presence of ligand, we detected robust recruitment (~11-fold) of wt PR to PRE1 (located just upstream of the Wnt1 TSS). Moderate levels of PR recruitment (~4-fold with ligand) were detected at two other PREs located downstream of the Wnt1 TSS (PREs 2 and 4; Fig 7B). Statistically significant levels of ligand-induced recruitment were not seen at PRE3, although PR is present at this site in wt PR-expressing cells (ligand-independent; as compared to IgG controls). Notably, PR was also detected at the four putative GAS sites (GAS1-4) identified in the Wnt1 promoter/enhancer region.
region; levels of PR at the GAS sites were unchanged in response to ligand (Fig 7B). These data indicate that PR is recruited to regulatory regions in the Wnt1 promoter/enhancer in response to ligand, and that PR is present in a ligand-independent fashion at GAS sites within those same areas. Immuno-precipitation of lysates from T47D cells stably expressing wt PR, or PR-null cells (not shown), using a STAT5-specific antibody showed complex patterns of STAT5 binding. STAT5 was present (unchanged with ligand) at two of the four GAS sites (GAS 2 and 3), present (unchanged with ligand) at three of the four PREs (PREs1, 2 and 4), and not present at the other sites (GAS1 and 4, PRE3) (Fig 7B). Finally, we performed ChIP analysis on lysates from EtOH- or R5020-treated T47D cells stably expressing wt PR, or from PR-null cells (negative control; not shown), using ck2 and MKP3-specific antibodies, proteins previously shown to be critical for PR Ser81-phosphorylation, and therefore, regulation of these genes (see Fig 5 and [23]). In cells expressing wt PR, MKP3 and ck2 were both recruited to PRE1 in a ligand-dependent manner (Fig 7C). Cumulatively (summarized in Table 1), these data suggest that regulation of the Wnt1 promoter is a complex process involving PR, STAT5, MKP3 and ck2.

Because STAT pathway inhibition affected multiple Ser81-regulated genes (Wnt1, STAT5; Fig 6B), we hypothesized that STAT5 regulation of PR-target genes is a global phenomenon. To test this hypothesis, we analyzed a publically available PR ChIP-Chip data set for the presence of STAT5 binding sites within PR binding sites [69]. This data set was created using T47D breast cancer cells treated with vehicle or estrogen (to increase levels of PR; PR expression in this cell line is dependent on estrogen, unlike the T47D variant cell lines used in the experiments presented herein) followed by progesterone treatment (45min), and represents all PR binding sites under these conditions (PR cistrome). Interestingly, a CEAS (cis-regulatory element annotation system; [70]) analysis revealed a 1.8-fold enrichment of STAT5 binding sites within the PR binding sites (as compared to the whole genome; p=8.22x10^{-11}), a finding similar to what has been seen for other nuclear receptors and their respective pioneer factors [11-14, 71]. These data suggest that STAT5 may be a pioneer factor for wt PR-B, and possibly, phospho-PR-B (as Ser81 PR mutants don’t regulate STAT5 similarly to wt PR, see above).
Discussion

Our studies identify a novel protein interaction domain (CD domain) in PR that mediates an interaction between PR, MKP3 and ck2. Formation of this complex is required to obtain ligand-activated phosphorylation on PR Ser81, which subsequently regulates PR-target genes known to be critically involved in mammary stem cell maintenance and proliferation (Wnt1, STAT5A). We show that mutation of the CD domain in PR abrogates an interaction between PR and MKP3 (Figs 4A and C); failure to form this complex results in lack of Ser81 phosphorylation in response to ligand (Fig 5A). MKP3 appears to play a scaffolding role in this complex, as phosphatase activity is not required to achieve PR Ser81 (ck2-dependent) phosphorylation (Fig 5C). Cumulatively, these data present a model for Ser81 phosphorylation in which MKP3, binding to both ck2 [49] and PR (via the CD domain; see Fig 4), facilitating ck2 phosphorylation of PR Ser81. We show that PR Ser81 phosphorylation is required for progestin-mediated transcription of Wnt1 and STAT5A (Fig 6), and the complicated interplay at the Wnt1 promoter involved binding of PR, STAT5, MKP3 and ck2 (Fig 7). Taken together, these data indicate that phospho-Ser81 PR is required for regulation of two genes (Wnt1 and STAT5) that are seminal to mammary gland biology and proliferation, and experiments shown herein outline a novel mechanism for phospho-dependent PR-B isoform-specific gene regulation.

CD domains and nuclear receptors

Data presented here characterize the CD domain in PR as a functional domain that contributes to protein interactions, regulates progestin-induced S-phase entry, facilitates PR phosphorylation, and thereby, dictates a subset of PR transcription. A similar, less specifically defined, domain has been identified in another member of the nuclear receptor family, PPARδ [72]. Burgermeister et al showed that PPARδ and MEK1 interacted through this domain, a 17-amino acid segment located in the C-terminal AF2 portion of PPARδ. The interaction between PPARδ and MEK1 leads to PPARδ nuclear export downregulation of nuclear action. It is possible that the interaction between PR and MEK1 serves a similar function as a potential mechanism to regulate PR nuclear-cytoplasmic shuttling, although the CD domain of PR does not appear to mediate the interaction observed between PR and MEK1 (data not shown) [25]. Additionally, preliminary confocal experiments showed similar cellular localization profiles for wt and mCD PR (data not shown), suggesting that the CD domain in PR serves a different function.
from that identified for PPARδ. Interestingly, deletion of the PPARδ region that contains the putative CD domain in PPARδ did not completely abrogate the interaction between PPARδ and MEK1, suggesting that other areas of PPARδ are necessary for binding to MEK1 [72]. These data are similar to what we observed for the interaction between PR and MKP3: mutation of PR's CD domain did not completely disrupt MKP3 binding (see Fig 4). These data suggest that binding to members of the MAPK regulatory family (MEKs or MKPs) may be modulated through, in addition to the CD domain, more/additional as yet unidentified domains.

Data presented here identified an interaction between PR and MKP3 mediated through the CD domain. It is likely that additional proteins interact with PR through this domain; large-scale screening studies are underway to identify additional binding partners. Additionally, as CD domains (or similar) have been identified now in two members of the nuclear receptor superfamily, it would seem likely that other receptors contain similar interaction domains. A cursory *in silico* analysis of the most closely related (steroid hormone-activated) nuclear receptors (ER, glucocorticoid receptor, androgen receptor) revealed weak potential candidate regions, however, none with the conserved Erk2-sequence similarity of PR's CD domain. This indicates that PR's interaction with members of the MAPK family may bestow upon PR unique regulatory actions and inputs to the MAPK family and kinase signaling in general relative to other steroid receptors, thus making PR a unique target for modulation of the MAPK pathway.

**Regulation of ck2-dependent PR phosphorylation**

As noted previously, ck2 is a ubiquitously expressed, constitutively active kinase that has over 300 substrates [20]. Unlike traditional kinases that require upstream inputs for full activation, ck2 is always active. Very little is understood about its regulation, however, it's thought that ck2 may be modulated through subcellular localization, substrate distribution/complex formation, ck2 enzyme formation, small molecule interactions and/or autophosphorylation [73]. Previous work from our lab detailed ck2-dependent phosphorylation on PR Ser81 and showed that this phosphorylation was sensitive to cell cycle phase (substrate distribution/complex formation) [23]. Data presented here indicates that MKP3 binding to PR is an additional mechanism through which ck2 phosphorylation of PR Ser81 may be regulated (Figs 4 and 5). PR must be bound to MKP3 to “accept” Ser81 phosphorylation by ck2, either because of proximity restrictions (MKP3 needed to bring ck2 into close proximity with its substrate, PR Ser81) or perhaps substrate conformation changes (MKP3 binding to PR causes conformational changes
in PR making it permissive to Ser81 phosphorylation). How the PR-MKP3 binding relationship is regulated is not understood, and is the topic of further study.

PR Ser81 phosphorylation is breast cancer

ck2, the kinase responsible for phosphorylation of PR Ser81, is upregulated in many human cancers, including breast cancer [20, 24]. Because regulation of ck2 activity is not well understood, understanding how PR Ser81 phosphorylation is regulated (see above) becomes critical to determining how phosphorylation by ck2 affects PR function in breast cancer. Preliminary data obtained from our lab using a small subset of clinically derived PR-positive breast tumors (data set described in [18]; 7 total PR-positive samples) showed that half had detectable levels of Ser81 phosphorylation (data not shown; A. Daniel). These data indicate that Ser81 phosphorylation is clinically relevant, and underscores the importance of further study of PR phosphorylation in human breast tumors.

PR modulation of MAPK signaling

The interaction between PR and MKP3 may not only affect regulation of PR, but modulation of the MAPK pathway as well. Preliminary data not presented here indicates that cells expressing wt PR-B had higher levels of phospho-Erk in response to EGF treatment as compared to cells lacking PR-B expression. These data, while preliminary, suggest that wt PR's capacity to bind to MKP3 may alter levels, and thereby activity, of phospho-Erk, perhaps through a simple mechanism of sequestration: MKP3 when bound to wt PR (through the CD domain) is no longer available to dephosphorylate and deactivate Erk. MAPK activation is complex and intricately regulated at various levels, therefore much work remains to test this potential mechanism of PR/MAPK regulation. However, complex formation between PR and MKP3, coupled with differences in phospho-Erk levels in mutant PR cell lines, makes this an intriguing avenue for investigation.

MKP3 as a scaffold protein

MKP3 is a potent phosphatase responsible for regulating levels of Erk1/2 phosphorylation, and therefore, activity. Interestingly, our data suggest (Fig 5) that MKP3 phosphatase activity is not required for PR Ser81 phosphorylation, rather, that MKP3 is acting as a scaffold protein to bridge the previously reported interaction between PR and ck2 [49], thereby bringing ck2 in close proximity with its target for phosphorylation: PR Ser81 [23]. Functioning as a scaffold represents a unique role for MKP3. Typically, MKP3 and other MAPK signaling molecules are
scaffolded by proteins such as KSR (Kinase suppressor of Ras) or Ste5 (in yeast) [74]. These data imply that classically defined roles of kinases and phosphatases may actually have broader scopes of action, such as behaving as scaffold proteins for pathways previously thought to be unrelated (i.e. PR regulation). Although MKP3 phosphatase activity is not necessary for scaffolding PR Ser81 phosphorylation, it may be critical for other PR/MKP3 mediated signaling, such as regulating the MAPK pathway itself (discussed above).

**PR Isoform specificity (PR-B vs. PR-A) determined by Ser81 phosphorylation**

PR-B is necessary for normal mammary gland development, while PR-A is required for uterine development [75-78]. Although PR-A and PR-B share structural and sequence similarity, they are functionally distinct transcriptional regulators with almost entirely non-overlapping transcriptional profiles, exhibiting recruitment to different subsets of PR-target gene promoters [79]. The structural difference between the two PR isoforms is the presence of the BUS in PR-B, which contains the AF3 (likely to confer a large amount of isoform-specific actions), CD domain and Ser81. Phosphorylation of Ser81 is likely a significant determinant of isoform-specificity between PR-A and PR-B.

Very little is understood regarding regulation of PR isoform-specific transcriptional activity. Progesterone actions vary depending upon the target tissue. Progestins are largely proliferative in the breast (PR-B), but conversely, anti-proliferative to estrogen-induced growth in the reproductive track (PR-A) [77]. PR-A and PR-B are usually co-expressed in the same tissues; cells that express only a single PR isoform are rare [80-82]. A 1:1 ratio of PR-A to PR-B seen in normal tissues is often altered in malignant tissues, suggesting that balanced isoform action is crucial to normal (adult) mammary gland biology [80, 83]. Understanding the critical differences between PR-A and PR-B-dependent gene regulation (i.e. as linked to phosphorylation on Ser81 by ck2) may allow for highly selective isoform-specific therapies that specifically inhibit PR-B (responsible for pro-proliferative signals in the breast), while preserving the anti-proliferative (protective) action of PR-A in reproductive tissues. Restoration of the balance between PR isoform actions may provide an innovative and complementary approach to existing endocrine therapies.

**Phospho-PR regulation of key PR-target genes**
STAT5A and Wnt1 are key target genes recently implicated in PR control of mammary stem cell maintenance. Like PR-B, STAT5A is required for mammary gland development; STAT5A and PR-B knockout mice have similar defects in this regard [64]. Progesterone is a known activator of STAT5A expression [29, 57], however the mechanism is not well understood. Similarly, Wnts are important mediators of progesterone action in the normal [58] and pregnant mammary gland [59]. Previous work published from our lab showed that phospho-PR-B-dependent upregulation of Wnt1 is required for breast cancer cell soft-agar growth in response to progestins [60]. Wnts have most recently been shown to be critical paracrine mediators of progesterone-induced expansion of mammary stem cells [61]. Importantly, deregulation of the Wnt/β-catenin signaling pathway has been found in many human cancers, including breast cancer [65]. Interestingly, unlike most other cancers, direct mutations of positive and negative regulators of the Wnt/β-catenin signaling pathway are rarely seen in breast cancer, despite the clear upregulation of downstream pathway endpoints (β-catenin stabilization and nuclear accumulation) [84, 85]. Progesterone/PR-B is a direct activator of this pathway [58, 61]. Potential involvement of these key mediators (Wnts, STAT5A) of mammary gland biology in progestin-induced early breast cancer development and/or tumor progression, underscores the need to understand how PR-B regulates these genes. The data presented here suggest that Ser81 phosphorylation, modulated by MKP3 binding through the CD domain, is critical to the ability of PR-B to regulate transcription of Wnt1. Moreover, because Ser81 phosphorylation is a major determinant of PR isoform-specificity, understanding how the two isoforms (only PR-B regulates Wnt1) are regulated is of critical importance to the development of isoform-specific therapies (discussed above). Finally, because mCD PR is deficient in ligand-induced S-phase entry (perhaps due to lack of Wnt1 or STAT5 expression, although many other genes likely contribute to this phenotype; see Fig 6C), this suggests that the CD domain is critical to the ability of wt PR-B to regulate breast cell growth and proliferation.

STAT5 as a PR pioneer factor

Data presented here indicates that STAT5 may be a putative pioneer factor for PR-binding. Experiments are underway to further test this hypothesis and determine is STAT5 is a pioneer factor specifically for phospho-PR-B, thereby making it a key determinant in isoform-specific transcription. Interestingly, PR-B appears to regulate its own putative pioneer factor; STAT5A is a phospho-Ser81 PR-B-specific target gene. Therefore, understanding how PR regulates STAT5A (via MKP3 and ck2), and in the future determining if STAT5A functions as a transcriptional “master regulator” for phospho-PR-B gene regulation, would significantly
enhance our understanding of PR isoform-specific actions, and subsequently, help us understand how deregulation of PR-B regulated pathways (i.e. Wnt, STAT5) contributes to breast cancer development and/or progression.


Figure 1

A. Consensus D X X D/E
   PR     D P S D
   ERK2   D P S D
   p38\(\alpha\) D P D D
   JNK1   D P S E

Consensus D X X D/E E X/E
   PR     D P S D E K
   ERK2   D P S D E P
   p38\(\alpha\) D P D D E P
   JNK1   D P S E A E

B. BUS
   DBD H
   HBD
   CD domain

68-DPSDEKTDQD-76
Figure 2

A. 

![](figure.png)

B. 

![](figure.png)

C. 

![](figure.png)

(graininess is from damaged film)
Figure 3

A. wt PR and mCD PR

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B. Vector, wt PR, and mCD PR

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<td>+</td>
<td>-</td>
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C. T47D-YB and T47D-mCD PR

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</tr>
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Ratio of S81 phospho-PR to total PR

Graph showing the ratio of S81 phospho-PR to total PR for wt PR and mCD PR over time.
Figure 4

A.

<table>
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<tr>
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<td>+</td>
<td>+</td>
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</tr>
</tbody>
</table>

B.

- CD: wt PR-B
- mCD: mCD PR-B
- wt PR-A
- CD: CD-PR-A
- mCD: mCD-PR-A

C.

<table>
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IP:PR
Figure 5

A.  

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T47D-YB

B.  

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<tr>
<td>Erk1/2</td>
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</table>

T47D-YB

C.  

EGF  

↑  

MEK1  

ERK1/2  

P  

→  

p-ERK1/2  

↓  

ROS (H₂O₂)  

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T47D-YB
Fig 7A

Schematic of Wnt1 promoter

Distance from TSS (kb) -10 -1 +1.2 +4.0 +27 +44 +65-66

PRE
STAT5 (GAS)
No Wnt1 regulatory sites in this category
Fig 7C

Fold recruitment to PRE1

Fold recruitment (R5020/EtOH)

0 1 2 3 4 5 6 7 8 9

MKP3 ck2

Protein IP'd from lysate
Mechanisms of Context Dependent Progesterone Receptor Action in Breast Cancer Models

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Summary

In the clinical setting, progesterone receptors (PR) are primarily used as biomarkers of luminal-A type or endocrine-sensitive breast cancers. PR expression in estrogen receptor (ER) positive breast cancers is associated with good prognosis, as it is indicative of the presence of functional ER; ER+/PR+ breast cancers are more likely to respond to anti-estrogen therapies relative to those (luminal-B) that have become PR-null [1]. However, historical dogma that PR serve largely as useful biomarkers of ER action is steadily being replaced with the concept that PR isoforms indeed contribute to breast cancer progression as key mediators of mammary epithelial cell proliferation and pro-survival as well as regulators of the mammary stem cell niche. As a result, considerable effort has been directed towards a more complete understanding PR biochemistry and mechanisms of signaling (i.e. promoter selection) as they relate to mammary gland development and during breast cancer initiation and early progression. PR/progestin action in breast tumors appears to be quite distinct from that in the normal adult breast [2; 3]. However, given the appropriate proliferative bursts directed by PR-B during mammary gland development, menstruation, and in pregnancy, it is likely that PR retains the potential to inappropriately drive dangerous growth programs in a malignant environment [4]. As such, anti-progestins represent a timely opportunity to improve modern endocrine therapies. Emerging evidence suggests that steroid hormone receptors are highly context-dependent transcription factors; these molecules are heavily post-translationally modified and influenced by a variety of cofactors. Given the heterogeneity of human breast cancer, perhaps the next challenge for the PR/progestin field is to identify which tumors are most likely driven by active PR, and thus well-suited for endocrine therapies that may include a highly selective anti-progestin. Herein, we discuss the context-dependent actions of PR, and provide biochemical
hints as to what molecular environments may drive PR signaling toward the emergence of malignant phenotypes.

**Introduction: PR structure and function**

Progesterone, an ovarian steroid hormone, is produced during the luteal phase of the menstrual cycle and throughout pregnancy. Progesterone binds progesterone receptors (PR) to elicit a biological response. PR are expressed in multiple human tissues including the uterus, mammary gland, brain, pancreas, thymus, bone, ovary, testes, and in the urinary tract [5]. Two primary PR protein isoforms exist (PR-A and PR-B) that are derived from a single gene with unique promoters [6]. PR-B is the full-length receptor that contains an additional 164 amino acids at the N-terminus that is absent in PR-A; otherwise these protein isoforms have the same peptide sequence [7]. Both receptors contain a DBD (DNA binding domain), a LBD (ligand binding domain) separated by a hinge region (also known as the CTE, carboxy terminal extension), and two AF (activation function) domains (Fig. 1). PR-B contains a third AF domain located in its N-terminus that is not present in PR-A [8]. The AF domains function to interact with coregulatory molecules to enhance PR transcriptional activation of genes. Both PR isoforms also contain an N-terminal inhibitory function (IF) domain that acts to recruit corepressor molecules; IF activity is only revealed in the context of PR-A [9]. Following ligand binding, PR undergo a conformational change that initiates unique actions. Isoforms dimerize (A:A, B:B, or A:B) and are retained in the nucleus. Nuclear PR activates transcription of target genes, either directly through binding to progesterone response elements (PREs), or indirectly through tethering interactions with other transcription factors (AP1, SP1, STATs) [10; 11; 12]. To initiate transcription (ligand-dependent or –independent) PR recruits co-regulator proteins and basal transcriptional machinery to enhancer and promoter regions of genes [13]. These responsive elements are located in chromatin at proximal and distal sites. Recent cistrome (whole genome binding site) analysis demonstrated that these responsive elements may be located up to 50Kb.
(kilobases) upstream or downstream of the TSS (transcriptional start site) [14]. PR genomic (transcriptional) activities are integrated with nongenomic actions where PR is a node within cytoplasmic kinase signaling cascades (for reviews, see [15; 16; 17; 18]); as reported for estrogen receptor (ER), PR is able to associate with signaling molecules to activate kinase pathways in response to progesterone and to enhance responses to growth factors [19; 20; 21].

