Modifications to glucocorticoid and progesterone receptors alter cell fate in breast cancer

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Abstract
Steroid hormone receptors (SRs) are heavily posttranslationally modified by the reversible addition of a variety of molecular moieties, including phosphorylation, acetylation, methylation, SUMOylation, and ubiquitination. These rapid and dynamic modifications may be combinatorial and interact (i.e. may be sequential, complement, or oppose each other), creating a vast array of uniquely modified receptor subspecies that allow for diverse receptor behaviors that enable highly sensitive and context-dependent hormone action. For example, in response to hormone or growth factor membrane-initiated signaling events, posttranslational modifications (PTMs) to SRs alter protein–protein interactions that govern the complex process of promoter or gene-set selection coupled to transcriptional repression or activation. Unique phosphorylation events allow SRs to associate or dissociate with specific cofactors that may include pioneer factors and other tethering partners, which specify the resulting transcriptome and ultimately change cell fate. The impact of PTMs on SR action is particularly profound in the context of breast tumorigenesis, in which frequent alterations in growth factor-initiated signaling pathways occur early and act as drivers of breast cancer progression toward endocrine resistance. In this article, with primary focus on breast cancer relevance, we review the mechanisms by which PTMs, including reversible phosphorylation events, regulate the closely related SRs, glucocorticoid receptor and progesterone receptor, allowing for precise biological responses to ever-changing hormonal stimuli.

Introduction
Steroid hormone receptors (SRs) belong to the nuclear receptor superfamily of ligand-activated transcription factors. Members include the mineralocorticoid receptors, androgen receptors (ARs), estrogen receptors (ERs), progesterone receptors (PRs), and glucocorticoid receptors (GRs). SRs are evolutionarily conserved and likely originated from a common ancestor via multiple gene duplication over 400 million years ago (Thornton 2001). They are modular proteins that share a common structure with three important functional domains defined as the N-terminal domain (NTD), DNA-binding domain (DBD), and C-terminal ligand-binding domain (LBD). The NTD contains a variable domain, which differs significantly in size and sequence between receptors. A strong transcriptional activating function (AF1) motif is typically found in the NTD and is responsible for binding cofactors and components of the basal transcription machinery; selected SR family members, including GR and PR, have two or three AF motifs (Fig. 1).
The DBD is centrally located and consists of two highly conserved zinc fingers that recognize and bind specific DNA sequences. It is the most conserved domain throughout the superfamily. The C-terminal region of steroid receptors contains the LBD, which is connected to the DBD via a hinge region (reviewed in Mangelsdorf et al. 1995). The LBD is composed of α-helices and β-sheets, which form a hydrophobic pocket to allow for ligand binding. In general, the binding of cognate ligands to the LBD of SRs classically acts as a ‘molecular switch’ that alters the conformation of the receptor, shifting the receptor to a transcriptionally active conformation capable of binding coactivators with high affinity.

SRs rapidly shuttle between the cytoplasm and the nucleus in both the absence and the presence of hormone ligands. Unliganded SRs are typically bound in multiprotein cytoplasmic complexes containing heat-shock protein (HSP) chaperone molecules, including HSP70 and HSP90. Upon ligand binding, receptors undergo a conformational change, resulting in dissociation of HSP-containing complexes and greater SR retention in the nucleus. Liganded SRs bind hormone response elements (HREs) in chromatin as dimers that activate or repress transcription of target genes via recruitment of coactivators or corepressors. In addition to directly binding DNA elements, SRs regulate gene expression via tethering to other transcription factors. For example, PR tethers to DNA-bound SP1 transcription factors to regulate the expression of genes such as p21 and EGFR, which lack conical progesterone response elements (PREs) within their proximal promoter regions (Favier et al. 2008). Additionally, SRs often regulate target genes via tethering and/or direct binding to HRE enhancer elements located great distances (i.e. kilobases) from the transcriptional start sites of the genes they repress or activate (Carroll et al. 2006).

In addition to their classical roles in tissue development and homeostasis, selected SRs have well-established functions as drivers of hormone-associated cancers. ERs and ARs are primary therapeutic targets for breast and prostate cancers, respectively, due to their role in cancer proliferation or survival and tumor progression. These receptors have recently been shown to interact as part of novel transcription complexes that function to promote endocrine therapy resistance (De Amicis et al. 2010, Ciupek et al. 2015, Daniel et al. 2015). In addition to ER:AR transcriptional complexes (Rechoum et al. 2014), GR:AR (reviewed in Narayanan et al. 2015) and ER:PR (Giulianelli et al. 2012, Daniel et al. 2015, Mohammed et al. 2015) cooperation also occur in breast and prostate cancer models. For example, PR is emerging as an important modifier of luminal (ER+) breast cancer development and progression, acting in part via novel ER/PR/PELP1 signaling and transcriptional complexes (Daniel et al. 2015). In the presence of both estrogen and progesterone, PR action in ER/PR complexes appears to be inhibitory in transient assays of breast cancer cell proliferation (Mohammed et al. 2015), but this effect is variable and
GRs are critical sensors of physiologic and cell stress

Physiologic homeostasis is maintained in the presence of environmental and physiological stresses primarily through the production and effects of the glucocorticoid stress hormones. In humans, cortisol is the primary adrenal-derived glucocorticoid secreted in response to stress. The production of cortisol is mediated by the hypothalamic–pituitary–adrenal axis and results in secretion of cortisol from the adrenal cortex following physical, emotional, or mental stress stimuli. The physiological action of cortisol is mediated by binding to the GR. GR is expressed in all cells of the body, except for nonnucleated red blood cells. Following ligand binding, GRs regulate diverse gene programs resulting in changes in metabolism, immune system function, and central nervous system function, among others, as part of the complex processes required to maintain homeostasis and adapt to the challenges of multicellular life.

