THEMATIC REVIEW

90 YEARS OF PROGESTERONE

Steroid receptors as MAPK signaling sensors in breast cancer: let the fates decide

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Abstract

Steroid hormone receptors (SRs) are classically defined as ligand-activated transcription factors that function as master regulators of gene programs important for a wide range of processes governing adult physiology, development, and cell or tissue homeostasis. A second function of SRs includes the ability to activate cytoplasmic signaling pathways. Estrogen (ER), androgen (AR), and progesterone (PR) receptors bind directly to membrane-associated signaling molecules including mitogenic protein kinases (i.e. c-SRC and AKT), G-proteins, and ion channels to mediate context-dependent actions via rapid activation of downstream signaling pathways. In addition to making direct contact with diverse signaling molecules, SRs are further fully integrated with signaling pathways by virtue of their N-terminal phosphorylation sites that act as regulatory hot-spots capable of sensing the signaling milieu. In particular, ER, AR, PR, and closely related glucocorticoid receptors (GR) share the property of accepting (i.e. sensing) ligand-independent phosphorylation events by proline-directed kinases in the MAPK and CDK families. These signaling inputs act as a ‘second ligand’ that dramatically impacts cell fate. In the face of drugs that reliably target SR ligand-binding domains to block uncontrolled cancer growth, ligand-independent post-translational modifications guide changes in cell fate that confer increased survival, EMT, migration/invasion, stemness properties, and therapy resistance of non-proliferating SR+ cancer cell subpopulations. The focus of this review is on MAPK pathways in the regulation of SR+ cancer cell fate. MAPK-dependent phosphorylation of PR (Ser294) and GR (Ser134) will primarily be discussed in light of the need to target changes in breast cancer cell fate as part of modernized combination therapies.

Key Words

- glucocorticoid receptors
- progesterone receptors
- breast cancer
- map kinases
- cancer stem cells
Introduction

Of the 48 ligand-activated and ‘orphan’ (i.e. ligands remain unidentified) nuclear receptors (NRs), six are related members of the SR superfamily whose steroid hormone ligands are primarily derived from cholesterol metabolism: estrogen receptor (ERα, ERβ), progesterone receptor (PR), glucocorticoid receptor (GR), androgen receptor (AR), and mineralocorticoid receptor (MR). PR is most well-studied as an estrogen-responsive gene, and PR expression is used as a clinical biomarker of intact estrogen signaling or functional ER. There are two predominant PR isoforms: full-length PR-B and N-terminally truncated PR-A that is missing the first 164 amino acids found in full-length PR-B, termed the B-upstream segment (BUS). Although PR-A and PR-B share structural and sequence identity downstream of the BUS, this segment contains regulatory components that account for major differences in PR-binding partners, promoter selectivity, and transcriptional activity between these two isoforms. A third uterus-specific isoform, termed PR-C, is a further truncated PR receptor composed of only the hinge region and hormone binding domain (HBD) that is incapable of binding DNA but inhibits PR transactivation. PR-C signaling occurs specifically in the fundal myometrium during the onset of labor and is likely mediated by direct competition for C-terminal binding partners (Condon et al. 2006). PR-A and PR-B are generally co-expressed in PR+ tissues such as the breast or uterus, but are not always found together in the same cells (Aupperlee et al. 2005). Along with ER and Her2, total PR levels, rather than individual PR isoforms, are measured clinically to classify the luminal A (ER+/PR+) and luminal B (ER+/PR-low/null) breast cancer subtypes. However, increasing studies have implicated differential PR isoform expression in the etiology of breast cancer (Mote et al. 2002, 2015, Rojas et al. 2017, Lamb et al. 2018). Furthermore, PRs dramatically influence ER transcriptional responses (Daniel et al. 2015, Mohammed et al. 2015, Singhal et al. 2018); PR-A can transrepress both ER and PR-B (Abdel-Hafiz et al. 2002), while PR-B is an important ER-binding partner (Daniel et al. 2015, Mohammed et al. 2015). Measuring PR isoform ratios by immunohistochemistry (IHC) has been reported (Sletten et al. 2019) but remains challenging due to the lack of reliable antibodies that are capable of accurately distinguishing PR-A vs PR-B since there are no unique amino acid sequences in PR-A. Thus, defining the context-dependent actions of PR isoforms is crucial to understanding how imbalanced PR-A/PR-B ratios can be exploited therapeutically in the clinic.