In the human breast, PR action is critical for maintaining the population of mammary stem cells (via paracrine signaling) [22; 23] and for coordinating the dynamic (proliferative) regulation of glandular structures during menstrual cycling and pregnancy (via both paracrine and autocrine signaling [24]) [20]. Deregulation of the normal proliferative actions of PR in the breast may contribute to the increased risk of breast cancer revealed in clinical trials using progestin containing hormone replacement therapy [25; 26]. In these large trials, estrogen alone, when given to postmenopausal women protected against breast cancer, while estrogen plus progestin (E+P) was associated with a higher breast cancer risk and the appearance of larger more aggressive tumors [27]. These effects were reversed upon cessation of E+P hormone use [28], suggesting a tumor-promoting effect of progestin and that E+P may have stimulated increased growth and progression of occult pre-existing breast lesions in these relatively short-term (~4 year) trials. However, lifetime exposure to elevated levels of steroid hormones (including estrogens, progestins, and androgens) also increases the relative risk of breast cancer incidence in post-menopausal women [29; 30]. Similarly, in mouse studies, inhibiting PR actions with RU486 (PR antagonist/partial agonist) during a 14-day window of time dramatically precluded virgin BRCA1/p53 mutant mice from developing mammary tumors [31]. In other mouse studies, treatment with the PR agonist MPA (Medroxyprogesterone Acetate) showed definitively that progestins/PR actions contribute to increased mammary tumor formation [32]. Furthermore, PR knockout mice were resistant to mammary tumor incidence after challenges with chemical carcinogens [33]. These and other studies (reviewed in [20; 34; 35]) have
provoked great interest in PR function and its role in breast cancer. Herein, we review basic studies on PR biochemistry as a means to provide further insight to its context-dependent actions and emerging relevance to mechanisms of (luminal) breast cancer initiation and progression.

Model systems: context for studying PR biochemistry

Studies of PR biochemistry have primarily used a limited number of ER+/PR+ (luminal) human breast cancer cell lines (MCF-7, T47D, and ZR75). Notably, PR is a classic ER target gene. Thus, PR mRNA expression in mammary epithelial cells is primarily driven by estrogen-bound ER, making the study of PR action (in isolation) difficult without the confounding (mitogenic) effects of added estrogen. The Horwitz lab derived a breast cancer cell model system for studying PR/progestins by first identifying a variant of T47D cells in which PR is constitutively expressed and independent of estrogen stimulation, termed T47Dco cells [36]. A naturally occurring PR-null variant of these cells, T47D-Y, provided a useful tool for making stable cell lines containing PR-A only, PR-B only, or various mutant version of PR isoforms [37]. An advantage of this model system is that PR expression levels in stable clones can be selected to closely resemble the levels found in unmodified parental cells (T47Dco). Indeed, PR expression in stable cell lines at levels similar to endogenous expression has been shown to be critical for appropriate PR transcriptional activation, particularly with regard to the actions of PR phospho-mutants. Namely, S294A (Ser294 to Ala) mutant PR-B is heavily sumoylated and transcriptionally repressed relative to wt PR-B when either receptor is expressed stably in T47D-Y cells and at levels similar to that seen in T47Dco cells. However, when overexpressed in transient systems such as COS or HeLa cells this mutant can activate transcription in PRE-luciferase reporter assays much like wt PR-B [38]. Notably, endogenous factors that may influence PR transcriptional activity are limiting (kinases, co-factors, SUMO molecules and/or PIAS proteins that act as SUMO conjugating enzymes, etc.) [39]. Others have reported major
functional differences between stably and transiently expressed PRs. For example, transiently expressed PR is unable to induce chromatin remodeling changes necessary to activate an integrated MMTV promoter, while stably expressed PR is fully capable of transactivation [40; 41; 42]. These data highlight the need for model systems that provide appropriate levels of myriad regulatory molecules that interact with and alter steroid receptors. Additionally, they caution against over-interpretation of exogenous expression models only, particularly in the case of receptor regulation by phosphorylation events and other dynamic post-translational modifications, such as sumoylation [43; 44].

Recently, PR mRNA expression in a selected sub-population of stem-like progenitor cells in the human breast was shown to be fully independent of ER [45], suggesting that PR expression in this specialized compartment is sensitive to factors other than estrogen (perhaps cAMP [46]). PR protein expression as measured by IHC (immunohistochemistry) was detectable in basal cells of the mammary gland, while PR and ER expression was only detected in adult luminal cells [45]. It will be important to further establish additional ex-vivo models to further understand mechanisms of ER-independent PR expression.

**PR signaling is context dependent**

PR participation in signaling complexes serves as a “fine-tuning” mechanism to precisely adjust hormone responses according to specific cellular conditions. Notably, PR-B, but not PR-A, functions outside of the nucleus to rapidly activate protein kinases (MAPK, Akt, c-Src) in part by a ligand-induced interaction between PR and c-Src kinase [47; 48; 49; 50]. Rapid progesterone-induced c-Src/MAPK activation serves to phosphorylate PR Ser345, and thereby potentiates nuclear PR tethering to SP1 transcription factors to regulate genes required for cell cycle progression and anchorage-independent growth [51; 52]. This “feed-forward” loop underscores the profound effect that activated kinases have on the nuclear functions of PR; particularly with regard to promoter selectivity (i.e. phosho-PRs target or select specific gene subsets, perhaps
in cooperation with co-factors that preferentially recognize receptor phospho-species). Progestin bound PR-B induces sustained activation of MAPK signaling (18-24 h) mediated by EGFR transactivation through matrix metalloproteinase mediated release of EGFR ligands, resulting in Wnt1 upregulation (genomic PR action) and proliferation [51]. PR rapid activation of MAPK signaling (Msk1) is also mediated via an interaction with ER/c-Src complexes in response to progestins [47; 53; 54]. These PR/ER/Msk1 complexes are recruited to the MMTV promoter in response to progestin [55]. In recent studies, progestin induced PR/ER complexes were shown to bind the c-myc and cyclin D1 endogenous gene promoters and enhance their transcriptional activity [56].

Additionally, progestin/PR activation of c-Src induces Jak1/2 phosphorylation leading to downstream STAT3 transcriptional activation [57]. PR interacts with FGFR2 and STAT5 to induce transcriptional changes in the cell [58], and also participates in signaling complexes with a variety of signaling molecules including cyclins, caveolin-1, and ErbB receptors [19; 21; 59; 60; 61; 62; 63; 64; 65]. These signaling events are influenced by cellular context (i.e. abundance/location of signaling molecules, cell cycle phase, growth factor availability) in addition to the availability of progesterone and incur specific biological outcomes for the cell.

**Post-translational modifications: mediators of context dependent PR action.**

Post-translational modifications (PTMs) are a class of covalently attached moieties that induce conformational changes in substrate proteins that can dramatically impact enzyme activity, location, and/or protein-protein interactions: PTMs have been defined in basic processes including DNA repair, replication, transcription, chromosome segregation, genomic stability, and intracellular trafficking (for reviews, see [66; 67; 68; 69]). PR isoforms are post-translationally modified by phosphorylation, ubiquitination, sumoylation, and acetylation [34; 70; 71; 72]. These
regulated modifications are highly dynamic, depend on cellular context, and refine PR transcriptional activity by altering PR subcellular localization, protein stability/turnover, and interactions with other proteins and with DNA [73].

Acetylation and nuclear localization

The hinge region of PR contains a conserved motif of lysine residues (RKXKK) that are rapidly acetylated in response to ligand binding [72]. These residues also make up part of the bipartite nuclear localization signal (NLS) that is required for efficient PR localization in the nucleus and thus required for PR transcriptional activity [74; 75; 76]. Mutations within the NLS that mimic acetylation (Q and T) also disrupt the ability of PR to accumulate in the nucleus, indicating that the regulation of PR acetylation and nuclear localization are intimately linked [77]. Rapid ligand induced PR nuclear accumulation is critical for certain PR phosphorylation events and for regulating PR early response genes (1 hr) such as c-myc. Additionally, acetylation of PR reduced progestin responsiveness on select latent (18 hr) target promoters [77]. Notably, other disruptions within this conserved sequence alter PR DNA binding and cofactor recruitment [78; 79; 80], indicating the PR hinge region is a critical regulator of PR genomic functions.

Upon ligand binding and nuclear accumulation, PR molecules aggregate with DNA and form transcription factor complexes in discrete foci to activate transcription [81; 82]. These complexes are tethered to the nuclear matrix and contain nascent RNA, activated RNAPolII, p300, and specific chromatin associated with transcriptional activation [3; 81]. Treatment of cells with the cdk2 inhibitor Roscovitine prevents formation of these foci, indicating a kinase signaling dependence (in addition to the presence of progestins) of PR transcriptional activity occurring in these complexes [5].

Constitutive Kinases
PR contains at least 14 serine residues that are phosphorylated by multiple kinases (e.g. MAPK, CK2, and CDK2) either basally or in response to ligand binding (Fig. 1) [83]. In conditions where kinases are upregulated, such as breast cancer, amplified signaling mediates PR-B hypersensitivity to hormone and ligand-independence, thus leading to inappropriate activation of PR-B dependent transcription and expression of growth and pro-survival genes [38; 64; 84]. Importantly, PR hypersensitivity to ligand may be increasingly relevant to cancer as evidence suggests local production of progestins may occur in the tumor microenvironment [85; 86].

Mechanisms of kinase induced PR hyperactivation are discussed below.

**CK2**: PR Ser81 is a known ck2 site in the PR-B N-terminus (not in PR-A) [87; 88]. ck2 is a ubiquitously expressed, constitutively active kinase that is overexpressed in every cancer examined thus far, including breast cancer. Our recent work (using Ser81-specific phospho-antibodies) demonstrated that PR-B was basally phosphorylated at Ser81 in breast cancer cells. Progestin exposure, in addition to synchronization of cells at the G1/S phase border, induced robust phosphorylation at this site; both effects were ck2-dependent [88]. Cells expressing a PR mutant (S79/81A PR) that cannot be phosphorylated at Ser81 had decreased ligand-independent cell survival in soft agar assays. Phospho-mutant-PR-B also exhibited defects in recruitment to select PR-B-target genes (i.e. *BIRC3*) important for proliferation and survival, both in the presence and absence of ligand. ChIP assays revealed that in contrast to wt PR-B, a phospho-Ser81 mutant receptor (S79/81A PR-B) was also impaired in its ability to recruit ck2 to PR-associated enhancer sites of the *BIRC3* gene. ck2 is not thought to be oncogenic on its own, but appears to increase the oncogenic potential of cancer-promoting substrates and pro-growth signals (such as progestin/PR in the breast) [89; 90]. In the context of breast cancer, where progestins have been implicated as a risk factor for tumor development and early progression [25; 26; 91], overexpressed ck2 could further enhance the oncogenic potential of PR through inappropriate phosphorylation (on Ser81), thereby directing phospho-Ser81 PR-B to
growth-promoting genes. These data suggest that the interaction of PR-B with kinases, and ultimately Ser81 phosphorylation, may be a key determinant in dictating PR-A vs. PR-B target-gene specificity.

**Cdk2:** There is a complex interplay between PR activity and cell cycle regulation. In response to progestin or mitogen treatment, CDK2 signaling is activated and the G1/S-phase transition is initiated. CDK2 has been shown to phosphorylate PR at multiple sites that regulate PR transcriptional activity [64; 92; 93]. PR is basally phosphorylated on Ser400 in resting cells and highly phosphorylated by CDK2 in response to progestins [92]. In addition, treatment with mitogenic growth factors known to activate CDK2 activity, or expression of constitutively active CDK2 *in vitro*, induces ligand-independent PR transcriptional activity. In T47D breast cancer cells, high expression of the cell cycle inhibitor p27 also blocked CDK2-induced PR Ser400 phosphorylation and ligand-independent PR transcriptional action [64]. These data suggest phosphorylation at PR Ser400 by activated CDK2 regulates the transcriptional activity of PR during the cell cycle and this may be deregulated in tumor cells, especially when cell cycle check point control is compromised by high CDK2, overexpression of cyclins D, A, or E, or loss of cell cycle inhibitor proteins (p15, p16, p21, p27).

PR also interacts with cyclin E/ckd2 [64; 65] and cyclinA/ckd2 complexes; PR bound to cyclinA/ckd2 increases SRC-1 phosphorylation and PR binding to the MMTV promoter [62; 63]. PR displays increased transcriptional activation during S phase, an effect dependent on cdk2 activity [62]. Cancer cells often downregulate cell cycle inhibitors (p27 and p21) thereby increasing cdk activity and traverse of the cell cycle, thus inducing rapid proliferation [94; 95]. A cancer cell environment with low cell cycle inhibitor activity predicts PR ligand-independent activity and hypersensitivity to low concentrations of progestins. In these circumstances, low levels of locally produced progesterone [85; 86] may be sufficient to drive tumor proliferation leading to increased tumor progression.
MAPK: PR phosphorylation at PR Ser294 has been intensely studied. In the presence of progestins or growth factors (EGF or HRG) [21; 38], PR-B but not PR-A is rapidly phosphorylated at Ser294, accumulates in the nucleus, and becomes highly transcriptionally active at multiple genes important for cell cycle progression, proliferation, and survival [39; 93; 96; 97]. Ser294 phosphorylation augments PR-B degradation via the ubiquitin-proteasome pathway [71]. Thus, when PR-B is phosphorylated and highly sensitive to low concentrations of ligand it undergoes rapid turnover and often becomes more difficult to detect at steady state (by western blotting protein) [71; 93; 96]. In the absence of Ser294 phosphorylation, PR is transcriptionally repressed and stabilized [39].

PR phosphorylation on Ser294 in response to progestins or growth factors antagonizes sumoylation on Lys388 [39]. Sumoylation of PR at this site results in a more stable and transcriptionally repressed PR relative to phospho-Ser294 PR that is desumoylated. The SUMO-deficient PR mutant (K388R) is hypersensitive to low (sub-activating for wt PR) concentrations of progestin [39]. A subset of PR target gene promoters is sensitive to PR phosphorylation status (*HBEGF*), while others are insensitive (*Muc1*) [39]. PR phosphorylation in the absence of ligand occurs in response to activated kinase pathways (MAPK or Cdk2) and increases (desumoylated) PR activity on select ligand-independent gene targets (*SRC1, IRS1*) [93]. This dynamic regulation of PR Ser294 phosphorylation coupled to Lys388 desumoylation alters PR promoter selectivity, allowing the cell to direct distinct genetic programs according to changes in the availability of extracellular signals (progesterone and growth factors) (Fig. 2) [39; 93].

Recently, an in depth analysis of the target gene profiles of wt PR versus a SUMO-deficient mutant (phospho-Ser294 mimic) PR was performed in breast cancer cells [4]. Sumo-sensitive PR target genes largely included molecules that function in pathways responsible for cellular proliferation and survival (Fig. 3). Knutson *et al* demonstrated that desumoylated or phospho-
Ser294 PR is recruited to select genes (including MSX2) along with coactivator and chromatin remodeling components to induce a permissive chromatin environment and thereby derepress selected target genes (Fig. 4). Namely, SUMO-deficient PR was associated with more CBP than PR that could be sumoylated and was able to recruit the methyltransferase MLL2 to promoter regions. PR target gene promoters that were sensitive to the sumoylation/phosphorylation status of PR also displayed increased H3K4 dimethylation and nucleosome remodeling in regions containing PR (desumoylated) bound PREs [4]; this methylation mark is associated with the relocation of nucleosomes in transcriptionally active regions of chromatin [98].

Notably, the gene signature specific to desumoylated (Ser294 phosphorylated) PR is associated with HER2 activation in human breast cancers [4]. In cell models with HER2 gene amplification, treatment with MEK inhibitors blocked PR SUMO-sensitive gene regulation and decreased cell proliferation in response to progestins [4]. In a cohort of ER+ breast tumors, patients whose tumors expressed the SUMO-deficient PR gene signature had reduced distant metastasis free survival [4]. These data indicate that PR phosphorylation and sumoylation are able to profoundly alter PR activity and target gene selection in breast cancer cells. Importantly, activated PR gene signatures may be used to identify patients whose tumors are driven by hyperactive PR species and thus likely to benefit from therapies that target PR and upstream kinase pathways in addition to ER.

**Tissue specific PR actions (breast vs. reproductive tract)**

Progestosterone actions vary depending upon the target tissue. Progestins, primarily acting through PR-B, are largely proliferative in the breast. However in the reproductive track, progestins may act primarily through PR-A as potent antagonists of estrogen-induced hyperplasia [99] and inducers of differentiation [100]. Recent cistrome analysis comparing PR
chromatin binding in T47D breast cancer cells and primary uterine leiomyoma cells in response to RU486 revealed little overlap [101]. PR-A and PR-B are most often co-expressed in the same tissues, and cells that express only a single PR isoform are rare [102; 103; 104]; the normal 1:1 ratio of PR-A to PR-B is often altered in malignant versus normal breast tissue [102; 105], suggesting that balanced isoform action is crucial to normal (adult) mammary gland biology. Given the equimolar ratios of human PR isoforms in most cells, little is known about the tissue-specific predominance of either isoform and thus the mechanism of tissue selective hormone response. Evidence suggests that PR-A can alter (transrepress) PR-B transcriptional responses to hormone; this may be relevant to altered isoform ratios found in breast cancer and/or to tissue-specific receptor dominance as part of normal physiology (see discussion below) [5]. In rat models, PR-A and PR-B are expressed in luminal epithelial cells of the mammary gland and PR-B alone is present in myoepithelial cells, suggesting a unique role for PR-B in regulating myoepithelial processes [106].

PR-A and PR-B have distinct transcriptional activities [107; 108] and mediate unique developmental processes as determined by PR knockout studies in mice. Mice with both PR-A and PR-B isoforms knocked out develop into adulthood but have drastically impaired female reproductive processes, including: anovulation, uterine hyperplasia, and complete loss of mammary gland ductal and alveolar expansion during pregnancy [109]. Selective ablation of PR-A in mice demonstrated that PR-B is the isoform responsible for mammary gland ductal side-branching and lobuloalveolar expansion during pregnancy and regulates a different set of reproductive functions from PR-A [99; 110]. Alternatively, PR-A primarily mediates ovarian and uterine development in response to progestins [99]. Additional work from the PR knockout mouse models showed definitively that PR contributes to mammary gland tumorigenesis (independent of ER action) [109].
While PR-A and PR-B share structural and sequence similarity, they are functionally distinct transcriptional regulators with almost entirely non-overlapping transcriptional profiles, exhibiting recruitment to different subsets of PR-target gene promoters [107]. Little is understood regarding regulation of PR isoform-specific transcription. The receptors do exhibit unique post-translational modification profiles. PR-B is robustly phosphorylated on Ser294 in response to ligand (and growth factors) while PR-A phosphorylation at this residue is undetectable in intact cells/cell lysates [111]. In turn, PR-B is distinctly less sumoylated relative to PR-A [39]. PR-A also lacks the N-terminal Ser81 phosphorylation site found in PR-B and is therefore blind to CK2 kinase inputs that dramatically alter PR-B gene selectivity [88]. Mutation of Ser81 to Ala in PR-B converts its transcriptional response on selected PR-target genes to that of PR-A. Notably, structure-function studies propose that the presence of the BUS (B-upstream segment, including AF3) allows PR-B to adopt distinct conformations which support coactivator binding while PR-A displays an increased affinity for corepressors [5]. Additionally, PR-B, and not PR-A, displays the ability to rapidly signal to c-Src/MAPK [50]. It is thus hypothesized that differential abundance/localization of coregulator molecules and varying kinase pathway utilization may account for some of the tissue specificity and context-dependent action of progesterone/PR.