Cellular responses to glucocorticoids and GR signaling are not uniform. In fact, the effects of GR signaling are highly cell type specific. Glucocorticoids have powerful anti-inflammatory and immunosuppressive actions, as GR signaling causes apoptosis of lymphocytes and monocytes (Schmidt et al. 2004). As such, steroids are widely prescribed for reduction of inflammation and for treatment of hematological malignancies and following organ transplantation. In sharp contrast to their effects on the above blood cell types, glucocorticoids promote survival in a variety of epithelial cell types, including cells of the liver, ovarian follicle, endometrium, and mammary glands. In the context of wound healing, this dichotomy results in apoptosis and repression of the immune response, while simultaneously helping to preserve and stimulate regrowth in damaged organs eliciting the immune response. The impact of glucocorticoid signaling on epithelial cell survival is clinically relevant, as patients with breast cancer who receive adjuvant or neoadjuvant chemotherapy treatment are typically given high-dose glucocorticoids 1 h before receiving chemotherapy, in order to alleviate adverse side effects, including inflammation, edema, and nausea. However, studies have shown that in the context of tumors of epithelial origin, glucocorticoids lessen the effectiveness of chemotherapy treatments. This phenomenon has now been demonstrated in numerous in vitro and in vivo models of solid tumors (Wu et al. 2005, Pang et al. 2006, Zhang et al. 2007, Pan et al. 2011).
Multiple isoforms of human GR have been identified. The classic GRα protein consists of all nine exons in the GR gene and exerts the canonical function of GR. Further diversity among GR isoforms is generated via alternative translation initiation of GRα mRNA, as multiple AUG start codons exist in the GRα mRNA sequence (Zhou & Cidlowski 2005, Galliher-Beckley & Cidlowski 2009, Oakley & Cidlowski 2013). These GRα isoforms differ in their NTDs and have distinct cellular localization and gene regulatory profiles (Zhou & Cidlowski 2005, Galliher-Beckley & Cidlowski 2009, Oakley & Cidlowski 2013).

The full impact of expression of the various GR isoforms remains to be discovered. However, as numerous serine and threonine residues in the GR NTD are posttranslationally phosphorylated, differences in phosphorylation may also significantly alter isoform function. Additional PTMs to GRs include ubiquitination, acetylation, and SUMOylation. These reversible PTMs ultimately alter the receptor activity and enhance the functional diversity of the receptors and thus profoundly impact subsequent signaling. For example, ubiquitination at Lys-419 in a conserved PEST degradation motif targets GR for proteosomal degradation in a ligand-dependent manner, thus altering receptor half-life and transcriptional activity (Wallace & Cidlowski 2001, Dereoo et al. 2002). GRs are also modified by acetylation at Lys-494 and Lys-495 in response to glucocorticoids. This modification impairs the ability of GR to interfere with NF-κB signaling (Ito et al. 2006).

**Posttranslational modifications of GR**

Posttranslational modifications (PTMs) are critical for GR complex formation and recruitment of cofactors. Differential recruitment of coactivators or corepressors to the transcriptional complex is a critical step in expression or repression of SR target genes. SUMOylation, the addition of small ubiquitin-related modifier (SUMO) peptides, at numerous lysine residues diminishes GR transcriptional activity by the recruitment of corepressors and promotes GR degradation through the proteasome. Interestingly, phosphorylation of GR at Ser246 by c-Jun N-terminal kinase facilitates subsequent SUMOylation at Lys277 (rat 297) and Lys293 (rat 313) in the LBD, which function to repress GR transcriptional activity. These studies highlight the role of complex regulatory events occurring between PTMs and steroid receptor transcriptional activity (Iniguez-Lluhi & Pearce 2000, Tian et al. 2002, Holmstrom et al. 2003, Lin et al. 2006, Davies et al. 2008). Numerous corepressors have been found to interact with SUMOylated steroid receptors, including GR. One such protein is DAXX (death-domain associated protein), a transcriptional corepressor (Lin et al. 2006). However, this PTM (i.e. SUMOylation) is more complicated than simply leading to repression of receptor activity. A recent study demonstrated that SUMOylation of GR regulates occupancy of the receptor on chromatin of endogenous gene targets and influences target gene selectivity (Paakinaho et al. 2014). Similar to studies with deSUMOylated/SUMOylated PRs (Knutson et al. 2012), there are, in fact, both upregulated and downregulated genes in cells expressing SUMOylation-deficient GR (GR3KR), in which lysines 293, 297, and 703 were mutated to arginines, relative to cells expressing wild-type (WT) GR. For example, numerous anti proliferative genes are robustly induced in response to ligand in cells expressing WT GR, whereas these genes exhibit diminished expression in response to ligand in cells expressing SUMOylation-deficient GR. These data suggest that the sole function of SUMOylation is not gene repression. Indeed, Paakinaho and coworkers demonstrated that SUMOylation induces a genome-wide chromatin occupancy redistribution of GR (Knutson et al. 2012, Paakinaho et al. 2014). GR is also SUMOylated at Lys703 (K703) (rat 721). Druker and his coworkers have shown that interaction with the SUMOylation enhancer, RWD-containing SUMOylation enhancer, increases GR SUMOylation at K703 and subsequent transcription of target genes such as FKBP51 (Druker et al. 2013). It would be interesting to determine if differences in GR SUMOylation contribute to altered GR-induced gene expression in cancer (i.e. epithelial) cells relative to GR+ cells within the immune system (i.e. lymphocytes).