Closely related to PR within the SR superfamily, the glucocorticoid receptor (GR) is a ligand-activated transcription factor responsible for integrating the physiologic, pathological, and therapeutic functions of hormonal (cortisol, endogenous corticosteroids) and synthetic glucocorticoids like dexamethasone. GR is ubiquitously expressed and drives the expression of glucocorticoid-responsive genes in a cell/tissue- and promoter-specific manner after glucocorticoid binding to the GR C-terminal ligand-binding pocket (Miranda et al. 2013, Kumar & Thompson 2019). Consequently, GR activation regulates diverse gene programs controlling metabolism, immune system, and CNS function to maintain cellular homeostasis. Like other steroid hormone receptors, GR consists of three functional domains: intrinsically disordered N-terminal (NTD), DNA binding (DBD), and ligand binding (LBD). NTD and LBD contain transcription Activation Function regions, AF1 and AF2, respectively (Kumar & Thompson 2005). AF2 is strictly ligand-dependent, while AF1 can act constitutively in the absence of the LBD (Oakley & Cidlowski 2013), a property shared by both PR (Goswami et al. 2014) and AR (Dehm et al. 2008). Like other SRs, functionally distinct AF domains of GR mediate transcriptional activation by recruiting coregulatory complexes that remodel chromatin, target initiation sites, and stabilize RNA polymerase II for gene transcription (Miranda et al. 2013). Similar to PR isoform structure, a further complexity of GR action is that a large cohort of GR isoforms can be created from a single gene (known as NR3C1) by alternate splicing and at least eight alternative translational start sites within the GR N-terminal domain sequence (Turner & Muller 2005, Duma et al. 2006, Presul et al. 2007). The multiple GR isoforms are expressed in a highly tissue-specific manner and, when expressed as individual isoforms experimentally, GR isoforms regulate remarkably distinct transcriptomes (Lu & Cidlowski 2005).

Within the NTD of all SRs, serine, threonine, and lysine residues can be post-translationally modified (Fig. 1). Our prior studies have primarily defined MAPK-dependent phospho-PR transcriptomes (Knutson et al. 2012, 2017). While the extent to which phosphorylation events modify the GR cistrome is still being studied, such modifications significantly alter GR function (Gallagher-Beckley et al. 2011). For example, recent collaborative studies from our group (Regan Anderson et al. 2016, 2018) have established GR as an important sensor of both life stress (i.e. a collection of classical GR ligands acting via the LBD) as well as inducible cellular (hypoxia, reactive oxygen species (ROS), nutrient starvation), and microenvironmental
Studies of signal transduction

MAPK signaling pathways promote ligand-independent actions of phospho-SRs

Reversible post-translational modifications (PTMs) of SRs include phosphorylation, SUMOylation, acetylation, and ubiquitination. These PTMs may account for the functional diversity of receptor isoforms. This section will primarily focus on ligand-independent actions of PR and GR; the hormonal regulation of these SRs in breast cancer models have been reviewed (Diep et al. 2015, Scheschowitsch et al. 2017, Truong & Lange 2018). Ligand-independent SR activation has been proposed as a contributing factor to the gradual loss of hormone responsiveness in which SRs shift to hormone-independent or resistant states; this process is well-studied with regard to ER, while a similar paradigm is emerging for both PR and GR (Shen et al. 2001, Irusen et al. 2002, Matthews et al. 2004, Arpino et al. 2008, Giulianelli et al. 2008). One mechanism by which SRs become less dependent upon natural ligands occurs through hormone-induced rapid activation of cytoplasmic signaling pathways that, in turn, phosphorylate and sustain SR activation in diminishing ligand (Fig. 2). For example, constitutive ER/PR complexes exist in breast cancer cell lines and primary breast tumors (Daniel et al. 2015); trace levels of either estrogen (E2) or progestin (P4) induce rapid (3–15 min) activation of p42/p44 MAPKs (ERK1/2) via cytoplasmic or membrane-associated ER/PR/c-SRC complexes that have been shown to regulate breast cancer cell proliferation in vitro (Migliaccio et al. 1998, Boonyaratanakornkit et al. 2001, Ballare et al. 2003).