**PR action in normal vs. tumor settings**

Finally, normal versus neoplastic contexts appear to profoundly influence PR action in breast cancer cells, perhaps due, in part, to the biochemical mechanisms described above. Recent comparisons of malignant versus normal primary cell lines of the breast revealed largely distinct gene signatures in response to progestins [3]. Likewise, cistrome analysis of transformed cell lines expressing exogenous PR versus PR+ neoplastic cell lines confirmed these results. The relatively non-overlapping PR chromatin binding profiles were due, in part, to the differential expression of known steroid receptor pioneer factor FOXA1 [14] in the cell lines; the PR cistrome was significantly altered upon expression of FOXA1 in the ‘normal’ cell line, but this did
not predictably approach that found in cancer cells [2]. Levels of kinase activities increase dramatically in neoplastic settings [112; 113]. It is therefore likely that the alterations in PR post-translational modifications and participation in signaling pathways described above cooperate with altered co-factor availability to account for much of the changes seen in PR target genes within the malignant setting.

In the normal breast PR-A and -B are expressed in equimolar ratios, whereas in breast cancers these ratios can be disrupted; altered PR isoform ratios have been shown to be associated with tumor aggressiveness. In early breast lesions PR-A/B ratios are distorted, PR-A expression predominates in DCIS (ductal carcinoma in situ) and invasive lesions [102]. In breast cancer a high PR-A/B ratio is associated with an undifferentiated phenotype and a poorer prognosis [114], analysis of these tumors shows this altered ratio is likely due to decreased expression of PR-B rather than increased expression of PR-A [105]. In the face of phosphorylation events, steady state levels of activated phospho-PR-B are predicted to be lowered by rapid proteasome-dependent protein turnover [96]. This context (i.e. cancers with high kinases activities) predicts a higher PR-A/B ratio that favors activation of growth promoting gene programs [4].

Conclusion

As highly post-translationally modified transcription factors, PR molecules act as context-dependent “sensors” for the integration of ever-fluctuating mixtures of steroid hormones and growth factors. Under conditions where growth factors are abundant, phosphorylated PR-B becomes exquisitely sensitive to low concentrations of progesterone; in the normal mammary gland this serves to initiate developmentally appropriate proliferative programs in order to achieve rapid expansion of ductal structures during pregnancy. In the context of malignant transformation, the upregulation of kinase signaling coupled to downregulation of cell cycle
inhibitory molecules predicts PR hyperactivation of similar transcriptional programs. Most notably, phospho-PR target genes include the components of protein kinase pathways such as the growth factors themselves, their cell surface receptors (IGFR, EGFR/erbB2) and required downstream effectors. PR-driven remodeling of proliferative circuitry thus serves to further sensitize cancer cells to local hormones, creating a potent feed forward proliferative response. At peak PR (transcriptional) activity, rapid ligand-dependent down-regulation of phospho-PR predicts low steady state protein levels [71; 96]. Thus, the presence of a PR gene signature [4] rather than PR protein levels, provides a reliable biomarker of PR-driven breast cancer biologies (i.e. proliferation and pro-survival); breast cancer patients with this signature are good candidates for alternative endocrine therapeutics that include an anti-progestin.


[96] T. Shen, K.B. Horwitz, C.A. Lange, Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated


Fig. 1. The progesterone receptor is post-translationally modified by phosphorylation and sumoylation.

Both progesterone receptor (PR) protein isoforms, PR-A and PR-B, are heavily phosphorylated by multiple kinases, including MAPK, CK2, and CDK2. Both receptors contain DNA binding domains (DBD), ligand binding domains (LBD), hinge regions (H), and two activation function (AF) domains. PR-B contains an additional 164 amino acids at its N-terminus and a third AF domain. PR phosphorylation at Ser294 antagonizes PR sumoylation at Lys388.

Fig. 2. Kinase dependent PR Ser294 phosphorylation antagonizes PR Lys388 sumoylation and mediates rapid protein turnover.

Progestins diffuse through the plasma membrane and bind PR causing rapid sumoylation on Lys388 on a subset of receptors resulting in transcriptional repression at many cancer relevant genes. Persistent MAPK (or CDK2) pathway activation (e.g. EGF treatment) results in efficient PR Ser294 phosphorylation, inhibition of PR SUMOylation, and transcriptional activation. Phosphorylated PR is highly ubiquitinated and rapidly degraded by the 26S proteasome; whereas, sumoylated PR is highly stable with a longer half-life.

Fig. 3. PR Ser294 phosphorylation dependent desumoylation drives transcriptional activity at cancer relevant genes.

PR undergoes rapid sumoylation upon progestin binding via an enzymatic cascade (left). However, in conditions of high MAPK pathway activation (e.g. EGF treatment or within breast tumors), PR is phosphorylated at Ser294 which antagonizes PR sumoylation. Phosphorylated PR Ser294 (desumoylated) regulate unique gene signatures that contribute to cell proliferation and survival.
Fig. 4. PR regulates *MSX2* gene expression through coactivator recruitment and chromatin modification.

Schematic showing the *MSX2* gene and PRE-containing enhancer region located 15,094 bp upstream from the transcriptional start site. High levels of ligand-dependent *MSX2* expression occur in cells expressing SUMO-deficient PR recruitment. In addition, these cells have higher levels of histone acetyltransferase (CBP) and histone methyltransferase (MLL2) recruitment to the *MSX2* enhancer region. This results in higher levels of histone H3 Lys4 dimethylation, a marker of transcriptionally important enhancer regions.
Molecular and Cellular Biology

ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B Isoform-Specific Target Gene Expression in Breast Cancer Cells

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ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B Isoform-Specific Target Gene Expression in Breast Cancer Cells

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Progestosterone receptors (PR) are critical mediators of mammary gland development and contribute to breast cancer progression. Progestin-induced rapid activation of cytoplasmic protein kinases leads to selective regulation of growth-promoting genes by phospho-PR species. Herein, we show that phosphorylation of PR Ser81 is ck2 dependent and progestin regulated in intact cells but also occurs in the absence of PR ligands when cells enter the G1/S phase of the cell cycle. T47D breast cancer cells stably expressing a PR-B mutant receptor that cannot be phosphorylated at Ser79/81 (S79/81A) formed fewer soft agar colonies. Regulation of selected genes by PR-B, but not PR-A, also required Ser79/81 phosphorylation for basal and/or progestin-regulated (BIRC3, HSD11B2, and HbEGF) expression. Additionally, wild-type (wt) PR-B, but not S79/81A mutant PR, was robustly recruited to a progesterone response element (PRE)-containing transcriptional enhancer region of BIRC3; abundant ck2 also associated with this region in cells expressing wt but not S79/81A PR. We conclude that phospho-Ser81 PR provides a platform for ck2 recruitment and regulation of selected PR-B target genes. Understanding how ligand-independent PRs function in the context of high levels of kinase activities characteristic of breast cancer is critical to understanding the basis of tumor-specific changes in gene expression and will speed the development of highly selective treatments.

The ovarian steroid hormone progesterone acts by binding to and activating progesterone receptor (PR) A, B, and C isoforms expressed in target tissues. In the normal breast, PR-A and PR-B are typically expressed in a minority population (7 to 10%) of luminal epithelial cells. PR-B is required for mammary gland development during puberty and pregnancy and acts by contributing to lobulo-alveolar proliferation and ductal side branching (8, 46). Studies from PR-knockout mice show that these mice have significant defects in mammary gland morphology (primarily PR-B dependent) and reproductive abnormalities (primarily PR-A driven) (46, 54). Additionally, the presence of PR was shown to be required for the formation of mammary tumors in a carcinogen-induced mouse model of breast cancer (47). Finally, recent clinical data have shown that women taking hormone replacement therapy (HRT) whose regimens included both estrogen and a progestin, but not estrogen alone, experienced increased breast tumor numbers and sizes (1, 5, 12). Interestingly, the effect of combined HRT on breast cancer risk was reversible (5, 13), suggestive of epigenetic events.

In the absence of progesterone, PR molecules rapidly shuttle between the cytoplasm and the nucleus; cytoplasmic PRs contain membrane-associated species capable of direct binding and signaling to mitogenic protein kinases (c-Src, MAPK, PI3K) (3, 7, 25, 50). Following ligand binding, PRs dissociate from heat shock protein-containing chaperone complexes, undergo dimerization, and are largely retained in the nucleus. Nuclear receptors activate transcription of PR target genes, either directly through binding to progesterone response elements (PREs) or indirectly through tethering interactions with other transcription factors (AP1, SP1, STATs) (14, 61, 70). Notably, PR is highly posttranslationally modified, primarily on serine (phosphorylation) and lysine (acetylation, ubiquitination, and sumoylation) residues located in the N-terminal region (16, 17, 43, 76). These modifications are frequently ligand dependent but can also occur independently of progestin binding and significantly alter receptor stability, localization, tethering interactions, transcriptional activity, and promoter selectivity (18, 75). For example, MAPK and ck2 have previously been shown to phosphorylate and modulate the activity of both liganded and unliganded PR (43, 62, 79).

The serine-threonine protein kinase ck2 (formerly casein kinase II) is ubiquitously expressed with over 300 substrates, many of which are involved in proliferation, cell survival, and gene expression (49). Moreover, ck2 has been shown to be overexpressed in many different types of cancer, including breast cancer (31). ck2, a holoenzyme composed of two catalytic subunits (α and α') and two regulatory subunits (β), is a unique kinase in that it is constitutively active and does not require modifications or signaling inputs to modulate its kinase activity. In contrast, one mode of ck2 regulation likely occurs via altered subcellular localization of ck2 and/or its respective substrates (27). ck2 localization appears to be altered in a cell cycle-dependent manner, with nuclear accumulation occurring primarily in G1/S (51, 78). However, subcellular sequestration is not the only proposed mechanism for ck2 regulation. Others include regulated assembly of the ck2 holoenzyme, protein complex formation with substrates, autophosphorylation, and...
small molecule interactions (59); little is known about this topic.

Understanding how a cancer-associated kinase, like ck2, modulates PR function may provide insight into how PR promotes breast cancer cell proliferation (a PR-B-dependent action) and tumor progression (31). ck2 has previously been shown in vitro to phosphorylate human PR at Ser81, a residue located in the N-terminal region of PR unique to PR-B, termed the B-upstream segment (BUS) (80). Subsequent in silico analysis revealed 11 potential ck2 phosphorylation sites in PR (80). Mass spectrometry studies and in vitro kinase assays revealed that Ser81 was the primary site for ck2 phosphorylation; these studies failed to detect phosphorylation on any of the other ck2 consensus sites in PR (80). However, these studies were done using solely in vitro model systems; regulated phosphorylation at this site has not been studied in intact cells. Herein, we sought to understand the functional significance of ck2 regulation of PR-B Ser81 in breast cancer models.

MATERIALS AND METHODS

Cell lines. The estrogen-independent ER/PR positive T47Dco (T47D) variant cell line has been previously described (35). T47D-Y (PR negative), T47D-YB (stably expressing wild-type [wt] PR-B), and T47D-YA (stably expressing wt PR-A) cells were characterized by Sartorius et al. (66). HeLa-PR cells have been previously described (62). T47D-S79/81A PR cells were created by stable expression of pSG5-S79/81A PR and pSV-neo in T47D cells using FuGene HD (Roche). Individual colonies were selected in 500 μg/ml G418 and maintained in 200 μg/ml G418 after initial selection. The pSG5-S79/81A PR plasmid (containing serine-to-alanine mutations at Ser79 and Ser81) was generated by GenScript Corporation. T47D-Y and HeLa cells were maintained at 37°C in 5% CO2 in minimum essential media (MEM) (CellGro) supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, 1% nonessential amino acids, and 6 ng/ml insulin (cMEM). T47D-YB, T47D-YA, T47D-S79/81A PR, and HeLa-PR cells were maintained under the same conditions, with the addition of 200 μg/ml G418.

T47D cells containing an inducible PR expression system were created as follows using the ARGENT regulated transcription retrovirus kit (ARIAD Pharmaceuticals, Inc.). T47D-Y cells were first stably retrovirotransduced with the transcription factor vector pL-XR2SIH/2Z3 (necessary for activating subsequent transcription from the target gene vector). A clone from this cell line was stably retrovirally transduced with the target gene vector (pLH-Zp3-PL) containing wt PR-B (I-PR-B) or with the empty vector (I-EV). Upon addition of 10 μM 4T4Y-DB cells were isolated as described below.

Immunoblotting. For most of the immunoblotting presented here (exceptions noted in figure legends), cells were starved for 18 h in serum-free IMEM (modiﬁed improved MEM). Following starvation, cells were treated as noted in the respective ﬁgure legend and total cell lysates were isolated as described below.

Cell cycle analysis/flow cytometry.

Soft agar anchorage-independent growth assays. Soft agar assays were performed as previously described (16). Brieﬂy, cells were suspended in 0.6% SeaPlaque GTG agarose (Lonza) in IMEM supplemented with 5% DCC serum containing either ethanol (EtOH) or 10 nM R5020. Cells were plated in triplicate on 0.4% agarose. Cells were incubated under normal growth conditions for 21 days, following which colonies were counted in 15 ﬁelds/treatment group. The data are represented as an average number of colonies per ﬁeld ± standard error of the mean (SEM). Soft agar experiments were performed in triplicate.

qPCR experiments on G1/S synchronized cells, cells were plated at 2.5 × 105 cells/well in triplicate wells of a 6-well plate. Following 18 h starvation in serum-free IMEM, cells were treated for 18 h with 10 nM R5020 or EtOH (if applicable; see relevant ﬁgure legend). Total RNA was isolated using Trizol (Invitrogen); cDNA was created using the Transcriptor cDNA ﬁrst-strand cDNA synthesis kit (Roche) following the manufacturer’s recommendations. Real-time quantitative PCR (qPCR) was performed in equal amounts of cDNA using the LightCycler (Roche) in a 1× master mix on a Roche 480 light cycle. Results in triplicate for each gene were corrected for internal normalization to those for both β-actin, 18S, or GAPDH (as indicated in each respective graph) ± SD. For qPCR experiments on G1/S synchronized cells, cells were plated at 2.5 × 105 cells/well in triplicate wells of a 6-well plate. Cells were synchronized as described above, and RNA/cDNA was created and analyzed as described above.

ChIP assays. ChIP and ReChIP assays were performed using the ChIP-IT express or Re-ChIP-IT kit (Active Motif), according to the manufacturer’s instructions using sonication as the method for chromatin shearing. Lysates were immunoprecipitated (IP) overnight (18 h) with the following antibodies: PR (number MS-298-P; ThermoScientific), ck2α (number sc-12738; Santa Cruz); or an equal amount of mouse or rabbit IgG. Resulting DNA was analyzed using qPCR as described above, and data are represented as a percentage of input DNA. In silico analysis using MatInspector (Genomatix) identified potential PRE-binding sites using the following consensus sequence: RGNACANRTG TNCY. Primer sets used for qPCR analysis of ChIP data are as follows: BIRC3 PREI-F (5′-AAAAACAAATGCTGGACTCTGAGAC-3′), BIRC3 PREI-R (5′-ATGTCCTTCTGATGTTATCCG-3′), BIRC3 PREII-R (5′-TATCTGCTCCTGACTCCACG-3′), BIRC3 PREII-C (5′-TGCCAGCTTGATCAGG-3′), BIRC3 PREII-C (5′-TGCCAGCTTGATCAGG-3′), BIRC3 PREII-C (5′-TGTCATTGATCAGG-3′), BIRC3 PREII-C (5′-TGTCATTGATCAGG-3′), BIRC3 PREII-C (5′-TGTCATTGATCAGG-3′).

Luciferase transcription assays. Luciferase assays were performed as previously described (25) using the dual luciferase reporter assay (Promega). Relative luciferase units (RLU) were normalized to the mean ± standard deviation (SD) for Renilla luciferase.

Reagents. Cells were treated with the following reagents (when applicable): R5020 (10 nM; Sigma), RU486 (100 nM; Sigma), EGF (30 ng/ml; Sigma), TBB (1 to 100 μM; CalBioChem), DMAT (1 to 100 μM; CalBioChem), PP2 (10 μM; CalBioChem), roscovitine (100 μM; CalBioChem), U0126 (10 μM; CalBioChem), and AF21967 (1 nM; ARIAD Pharmaceuticals, Inc.).
control 1-R (5'-CCCTTAAGTGAAGGATCCTTTTG-3'), TF (neg control 2)-F (5'-CTAGATGTGGATGAAATGAGTTGG-3'), and TF (neg control 2)-R (5'-TTCTGAAGAAACTAAGGCCAAAAC-3').

Statistics. Statistical significance for all experiments was determined using an unpaired Student's t test.

RESULTS

Hormone- and ck2-dependent regulation of PR Ser81 phosphorylation. Previous studies have shown that PR is phosphorylated on Ser81 in vitro (80). However, regulation of this site in vivo has yet to be defined. Using custom-made polyclonal antibodies created to recognize PR phospho-Ser81, we measured progestin-induced phosphorylation of this site in T47Dco human breast cancer cells (Fig. 1A). T47Dco cells are unmodified breast cancer cells that naturally constitutively express both PR-A and PR-B, without the requirement of estrogen treatment to induce PR expression (35). We detected weak basal PR-B Ser81 phosphorylation that substantially increased in response to treatment with the synthetic progestrone R5020 (Fig. 1A). PR-A does not contain Ser81, located within the BUS domain of PR-B. As expected, our phospho-Ser81-specific antibodies detected PR-B but not PR-A.

In most steroid hormone receptor-positive breast cancer cell models, the levels of PR are primarily upregulated by estradiol, making experimental isolation of PR action (i.e., as studied independently of estrogen) very difficult (34). A naturally occurring PR-negative variant of the T47Dco human breast cancer cell line, termed T47D-Y, was first described by Sartorius and coworkers (66). This parental cell line was used to create stable cell lines constitutively expressing either wild-type (wt) PR-B (T47D-YB) or PR-A (T47D-YA) (66). As observed in unmodified T47Dco cells (Fig. 1A), we also detected low basal levels of Ser81 phosphorylation in T47D-YB cells (Fig. 1B). Again (as in T47Dco cells), the level of PR Ser81 phosphorylation increased significantly in response to R5020 (Fig. 1B). Control cells not expressing PR (T47D-Y) failed to exhibit any nonspecific bands with phospho-S81 or total PR antibodies, indicating a high degree of specificity.

T47D and HeLa cells (stably or transiently expressing PR isoforms) are routinely used as model systems for studying PR action; these cell lines behave similarly with regard to the regulation of posttranslational PR modifications and subsequent changes in receptor function (19, 24, 62). To determine the kinetics of PR Ser81 phosphorylation, we analyzed T47D and HeLa cells stably expressing PR-B. Following a time course of 10 nM R5020 treatment (0 min to 6 h), we observed increased Ser81 phosphorylation beginning at 10 min (T47D-YB) (Fig. 1C) to 15 min (HeLa-PR) (data available on request). This reached a maximum level in both cell lines at 30 to 60 min (Fig. 1C and data available on request). PR Ser81 phosphorylation preceded the ligand-dependent PR upshift primarily mediated by phosphorylation events on one or more unidentified residues (71). Note that ligand-dependent down-regulation of PR was observed after at least 4 h of R5020 treatment in both cell lines (58).