The most widely studied PTM of GR is phosphorylation. Multiple serine residues are conserved phosphorylation sites between human, mouse, and rat GRα, and include Ser113, Ser134, Ser141, Ser203, Ser211, Ser226, and Ser404. These serine residues typically display a basal level of phosphorylation that is enhanced upon ligand binding (Galliher-Beckley & Cidlowski 2009, Oakley & Cidlowski 2013). However, certain phosphorylation events are known to be regulated ligand independently, such as phosphorylation at Ser134 by p38 MAPKs in response to cellular stress stimuli (further discussed next) (Galliher-Beckley et al. 2011). As with PR (discussed next) GR phosphorylation profoundly impacts the transcriptional activity and promoter selectivity of GR. Notably, phosphorylation at specific residues globally alters the transcriptional profile of GR in response to ligand. Ser211 is phosphorylated in response to ligand binding in a robust and sustained manner. Moreover, the
transcriptional activity of GR is correlated with the level of Ser211 phosphorylation, suggesting this site as a marker for activated GR signaling. In accordance with these data, phospho-Ser211 GR localizes primarily to the nucleus (Wang et al. 2002). Importantly, phosphorylation of GR at Ser211 is required to mediate the induction of apoptosis in lymphocytes in response to glucocorticoids (Miller et al. 2005, 2007). Conversely, certain posttranslational phosphorylation events can cause diminished activity of steroid receptors. For example, GR is phosphorylated at serine 404 (Ser404) in response to hormone by glycogen synthase kinase 3 beta. This phosphorylation event induced dampened GR transcriptional activity and repression of GR target genes. Moreover, cell lines expressing mutant GR incapable of being phosphorylated at Ser404 exhibited altered target gene selectivity and global transcriptional activity (Galliher-Beckley et al. 2008). These studies showcase the importance of PTMs in generating diverse yet highly tissue-specific responses to hormones and their cognate SR partners.

High expression of GR predicts poor outcome in TNBC

Extensive work by Conzen and coworkers (Wu et al. 2004, Wu et al. 2005, Pang et al. 2006, Pan et al. 2011) has defined the context-dependent function of GR activity in breast cancer cells. GR signaling has distinct functions in ER-positive versus ER-negative subtypes of breast cancer. High expression of GR in the triple-negative subtype of breast cancer, which lacks the expression of ER, PR, and HER2 growth factor receptor amplification, is significantly associated with chemoresistance and recurrence of disease. Therefore, in ER-negative breast cancers, high GR expression is a poor prognostic marker (Pan et al. 2011). However, in luminal breast cancer subtypes that are ER positive, as with PR expression, high GR expression is associated with an increased overall survival and a more favorable prognosis. Cross talk between ER and GR likely accounts for the differential impact of GR expression and activity across breast cancer subtypes. In breast cancer cells lacking ER expression, GR signaling promotes the expression of multiple gene products that have known functions in promoting cell survival, including SGK1, DUSP1, and BCL family proteins (Wu et al. 2004) and PTK6/Brk (Regan Anderson 2016). Moreover, in xenograft models of epithelial-derived tumors, including breast, treatment with the synthetic glucocorticoid dexamethasone significantly impairs chemotherapy (Zhang et al. 2007). Thus, in cells lacking ER expression, GR signaling has been shown to enhance cell survival and promote resistance to chemotherapy in vitro and in vivo. These studies underscore the need to fully understand the biological actions and clinical implications of SR expression and SR cross talk across breast cancer subtypes.

Of further relevance to PTMs and context-dependent actions of GR in breast cancer cells, ligand-independent phosphorylation of GR at Ser134 has been recently reported using an osteocarcoma cell line model (Galliher-Beckley et al. 2011). This unique phosphorylation event was found to be constitutive, ligand independent, and greatly enhanced following exposure to physiologic cell stress, such as UVC radiation, glucose starvation, and exposure to reactive oxygen species. Specifically, cell stress stimuli resulted in the activation of p38 MAPK, which in turn phosphorylated GR at Ser134 (Galliher-Beckley et al. 2011). High levels of activated phospho-p38 MAPK expression in breast tumors are significantly associated with increased Ki67 staining and decreased progression-free survival in HER2-negative patients (Esteva et al. 2004). Moreover, inhibition of p38 MAPK signaling in TNBC cells inhibited cell proliferation and anchorage-independent growth, suggesting that this kinase has important prosurvival roles in breast cancer (Chen et al. 2009). Phosphorylation of GR at Ser134 was found to significantly enhance GR association with the 14-3-3 zeta adaptor protein (Galliher-Beckley et al. 2011). 14-3-3 zeta is a potent mediator of signal transduction pathways, thus linking phosphorylation of GR at Ser134 with robust activation of downstream pathways. Notably, 14-3-3 zeta has also been implicated as a driver of aggressive phenotypes in breast cancer. High expression of 14-3-3 zeta in patient tumors was predictive of significantly decreased overall survival. The association of phospho-Ser134 GR with 14-3-3 zeta dramatically altered global GR promoter selectivity and subsequent target gene expression (Galliher-Beckley et al. 2011). Although this finding must be confirmed in breast cancer models, targeting 14-3-3 zeta/phospho-Ser134 GR complexes may offer exciting new therapeutic avenues for patients with TNBC.

Recent studies from our laboratory have investigated the function of phospho-Ser134 GR in TNBC. Notably, we have discovered a novel interaction between GR and the steroid receptor coactivator proline-, glutamic acid- and leucine-rich protein 1 (PELP1). PELP1 and GR interact basally, and this interaction is enhanced upon dexamethasone treatment (Regan Anderson 2016). Studies in which Ser134 has been mutated to alanine, which cannot be phosphorylated, demonstrated the requirement of
phospho-Ser134 for the interaction of GR and PELP1. Importantly, a novel PELP1 inhibitor, D2, blocks dexamethasone-induced GR and PELP1 recruitment and subsequent induction of mRNA expression of a novel GR/PELP1 target gene, breast tumor kinase (Brk/PTK6), a known driver of aggressive phenotypes in breast cancer cells (Fig. 2). Taken together, these studies suggest that targeting phospho-Ser134 GR (or the mediator of this PTM to GR) in TNBCs with high p38 MAPK activity and/or PELP1 and 14-3-3 zeta expression (i.e. as novel biomarkers of GR-driven disease) may be a useful strategy.

Structure and isoform-specific actions of PRs

PGR (i.e. the gene name for PR) is an estrogen-responsive gene, largely reliant on estrogen and ER signaling for robust expression, although exceptions exist (Hilton et al. 2014a,b) (discussed later). Two predominant PR isoforms (A and B) are created from the same gene and mRNA precursors via the use of alternate internal translational start sites (truncated PR-A is missing the first 164 amino acids found in full-length PR-B). Thus, PR-A lacks the N-terminal B-upstream segment (BUS) unique to PR-B, which contains important structural and regulatory components that alter PR-binding partners, promoter selectivity, and transcriptional activity (Fig. 1). For example, located within the BUS lies a common docking (CD) domain, numerous phosphorylation sites (including Ser81 discussed later), and an additional (third) activating domain (AF-3), all of which have important roles in PR-B-specific actions. Although both PR isoforms are generally coexpressed in PR+ tissues, they are not always found together in the same cells (Aupperlee et al. 2005). Knockout mouse studies demonstrated that PR-B (120 kDa) is required for mammary gland development, whereas PR-A (94 kDa) is required for uterine development. A third isoform (primarily expressed in the uterus) termed PR-C (60 kDa) is a further truncated receptor lacking the DBD that appears to antagonize uterine-specific actions of PR-B (Condon et al. 2006).

Liganded ER has been termed a ‘permissive’ factor for PR-dependent ductal side-branching and proliferative expansion of the mammary epithelial cell (MEC) compartment during puberty and pregnancy (Tanos et al. 2013). Though ER and PR are expressed in only ~10–15% of MECs (i.e. in nonpregnant adults), PR-regulated genes include both autocrine and paracrine signals. Proliferative signaling occurs via an initial (autocrine) wave within PR+ cells that express cyclin D1 in response to progesterone, followed by a larger (paracrine) wave that stimulates proliferation of nearby PR-negative cells (Fata et al. 2001), including mammary stem or progenitor cells (Asselin-Labat et al. 2010, Joshi et al. 2010). Specifically, PR-positive cells secrete Wnt4 and RANKL, which act on PR-null mammary stem cells to activate NF-κB signaling, leading to their self-renewal (Joshi et al. 2010). A similar...
scenario is emerging in luminal (ER+) breast cancer models, in which PR signaling drives the appearance of luminal breast cancer stem cell markers (CK5, KLF4) that ultimately contribute to increased tumor heterogeneity and endocrine resistance (Axlund et al. 2013, Cittelly et al. 2013). In breast cancer models (primarily maintained in 2D cultures), progesterone has both proliferative and antiproliferative actions (Musgrove et al. 1991, Del Vecchio & Sutherland 1997, Groshong et al. 1997). In 3D or soft agar conditions that maintain epithelial cell polarity, progesterone is proliferative (Manni et al. 1991, Faivre & Lange 2007, Tanos et al. 2013). Similarly, in the presence of estrogen, PR-B is primarily proliferative, whereas PR-A inhibits the proliferative actions of progesterone (Fig. 3). The proliferative actions of progesterone are entirely dependent on PTMs to PR-B (namely, phosphorylation events) (Faivre & Lange 2007, Qiu et al. 2003). PR phosphorylation at Ser294 is a common event in breast tumors (T P Knutson and C A Lange, unpublished results). Recent studies highlighting the importance of ER/PR cross talk in luminal breast cancer confirmed the transient growth inhibitory actions of progestins in the presence of estrogen but did not measure time-dependent accumulation of heterogeneous cell types, including progenitor cells expressing stem cell markers (Daniel et al. 2015, Mohammed et al. 2015); and further discussed below). In the uterus, stromal PR (namely, PR-A) blocks the proliferative effects of epithelial ERα. The detailed actions of PR isoforms in the reproductive tract are the topic of a recent review (Diep et al. 2015a).