PR phosphorylation on Ser294, Ser345, and Ser400 occurs in response to either progestins (i.e., R5020) or mitogenic inputs to MAPKs and/or cdk2 (i.e., EGF, serum) (24, 62, 79). To determine the potential for mitogenic inputs to regulate Ser81 phosphorylation, we performed a time course of EGF treatment in HeLa-PR cells (data available on request). PR Ser81 phosphorylation was not affected by this mitogen, following up to 60 min of EGF treatment, despite significant activation of Erk1/2 over the same time course. To test a broader spectrum
of mitogens, we used fetal bovine serum (FBS; 20%) as a rich source of multiple growth factors. HeLa-PR cells were grown overnight either in serum-free medium, medium supplemented with 5% DCC (charcoal-stripped steroid-free medium), or full growth medium (5% FBS), followed by treatment with either R5020 (positive control for Ser81 phosphorylation; 60 min) or 20% FBS (15 or 60 min). Only R5020 treatment induced robust PR Ser81 phosphorylation (data available on request); no phosphorylation was detected following any of the serum treatments. MAPK (Erk1/2) phosphorylation served as a positive control for serum/mitogenic treatment. Finally, we used the synthetic PR antagonist/partial agonist, RU486, to demonstrate the specificity of PR ligand induction of Ser81 phosphorylation. T47D-YB (Fig. 1D) and HeLa-PR (data available on request) cells were treated with R5020, RU486, or a combination of both. Both ligands induced potent PR Ser81 phosphorylation, while the combination of R5020 plus RU486 was neither additive nor inhibitory. Cumulatively, these data suggest that PR Ser81 phosphorylation occurs primarily in response to progestins, although we frequently observed a low level of basal phosphorylation at this site (see Fig. 1; addressed below).

In vitro kinase assays suggest that ck2, a ubiquitously expressed Ser/Thr protein kinase, directly phosphorylates PR on Ser81 (80). We probed the requirement for ck2 kinase activity in intact cells using two different synthetic, highly specific ck2 kinase inhibitors, TBB and DMAT (23). HeLa-PR and T47D-YB cells were pretreated with increasing concentrations of either TBB or DMAT (or dimethyl sulfoxide [DMSO] vehicle alone) for 30 min, followed by 10 nM R5020 for 30 min. Alternatively, cells were treated with R5020 for 30 min or vehicle (EtOH) with no pretreatment. Lysates were analyzed by Western blotting using p-S81, PR, and Erk1/2 antibodies. (C) HeLa-PR cells were starved for 18 h in serum-free medium. Cells were then pretreated with increasing doses of TBB (1 to 100 μM), DMAT (1 to 100 μM), or DMSO (vehicle) for 30 min, followed by 10 nM R5020 for 30 min. Alternatively, cells were treated with R5020 for 30 min or vehicle (EtOH) with no pretreatment. Lysates were analyzed by Western blotting using p-S81, PR, and Erk1/2 antibodies. (C) HeLa-PR cells were starved for 18 h in serum-free medium. Cells were then pretreated (30 min) with TBB (10 μM), DMAT (10 μM), PP2 (10 μM), Roscovitine (100 μM), U0126 (10 μM), or vehicle (DMSO) or left untreated. Following kinase inhibitor pretreatments, cells were treated with 10 nM R5020 or vehicle (EtOH) for 30 min. Lysates were analyzed by Western blotting as described for panel A. (D) Left: T47D-YB cells were serum starved for 18 h and treated with TBB (10 μM) or vehicle (EtOH) for 60 min (left two lanes). Alternatively, cells were treated sequentially as follows: 18 h with thymidine (2.5 μg/ml) or vehicle (PBS), iMEM plus 5% DCC for 7 h, iMEM-5% DCC-mimosine (50 μg/ml; G1/S Sync.) or vehicle (EtOH; Unsyc.) for 18 h. Following synchronization (confirmed by flow cytometry; data not shown), protein was analyzed via Western blotting with antibodies for p-S81, phospho-Ser294 (p-S294), or PR. Right: T47D-YB cells were synchronized as just described (or treated with vehicle; Unsyc.). Following synchronization, cells were treated for 60 min with vehicle (DMSO) or TBB (10 μM). Protein was analyzed via Western blotting with antibodies for p-Ser81, PR, or Erk1/2 (loading control).
ck2 has been shown to be regulated in part by cell cycle-dependent localization to the nucleus (51, 78). Steroid receptors rapidly shuttle between the cytoplasm and nucleus; in the presence of progestins, PRs are primarily nuclear. To further address the potential for ck2-mediated regulation of PR Ser81 in the absence of progestins (i.e., basal phosphorylation levels observed above), we tested the cell cycle dependence of this event. For these studies, T47D-YB cells were synchronized at the G1/S transition using mimosine, a chemical inhibitor of DNA replication; synchronization of control (vehicle) and mimosine-treated T47D-YB cultures was confirmed by flow cytometry (data not shown). In G1/S-synchronized T47D-YB cells, but not vehicle controls, we observed robust PR Ser81 phosphorylation in the complete absence of ligand (Fig. 2D, left), but it was comparable in magnitude to levels induced following progestin (R5020 or RU486) treatment of unsynchronized cells (Fig. 1D and Fig. 2D, left). Ser294, a MAPK site primarily regulated only in PR-B, was unaffected by mimosine-induced synchronization (Fig. 2D, left). To confirm the ck2 dependence of PR Ser81 phosphorylation in G1/S phase cells, we treated synchronized populations of cells with or without the ck2 inhibitor, TBB. As in progestin-treated cells above (Fig. 2A to C), ligand-independent PR Ser81 phosphorylation in G1/S phase cells was completely blocked by addition of the ck2 inhibitor (Fig. 2D, right). Cumulatively, these data suggest that phosphorylation of PR Ser81 occurs independently of the ck2 inhibitor (Fig. 2D, right). Notably, Ser81 is mutated. Phospho-Ser81 PR antibody specificity was verified using the double phospho-mutant receptor (S79/81A) (58), followed a similar time course in cell lines expressing either wt or phospho-mutant S79/81A PR. To verify that ck2 expression levels remained equal among the clonal

**FIG. 3.** S79/81A PR phospho-mutant is transcriptionally active. (A) HeLa cells were transiently transfected with wt PR-B, S79/81A PR, or empty vector. At 24 h following transfection, cells were starved for 18 h in serum-free medium and then treated with 10 nM R5020 for 60 min. Lysates were analyzed via Western blotting using p-S81, PR, and Erk1/2 antibodies. (B) HeLa cells were transiently transfected with plasmids expressing wt PR-B, S79/81A PR, or vector only, as well as a firefly PRE-luciferase reporter construct and Renilla expression control. At 24 h following transfection, cells were starved for 18 h in serum-free medium, followed by an 18-h 10 nM R5020 (or vehicle) treatment. Fold relative luciferase units (RLU: PRE-luciferase over Renilla luciferase controls) of R5020-treated cells over EtOH-treated cells is plotted. Error bars represent means ± standard deviations (SD) of results from three independent experiments.

**PR Ser81-dependent transcriptional activity and promoter selectivity.** To investigate the functional consequences of PR Ser81 phosphorylation by ck2, we created a phospho-mutant receptor. Point mutation of phosphorylated residues within phospho-proteins can shift specificity to adjacent or very nearby phospho-acceptor sites that are not detected using mass spectrometry of the wt protein (63). Thus, both PR residues (Ser79 and Ser81) were mutated to ensure that nearby Ser79 is not weakly targeted by highly active kinases (in vivo) when Ser81 is mutated. Phospho-Ser81 PR antibody specificity was verified using the double phospho-mutant receptor (S79/81A PR). Western blotting showed that when transiently transfected into HeLa cells, wt PR and S79/81A PR-B were expressed at equal levels; following treatment with R5020, Ser81 phosphorylation was detected only in cells transfected with wt PR (Fig. 3A). Notably, wt and S79/81A PRs were similarly phosphorylated on all other PR phosphorylation sites tested (Ser190, Ser294, Ser345, and Ser400; data not shown), suggesting that mutant receptors fold properly and bind ligand. To determine if phospho-mutant S79/81A PR was capable of binding DNA and subsequently activating transcription, we analyzed wt and mutant PRs using PRE-luciferase reporter gene assays. In transiently transfected HeLa cells treated with vehicle or R5020, wt and S79/81A PRs behaved similarly (Fig. 3B); each receptor activated PRE-luciferase transcription to similar levels (~15- to 20-fold) in the presence of progestin (Fig. 3B). Additional characterization of the S79/81A PR mutant using confocal microscopy showed no apparent differences in subcellular localization of S79/81A PR relative to wt PR, in both the presence and absence of ligand (data not shown). Single mutant receptors (S79A and S81A) behaved similarly to the double mutant (not shown).

We then created multiple clones of stable T47D-Y cell lines expressing S79/81A mutant PR (T47D-S79/81A). Cells expressing wt PR (T47D-YB) in the same parental cell line background served as controls. Western blotting demonstrated that S79/81A PR-B is expressed at similar levels relative to wt PR-B in this model system (Fig. 4A). Again, upon progestin treatment, we detected robust Ser81 phosphorylation in wt, but not S79/81A, PR-B-expressing cells. Additionally, ligand-dependent receptor downregulation, which has been shown to be augmented by MAPK-dependent PR phosphorylation (i.e., at Ser294) (58), followed a similar time course in cell lines expressing either wt or phospho-mutant S79/81A PR. To verify that ck2 expression levels remained equal among the clonal
cell lines, we analyzed ck2α and β protein levels via Western blotting (Fig. 4B). T47D-Y cells stably expressing wt PR-B, mutant S79/81A PR, or PR-null exhibited equal levels of both ck2 subunits; neither subunit appeared to be affected by treatment with R5020.

In soft agar assays performed in vitro, the proliferative and survival effects of progestins are mediated by PR-B but not PR-A (25). We therefore assayed the ability of S79/81A mutant PR to induce breast cancer cell growth in anchorage-independent soft agar assays. Stable T47D cell lines expressing either wt PR or S79/81A PR-B or PR-null were plated for soft agar colony formation assays in the presence of either vehicle or R5020 (10 nM). Following 21 days, established colonies were counted. Cells stably expressing S79/81A PR retained their ability to form colonies in response to R5020; total numbers of R5020-induced colonies were similar between cells expressing wt or S79/81A PR by the end of the 21-day assay, while PR-null cells failed to grow well in either condition (Fig. 4C; data from additional clones are available on request). Interestingly, however, cells expressing S79/81A PR formed significantly fewer colonies in the ligand-independent condition than cells expressing wt PR-B; S79/81A PR cells resembled PR-null cells in this regard (Fig. 4C). These data suggest that in the absence of exogenously added progestin, phospho-Ser81 PR may regulate genes that primarily contribute to cell survival and/or proliferation. Ligand binding is able to overcome this deficit, perhaps because the same set of genes are also highly responsive to hormone (addressed below).

Although our PRE-luciferase reporter gene analysis (Fig. 3B) indicated that S79/81A PR behaved similarly to wt PR, transcriptional activity on endogenous PR target genes offers a more sensitive and relevant readout of PR genomic action (i.e., PR-dependent regulation of complex promoters/distant enhancer elements arrayed in chromatin). Additionally, we have shown that PR phosphorylation by rapidly activated cytoplasmic protein kinases provides a mechanism for altered PR target gene selectivity, recruiting differentially phosphorylated PR species to specific gene subsets (reviewed in reference 18).

Using our stable T47D cell line models, we surveyed mRNA expression of known PR target genes in the absence and presence of progestin (R5020; 0 to 18 h) by quantitative real-time PCR (qPCR). While many progestin-regulated genes were similarly expressed in cells containing either wt PR or S79/81A PR-B, others were differentially regulated (see below, Fig. 5; data from additional clones are available on request). These included the previously identified progestin-regulated genes BIRC3 (64), HSD11β2 (2), and HbEGF (4, 20, 81).

Notably, in the absence of progestin, BIRC3 (baculovirus inhibitor of apoptosis repeat 3), an antiapoptosis gene recently identified as a PR target gene (64), exhibited decreased levels of basal transcription in cells stably expressing S79/81A mutant PR relative to cells stably expressing wt PR-B (Fig. 5A, top). Unliganded PR appears to contribute to basal BIRC3 expression, as PR-null cells (T47D-Y) also contained lower levels of BIRC3 mRNA relative to cells expressing wt PR-B (T47D-YB). Thus, mutation of the Ser81 phosphorylation site in PR appears to have abrogated basal expression of this gene. Additionally, although mutant S79/81A PR was able to weakly induce BIRC3 mRNA in response to ligand, levels of this transcript never reached those observed in R5020-treated cells.

**FIG. 4.** Stable S79/81A PR cell lines have impaired anchorage-independent survival in soft agar. (A) T47D-Y cells stably expressing wt PR-B (T47D-YB) or S79/81A PR (T47D-S79/81A) were serum starved for 18 h and then treated with 10 nM R5020 for 0 to 18 h or vehicle (EtOH; 18 h). Lysates were analyzed by Western blotting using p-S81, PR, and Erk1/2 antibodies. (B) T47D-Y cells stably expressing wt PR-B (T47D-YB) or S79/81A PR (T47D-S79/81A) or unmodified were serum starved for 18 h and then treated with 10 nM R5020 or EtOH for 60 min. Lysates were analyzed via Western blotting using antibodies against ck2α, ck2β, PR, and Erk1/2 (loading control). (C) T47D-Y cells (PR-null) or T47D cells stably expressing PR-B or S79/81A PR were plated in soft agar containing 5% DCC medium and either EtOH or 10 nM R5020 for 21 days. Colonies were counted in 15 fields/treatment group, and error bars represent the standard errors of the means (SEM) of these measurements. Soft agar assays were performed in triplicate with similar results. Asterisks indicate statistical significance (P < 0.05; determined using an unpaired Student’s t test) compared to the respective treatment group (EtOH or R5020) in control cells (PR-null).
containing wt PR-B. Finally, T47D cells stably expressing PR-A (T47D-YA), and thus lacking the BUS region containing Ser81, displayed significantly lower basal expression of BIRC3 and failed to respond to progestin relative to cells expressing wt PR-B (Fig. 5A, bottom), indicating that the structural requirements for regulation of this gene (basal and ligand dependent) are localized to the segment of PR unique to the B isoform, which includes the Ser81 phosphorylation site. Together, these data indicate that phosphorylation at PR-B Ser81 significantly contributes to the basal expression of BIRC3 and is also required for robust responses to ligand.

HSD11β2 (11β-hydroxysteroid dehydrogenase type 2), a dehydrogenase enzyme that mediates tissue-specific metabolism of glucocorticoids (9), has previously been identified as a cancer-associated proliferative protein (40) and a progestin-responsive gene (2, 21). HSD11β2 behaved similarly to BIRC3 in that basal mRNA levels were significantly decreased in cells containing mutant S79/81A PR, as well as in PR-null cells, relative to wt PR-B-expressing cells, again strongly suggesting that wt PR Ser81 phosphorylation is responsible for the maintenance of basal transcription of this gene (Fig. 5B, bottom). Similar to the regulation of BIRC3, cells containing S79/81A PR further enhanced HSD11β2 mRNA expression in response to ligand, while overall transcript levels remained significantly lower relative to those induced in cells expressing wt PR-B. Finally, cells stably expressing PR-A contained HSD11β2 mRNA levels similar to those seen in S79/81A PR cells (both basally and in response to ligand), again suggesting that regulation of this gene is linked to PR-B-specific phosphorylation of Ser81 (Fig. 5B, bottom). These data indicate that PR-B Ser81 phosphorylation primarily regulates the basal expression of these genes (BIRC3, HSD11β2) but can also alter the magnitude of their response to hormone. Taken together with the above effects on soft agar colony formation (Fig. 4C), our data suggest that phospho-Ser81 PR contributes to gene regulation and breast cancer cell survival, even when progestins are absent or limiting.

HbEGF (heparin-binding epidermal growth factor-like growth factor) is a well-characterized phosphorylation-sensitive PR target gene shown to be important for the growth of mammary epithelial cells (4, 16, 81). In cells expressing wt PR-B, HbEGF mRNA levels were responsive to ligand (Fig. 5C, top). In contrast, cells expressing mutant S79/81A PR failed to induce HbEGF mRNA in response to R5020. Interestingly, in contrast to the previously discussed genes (Fig. 5A and B), basal HbEGF transcript levels remained comparable in containing wt PR-B. Finally, T47D cells stably expressing PR-A (T47D-YA), and thus lacking the BUS region containing Ser81, displayed significantly lower basal expression of BIRC3 and failed to respond to progestin relative to cells expressing wt PR-B (Fig. 5A, bottom), indicating that the structural requirements for regulation of this gene (basal and ligand dependent) are localized to the segment of PR unique to the B isoform, which includes the Ser81 phosphorylation site. Together, these data indicate that phosphorylation at PR-B Ser81 significantly contributes to the basal expression of BIRC3 and is also required for robust responses to ligand.

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the absence of ligand in cells expressing either wt PR-A or PR-B, mutant S79/81A PR, or no PR, suggesting that PR does not influence basal transcription of this gene. Cells expressing PR-A and treated with progesterin failed to induce HbEGF, again implicating the Ser81-containing region unique to PR-B in the progesterin-dependent regulation of this gene (Fig. 5C, middle). Finally, cells treated with the ck2 inhibitor, TBB, also failed to induce HbEGF mRNA in response to ligand (Fig. 5C, bottom). Together, these data implicate the kinase activity of ck2, presumably through direct phosphorylation of PR Ser81, in progesterin-induced upregulation of HbEGF mRNA expression.

To verify that the transcriptional differences described above (BIRC3, HSD11β2, and HbEGF) between cells expressing wt PR and S79/81A PR indeed reflect a functional requirement for phosphorylation of PR Ser81 in gene activation, rather than altered kinetics of gene activation, we analyzed mRNA isolated from cells following a time course of R5020 treatment (0 to 18 h) (Fig. 6). Impaired transcription observed in S79/81A PR-B-expressing cells relative to cells containing wt PR-B remained significant throughout this time course. Absolute mRNA levels (HbEGF and HSD11β2) became equal only after the peak of transcriptional activation, when mRNA levels began to decline. These data support the conclusion that PR Ser81 is required for absolute regulation of selected PR target genes over an extended time course.

Notably, the expression of well-characterized PR target genes, including c-Fos, tissue factor (TF), and EGFR (epidermal growth factor receptor) (38, 55, 56) was not differentially affected either basally or in response to ligand in cells expressing mutant S79/81A PR compared to expression of those expressing wt PR (data not shown). These genes represent a diverse spectrum of progestin-responsive promoters that display a variety of transcriptional kinetics (i.e., peak mRNA expression) following ligand treatment at 1 h (c-Fos), 6 h (TF), and 18 h (EGFR). These data suggest that mutation of the Ser81 phosphorylation site has not disrupted the ability of PR to activate endogenous target genes via general mechanisms (i.e., that may alter all PR transcriptional complexes or effect PR localization), indicating that the genes discussed above are uniquely regulated by phospho-PR Ser81. Results repeated in multiple clones of T47D cells stably expressing wt and phospho-mutant PRs (data available on request).

There are few reports of ligand-independent PR action. Surprisingly, both BIRC3 and HSD11β2 exhibited basal upregulation in cells expressing wt but not phospho-mutant PR-B (Fig. 5A and B and data available on request). To confirm that these genes are regulated by phospho-PRs independently of progesterin, we employed an isogenic model of inducible PR-Ligand-independent regulation of selected PR target genes provides a mechanism for PR coupling to cell cycle regulation in rapidly dividing cells. To link ck2-induced (ligand-independent) PR Ser81 phosphorylation (occurring in G1/S phase; Fig. 2D) to functional changes in gene expression, we examined BIRC3 mRNA levels during the G1/S phase of the cell cycle in synchronized populations of T47D cells either lacking PR or stably expressing wt or S79/81A PR-B. Upon G1/S phase synchronization of PR-null cells, we observed PR-independent (G1/S-dependent) increased BIRC3 mRNA expression (Fig. 7B, left). However, cells containing wt PR-B, but not phospho-mutant S79/81A PR, exhibited a further significant increase in BIRC3 mRNA levels (relative to PR-null cells). TF mRNA significantly affected by PR expression (data not shown). These data confirm that basal transcription of these phospho-Ser81-regulated genes is indeed PR dependent, but independent of exogenously added progesterins.