PR-A and -B isoforms have distinct but overlapping transcriptional functions that are highly dependent on receptor PTMs. Although PR-B typically acts as a more potent transcription factor relative to PR-A on hormone-induced (i.e. activated) target genes, the ratio of PR isoforms and their specific PTMs may dramatically alter hormone sensitivity. For example, PR SUMOylation at K388 is a repressive modification (on a majority of PR target genes, while a subset is activated as with GR) that is opposed by phosphorylation at Ser294 (Daniel et al. 2007a). SUMOylated PR-A transrepresses PR-B (Daniel et al. 2007a), while phosphorylated (Ser294) PR-A is capable of transactivating PR-B on selected target genes (Diep et al. 2015b). PR isoforms may undergo differential rates of ligand-dependent downregulation (Faivre & Lange 2007) that is impacted by PTMs (Lange et al. 2000). When phosphorylated at Ser294, deSUMOylated PR-B is rapidly degraded by the ubiquitin pathway relative to PR-A (Faivre & Lange 2007). Imbalanced protein expression of PR isoforms away from the normal 1:1 ratio (i.e. via loss or gain of either isoform) appears to be an early event in breast cancer (Mote et al. 2002) and may signify the emergence of activated growth factor signaling pathways that drive unequal PR isoform activity and loss (Shen et al. 2001, Cui et al. 2002). However, the clinical significance of altered PR isoform expression is unknown (individual PR isoforms are not routinely assayed as part of luminal breast cancer diagnosis).

As noted previously, SRs, including PR isoforms, typically undergo rapid and dynamic nuclear-cytoplasmic shuttling, an action that is required for their transcriptional activity (Guiochon-Mantel et al. 1991). Cytoplasmic receptors associate with HSPs, chaperones, and signaling molecules such as PI3K, c-Src, and other downstream protein kinases (MAPKs). Like ER, PR contains a palmitoylation motif important for tethering to the inner surface of the plasma membrane (Pedram et al. 2007).
A fraction (~5%) of ER and PR appears to be membrane associated, and membrane localization may vary according to cell type and hormonal context (Razandi et al. 2010). Ligand binding to ER or PR triggers rapid ‘extranuclear’ signaling via direct interaction with cytoplasmic and membrane-associated protein kinases. Specifically, PR-B/c-Src complexes that likely also contain ERα (Daniel et al. 2015) initiate rapid activation of MAPKs and engagement of IGFR- and EGFR-linked pathways (Faire & Lange 2007). Notably, both ER and PR are key substrates for rapidly activated (i.e. by progestin or estrogen) AKT and MAPKs. Thus, these SRs act as ‘antennas’ for sensing both the intracellular and extracellular environments. Target gene selection is highly dependent on PTMs to receptors, suggesting that these dynamic events regulate SR interactions with specific pioneer factors as well as alter the ability of coactivators or corepressors to participate in functional transcriptional complexes at HREs, thus impacting the activation or repression of specific gene sets by allowing rapid adaptation to ever-changing cell contexts.

**Post-translational modifications of PRs**

Like GR (discussed earlier), PR is heavily modified (Clemm et al. 2000), and PTMs to PR greatly alter its transcriptional activity at selected target genes or gene subsets (Daniel et al. 2010, Knutson et al. 2012, Hagan et al. 2013), enacting expression of highly context-dependent transcriptomes by complex mechanisms. Notably, the concentration of steroid hormone required for half-maximal induction or repression by a given receptor–steroid complex (i.e. the EC_{50}) is not constant for all responsive genes, allowing for the differential expression of genes by a common hormone concentration during development, differentiation, and homeostasis (Simons 2006, Simons & Chow 2012). Phosphorylation events may lower the EC_{50} for a given gene or gene subset (Daniel et al. 2007b) but rarely alter hormone/receptor binding affinity. Instead, these events facilitate protein–protein interactions required for SR target gene selection and receptor coactivation (Faire & Lange 2007). ER+PR+ luminal breast cancer development and progression are often accompanied by persistent activation of mitogenic and cell cycle-dependent protein kinases (MAPKs, AKT, CK2, cyclin-dependent kinases (CDKs)) classically regulated downstream of Tyr kinase growth factor receptors or during cell cycle (S phase) traverse. These kinases act as direct inputs to key transcription factors, including SRs and their coregulators. Interestingly, molecular antagonism between PR phosphorylation at S294 and SUMOylation at K388 profoundly alters PR behavior and progestin-induced gene expression. Phosphorylated PRs (i.e. at Ser294 by MAPK or CDK2) are readily ubiquitinylated (i.e. undergo rapid ligand-induced protein downregulation) and less SUMOylated relative to their dephosphorylated species (Salghetti et al. 2001, Daniel et al. 2007a,b). As SR SUMOylation is a predominantly repressive PTM (i.e. of transcription), these phosphorylated/deSUMOylated receptors exhibit transcriptional hyperactivity (i.e. are activated in subthreshold concentrations of ligand) at a subset of ‘phosphorylation-dependent’ target genes important for cell cycle regulation, proliferation, and survival, whereas other PR target genes remain insensitive to changes in PR SUMOylation but are rigidly sensitive to hormonal regulation (as reviewed in Knutson & Lange 2014). Breast cancer cells expressing K388R (SUMOylation-deficient or phospho-mimic) PRs exhibited an alternative gene program significantly associated with luminal B (HER2-positive) breast cancers (Knutson et al. 2012). These findings suggest that in breast cancer cells with increased MAPK or CDK2 signaling, phospho-Ser294 PRs (namely, PR-B) promote the expression of a unique transcriptional program that contributes to tumor heterogeneity (i.e. appearance of Her2+ cells) during luminal tumor progression (Knutson et al. 2012).

Interesting cross talk occurs between PTMs of PR, illustrating the complexity and expanded potential for SR flexibility conferred by concurrent regulation of multiple modifications. For example, methylation of Lys464 in the AF-1 domain of PR has been recently identified by mass spectroscopy (Chung et al. 2014). This modification repressed PR transcriptional activity by antagonizing PR phosphorylation at Ser294 and Ser400, and via decreasing PR interaction with NCoR and SRC-1 (Chung et al. 2014). Monomethylation of PR at Lys464 inhibited ligand-independent PR activity as well as dampened PR sensitivity to ligand, leading to reduced transcriptional activation of PR target genes (Chung et al. 2014). Additionally, progestin-induced breast cancer cell proliferation and spreading/focal adhesion were impaired upon loss of PR monomethylation (Chung et al. 2014). Similarly, ligand-induced PR acetylation at Lys138 and at residues 638–641 in the hinge region of PR has been studied (Daniel et al. 2010, Chung et al. 2014). Notably, acetylation of PR at Lys138 by p300 resulted in increased Ser294 phosphorylation. These events culminated in
enhanced PR binding to PREs and increased transcriptional activity at PR target genes, including FKBP5, HSD11B2, and MUC1 (Chung et al. 2014). The hinge region of PR is important for nuclear retention, early (i.e. rapid) gene transcription (i.e. PR-dependent induction of c-Myc mRNA occurs in minutes) and global PR phosphorylation. Acetylation in this region of PR is likely important for promoter selectivity, nuclear retention, and efficient Ser400 phosphorylation (Daniel et al. 2010).

PTMs are major determinants of PR isoform specificity. Notably, the NTD of PR-B contains a CD domain that is required for phosphorylation at PR Ser81 by CK2 (Hagan et al. 2011). Phosphorylation at Ser81 is unique to the PR-B isoform, as this site is not found in the N-terminally truncated PR-A isoform. PR-B Ser81 is phosphorylated in the presence of ligand or in the absence of ligand during the G1/S-phase transition of the cell cycle when CK2 is predominantly nuclear and thus colocalized with PR (Hagan et al. 2011). In the presence of ligand, phosphorylation at PR-B Ser81 by CK2 requires the scaffolding action of DUSP6 (MKP3), which binds directly to the PR CD domain and also to CK2, thus linking the required kinase to the BUS domain of PR-B (i.e. in close proximity to Ser81) (Hagan et al. 2011, Hagan et al. 2013). This tripartite complex is recruited to PRE-containing regions of PR-B-specific target genes such as STAT5. Notably, in the presence of progestin, and dependent on an intact CD domain or phosphorylated Ser81, PR-B but not PR-A promotes transcription of STAT5, initiating a feed-forward loop for PR-B/STAT5-dependent gene expression that includes regulation of WNT1 (Hagan et al. 2013). Secreted Wnt1 is required for progestin-induced breast cancer cell growth in soft agar (Faivre & Lange 2007) and has been implicated in progesterone-dependent mammary stem cell self-renewal (Joshi et al. 2010). Taken together, these studies demonstrated that PR Ser81 dictates PR-B-specific target gene selection at STAT5-dependent genes. STAT5 likely serves as a pioneer factor for phosho-Ser81 PR in breast cancer cells (Hagan et al. 2013).

In dividing cells, PRs are phosphorylated in a ligand-independent but cell cycle-dependent manner. Notably, PR Ser345 is rapidly phosphorylated by MAPKs in the presence of progestins, an event that leads to SP1 binding to PR and increased cyclin D1 mRNA and protein expression followed by cell cycle entry (Faivre et al. 2008). However, in the absence of progestins, this site is primarily targeted by CDK2 during the G2/M phase of the cell cycle (Dressing et al. 2014). Phospho-Ser345 PRs interact directly with SP1 and cyclin D1 at PR target genes important for breast cancer cell survival (Dressing et al. 2014). PR Ser81 is also required for ligand-independent regulation of PR target genes expressed during S-phase in PR+ proliferating cells, such as the antiapoptotic protein, BIRC3 (Hagan et al. 2013). It is tempting to speculate that these and other PR-B target genes relevant to cancer cell biology are primarily regulated in a cell cycle-dependent manner by phosphorylation events rather than strictly in response to hormonal stimuli (i.e. specifically in dividing PR+ cancer cells and in conditions of low progesterone ligand such as during postmenopause or during therapeutic hormone ablation).