![FIG. 6. Time course of endogenous gene expression in wt and S79/81A PR-expressing cells. T47D-Y cells stably expressing either wt PR-B or S79/81A PR were starved for 18 h in serum-free medium, followed by treatment with 10 nM R5020 for 0 to 18 h. BIRC3 (A), HSD11β2 (B), HbEGF (C), or β-actin (internal control) mRNA levels were analyzed by qPCR. Statistical significance (P < 0.05; determined using an unpaired Student’s t test) was achieved for all time points when comparing wt PR-B- and S79/81A PR-expressing cells with the following exceptions: HSD11β2 (18 h) and HbEGF (0 and 18 h). Error bars represent means ± SD of triplicate measurements.](http://mcb.asm.org/)
levels were similar among G1/S-synchronized cells, independent of their PR status (Fig. 7B, right). These data indicate that BIRC3, a gene regulated basally in response to Ser81 PR phosphorylation, is transcriptionally activated during G1/S phase, a period when ck2-dependent PR Ser81 phosphorylation occurs in the absence of progestins.

Recruitment of phospho-Ser81 PR and ck2 to target gene promoters. To confirm direct regulation of PR target genes by phospho-Ser81 PR-B, we performed chromatin immunoprecipitation (ChIP) assays. In silico analysis of promoter and enhancer regions of the BIRC3 gene revealed several putative full-length PRE binding sites, including sites located just after the transcriptional start site (Fig. 8A). ChIP analysis was performed on lysates from EtOH- or R5020-treated cells stably expressing wt or S79/81A PR, or from PR-null cells, using PR-specific antibodies. In the presence of ligand, we detected robust recruitment (≈70-fold) of wt PR to a full-length PRE (PRE1) located within 4 kb (downstream) of the BIRC3 transcriptional start site (Fig. 8B). This is in contrast to much-decreased S79/81A PR recruitment (~10-fold) to the same area observed in side-by-side assays performed from R5020-treated cells (Fig. 8B). PR-B recruitment to PRE1 appeared to be highly specific, as other areas tested within the proximal and distal promoter regions were negative for PR binding (data not shown). In the presence of progestin, wt and S79/81A PR-B were equally recruited to a PRE located in the TF promoter region (data available on request), a gene shown earlier not to be regulated by Ser81 phosphorylation. These data indicate that decreased recruitment of S79/81A PR to the PRE1-containing region of BIRC3 is specific to this phospho-Ser81-responsive gene and does not represent a general defect in DNA-binding and/or tethering to general transcription factors by mutant S79/81A PR. Interestingly, although we observed significant differences in the basal levels of BIRC3 mRNA expression between cells containing wt and S79/81A PR (Fig. 5 and 6), we did not detect appreciable recruitment of PR to PRE1 in the absence of progestin. It is possible that PRE1 primarily regulates the ligand-activated transcriptional response of this gene, whereas another PRE(s) in the region may regulate basal activities and would, therefore, not be detected in our ChIP analyses (focused on PRE1).

To determine if ck2, the kinase responsible for PR Ser81 phosphorylation and, therefore, functional activation of PR-B at Ser81-dependent target genes, was also present at this site, we repeated our ChIP assays using antibodies directed against ck2α, one of the active subunits comprising the ck2 holoenzyme. Interestingly, ck2α was also strongly recruited to PRE1 in cells containing wt PR-B (~8-fold), but not in those con-
containing S79/81A PR (−1-fold) (Fig. 8C). These data indicate that in the presence of progestin, both wt PR-B and its activating kinase, ck2, are recruited to PR-binding sites within transcriptional regulatory regions of BIRC3. Moreover, surprisingly, mutation of PR Ser81 greatly diminished not only PR-B recruitment to this PRE but recruitment of ck2 as well, suggesting that phosphorylation of this residue is important for the formation of stable protein complexes that are associated with direct regulation of this gene.

To determine if PR and ck2 were corecruited to this site in the BIRC3 enhancer, we performed ChIP-reChIP analysis (Fig. 8D). In cells expressing wt PR-B, sequential immunoprecipitations using PR antibodies (Fig. 8D, left) followed by ck2α antibodies (Fig. 8D, right) showed that the two proteins were present together at PRE1. This interaction was detected only in cells following treatment with R5020. Reversing the order of the antibodies for the ChIP-reChIP experiment yielded similar results (data not shown). We conclude that phospho-Ser81 PR-B provides a platform for the early recruitment of ck2-containing transcriptional complexes that direct promoter-specific PR target gene regulation.

Discussion

Our studies reveal novel hormone and cell cycle-dependent regulation of PR Ser81 by ck2, a protein kinase tightly associated with prosurvival and uncontrolled proliferative phenotypes that characterize human malignancy. We show that progestin induces robust ck2-dependent phosphorylation of PR Ser81. Interestingly, this ck2-dependent event also occurs in the absence of added PR ligands, during the G1/S transition point of the cell cycle (Fig. 2). This result highlights the im-

FIG. 8. Decreased recruitment of S79/81A PR and ck2α to a PRE-containing BIRC3 enhancer region. (A) Schematic of PRE1 location in BIRC3 gene. PRE1 is located 3.4 kb downstream of the transcriptional start site (denoted with the arrow). The sequence of PRE1 is shown. (B and C) T47D-Y cells stably expressing either wt PR-B or S79/81A PR or unmodified cells (PR-null) were serum starved for 18 h. Cells were then treated with EtOH or 10 nM R5020 for 60 min. Fixed lysates were subjected to ChIP with antibodies against PR-B (B) or ck2α (C), and qPCR was performed on the isolated DNA using primers designed to amplify PRE1. Fold recruitment of PR or ck2α in R5020 conditions over EtOH is shown. Error bars represent means ± SEM of results from triplicate experiments. (D) T47D-Y cells stably expressing wt PR-B were serum starved for 18 h. Cells were then treated with EtOH or 10 nM R5020 for 60 min. Fixed lysates were subjected to ChIP with antibodies against PR-B (left), followed by ck2α (right), and qPCR was performed on the isolated DNA using primers designed to amplify PRE1 in BIRC3. Species-specific IgG antibodies were used as controls (IgG). ChIP-reChIP experiments were performed in duplicate, and a representative experiment is shown.
PR-B mimics PR-A in this regard, suggesting that Ser81 may be part of the Ser81 phosphorylation site, and that mutant S79/81A gene transcription is lost on selected genes following mutation. In epithelial cells, but the ratio of expression is often altered in expressed at a 1:1 ratio (i.e., equal levels) in normal mammary different subsets of target genes. These receptors are generally distinct transcriptional profiles and have tissue-specific dominant PR isoforms, PR-B and PR-A, have overlapping but ther discussed below).

 Altering early events in promoter recruitment (Fig. 8 and fur- through which Ser81 phosphorylation alters PR-B target gene regulation in the absence of ligand. The precise mechanism(s) via hormone-regulated PR Ser81 phosphorylation), lacking phosphorylation at this site. One class of genes is altered in the presence and the absence of ligand (i.e., in proliferating/cycling cells). Our findings provide novel insight into how PR-B may contribute to breast cancer prosurvival and tumor progression, even when hormone concentrations are limiting.

 **Role of PR phosphorylation events in breast cancer models.** Phosphorylation can impact diverse properties of the respective substrate. Direct phosphorylation of PR at specific amino-terminal Ser residues has been shown to alter receptor stability, localization, protein complex formation, dimerization, transcriptional activity, and promoter selectivity (18, 75). Data presented here indicate that tightly regulated (i.e., in response to hormone-binding and/or during G1/S transition) Ser81 phosphorylation directs target gene specificity; we identified at least three PR target genes that are differentially regulated by phosphorylation at this site. One class of genes is altered in both the presence and the absence of progesterin (BIRC3 and HSD11B2), while HbEGF is an example of a gene whose expression is primarily ligand and ck2-dependent (i.e., induced via hormone-regulated PR Ser81 phosphorylation), lacking regulation in the absence of ligand. The precise mechanism(s) through which Ser81 phosphorylation alters PR-B target gene specificity is not clear, but such phosphorylation might occur via complex mechanisms that may include altered formation of transcriptional complexes and/or recognition/binding affinity for PRE elements and associated regulatory elements, thus altering early events in promoter recruitment (Fig. 8 and further discussed below).

 Related to this finding, phosphorylation on Ser81 contributes in part to PR isoform specificity (Fig. 5). The two predominant PR isoforms, PR-B and PR-A, have overlapping but distinct transcriptional profiles (64) and have tissue-specific effects on growth (54), presumably through activation of different subsets of target genes. These receptors are generally expressed at a 1:1 ratio (i.e., equal levels) in normal mammary epithelial cells, but the ratio of expression is often altered in breast cancers (53). The full-length receptor, PR-B, contains an N-terminal region (the BUS) unique to PR-B where Ser81 is located. Data presented here showing that PR-B-activated gene transcription is lost on selected genes following mutation of the Ser81 phosphorylation site, and that mutant S79/81A PR-B mimics PR-A in this regard, suggest that Ser81 may be critical for PR-B versus PR-A target gene specificity. Related to this concept, we have begun to explore the possibilities of altered PR-A/B protein-protein interactions with associated transcriptional coactivators, corepressors, and other cofactors. Changes in further posttranslational modifications of PR (sumoylation, acetylation, ubiquitination, subsequent multisite phosphorylation events) may also be isoform specific and dictated in part by early phosphorylation events (16) and/or sequential events (15) but are outside the scope of the present study.

 Transcriptional mechanisms are highly ordered and dynamic processes, characterized by waves of interactions between DNA and dozens of regulatory molecules. Given this enormous complexity, the precise role of ck2-dependent PR Ser81 phosphorylation may remain elusive. Notably, preliminary cell fractionation and confocal experiments suggested identical subcellular localization of wt PR and S79/81A PR, independent of ligand (data not shown). Additionally, the rate of ligand-dependent downregulation/receptor turnover appeared to be unaltered by Ser79/81 mutation (Fig. 4). Effects on PR dimerization are unlikely, as S79/81A PR was able to activate PRE-luciferase transcription (Fig. 3) as well as regulate other endogenous PR target genes to levels equal to that of wt PR (c-Fos, TF, EGFR). These data indicate that mutant S79/81A PR is a fully functional transcription factor for some promoters but not others (i.e., promoter selectivity is primarily altered). Interestingly, much less phospho-mutant PR protein appeared to be recruited to a PRE located in the BIRC3 enhancer region relative to wt PR-B (Fig. 8), while recruitment to other Ser79/81-independent genes (TF: data available on request and Gary-bedian) was unaffected. This finding suggests a block at some early event required for efficient PR/DNA recognition and/or interaction. Recent work from Blind and Garabedian (6) also suggests that phospho-specific steroid receptor isoforms are differentially recruited to the promoters of specific genes based on their phosphorylation status. Using ChIP analysis, the authors showed that phosphorylation patterns on the glucocortico-roid receptor (GR) dictate which gene promoters those phospho-GRs were recruited to, the kinetics of that respective recruitment, and, therefore, which GR target genes were subsequently activated (6). Our data showing decreased recruitment of mutant S79/81A PR to select PR target genes (Fig. 8) are in concordance with this finding and suggest that this mechanism of transcriptional regulation may be a characteristic shared by many steroid receptors.

 In addition to PR recruitment to the BIRC3 enhancer region, data presented here also show that ck2α, the kinase responsible for Ser81 phosphorylation of PR, is similarly recruited to the same region in the presence of progesterin (Fig. 8). ChIP-recIP experiments demonstrated that wt PR and ck2α reside together in the same DNA-bound protein complexes. Surprisingly, less ck2α is recruited to the BIRC3 enhancer region in cells expressing mutant S79/81A PR. These data suggest that PR Ser81 phosphorylation mediates the formation of stable transcriptional complexes that may contain multiple proteins/phospho-proteins. Other factors (not assayed herein), functioning similarly to estrogen receptor (ER) or AR-associated pioneer factors (45), may require ck2-dependent PR Ser81 phosphorylation for assembly and/or stable association (i.e., that can be detected upon cross-linking); no obvious se-
quences that could serve as binding sites for additional PR- or ck2-associated factors were noted in the BIRC3 or HSD11β2 gene regulatory regions. Notably, Narayanan et al. (57) showed that cyclin A and PR are recruited to PRE regions within the MMTV promoter (stably incorporated into the T47D cell genome). In these studies, the interaction between cyclin A and active ck2 was necessary to stimulate PR transcriptional activity, primarily via phosphorylation of SRC-1 coactivator molecules (57). These findings using an exogenous MMTV promoter system, and our data presented herein, performed on endogenous PR target genes expressed in breast cancer cells, suggest that phosphorylation events and subsequent transcriptional activation of PR are tightly linked at selected promoters and that the protein kinases responsible for these modifications (of PR and/or coregulators) are an integral part of PR-containing transcriptional complexes. ER was recently shown to associate with ERK2 and CREB at selected estrogen-responsive genes important for breast cancer cell proliferation, although the required substrate(s) in transcriptional complexes that are phosphorylated by ERK2 activity (i.e., possibly CREB) has yet to be defined (48).

Notably, weak PR Ser81 phosphorylation occurred in the absence of progesterins (Fig. 1 to 4). However, this site was potently phosphorylated in cells entering the G1/S boundary (Fig. 2D), as in response to progesterin. Ligand binding to PR sets up an exquisite program of cell cycle synchronization wherein cells enter S phase following precisely timed regulation of cell cycle mediators (reviewed in reference 22). Indeed, PR target genes include cyclins (D, E, and A) and cdk inhibitors (p21 and p27), and progesterin-treated breast cancer cells are known to pause or accumulate at the G1/S boundary (30). Given the tight coupling of PR to cell cycle control, it is perhaps not surprising that selected PR target genes depend upon PR Ser81 phosphorylation for regulation both in the presence (HbEGF) and absence (BIRC3 or HSD11β2) of ligand. Ligand-independent PR gene regulation may provide important clues to how ck2 is regulated during cell cycle traverse. Protein complex formation involving Ser81-phosphorylated PR and ck2 is the topic of future studies.

**Functional significance of ck2 and PR Ser81 target gene regulation in breast cancer.** The Ser/Thr protein kinase ck2 is upregulated in every cancer studied thus far (72). Although ck2 itself does not appear to be an oncogene, it is thought that ck2 works in an oncogenic fashion by potentiating the activity of other oncoprogens and progrowth signaling molecules that function as its major substrates (reviewed in reference 74). For example, numerous studies have shown that ck2 overexpression promotes tumorigenesis in existing transgenic mouse models of cancer (11, 39, 41, 42). In the context of breast cancer, where progesterins have been implicated as a risk factor for tumor development and early progression (1, 5, 12), overexpressed ck2 could further enhance the oncogenic potential of PR through inappropriate phosphorylation (on Ser81). Notably, the genes that are transcriptionally regulated by PR Ser81 phosphorylation have been shown to be important in cell growth and have each been identified in various types of cancer, including breast cancer. BIRC3 is an anti apoptosis protein belonging to the inhibitor of apoptosis (IAP) family of proteins (65). IAPs bind to and inhibit other pro-death-associated proteins, such as caspases, thereby preventing apoptosis (44). BIRC3, a mammalian-specific IAP also known as cellular IAP2 (cIAP2), is overexpressed, along with other closely related IAP family members, in breast cancer (28). HSD11β2 is a dehydrogenase enzyme that is responsible for the tissue-specific metabolism of glucocorticoids (reviewed in reference 9). Specifically, HSD11β2 expression has proliferative effects, especially in tumors, through inactivation of the anti proliferative effects of GR (36). Of note, HSD11β2 is upregulated in many different cancers, including breast, whereas the corresponding normal nonneoplastic tissue normally lacks HSD11β2 expression (36, 40). As a PR target gene, HSD11β2 may be an important mediator of progestin action. Finally, HbEGF, a gene shown here to be regulated by ligand-induced PR Ser81 phosphorylation, has been shown to contribute to mammary cell proliferation and breast cancer cell growth (4, 20). Moreover, ck2 is frequently upregulated in breast cancer. This fact, coupled with our findings that phospho-Ser81 PR can drive the expression of genes that clearly contribute to breast cancer biology, suggests a scenario for ck2-high breast tumors, in which PR may be inappropriately or persistently phosphorylated on Ser81 (i.e., either basally or in response to ligand) and thereby contribute to a hyperproliferative state. Indeed, we observed increased ligand-independent soft agar colony formation in cells expressing wt PR-B relative to cells expressing S79/81A PR and PR-null cells. Thus, the basal level of anchorage-independent growth was abrogated in cells expressing phospho-mutant S79/81A PR (Fig. 4C); cells expressing PR-A also fail to grow in soft agar (25). Related to this finding, we suspect that many additional prosurvival and/or proliferative genes are regulated by phospho-Ser81 PR. The identification of a more complete Ser81-regulated gene signature in breast cancer cells awaits detailed gene array analyses. Additionally, the presence of phospho-PR Ser81 in breast tumors may provide a marker of activated PRs in S-phase cells (in progress).

Due to the diverse nature and subcellular distribution of the >300 substrates of ck2, it is not surprising that ck2 has been localized to nearly every cellular compartment, including, but not limited to, the nucleus, cytoplasm, plasma membrane, and mitochondria (reviewed in reference 26). Conflicting reports exist regarding a correlation between ck2 localization and cell cycle; this discrepancy is likely due to cell type-specific differences in ck2 distribution. Reports indicate that ck2 localization (either the holoenzyme or specific subunits) shifts to predominantly nuclear during the G1 phase of the cell cycle and at the G1/S border (51, 78); we have also detected a similar shift in PR localization in G1/S synchronized cells (data not shown). Phosphorylation of PR Ser81 in the absence of ligand (observed in cells arrested at the G1/S transition; Fig. 2D) may be regulated as a consequence of increased nuclear accumulation of ck2 and PR observed at this stage of the cell cycle. In addition, work from the Ahmed lab (reviewed in reference 32) showed that in response to androgenic or growth factor signals in prostate cancer cells, ck2 localization was strongly nuclear and specifically associated with the nuclear matrix and chromatin, areas of high transcriptional activity (33). Progestins may work similarly to their androgenic counterparts and direct PR to the ck2-containing nuclear compartment, subsequently inducing prolonged phosphorylation of PR Ser81. Interest-
ingly, PR nuclear entry appears to precede Ser81 phosphorylation (data not shown), similar to the pattern recently described for PR phosphorylation on Ser294 and Ser400 (17).

Significantly, nearly 70% of breast cancers express both ER and PR at the time of diagnosis, in contrast to PR/ER expression in just 7 to 10% of normal breast luminal epithelium (67). As steroid hormone receptor (SR)-positive tumors progress, they frequently become hormone independent while retaining receptor expression, indicating an early switch to autocrine or paracrine growth factor signaling (60). In addition, many breast cancers have upregulated protein kinases, such as MAPK, c-Src, cdk2, and cdk2, which can modify and hyperactivate PR (29, 69, 72, 77). Recently, progesterone was shown to mediate mammary stem cell self-renewal via paracrine mechanisms in which secreted factors (Wnt, RANKL) derived from PR-positive cells influence the PR-null stem cell niche (37). In PR-negative breast cancer cells, PR action drives proliferation, prosurvival signaling, and early invasion primarily by autocrine mechanisms (10, 25, 61). In an environment where steroid hormones are no longer required to drive cellular proliferation (i.e., during SR-positive tumor progression), the increased expression and constitutive activation of PR-activating protein kinases may promote increased cell survival and uncontrolled growth (i.e., in the face of endocrine therapies primarily directed against ER). Understanding how mitogenic protein kinases promote increased cell survival and uncontrolled growth (i.e., in the face of endocrine therapies primarily directed against ER), understanding how mitotogenic protein kinases, such as cdk2, alter PR phosphorylation and function is critical to fully understanding breast tumor etiology and developing better targeted therapies. Due to the ubiquitous nature of cdk2 and its prevalence in many different types of cancer, there has been much interest in the development of cdk2 inhibitors as anti-cancer agents (73). Clinical cdk2 inhibitors, in combination with more specific anti-progestins (new classes of selective progesterone receptor modulators or SPRMs), could provide an effective combination of targeted therapy for breast cancer treatment.

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Thank you for your assistance.
Role of phosphorylation in progesterone receptor signaling and specificity

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Progesterone receptors (PR), in concert with peptide growth factor-initiated signaling pathways, initiate massive expansion of the epithelial cell compartment associated with the process of alveologenesis in the developing mammary gland. PR-dependent signaling events also contribute to inappropriate proliferation observed in breast cancer. Notably, PR-B isoform-specific cross talk with growth factor-driven pathways is required for the proliferative actions of progesterone. Indeed, PRs act as heavily phosphorylated transcription factor "sensors" for mitogenic protein kinases that are often elevated and/or constitutively activated in invasive breast cancers. In addition, phospho-PR-target genes frequently include the components of mitogenic signaling pathways, revealing a mechanism for feed-forward signaling that confers increased responsiveness of, PR + mammary epithelial cells to these same mitogenic stimuli. Understanding the mechanisms and isoform selectivity of PR/kinase interactions may yield further insight into targeting altered signaling networks in breast and other hormonally responsive cancers (i.e. lung, uterine and ovarian) in the clinic. This review focuses on PR phosphorylation by mitogenic protein kinases and mechanisms of PR-target gene selection that lead to increased cell proliferation.

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1. PR structure and function

The ovarian steroid hormone, progesterone, acts by binding to and activating progesterone receptor (PR) A-, B-, and C-isoforms expressed in target tissues. Isoform-specific expression results from selection of alternate promoters encoded by a single gene (Kastner et al., 1990). The full-length receptor, PR-B (116 kDa), contains a unique N-terminal segment, termed the B-upstream segment (BUS), that is not present in the truncated isoforms, PR-A (94 kDa), or PR-C (60 kDa). PR-C lacks both the BUS and a portion of the DNA-binding domain (DBD), rendering it transcriptionally inactive (Wei et al., 1996). In addition to intact DBDs, the two transcriptionally active isoforms, PR-B and PR-A, contain the following structural/functional domains: a flexible hinge region (H); also referred to as the carboxy terminal extension or CTE) that functions, in part, to aid DNA binding (Roemer et al., 2008), a ligand-binding domain (LBD), and multiple activating function (AF) domains required for transcriptional activity (Roemer et al., 2008). Studies from knockout-mice have shown that PR-B is necessary for the alveologenesis phase of normal mammary gland development, while PR-A is required for uterine development (Conneely et al., 2001; Lydon et al., 1995; Mulac-Jericevic et al., 2003; Shyamala et al., 1998). PR-C, lacking transcription activity, has been shown to inhibit PR-B function in the uterus (Condon et al., 2006), and conversely, appears to potentiate the transcriptional activity of the other PR isoforms in the breast (Wei et al., 1997).
PR isoforms rapidly shuttle between the cytoplasm and the nucleus; unliganded receptors reside in both compartments and exist as part of multi-protein complexes in association with heat-shock protein chaperone molecules, such as Hsp70 and Hsp90 (Pratt et al., 1989; Kost et al., 1989). Additionally, unliganded and liganded PRs (primarily PR-B; (Boonyaratanakornkit et al., 2007)) participate in cytoplasmic or membrane-associated signaling complexes that activate mitogenic protein kinases, such as c-Src, MAPK and PI3 K (Boonyaratanakornkit et al., 2001; Migliaccio et al., 1998; Bagowski et al., 2001; Faivre and Lange, 2007; Carnevale et al., 2007).

Fig. 1. Progesterone receptor structure and phosphorylation sites. All three human PR isoforms (hPRα, hPRβ, and hPRγ) are transcribed from the same gene, containing distal and proximal promoters. Shown are three transcription activation function (AF) domains, the B-upstream segment (BUS), the DNA-binding domain (DBD), the hinge region (H) and the hormone-binding domain (HBD). PR is phosphorylated basally, as well as in response to hormone. Shown here are known PR phosphorylation sites as determined in vitro and in vivo, and the protein kinases that are likely responsible for direct phosphorylation at these sites.

Fig. 2. Integration of PR rapid signaling and transcriptional activities. Progesterone (P4) binding to PR induces the rapid association of PR and c-Src. This interaction leads to a c-Src-dependent activation of the MAPK module through Ras/Raf signaling. This MAPK activation can lead to phosphorylation (P) of PR, transcriptional coactivators, and/or activation of downstream MAPK target genes (i.e. Cyclin D1). Phosphorylated PRs can activate transcription directly by binding to progesterone response elements (PREs) or indirectly though tethering interactions (i.e. SP1). Extracellular and classical actions of PR are likely highly integrated actions, rather than separable events mediated by discrete populations of receptors.
ties on a given promoter, but also appear to select different pro-
(Elend et al., 2004).

However, PR-A and PR-B isoforms are highly post-translationally
modified, primarily on serine (Ser; phosphorylation) and lysine
(Lys; acetylation, ubiquitination, and sumoylation) residues lo-
cated in the N-terminal region (Lange et al., 2000; Weigel et al.,
1995; Daniel et al., 2010; Abdel-Hafiz et al., 2002; Hagan et al.,
2011). These modifications are frequently ligand-dependent, but
can also occur independently of progestin-binding (i.e. in response
to kinase activation), and significantly alter receptor stability,
localization, tethering interactions, transcriptional activity, and
promoter selectivity (Daniel et al., 2009; Ward and Weigel,
2009). For example, PR-phospho-species exhibit differential activi-
ties on a given promoter, but also appear to select different pro-
motors (reviewed in Daniel et al., 2009). Although the mecha-
nisms are not entirely clear, unique PR-phospho-species are
likely directed to distinct PR target gene subsets in part via
phosphorylation-dependent protein–protein interactions with the
same set of protein kinases that may occur within so-called “rapid
signaling complexes”. Thus, the non-genomic and genomic actions
of PRs are highly integrated functions that serve to coordinate iso-
form-specific PR actions and mediate PR-target gene promoter
selection; mechanisms of PR integration with signaling pathways
is the topic of this review (Fig. 2).

2. MAPK activation alters PR function

MAPK signaling modulates PR activity directly by phosphoryl-
ating the receptor on consensus site serine residues Ser294 and
Ser345 (Faivre et al., 2008; Lange et al., 2000). These distinctively reg-
ulated phosphorylation events have unique functional con-
sequences for PR that ultimately regulate cell fate. Upon growth
factor stimulation, PR phosphorylation of Ser294 primes the recep-
tor for robust transcriptional activation in response to ligand by
ensuring retention in the nucleus (discussed below) (Qiu et al.,
2003), association with DNA (Daniel et al., 2007a,b), and removal
of repressive modification by sumoylation (Daniel et al., 2007b).
Ser294-phosphorylated receptors are transcriptionally hypersensi-
tive to low concentrations of ligand on a select set of promoters
(Qiu and Lange, 2003); the mechanism of growth factor-induced
PR hypersensitivity maps to phospho-Ser294 antagonism of
Lys388 sumoylation (Daniel et al., 2007a,b). Likewise, phosphory-
lation on Ser294 increases PR ubiquitinization, an activation step
for many transcription factors (Salghetti et al., 2001), and also aug-
ments its downregulation (Lange et al., 2000). Therefore Ser294
phosphorylation in response to MAPK activation (by either pro-
gestins or growth factors) generates receptors that are hyperactive on
select promoters in response to ligand and serves to couple this
activity to rapid protesome-dependent turnover. In addition,
phosphorylated/sumoylated receptors are active on a subset of
ligand-independent PR-target gene promoters whose expressed
protein products (IRS-1 and STC1) contribute to breast cancer cell
proliferation and pro-survival (Daniel and Lange, 2009). Further-
more, Ser294 appears to be a “hot-spot” for the regulation of
PR-B transcriptional activity as phospho-mutant PR-B (Ser294 to
alanine) is virtually transcriptionally inactive when measured on
endogenous genes (i.e. in cells stably expressing S294A PR-B rel-
tive to cells containing wild-type PR) (Shen et al., 2001). Notably,
PR-A is not appreciably phosphorylated on Ser294 in intact cells,
while this site in PR-A can be phosphorylated in vitro using recom-
binant PR-A proteins (Clemm et al., 2000). This finding underscores
the role of protein–protein interactions between PRs and associ-
ated signaling complexes that contain protein kinases as major
determinants of PR isoform specificity.

In an alternative route to phosphorylation-dependent PR pro-
motor selection, rapid progestin-mediated MAPK activation drives
specific phosphorylation of PR-B on Ser345, a site shown to be crit-
ical for PR tethering to SP1 transcription factors (Faivre et al.,
2008). PR/c-Src/EGFR rapid signaling complex formation precedes
PR Ser345 phosphorylation and PR/SP1 association with non-classi-
cal promoters (lacking PREs), such as p21 and EGFR (Fig. 2). This
unique mechanism of steroid receptor activation by MAPK signal-
ing (i.e. non-genomic/genomic signaling integration) is required
for progestin-induced breast cancer cell entry into S-phase (Faivre
et al., 2008).

3. Cyclin dependent kinase 2 (CDK2) regulation of PR function

Studies using both in vitro and in vivo techniques have identified
multiple CDK2-dependent phosphorylation sites on PR (reviewed in
(Moore et al., 2007)). These sites include PR serines 25, 162,
190, 213, 400, 554, 676 (Zhang et al., 1997; Knotts et al., 1997;
Knotts et al., 2001) and threonine 430 (Knotts et al., 2001). Additionally, while
Ser294 is phosphorylated by MAPK (discussed earlier), it can also
be phosphorylated by CDK2 (Daniel and Lange, 2009). Although
only a fraction of these CDK2 sites have been studied in depth,
PR phosphorylation by CDK2 has specific implications for PR func-
tion and activity. Phosphorylation of PR on serines 190, 294, 554
and 676 clearly contributes to PR hormone-dependent transcrip-
tional activity (Shen et al., 2001; Takimoto et al., 1996). Individual
mutation of each of these sites results in significant decreases
(20–90%) in overall PR transcriptional activity, as measured using
PRE-reporter gene constructs. While Ser 190, 554 or 676- phos-
pho-mutant PRs exhibit significant decreases in transcriptional
activity, these mutant PR species are each able to bind DNA
similarly to wild-type PR, suggesting that phosphorylation at these
serines may contribute to recruitment of co-activators to PR-
containing transcriptional complexes (Takimoto et al., 1996).

Phosphorylation of PR Ser400 by CDK2 has been linked to en-
hanced ligand-independent PR transcriptional activity, as mea-
sured using PRE-reporter gene constructs (Pierson-Mullany
and Lange, 2004). In the presence of high CDK2 kinase activity and/or
low cell cycle inhibitors (namely, p27), PR Ser400 is constitutively
phosphorylated and thereby drives heightened PR transcriptional
activity in the absence of progestins (Pierson-Mullany and
Lange, 2004). This particular interaction of CDK2 with PR has important
implications for deregulated PR activity in the context of breast
cancer, as transformed cells often exhibit loss of cell cycle control
that is characterized by Rb-inactivation, elevated CDK4/6 activity,
high expression of cyclins D, E, or A, and/or low expression of cell
cycle inhibitors (Slingerland and Pagano, 2000; Cariou et al., 1998;
Musgrove et al., 2004; Alkarain et al., 2004; Tawfic et al., 2001;
Wilson et al., 2006) ultimately leading to increased, deregulated
CDK2 activity. Notably, PR-target genes include key cell cycle
mediators (reviewed in (Dressing and Lange, 2009)) such as
D-type cyclins and cyclin E, the regulatory subunits of CDK4/6 and
CDK2, respectively. Thus, activation of unliganded PRs in this setting
(cell cycle deregulation leading to high CDK2 activity) may produce
a “feed forward” mechanism of persistent CDK2 activation early in
breast tumor development. This unliganded activity of PR can be
blocked by anti-progestins (Pierson-Mullany and Lange, 2004), suggesting that selective PR modulators could be used to block CDK2-driven cell proliferation and pro-survival in PR + tumor cells.

Phosphorylation events also contribute to PR nuclear localization. Recent studies suggest that mutant PRs unable to enter the nucleus (devoid of nuclear localization signals; ANLS PR) are phosphorylated on Ser190, but not on Sers 81, 294, 345 and 400 (Daniel et al., 2010) and data not shown). However, ANLS PR is phosphorylated on these sites upon coexpression and dimerization with wt PR, forcing ANLS PR nuclear entry. Other studies showed that Ser400 phosphorylation (CDK2-dependent) enhanced ligand-induced nuclear accumulation (Pierson-Mullany and Lange, 2004), while Ser294 phosphorylation was required for growth factor (EGF, MAPK), but not progestin-mediated nuclear accumulation (Qi et al., 2003). These data suggest that phosphorylation of PR on these residues occurs upon nuclear entry and serves to promote nuclear retention. Phosphorylation on Ser190, another CDK2 site, likely occurs in the cytoplasm and does not contribute to nuclear entry or retention (Daniel et al., 2010). Together, these data demonstrate that CDK2 is able to phosphorylate PR in both the cytoplasm and the nucleus and that, once in the nucleus, phosphorylation at some CDK2 sites (Sers 294 and 400) promotes PR nuclear retention, perhaps via protein–protein interactions requiring these specific phosphorylation events. Moreover, rapid nuclear translocation and retention of PR appears to be critical for proper execution of rapidly-activated (i.e. c-myc mRNA expression is induced by liganded PR within minutes) PR-target genes (Daniel et al., 2010). Properly timed PR nuclear entry/retention in response to phosphorylation events likely ensures robust execution of PR transcriptional activity at such “early genes” perhaps, in part, by ensuring that both PR and its co-regulators are activated (i.e. appropriately phosphorylated) and co-localized in the nucleus. Indeed, latent nuclear localization of PR is associated with delays in PR-induced immediate early genes (i.e. c-myc) but not in overall PR transcriptional activity measured at late time points (i.e. on reporter genes) and/or on endogenous genes that are not particularly sensitive to changes in phosphorylation events (Daniel et al., 2010).

Interestingly, CDK2 not only acts to phosphorylate PR but may also act as an integral part of PR transcriptional complexes. Cyclins A and E, the regulatory subunits of CDK2, bind to both unliganded and liganded PRs; these constitutive interactions may serve to recruit and sustain CDK2 activity at active sites of transcription (reviewed in Dressing and Lange, 2009). Although endogenous genes have not been extensively studied, Cyclin A is clearly recruited along with PR to stably embedded (i.e. in chromatin) MMTV promoter regions (Narayanan et al., 2005). Thus, CDK2 (a cyclin A-binding partner) is also likely present at PR-bound PRE-containing enhancers (Moore et al., 2007; Narayanan et al., 2005; Weigel and Moore, 2007). Inhibition of CDK2 activity using a small molecule CDK2 inhibitor, roscovitine, decreased phosphorylation of SRC-1 (steroid receptor co-activator-1) and blocked recruitment of both PR and SRC-1 to the PR transcriptional complex on the MMTV promoter (Narayanan et al., 2005). In these studies, mutation of PR at multiple CDK2 phosphorylation sites had no effect on reporter gene transcription. Thus, CDK2 appears to mediate SRC-1 co-activator phosphorylation (independently of PR phosphorylation). The scaffolding function of PR/cyclin interactions likely serves to recruit and sustain CDK2 activity (wherein the primary substrate is SRC-1); this model awaits confirmation on endogenous genes and during cell cycle traverse.

4. cd2 modification of PR

Initial in vitro work showed that PR Ser81 (unique to the BUS region of PR-B) was phosphorylated by cd2, a ubiquitously expressed, constitutively active protein kinase (Zhang et al., 1994). Recent published work from the Lange lab has shown in breast cancer cells that basal levels of PR Ser81 phosphorylation are rapidly increased in response to either agonist or antagonist ligands (Hagan et al., 2011); an effect shown to be dependent on cd2. However, unlike other PR phosphorylation sites (i.e. Ser294), PR Ser81 phosphorylation is unresponsive to growth-factor or serum treatment of cells. Interestingly, in the absence of ligand, PR Ser81 phosphorylation is increased in cells that are synchronized at the G1/S phase border, suggesting that phosphorylation at this site is regulated in a cell-cycle dependent manner (Hagan et al., 2011). In line with this finding, ligand-independent cell survival, as measured by soft-agar colony formation, was decreased in cells expressing a PR phospho-mutant (S79/81A PR) that cannot be phosphorylated at Ser81 (Hagan et al., 2011). Moreover, this mutant displayed defects in recruitment to selected PR-B-target genes important for proliferation and pro-survival, and was impaired in its ability to recruit cd2 to PR-associated enhancer sites (Fig. 3) (Hagan et al., 2011). cd2, a kinase shown to be upregulated in every cancer studied thus far, including breast cancer, is not thought to be oncogenic on its own, but appears to increase the oncogenic potential of cancer-promoting proteins and pro-growth signals that are its substrate molecules (Tawfic et al., 1999; Trembley et al., 2009). In the context of breast cancer, where progrestins have been implicated as a risk factor for tumor development and early progression (Beral, 2003; Anderson et al., 2004; Chlebowski et al., 2005), overexpressed cd2 could further enhance the oncogenic potential of PR through inappropriate phosphorylation (on Ser81), thereby directing phospho-Ser81 PR-B to growth-promoting genes.

5. PR-dependent activation and amplification of kinase signaling pathways

Several studies illustrate the emerging concept that PR and associated signaling pathways are fully integrated, from membrane-initiated events to genomic actions (Fig. 2). Upon progesterin treatment, PR rapidly associates with signaling complexes via two distinct domains: a consensus poly-proline rich region (PR amino acids 396–456) known to interact with consensus SH3 domains (Boonyaratankornkit et al., 2001) and unique (to PR) regions termed Estrogen Receptor Interacting Domains or ERIDs; ERID1 (amino acids 165–345) and ERID2 (amino acids 456–546) are located in the PR N-terminus (Ballare et al., 2003). Progestin-binding induces direct interaction of PR with the SH3 domain of c-Src, or to ER (via the ERID domains), causing rapid (5–10 min) activation of the EGFR/c-Src/Ras/Erk pathway (Boonyaratankornkit et al., 2001; Migliaccio et al., 1998; Faivre et al., 2008; Ballare et al., 2003) and the PI3K/Akt pathway (Carnevale et al., 2007). These signals, shown to be critical for progesterin-induced proliferation of breast cancer cells (Boonyaratankornkit et al., 2007), provide a feedforward signaling mechanism for PR/progesterin-dependent genomic events in addition to activating other transcription factors (Fairev et al., 2008). Phosphorylation of PR and co-activator molecules enhances PR transcriptional activity on classical (Qi and Lange, 2003) and non-classical promoters (Fairev et al., 2008). Progestin-activated Erk is recruited to PR-containing transcriptional complexes in chromatin (Vincent et al., 2006) and PR devoid of ERIDs activates a gene expression profile distinct from wt PR (Quiles et al., 2009), indicating that PR-induced kinase signaling contributes directly to promoter activation and selectivity. Notably, progesterin treatment also elicits delayed (18 h) and sustained activation of MAPK signaling, whereby MAPK-dependent upregulation of PR-target genes (Wnt1, MMPs, and EGFR) completes an autocrine signaling pathway that culminates in high cyclin D levels and breast cancer cell growth/survival in soft agar (Fairev and Lange).
Thus, progestin/PR-mediated rapid activation of MAPK signaling ultimately functions to amplify PR genomic actions, modulate PR target gene selectivity (i.e. by directing phospho-PRs to selected promoters), and induce sustained MAPK signaling (i.e. downstream of activated EGFR) capable of activating multiple (PR-independent) transcription factors that serve to perpetuate the proliferative signal (long after liganded PRs have been downregulated). In this manner, progesterone/PRs may confer greatly increased sensitivity of target tissues to the actions of peptide growth factors. These interactions clearly allow for rapid expansion of the mammary epithelium during puberty and pregnancy (in preparation for lactation), but may inappropriately drive early breast cancer progression of steroid hormone receptor positive tumors.