**PR signaling cross talk is relevant to ER+ luminal breast cancer**

Context-dependent PR signaling, including the role of PTMs, has important implications for cellular outcome in ER+ luminal breast cancer. Seminal clinical studies demonstrated that hormone replacement therapy (HRT) that contains both estrogen and a progestin, but not estrogen alone, increased breast cancer risk in postmenopausal women (Collaborative Group on Hormonal Factors in Breast Cancer 1997, Rossouw et al. 2002, Beral & Million Women Study 2003, Chlebowski et al. 2003). Tumors were larger and of higher grade, suggesting that PR signaling is mitogenic in adult breast tissues and promotes tumor progression if taken as part of HRT. These findings lead to decreased use of HRT that has resulted in reduced breast cancer incidence (1997) but remain controversial due to the use of the synthetic progestin with androgenic actions, MPA, in women who were also many years into the menopause upon the start of the trial. However, the critical findings have recently been extended in an appropriately powered (855,324 women-years) clinical report in which young (premenopausal) women were treated for menorrhagia via single or multiple doses of intrauterine levonorgestrel (Soini et al. 2014). These studies demonstrated the predicted progestin-induced protection from uterine and ovarian cancers but also reported a dose-dependent increase in breast cancer incidence within this cohort. Surprisingly, intrauterine progestin also protected women from liver, pancreatic, and lung cancers by unknown mechanisms. Although the mechanisms are unclear, current modeling suggests that HRT (estrogen and a progestin) regimens induced the outgrowth of preexisting slow-growing or ‘indolent’ mammary lesions that developed into ‘measurable’ breast tumors in women taking progestin-containing HRT (Horwitz & Sartorius 2008). Most notably, a growing
body of literature suggests that progesterin stimulation of PR+ MECs may contribute to expansion of mammary cancer stem cells and increased tumor heterogeneity. As mentioned previously, progesterone/PR signaling is now recognized as a primary mediator of normal mammary stem cell self-renewal (Lydon et al. 1999, Asselin-Labat et al. 2010, Joshi et al. 2010). PR-induced expansion of the mammary stem cell compartment occurs via paracrine Wnt (requiring phospho-PR as discussed previously) and RANKL signaling (Joshi et al. 2010, Tanos et al. 2013). In breast cancer models, PRs also induce Wnts (Fairvre & Lange 2007) and drive the emergence of CK5+ but ER/PR-null stem-like progenitor cells (Kabos et al. 2011). Progestin-mediated downregulation of miR-29 led to increased expression of Kruppel-like factor 4 (KLF4) and reprogramming to a stem cell-like state (Cittelly et al. 2013). Downregulation of miR-141 by progesterone increased the expression of both PR and Stat5 (requiring phospho-PR as discussed previously), increased stemness, and promoted increased tumor initiation and growth in xenograft (mouse) models (Finlay-Schultz et al. 2015). Thus, PRs appear to regulate luminal progenitors in both the normal and cancerous mammary glands. In sum, in preexisting or indolent early breast tumors, heightened hormonal stimulation or other events leading to inappropriate PR signaling (via PTMs) may initially either promote tumor growth (Knutson & Lange 2014) or reverse progesterin or antiprogesterin-induced inhibition of tumor growth (Groshong et al. 1997, Wilcken et al. 1997), but may ultimately contribute to increased tumor heterogeneity, in part by driving the appearance of ER/PR-null cells with basal characteristics (i.e. markers such as CK5 or KLF4) and associated basal-like gene signatures (Knutson et al. 2012).

It will be essential to fully define the role of PTMs to PR isoforms and/or ER that relate to luminal breast cancer biology and cell fate. Important mechanistic hints are emerging from in vitro and in vivo studies, including those that model the role of specific PTMs. For example, phospho-Ser294 PR-B signaling (i.e. as modeled via the expression of K388R deSUMOylated PR-B or S294D PR-B) drives a gene signature initially discovered using T47D and MCF-7 cell line models, which is significantly associated with publicly available gene sets known to define poor prognosis in patients with ER+/PR+ luminal breast cancer (Knutson et al. 2012). Surprisingly, in these studies, many PR target genes were previously defined as primarily estrogen-induced genes. Significantly, ER and PR interact in both rapid signaling (Razandi et al. 2003, Boonyaratanaokornkit et al. 2007) and transcriptional contexts (Ballare et al. 2003, Giulianelli et al. 2012, Daniel et al. 2015, Mohammed et al. 2015). Indeed, cross talk between ER and PR appears to be extensive (Migliaccio et al. 1998) and clearly impacts global gene expression and breast cancer cell fate (Giulianelli et al. 2012, Daniel et al. 2015, Mohammed et al. 2015, Need et al. 2015) (further discussed later). For example, classic studies demonstrated that in the presence of progesterin, ER augments PR-dependent gene expression and tumor growth. In an MPA-dependent murine mammary tumor model, inhibition of ER with fulvestrant inhibited PR-dependent tumor formation. Giulianelli and his colleagues confirmed earlier studies that ER and PR interact and colocalize (Migliaccio et al. 1998, Giulianelli et al. 2012), but extended ER/PR cross talk studies with regard to the regulation of gene expression in human breast cancer cell lines and mouse tumors. Both receptors were detected (via ChIP assays) at the promoters of CCND1 and MYC genes in T47D cells. Although global gene expression was not performed, many genes (in addition to CCND and MYC) likely require PR and ER for optimal hormone responsiveness.

Similar to the above studies demonstrating that ER contributes to transcriptional activation of PR target genes, PR is essential for the estrogen-induced expression of selected ER target genes. In ER+ luminal breast cancer models, PR participates in signaling and transcriptional complexes whose formation and function are entirely independent of progesterone. For example, global gene expression analysis of estrogen-treated ER+ breast cancer cells revealed that the presence of PR-B increased phosphorylation of ER and was absolutely required for estrogen induction of gene expression on select promoters previously thought to be entirely ER driven. Notably, the expression of these genes was unaffected by progesterin agonists but blocked by pure antagonists or PR knockdown (Daniel et al. 2015). PR-B, but not PR-A, promoted estrogen-induced expression of novel ER/PR target genes characterized by recruitment of signaling and transcriptional complexes containing ERα, PR-B, IGF1R, and PELP1 to ERE enhancers within estrogen-regulated target genes (Fig. 4). In vitro (i.e. intact T47D and MCF-7 cell lines) forced the expression of PR-B (i.e. in PR-lowcells) promoted estrogen-induced anchorage-independent growth and enhanced phosphorylation of ER at serine residues (Ser118, Ser167) targeted by MAPK and AKT, and implicated in endocrine resistance (Anbalagan & Rowan 2015). The ligand-independent action of PR-B likely involved the coordination of the IGF1R signaling axis to enhanced AKT signaling and ER phosphorylation (note that IRS1 is an important phospho-PR-B target
gene) (Byron et al. 2006). Inhibition of PR via shRNA knockdown or onapristone inhibited estrogen-induced gene expression, blocked anchorage-independent growth, and partially reversed tamoxifen resistance. The combination of onapristone and AEW541, an inhibitor of IGF1R signaling, greatly inhibited cell growth relative to either agent alone. Importantly, the components of the ER/PR/PELP1 complex readily co-purified (via immunoprecipitation assays) in 10/10 human luminal breast tumor samples, suggesting that this complex exists in vivo and is relevant to tumor biology (Fig. 4). Recently, Mohammed and his coworkers have confirmed that ER/PR complexes mediate global changes in estrogen-dependent gene expression, but focused on the short-term growth inhibitory actions of progestin in estrogen-treated models (Mohammed et al. 2015). These studies highlight the importance of PR as a modifer of ER action rather than simply a bystander or marker of estrogen responsiveness in ER+/PR+ luminal tumors.

Relevant to mechanisms of altered PTMs to SRs in breast cancer, PELP1 is emerging as a driver of altered SR action (including ER, PR, GR, and AR) and endocrine resistance (Daniel et al. 2015, Girard et al. 2015). In breast cancer studies, PELP1 is restricted to the nucleus of normal MEC but is partially to largely cytoplasmic in a significant number of breast tumors in which its altered localization is associated with tamoxifen resistance (Vadlamudi et al. 2005, Kumar et al. 2009). Mechanistic studies conducted in cancer cell lines demonstrated that altered localization of PELP1 to the cytoplasm augments intracellular c-Src, MAPK, and AKT signaling (Fig. 4). As these kinases are major inputs to SR phosphorylation, altered PELP1 location likely drives inappropriate expression of phospho-SR target gene sets important for cell fate. For example, MCF-7 cells expressing cytoplasmic PELP1 exhibited increased estrogen and progesterone-induced expression of ER/PR target genes relative to cells expressing nuclear PELP1. PELP1 most likely acts as a scaffold that tethers ER, PR, and

**Figure 4**
Phospho-PR signaling mediates proliferative gene programs and ER signaling. Specific phosphorylation events at Ser81, Ser294, and Ser345 dictate PR target gene selection and expression to drive phospho-specific gene programs, including genes known to drive breast cancer cell proliferation and survival (Faivre et al. 2008, Knutson et al. 2012, Hagan et al. 2013). PR-B expression and signaling are required for estrogen-induced expression of specific ERα target genes (Daniel et al. 2015) via the formation of ER/PR/PELP1 signaling and transcriptional complexes.
IGF1R in order to facilitate extranuclear signaling, which in turn enhances SR-dependent transcriptional responses via phosphorylation events that, when inappropriately amplified, function to promote escape from normal hormonal cues (i.e. controls) and endocrine resistance. Interestingly, ER and GR signaling pathways also converge to mediate the distinct functions of GR in luminal breast cancer models. In contrast to its role in TNBC, the presence of GR in ER+ luminal breast cancers predicts good outcome (Pan et al. 2011). Estrogen treatment dampened GR transcriptional activity (Zhang et al. 2009), whereas GR directly interfered with ER function, in part via competitive binding to ERα-response elements in DNA (Karmakar et al. 2013). In addition to ER and PR (or GR), other SRs (namely, AR) also likely cooperate and interact extensively (including within PELP1 complexes) as part of an emerging paradigm of how cells, including cancer cells, continuously sense and respond to their changing hormonal milieu, and PTMs to SRs and their binding partners likely figure prominently into the mechanisms involved.

Summary

We conclude that via their repertoire of dynamically regulated PTMs, SRs act as highly context-dependent sensors of the cellular signaling environment. It is through a diverse array of interacting PTMs that SRs rapidly adapt and adjust in a perpetual state of homeostatic flux (both outside and inside the cell). In principle, selected SR-dependent actions that also require PTMs may theoretically be targeted according to context and to achieve the desired change in cell fate. It is now clear that there are no truly SR-negative breast cancer subtypes. Of note, a majority of TNBCs contain elevated GR and/or AR, whereas a more accurate view of ER+ luminal cancers is that they may also contain GR and AR, but that most tumors represent mixed populations of both luminal and basal cells, especially in the context of hormone exposure or following prolonged endocrine treatment. In this article, we have focused on PTMs to highly related GR and PR as emerging SRs acting downstream of major signaling pathways with significant breast cancer relevance (and as understudied SRs relative to ER and AR). A complete understanding of the mechanisms governing the regulation of PTMs to GR and PR and their complex roles in cell fate and transformation, including tumor cell heterogeneity, is essential not only for forging inroads into cancer prevention via hormonal modulation, but in order to further leverage and refine existing endocrine therapies for best treatment outcome. Achieving this goal may involve boldly expanding our treatment options to include novel SR (i.e. non-ER) targeted therapies that go one step beyond the relevant kinase inhibitors to specifically block the deleterious actions of phospho-GR or phospho-PR while preserving or promoting their protective functions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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