In addition to scaffolding MAPK pathway signaling events, PR also participates in signaling complexes with cell cycle regulators. PR contains numerous consensus CDK binding motifs, and has been shown to associate with CDK2, perhaps mediating its interactions with cyclins E and A (discussed above) (Narayanan et al., 2005; Faiivre et al., 2005). This complex formation, in addition to PR transcriptional upregulation of cyclins and CDK inhibitors (p21, p27) that appears to be required for initiating CDK kinase activity, may account for the rapid (15 min) and sustained (days) activation of CDK2 observed in breast cancer cells upon a single treatment with progestin (Pierson-Mullany and Lange, 2004). Again, these studies indicate that phospho-PRs are capable of robust positive feed forward or self-regulation of the very same signaling pathways that they rapidly activate.

A number of studies have illustrated further cross-talk between PRs and Signal Transducers and Activators of Transcription (STATs), involving PR-mediated activation of both STAT3 and STAT5. Cumulative work from the Elizalde lab has shown that STAT3 activation by the heregulin/ErbB-2 pathway is mediated by ligand-independent functions of PR, and requires phosphorylation of PR Ser294 (in response to growth factor stimulation) (Proietti et al., 2009). Further work has defined a bi-directional transcriptional co-activator relationship between PR and STAT3, each appearing to activate the transcriptional capacity of the other (Beguelin et al., 2010; Proietti et al., 2010). A similar story has emerged for STAT5 and PR. Progesterone treatment induces PR-dependent STAT5 nuclear translocation and transcriptional activity, potentially mediated by a direct interaction between PR and STAT5 (Richer et al., 1998), at times involving other signaling molecules that serve as co-regulators like FGFR-2 (Fibroblast growth factor receptor-2) (Cerliani et al., 2011). PR-dependent regulation of (downstream) STAT5 activity is well established as critical for normal mammary gland development (Santos et al., 2010; Santos et al., 2008).

Indeed, the end point of mitogenic signaling pathway activation is often the regulation of transcription factor substrates. Notably, phospho-PR target genes most often include the components of signal transduction pathways (T. Knutson and C. Lange, unpublished results). Thus, PR is directly responsible for modulating/maintaining kinase signaling in cells via transcriptional upregulation of growth factor receptors, their ligands, and their downstream effectors and associated adaptor molecules. Direct PR target genes include EGFR, IRS1, STAT5A, numerous Ras pathway members (including adaptors and exchange factors), many kinases, as well as peptide growth factors (Hb-EGF, Wnt1) and other secreted signaling molecules (Daniel et al., 2007a,b; Jacobsen et al., 2003). Ultimately, kinase pathway “restructuring” by PR may serve to prime mammary epithelial cells for the rapid proliferation stage associated with massive expansion of the (pregnant) mammary gland that occurs in preparation for lactation. Similarly, the deregulation of these events during breast cancer development and/or early progression is suspected to contribute to advanced malignant breast cancer phenotypes.

6. PR significance in breast cancer

Highly publicized and controversial clinical data has demonstrated that women taking hormone-replacement therapy (HRT) whose regimens included estrogen and synthetic progesterone, but not estrogen alone, experienced increased breast tumor number, size, and aggressiveness (Beral, 2003; Anderson et al., 2004) increased breast cancer risk was reversed upon cessation of HRT.
(Beral, 2003; Chlebowski et al., 2009). Significantly, nearly 70% of breast cancers express both ER and PR at diagnosis, in contrast to PR/ER expression in just 7–10% of normal (non-pregnant) breast luminal epithelium (Seagraves et al., 2000). As these steroid receptor (SR)-positive tumors progress, many of them become hormone-independent (refractory to estrogen- or ER-targeted endocrine treatments) while retaining high SR expression, suggesting an early switch to autocrine and/or paracrine growth factor signaling (Osborne et al., 2005). In addition, a majority of these cancers have upregulated and activated protein kinases, such as MAPK, Akt-c-Src, cyclin/CDKs, and ck2, all of which modify and/or activate PR and/or its co-regulators (discussed in detail above) (Tawfic et al., 2001; Wilson et al., 2006; Gregory et al., 2004; Steeg and Zhou, 1998). In breast cancer cells, PR-B action clearly drives proliferation and pro-survival signaling. Interestingly, PR (mRNA expression) was recently identified as an independent-(single-gene) predictor of poor outcome in non-small cell lung cancer, implicating PR and hormone-responsiveness in cancers other than breast (Jeong et al., 2010). In an environment where progesterone is no longer required to drive cellular proliferation (i.e. ligand-independence), constitutive activation of PR-activating protein kinases may promote uncontrolled cell growth that is primarily driven by deregulated phospho-PR-target genes. Most recently, progesterone was shown to mediate mammary gland stem cell self-renewal via paracrine mechanisms in which secreted factors (Wnt, RANKL) derived from PR-positive cells influenced the PR-null stem cell niche (Joshi et al., 2010; Asselin-Labat et al., 2006). Progesterone/progesterone antagonists may alter breast cancer stem cell behavior by similar mechanisms. In sum, in light of the cumulative data discussed herein, understanding how mitogenic protein kinases alter PR (and vice versa) is critical to fully understanding breast tumor etiology with the goal of developing superior approaches for the prevention or treatment of endocrine resistance in SR-positive breast cancers.

References


Progesterone receptor action: defining a role in breast cancer

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Abstract

The ovarian steroid hormones, estradiol and progesterone, and their nuclear receptors (estrogen receptor [ER] and progesterone receptor [PR]), are involved in breast cancer development. As ER-positive/PR-positive tumors progress, they are likely to become steroid hormone-resistant/independent, yet often retain expression of their steroid receptors. Notably, up to 40% of women with steroid receptor-positive tumors exhibit de novo resistance or eventually fail on estrogen- or ERα-blocking therapies (acquired resistance). Indeed, most of the research on this topic has centered on mechanisms of ER ‘escape’ from endocrine therapy and the design of better ER-blocking strategies; signaling pathways that mediate endocrine (i.e., anti-estrogen) resistance are also excellent therapeutic targets. However, serious consideration of PR isoforms as important drivers of early breast cancer progression and ER modulators is timely and significant. Indeed, progress has been hindered by ER-centric experimental approaches. This article will focus on defining a role for PR in breast cancer with hopes of providing a refreshing PR-focused perspective.

Keywords

breast cancer; estrogen receptor; hormone replacement therapy; mammary gland biology; progesterone receptor; protein kinases; stem cells

Progesterone receptor isoforms are multifunctional transcription factors

Progesterone receptors (PRs) are ligand-activated transcription factor members of the steroid hormone receptor (SR) subfamily of nuclear receptors (Figure 1). Two common isoforms (A and B) are created from the same gene via alternate translational start sites; PR-B refers to the full-length receptor, while PR-A is an N-terminally truncated version (missing the first 164 amino acids found in PR-B). The PR gene is differentially regulated by two independent (isoform-specific) promoters. A and B isoforms can act as homo- (A:A or B:B) or heterodimers (A:B) and are capable of binding DNA at progesterone response elements [1] and/or via tethering to other transcription factors (signal transducers and activators of transcription [STATs], specificity protein 1 [SP1] and activator protein 1) [2–5]. PR-A and -
B can regulate the same or different (isoform-specific) sets of target genes and exhibit both ligand-dependent and -independent activities [6,7]; these PR functions are heavily influenced by cross-talk/input from peptide growth factor-initiated signal transduction pathways [8]. A third PR isoform termed PR-C is truncated still further downstream by use of an additional AUG codon within the DNA-binding domain; this highly tissue-specific receptor inhibits the actions of PR-B in the uterus and is important for the induction of labor [9].

Steroid hormone receptors function as signal transduction molecules. PRs function not only as critical regulators of transcription but also to activate signal transduction pathways, many of which are involved in pro-proliferative signaling in the breast. Because normal (cycling) mammary epithelial cells are devoid of estrogen receptor (ER) and PR, studies on the biochemistry of PR action have largely employed ER-positive (ER+)/PR-positive (PR+) human breast cancer cell lines (MCF-7, T47D and ZR-75). Emerging in vitro data suggest that PR extranuclear (nongenomic) actions lead to rapid activation of protein kinases (MAPK, PI3K/Akt and c-Src) in part by a ligand-induced interaction between PR and c-Src kinase [10–12]. Seminal work from Migliaccio et al. demonstrated that synthetic progesterone treatment rapidly activated c-Src and ERK2 (MAPK) in breast cancer cells (T47D), and this MAPK activation translated into an increase in T47D cell growth (Figure 2) [10]. These data showed that c-Src activation was dependent upon an interaction between PR, c-Src and, surprisingly, ERα; treatment with anti-estrogens blocked progesterone-induced MAPK activation. Interestingly, in these studies, no direct interaction between PR and c-Src was observed, implicating ER as a linker molecule in heterotrimeric signaling complexes. Subsequent work from this group identified two ER-interacting domains within PR that are responsible for mediating PR/ER/c-Src interactions [13]. Complementary work from Boonyaratanakornkit et al. reached a similar conclusion; rapid activation of c-Src/MAPK was observed following treatment with progestins [11]. However, in vitro, signaling occurred independently of PR interaction with ER. These researchers identified a direct interaction between an N-terminal proline-rich region of PR and the SH3-domain of c-Src. In contrast to what was previously observed (described earlier), progestin-induced MAPK levels were low (25% of EGF-treated positive control), and did not translate to increased cell growth; this group observed a drop in progesterone-induced cell growth inhibition in PR-null normal mammary epithelial cells (MCF10A) stably expressing mutant PR-B incapable of interacting with c-Src (relative to cells expressing wild-type PR-B) [11].

The rapid signaling and transcriptional activities of PR are integrated events. Although the rapid signaling actions of SRs take place independently of transcription (i.e., in seconds to minutes), it is becoming increasingly clear that membrane-initiated and nuclear functions of SRs are fully integrated events (Figure 2). For example, Faivre et al. first demonstrated a mechanism of progestin/PR-induced autocrine signaling in which rapid signaling complexes (containing PR and c-Src) are required for subsequent expression of PR-target genes (including EGF receptor [EGFR] and WNT1) [14]. In response to progestins, secreted WNT1 activates frizzled receptors on the cell surface, leading to matrix metalloproteinase production and cleavage of heparin-binding EGF molecules (i.e., to produce free EGF). Progestin-dependent transactivation of EGFR ultimately induces sustained MAPK activation, cyclin D1 expression, and increased cell proliferation and survival [14]. In this model, rapid or membrane-associated PR signaling induces c-Src- and MAPK-dependent phosphorylation of PR Ser345 [15]. Phosphorylation of PR Ser345 is required for PR tethering to SP1, a transcription factor mediator of progestin-responsive genes, such as p21 and EGF. These data demonstrated that PR-containing rapid signaling complexes function to transmit specific information (i.e., in the form of phosphorylation events) to genomic transcriptional complexes. Related to this concept, intriguing new data from Béguelin et al. defined a novel model for PR cross-talk with signaling complexes that involved progestin-
induced activation and nuclear translocation of ErbB2, a membrane-associated receptor tyrosine kinase [16]. Once localized in the nucleus, ErbB2 formed a transcriptional complex with PR and STAT3, serving as a transcriptional coactivator for STAT3 and controlling genes such as cyclin D1. Inhibiting formation of this transcriptional complex prevented progestin-driven PR/ErbB2-positive tumor growth in mouse models. Taken together, these data support a novel role for PR involving a hybrid of extranuclear and genomic actions: ligand-activated PR induces EGFR [14] or ErbB2 [16] transactivation and subsequent transcriptional complex formation, with nuclear PR being a critical component of this protein complex at selected gene promoters.

Whereas the protein complex components that are critical to support progestin-induced MAPK activation remain somewhat controversial (discussed earlier), all models tend to agree that rapid activation of MAPKs by progestins is mediated by membrane-associated PR, either directly or indirectly. Notably, SRs (ER, PR and androgen receptor) traffic to the plasma membrane, in part via heat-shock protein 27-dependent tethering, where they are reversibly palmitoylated in order to facilitate and prolong membrane location and function [17]. Work from these groups and others [12] underscored the important extra-nuclear role that SRs play in the rapid activation of cytoplasmic or membrane-associated protein kinases (c-Src and PI3K/Akt), and downstream signaling cascades (MAPKs). Importantly, these kinases modify regulatory sites on SRs, including ER and PR [15], and their coregulators [18], thereby integrating both rapid signaling and genomic actions.

Like other SRs, PRs are significantly post-translationally modified by phosphorylation, acetylation, sumoylation and ubiquitination [19–23]. These modifications are often ligand dependent, but can also occur independently of ligand binding (primarily in response to protein kinase activation), and significantly alter receptor stability, localization, tethering interactions, transcriptional activity and promoter selectivity [24]. For example, MAPK and cdk2 have previously been demonstrated to phosphorylate and modulate the activity of both liganded and unliganded PR [21,25–27]. Phospho-PRs are targeted to specific gene subsets, and subsequent specific transcriptional profiles depend on the phosphorylation status of PR [15,19,28]. Thus, a feed-forward loop between progestin-activated protein kinases and subsequent phosphorylation of PRs (by those same kinases) underlies the profound effects that activated kinases have on the nuclear functions of PR, particularly with regard to promoter selectivity [14,15,28]. With the exception of K303A ERα, a hyperactive mutant ER found in a subset of human breast cancers [29], one reason that ER and PR are seldom mutated is because these receptors are subject to intense epigenetic regulation (i.e., phosphorylation most often translates to gain of function) by the same protein kinases that are most often upregulated or constitutively activated in breast cancer. Because a myriad of post-translational inputs are capable of driving receptor and/or coregulator behaviors, there may be little pressure for adaptive mutations that accomplish that same task (however, the receptors are frequently overexpressed).

Growth factor- or SR-induced rapid signaling provides a mechanism for PR promoter selection. These types of data underscore the concept that so-called rapid signaling actions of SRs simply constitute a required step in the pathway to gene regulation and, specifically, promoter selection (i.e., by the very same receptors). That is, rapid and dynamic shuttling of SRs between the cytoplasmic and nuclear compartments allows for constant interaction with protein kinases; SRs are in fact sensors for the actions of growth factors and signaling molecules stationed within and at the plasma membrane. Thus, although extranuclear PR actions are often considered to be functionally distinct from downstream genomic PR events (they are most often studied separately), cytoplasmic and nuclear receptors are probably part of the same dynamic or ‘fluxing’ population (Figure 2). In response to hormonal cues, cycling populations of transiently membrane-localized PRs rapidly activate appropriate
protein kinase cascades. These kinases phosphorylate nearby substrates (i.e., membrane-tethered PRs and cytoplasmic coregulators). Entire complexes containing steroid receptor phospospecies, coregulators and signaling molecules (including kinases) then associate dynamically with regulatory regions/enhancers in DNA to activate or inhibit gene expression. This scheme explains why some SR-dependent promoters are exquisitely sensitive to alterations in protein kinase activities (a minority of receptors are membrane associated at any given time), while others are much more tightly regulated by steroid hormone alone [15]. Overall, kinase signaling (including SR-dependent rapid signaling) is a mechanism for promoter selection; it provides a means of quickly altering hormone responsiveness at some, but not all, promoters. This is an important facet of PR action and explains why PR gene signatures differ in normal versus neoplastic mammary epithelial cells [30]; under the influence of signal transduction pathways commonly activated in breast cancer cells, PR signaling and thus promoter selection, differs dramatically, resulting in altered cell/tumor biology.

Progesterone is a potent breast mitogen. Once a controversial notion, it is now well accepted that progesterone acts as a proliferative hormone in the breast, although it is paradoxically inhibitory in the reproductive tract and ovaries. A primary function of progesterone/PR is to mediate the massive expansion of epithelial-derived mammary alveoli (alveologenesis and organization of alveoli into lobules) during puberty and pregnancy in preparation for lactation. Increased serum levels of progesterone during the luteal phase of the menstrual cycle are coincident with a high proliferative index of epithelial cells in the milk duct system [31]. Likewise, during diestrus in mice, when progesterone levels rise by approximately fourfold, an increase in ductal structures is visible in mammary gland whole mounts [32]. Mouse knockout studies demonstrated that PR-B, rather than PR-A, is specifically required for the epithelial cell proliferation that is the basis of extensive mammary gland ductal side branching and alveologenesis [33]. Studies in receptor activator of NF-κB ligand (RANKL) and cyclin D1 (i.e., both major downstream effectors of PR)-deficient mice show similar blocks in alveologenesis [34,35], while receptor of activator of NF-κB (RANK)-transgenic mice express increased cyclin D1 and undergo increased hormone-driven proliferation and mammary tumor formation [36]. In contrast to PR-B, ERα is required for mammary ductal elongation prior to pregnancy when the gland is highly responsive to estrogen, but relatively unresponsive to progesterone [37]. Estrogen/ER also contributes to alveolar development, in part via induction of PR expression [38].

Steroid receptor action is required for normal mammary gland development. Like ERα, PR isoforms are found in a minority of mammary epithelial cells (MECs). These receptors are most often coexpressed, occurring in only approximately 10–20% of luminal epithelial cells in the normal mammary gland [37]. Multiple studies have concluded that SR-negative (SR-) cells comprise the majority of the proliferating (nonpregnant) normal MEC cohort [39–41]. Thus, in response to progesterone, it has been proposed that PR+ cells provide mitogenic paracrine signals that direct neighboring SR- cells to divide (Figure 3) [42]. Recently, Beleut et al. described two distinct mechanisms of progesterone-induced MEC proliferation that occurred in waves following progesterone administration to adult ovariectomized mice [43]. Initially peaking approximately 24 h post-treatment, a subset of PR+ cells (5% of MECs) in the luminal compartment were stimulated to divide. Cyclin D1, a PR target gene, was required for this cell-autonomous proliferative response. After approximately 3 days of progesterone treatment, there was a second wave of proliferation peaks (27% of MECs); this fraction of cells is PR null but dependent upon the PR-induced paracrine factor, RANKL, for mitogenic stimulation (Figure 3). Similarly, WNT4, another paracrine mitogen induced by PR, is required for progesterone-induced side-branching during the development of mammary ducts [44]. Other studies performed in mice and rats also illustrate that a small percentage of PR-B, but not PR-A, expressing MECs actively undergo cell division, as
measured by bromodeoxyuridine incorporation and PR co-staining; proliferation of PR-B containing cells becomes extensive during pregnancy [45]. Regulation of PR isoform expression is poorly understood in humans. However, in rodent models, estrogen induced PR-A expression, while progesterone alone or estrogen plus progesterone were required for significant PR-B expression [37]. In summary, in the normal breast, estrogen/ER may primarily act to increase PR-A expression [37], while progesterone/PR-B initiates a series of potent proliferative factors (WNT4, cyclin D1 and RANKL) for exquisitely timed expansion of the mammary gland. Hormone-dependent breast cancers undergo an early switch to autocrine growth signaling. Despite the relatively low abundance of MECs in the normal (i.e., nonpregnant) breast that express SRs, the majority of breast cancers are ER+/PR+ upon initial diagnosis [46]. Numerous models, both in vitro and in vivo, demonstrate that progesterone/PR remains a strong mitogenic and prosurvival stimulus within the context of breast cancer [8]. PR, in the presence and absence of ligand, induced anchorage-independent growth and increased survival in breast cancer cell lines [14,28,47]. In mouse models, mammary tumors induced by chemical carcinogens and genetic disruption of the tumor suppressor, BRCA1, were dependent on PR action [48,49]. In addition, administration of medroxyprogesterone acetate induced mammary carcinogenesis in multiple species, including mice [50]. Furthermore, in rats, CDB-4124, a clinically used (for uterine fibroids and endometriosis) antiprogestin/PR modulator (PRM), inhibited the appearance of spontaneous preneoplastic mammary lesions and N-methyl-N-nitrosourea-induced (ER+) mammary tumors, primarily via suppression of proliferation and induction of apoptosis [51]. A few small clinical trials have used additional PRMs to target PR in breast cancer with good success, despite cross-reactivity with glucocorticoid receptors [52,53]. Finally, large clinical trials have demonstrated that progestin added to hormone replacement therapy significantly increased the incidence and grade of breast tumors in post-menopausal women [54]. No increased risk was associated with estrogen alone [54,55], and estrogen-only hormone replacement therapy may be protective in some women. Synthetic progestins used in hormone replacement therapy clinical trials and progesterone have overlapping effects on PR [50]; therefore, progesterone is not considered an entirely safe alternative. Interestingly, gene-expression analysis of normal human MECs cultured in 3D relative to similarly cultured T47D human breast cancer cells showed distinct genetic profiles upon progestin treatment, indicating that progesterone-induced proliferative programs differ between normal and cancer cells [30]. This is not entirely surprising, considering that in the normal (non-pregnant) breast, the majority of proliferating cells are devoid of SRs and instead primarily divide in response to paracrine signals; in SR-positive breast tumors, PR-containing cells proliferate, presumably via autocrine mechanisms that may be WNT1-, EGFR- and cyclin D1-dependent [14]. In addition, mitogenic protein kinases (CDK2, c-Src, CK2 and MAPK), often upregulated in breast cancer, drive PR hypersensitivity to ligand and ligand-independent activity, and can also redirect phospho-PR to alternate promoters ([10,15,19,28,56]; discussed further later).

Progesterone mediates mammary gland stem cell self-renewal. Lifetime exposure to steroid hormones (either exogenous or endogenous) is a critical risk factor for the development of breast cancer. For example, a greater number of menstrual cycles (experienced over an individual’s lifetime) is correlated with increased breast cancer incidence [57]. Accumulating evidence implicates progesterone/PR in the maintenance and expansion of breast stem and progenitor cells. It has been proposed that mammary stem cells (MaSCs) comprise a population of putative primary targets for transformation to breast malignancies [58,59]. Quiescent MaSCs are thought to be activated during periods of glandular expansion, such as puberty and pregnancy [59–61], when progesterone levels are high. Early reports
described hormone receptor-positive (30–40%) and -negative cells that divide asymmetrically (as measured by DNA labeling) in mice undergoing puberty, and proliferate again in adulthood upon hormone administration [61–63]. Others reported mouse MaSCs to be ER-/PR- cells surrounded by myoepithelial and luminal cells, some of which express both ER and PR [64]. Similarly, in humans, the cell populations enriched for MaSCs have been reported to be both SR+ [65] and SR- [66]. It is likely that MaSCs are SR-, yet require local SR+ cells to provide paracrine signals [58]. Shackelton et al. were able to generate functional mammary glands from MaSCs isolated from a niche in the basal epithelial layer [60].

Recently, progesterone was shown to induce basal MaSC (CD49fhi) expansion in the diestrus phase of cycling female mice [32]. The authors suggest that PR induction of WNT4 and RANKL in the luminal compartment act in a paracrine manner to enrich the basal MaSC population. Genetically engineered mice with RANK deleted from mammary epithelial cells were resistant to progestin-induced epithelial proliferation and expansion of CD49hi stem cells; these mice also exhibited sensitization to DNA-damaging agents [67]. While these are intriguing results, the contribution of RANK to human breast development and cancer awaits confirmation [68]. In primary human breast cultures, Graham et al. described an increase in progenitor cell populations in response to progesterone treatment [30]. Recent work in human MECs showed that WNT1, a progesterone-regulated gene [14], is located upstream of Notch signaling [69], which is implicated in affecting stem cell self-renewal and lineage-specific differentiation in the mammary gland [70]. It is thus reasonable to predict that progesterone may also drive the expansion of breast cancer progenitor cells, a hypothesis examined by Horwitz et al. [71,72]. In these studies, T47D human breast cancer cell xenografts were reported to contain a rare population of basal-like CD44+ tumor-initiating cells (ER−PR−CK5+), an intermediate cell population (ER−PR−CK5−) and an expanding population of luminal-like cells (ER+PR+CK5−). Upon treatment with progestin, ER−PR−CK5+ cells were observed and ER−PR−CK5− cells were enriched. The authors propose that the ER+PR−CK5+ cells comprise a transitional cell population present in tumors that may retrogress to ER−PR−CK5+ cells in response to progestins [71,72]. As a result, progesterone maintenance and expansion of MaSCs may have implications for breast tumor stem cell populations; these cells are likely to be more resistant to traditional cancer therapies due to their ability to undergo quiescence, a state characterized by a high degree of resistance to apoptosis and agents that primarily target properties of rapidly dividing cells (i.e., classical chemotherapies). Going forward, it will be critical to delineate important similarities and differences between the various models used to study these hormone-dependent aspects of mammary gland biology; significant differences exist between mice (the primary genetic model employed in breast cancer research), humans and rats. The inclusion of more rat models may provide further insight into steroid receptor biology in mammary gland development and tumor progression (reviewed in [37]).

**Expert commentary**

Is the action of PRs in breast cancer a missed opportunity? Owing to a convergence of factors, PR action in breast cancer has been almost entirely overlooked. First, the topic is complex. The natural hormone, progesterone, has opposing effects according to target tissue and cell context. Progestins are mitogenic in the breast, but inhibitory in the uterus and ovaries; the basis for this divergence is still unknown. Human breast cancer cells cultured in 2D (plastic dishes) exhibit a biphasic pattern of growth in response to progesterone (when subjected to continuous progesterone treatment, they undergo one or more rounds of cell division and are then growth inhibited [73]). In addition, genetically engineered mice are the primary animal models used in breast cancer research. As both ER and PR are required for mammary gland development, interpretation of studies using ER- or PR-knockout mice are
limited in that these animals lack the structures/cells that give rise to breast cancer (i.e., mammary gland development is severely impaired). Unlike the human breast, the mouse mammary gland does not fully develop until pregnancy; virgin glands are relatively unresponsive to progesterone and primarily express PR-A, but contain very little PR-B. Indeed, few genetic mouse models develop ER+/PR+ mammary tumors [74]. Furthermore, studies of human breast oncogenes (i.e., transgenic mouse models) frequently evaluate virgin animals, making it impossible to implicate PR-B (i.e., the proliferative receptor) in tumor biology. This may partly explain why treatment of well-established animal tumor models with progestin (agonists) rarely augments tumor biology (although the use of antiprogestin [antagonists] is often inhibitory; discussed later). Antiprogestins were rejected in early human clinical trials not because they were not highly effective [53], but because they had significant cross-reactivity with their glucocorticoid receptor close cousins, resulting in intolerable side effects (reviewed in [75]). Finally, considerable political resistance has discouraged mainstream use of antiprogestins within the USA for any indication (i.e., the antiprogestin, RU486, is clinically known as ‘the abortion pill’); drug companies avoid the development of agents perceived to be unpopular or not sufficiently lucrative/patentable. For these unfortunate reasons (few of which are relevant to peer-reviewed science on this topic), PR isoforms are grossly understudied relative to ERα in the breast and breast cancer. In fact, experts suggest that PR is a highly relevant SR with respect to both normal and neoplastic breast epithelial cell proliferation [30], early breast cancer progression [51,76,77] and, more recently, mammary gland stem-cell biology [32]. Like ER, PR mutations are not commonly seen in the majority of breast cancers, although the normal 1:1 ratio of PR-A to PR-B is frequently altered [78]; the significance of this finding is unknown but probably relates to altered homeostasis and rapidly changing patterns of gene expression during early tumor development [30].

Why study progesterone/PR in the breast? ER is the first example and the primary focus of very successful ‘targeted’ breast cancer therapies. However, the actions of ER and PR are intimately linked in biology. PR is an important ER target gene and thus acts as a major downstream effector of estrogen action. As mentioned previously, historically, progesterone was assumed to have little to no effect on breast tumorigenesis, partly owing to its well-established inhibitory and differentiative role in the uterus and reproductive organs. However, more recently, progesterone has been implicated as a proliferative hormone in the normal breast [30] and a lifelong risk factor for breast cancer [55,79–84]. Notably, as with ER, there is extensive cross-talk between PR and the same signal transduction pathways that are required for mammary gland development and are most often elevated in breast cancer. For example, the proliferative effects of progesterone are highly dependent upon tyrosine kinase growth factor receptors (EGFR family members) and their downstream protein kinase effectors (c-Src and MAPKs); these effects (i.e., cell proliferation) map to direct phosphorylation of PR-B, but not PR-A [14]. Cross-talk between PR-B and the EGFR pathway provides a basis for understanding mechanisms of transcriptional synergy between progestins and EGF on numerous endogenous genes that are highly relevant to breast cancer biology [85]. PR target genes, such as WNTs [14,44], are secreted factors that may contribute to paracrine and autocrine proliferation signals during progression to malignant transformation [69]. The physiological significance of EGF-induced PR-B hyperactivation relates to the key role of both molecules, along with ERα, as mediators of massive alveolar proliferation during mammary gland development/early pregnancy [86]. This interplay between growth factors and both SR (ER/PR) functions (inappropriately) during breast cancer progression, when tyrosine kinase activities are elevated and hyperactive SRs are still present and functional (although frequently at low abundance; discussed further later). For this reason, targeted therapies against ER and ErbB (EGFR/ErbB2) family members are now a clinical mainstay, but their success can be limited by mechanisms of tumor progression. The addition of PR-blocking therapies to this list could be life saving; anti-progestins are
predicted to severely impair the process of tumor progression (i.e., by blocking PR-induced upregulation of signaling pathway intermediates that include known mediators of endocrine resistance), which invariably occurs upon exposure to anti-estrogens or estrogen blockers [87–89]. Indeed, this is a missed opportunity for women facing fewer and fewer treatment options as they fail classical endocrine therapies.

More abundant PR may not translate to increased transcriptional activity. An early event in tumor development includes an altered ratio of coexpressed PR-A to PR-B (normally observed to be 1:1), with loss of PR-B (i.e., apparent predominance of PR-A) occurring most often [78,90]. The natural assumption is that PR-A is thus the dominant isoform, perhaps even driving tumor phenotype. However, it is also well appreciated that liganded SRs are rapidly downregulated relative to their inactive forms. Thus, the expression of phosphorylated receptors (namely PR-B) may appear to be low in PR-driven tumors due to increased phospho-PR ubiquitinylation and rapid protein ‘loss’ by proteasome-mediated turnover of activated receptors [21]; growth factors also ultimately lower PR mRNA expression via reversible mechanisms [21,91,92]. SR proteins and their coregulators are direct targets of growth factor-activated cytoplasmic protein kinases. Thus, a ‘vicious cycle’ is created, wherein growth factors induce phosphorylated and transcriptionally hyperactive PRs that turn over even more rapidly, making low-abundance receptors nearly ‘invisible’ at the protein level. However, their robust nuclear activity is clearly detected in reporter gene assays and at endogenous genes using subphysiologic hormone concentrations [19,21,85]. In fact, apparent ‘loss’ of PR is an excellent clinical marker of high growth factor receptor expression and activity [92]. This high-kinase condition is responsible for phosphorylating PR and increasing both its transcriptional activity and rate of turnover. Hyperactive PR protein may be relatively undetectable by clinically employed antibody-binding assays; when protein levels are measured, clinicians may mistakenly conclude that apparently ‘PR-null’ tumors have escaped hormonal regulation. Instead, ‘loss’ of PR-B may in fact be an excellent early marker of PR-B-driven biology; similar mechanisms have been reported for ER in breast cancer cells containing activated c-Src kinase [93]. Importantly, we reported that hyperactive (deSUMOylated) phospho-PR-B is capable of driving breast cancer cell proliferation and survival via the transcriptional regulation of novel PR target genes that are not known to be particularly responsive to progestin alone, but are very responsive to high kinase activities [94]. Surprisingly, these genes include novel phospho-PR-regulated genes and ER-regulated genes. Because hyperactive phospho-PR-B is largely deSUMOylated [19], it also fails to transrepress ER [23]; we suspect that the two receptors (PR and ER) cooperate at many of the same genes.

The development of the ER+/PR-null tumor phenotype may be PR driven. There is considerable functional overlap between ER and PR. Notably, many ER-regulated genes are also PR regulated (including c-myc, cyclin D1, c-fos, STATs and IGF pathway components), and these receptors even tether to the same transcription factors (activator protein 1 and SP1) to regulate nonclassical target genes (which contain no hormone-responsive element). ERα and PR-B also participate in similar membrane-associated, cytoplasmic (or ‘rapid’) signaling complexes (discussed previously) in association with EGFR and c-Src kinase upstream of the ERK1/2 MAPK module [10]. ERα or PR-B localized near the cell membrane are both capable of transactivating EGFR [14,95]. In fact, steroid hormone-induced rapid activation of MAPK appears to be most robust when both ERα and PR-B are coexpressed in model cell lines [10,13]. The end point of MAPK signaling is most often the regulation of nuclear transcription factors. Indeed, ER and PR are direct targets (substrates) of mitogenic protein kinases, including MAPK. This cross-talk even extends to the regulation of ER/PR interactions (Figure 4). In response to progesterone binding, SUMOylated PR isoforms (both A and B) transrepress ER [23]; MAPK-dependent phosphorylation events (namely PR Ser294) lift this repression by blocking PR.

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SUMOylation [19]. ER and PR are most often coexpressed in early-stage breast cancer. Loss of PR mRNA and protein can indicate a functional loss of ER (ER+/PR-low or -null); this is a common assumption. However, an alternative pathway exists in which phospho-PR is under-SUMOylated and thus no longer able to transrepress ER (Figure 4). Phospho-PR instead behaves as a hyperactive or constitutive (i.e., ligand-independent) transcription factor at selected gene promoters, including those classically regulated by ER [28].

**Five-year view**

Future studies should focus on the goal of defining the contribution of protein kinase inputs to PR-dependent signaling and PR/ER cross-talk in breast cancers that are classically believed to be ‘ER driven’ but are resistant to anti-estrogen therapy (and may in fact be PR driven). SR-specific gene signatures, rather than protein levels (often limited to a small sampling of the tumor), should be used clinically to assess hormone responsiveness. With the development of more selective antiprogestins [51], the opportunity to understand and target the ER+/PR ‘loss’ phenotype as a means of combating early progression to hormone-refractory breast cancer is within reach; this phenotype can be clearly defined by the presence of a phospho-PR-B gene signature, predicted to be a sensitive and reliable readout of PR activity when PR protein levels appear to be reduced. Related to this idea, we recently defined a phospho-PR gene signature that includes both ligand-dependent and -independent PR-regulated genes; our signature predicts a high likelihood of rapid progression to breast cancer metastasis [Knutson T, Lange C, Unpublished Data]. It will now be important to validate this exciting finding in preclinical models of human breast cancer.

A wealth of basic and clinical studies have implicated PR action in breast cancer. However, only a fraction of information is known compared with what is known about ER, which was the first example of highly successful targeted therapy. A few tenants of PR action have emerged: PRs behave quite differently with regard to isoform specificity and cellular context (i.e., breast vs uterus or normal vs neoplastic cells); altered PR behavior is in large part conferred by the actions of activated protein kinases; PR hypersensitivity that approaches ligand independence is driven by phosphorylation events and may be significant in certain contexts; and phospho-PR may precede/mark the near complete loss of PR protein and later growth factor-driven suppression of PR mRNA that occurs during the development and progression of endocrine-resistant luminal B-type (ER+/PR−) breast cancers. Indeed, the most appropriate use for PRMs may be during early breast cancer development or very early tumor progression (i.e., before PR levels drop precipitously). There is an increasingly recognized need to prevent or reverse the development of early lesions (i.e., that may or may not ever progress); this is a largely untapped area that warrants intense scrutiny of PRs as potentially important drivers of an early switch from SR-dependent paracrine to autocrine signaling mechanisms. The ultimate degree of aggressiveness of progressing tumors may be determined early on, partly dictated by the influence of progesterone/PR on the stem-cell compartment. An increased understanding of PR function and cross-talk with ER in normal, pre-neoplastic and neoplastic settings, as well as stronger advocacy from scientist-, clinician- and patient/survivor-led groups are needed to overcome remaining resistance to the goal of including PR-targeted strategies as part of the repertory of mainstream endocrine/ER-based therapies.

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Papers of special note have been highlighted as:
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Key issues

- While mouse models have significantly expanded our knowledge of breast disease, through the development and utilization of rat models we may achieve a more balanced understanding of steroid receptor regulation in breast cancer. Such models provide insight into the complex hormone-driven mechanisms of human breast cancer development and early progression, which represents a significant gap in our knowledge.

- Clinicians need to consider progesterone receptor (PR)-A and -B isoform-specific expression and action in human tumors (rather than total PR levels). Assay of well-characterized phosphorylated residues on both estrogen receptor (ER) and PR may predict clinical outcome more accurately; incorporation of steroid receptor-specific gene signatures as indicators of transcriptional activity and thus steroid receptor-driven biology is timely and feasible, and may provide the ultimate readout of endocrine status.

- Important cross-talk between growth factors and PR and between both PR isoforms and ER exists (and is the subject of highly valuable targeted therapies); PR action has been widely overlooked in this scheme. Scientists and clinicians need to work together on the development of preclinical models that clearly evaluate PR action and PR cross-talk with ER, with the goal of advancing towards routine use of PR-targeted therapies as a significant and life-saving improvement to classical endocrine therapy.
Figure 1. **Progesterone receptor isoforms are sensors for growth factor-induced signaling**

PR-B and truncated PR-A are substrates for mitogenic protein kinases, including CDK2 (up to eight sites, including Ser400), MAPKs (Ser294 and Ser345) and CK2 (Ser81). Phosphorylated receptors and/or coregulators of transcription (such as steroid receptor coactivators) mediate promoter selection and sensitivity of PR target genes to progesterone and other hormones, including peptide growth factors (EGF, FGF receptor or IGF). Up to 14 sites (stars) in PR-B are phosphorylated either basally and/or in response to hormone action; MAPK- or CDK2-dependent phosphorylation of PR Ser294 facilitates ligand-dependent nuclear export and receptor downregulation via targeting to the ubiquitin–proteasome pathway.

Figure 2. Progesterone receptor-B, but not progesterone receptor-A, and estrogen receptor-α participate in membrane-tethered protein complexes capable of rapidly activating c-Src and MAPKs

Progesterone/PR and estrogen/ER transactivate EGFR and/or ErbB2; phosphorylated steroid hormone receptors and signaling molecules, including protein kinases and surface receptors, enter the nucleus and participate in transcription complexes at selected gene promoters.

E2: Estradiol; EGFR: EGF receptor; ER: Estrogen receptor; P: Phosphorylation; P₄: Progesterone; PR: Progesterone receptor; Shc: Src homology domain II containing.
Figure 3. Proliferating cells in the normal (non-pregnant) mammary gland are typically steroid hormone receptor null
ER and PR isoforms are coexpressed in a minority population of mammary epithelial cells that lie adjacent to proliferating (cyclin D1-positive) steroid receptor-negative cells. Progesterone/PR-dependent paracrine factors (WNTs, RANKL and IGF-II) induce neighboring (PR-null) cells to undergo proliferation. An early switch to autocrine signaling mechanisms occurs in the development of ER-positive/PR-positive breast cancers.
ER: Estrogen receptor; PR: Progesterone receptor.
Figure 4. Reversible progesterone receptor Ser388 SUMOylation provides a mechanism for rapid changes in hormone responsiveness according to extracellular cues
PRs are rapidly SUMOylated in response to progesterone binding. SUMOylated PR species are tenfold less active on selected gene promoters and capable of ER transrepression (by unknown mechanisms). Growth factor-induced MAPK activation leading to phosphorylation of PR Ser294 prevents PR Ser388 SUMOylation, thereby lifting SUMO-dependent repression of both PR and ER transcriptional activities. Phosphorylated and deSUMOylated PR-B drives breast cancer cell proliferation and survival.

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