We and others have shown that the SR coactivator, PELP1, functions as a cytoplasmic scaffold for docking of multiple SRs including GR (Kayahara et al. 2008, Regan Anderson et al. 2016), AR (Yang et al. 2012) and both ER and PR (Vadlamudi et al. 2001, Truong et al. 2018) as well as the SR coactivator, SRC-3 (Truong et al. 2018). Indeed, a common link between GR signaling in TNBC (discussed subsequently) and PR signaling in ER+ luminal breast cancer is the functional role of PELP1 as a scaffold to bring cytoplasmic kinases and SRs into close proximity. In TNBC models, PELP1 expression is upregulated in response to chemotherapy and cellular stress including hypoxia and ROS production. PELP1 and GR interactions increase in response to the glucocorticoid dexamethasone and creation of ROS by hydrogen peroxide (Regan Anderson et al. 2016). Furthermore, a transcriptional complex containing PELP1, GR, and hypoxia inducible factors (HIFs) is recruited to the breast tumor kinase (Brk/PTK6) promoter; stress-induced Brk/PTK6 signaling promotes breast cancer cell survival, migration, and metastasis (Castro & Lange 2010, Lofgren et al. 2011, Regan Anderson et al. 2013). Similarly, in ER+ breast cancer, PELP1 scaffolds SRC-3, ER, and PR to promote ER phosphorylation (i.e. at Ser118 and Ser167) and expression of E2-induced genes involved in cell survival and stem/progenitor cell formation (Truong et al. 2018). Notably, expression of PR-B (but not progesterone) was required for E2-induced expression of a distinct ER/PR target gene signature that predicts greatly shortened survival in luminal breast cancer patients (Daniel et al. 2015). Treatment of breast cancer cells with SI-2, an SRC-3 inhibitor, disrupts ER/PR/PELP1 complexes and blocks...
PELP1-induced tumorsphere formation. Importantly, the PELP1/GR interaction is enhanced by GR Ser134 phosphorylation, while PR Ser294 phosphorylation is required for the PELP1/PR/ER interaction. Thus, SRs participate in cytoplasmic signaling complexes that enable them to rapidly sense changes in the signaling microenvironment through direct PTMs. All SRs can be phosphorylated at multiple proline-directed sites within the NTD, and PR and AR also contain these sites in the hinge region (Fig. 1). Specifically, upregulation of MAPK-associated signaling pathways (e.g., Akt, ERK1/2, and p38 MAPK) in cancer states can lead to ligand-independent activation of SRs via phosphorylation at their N-terminal phosphorylation sites (Fig. 1). Ligand-independent phosphorylation at Ser118 (via MAPK) or Ser167 (via Akt) are two of the most well-studied phosphorylation sites in ER (Stellato et al. 2016). Additionally, PR (Ser294, Ser345, and Ser400) and GR (Ser134) also undergo ligand-independent phosphorylation in response to growth factor or cytokine induced activation of cytoplasmic kinase (MAPKs and CDKs) pathways. As discussed subsequently, these signaling inputs to SR N-termini phospo-sites can dramatically impact cell fate decisions that are highly relevant to breast cancer progression.

**Mitogenic signaling inputs to PR phosphorylation and transcriptional activity**

PR transcriptional activity and promoter selection are dramatically influenced by activation of mitogenic and cell cycle-dependent protein kinases (i.e., MAPK, Akt, CK2, and cyclin-dependent kinases (CDK)) that alter PR phosphorylation (Zhang et al. 1997, Knotts et al. 2001, Pierson-Mullany & Lange 2004, Daniel et al. 2007, Faiivre et al. 2008, Hagan et al. 2011, Dressing et al. 2014), SUMOylation (Daniel et al. 2007, Daniel & Lange 2009), or acetylation (Daniel et al. 2010). Modification of SRs may not appreciably alter transcriptional activity, as measured using reporter gene assays in vitro, but is a major mechanism for regulating endogenous target gene
selection. PTM sites identified on PR that are known to alter its transcriptional activity at selected endogenous target genes include phosphorylation (S81 unique to PR-B, S294, S345, and S400), SUMOylation (K388), and acetylation (K183, K638, K640, and K641). These PTMs enable PR to induce diverse biological outcomes such as anchorage-independent growth/survival, tumorsphere formation as a readout of stemness properties, and cell cycle-dependent gene regulation. These biologies are context-dependent as determined by the presence/absence of activated signaling pathways and the availability of coregulators.

For example, PR Ser294 phosphorylation is ligand-induced (progestins), but also occurs through ligand-independent pathways in response to tyrosine kinase growth factor receptor-induced MAPK and/or CDK activation. Phospho-Ser294 PR-B species are deSUMOylated at Lys388 and are subject to more rapid ligand-dependent receptor downregulation relative to dephosphorylated and heavily SUMOylated PR species (Daniel et al. 2007). Lys388 SUMOylation is a transcriptionally repressive modification at PR target genes associated with cancer biology that also stabilizes PRs in the presence of progesterin by slowing their 26S proteasome/ubiquitinoylation-dependent degradation. However, a number of genes (i.e. including numerous tumor suppressor genes) are induced by SUMOylated PRs. Co-mutation of PR-B Lys388 (K388R) rescues transcriptional deficiencies of phospho-mutant PR-B S294A receptors in luciferase assays (Daniel et al. 2007). Interestingly, studies with SUMO-deficient mutant PR-B (K388R; a biochemical phospho-mimic) demonstrated that SUMO-deficient PR-B exhibited transcriptional hyperactivity at a unique subset of PR target genes crucial for cell cycle regulation, proliferation, and survival. This occurred independently of progesterin and was increased by activated CDK2 (Daniel & Lange 2009).

Additionally, breast cancer cell models expressing K388R PR exhibited an alternative gene program significantly associated with Her2 signaling in luminal B (HER2+) breast cancers and the emergence of endocrine resistance (Knutson et al. 2012). These studies suggest that, in breast cancer cells with increased MAPK or CDK2 signaling, phospho-Ser294 PR-B (i.e. SUMO-deficient) species promote the expression of transcriptional programs that can contribute to tumor heterogeneity (e.g. emergence of HER2+ cells) during luminal tumor progression (Knutson et al. 2012). Crosstalk between Ser294 and Lys388 in PR-A was less apparent relative to that in PR-B (Daniel et al. 2007). Additional studies are ongoing to define the isoform-specific contributions of SUMO-deficient (i.e. K388R mutant) and Ser294-phosphorylated PR-A.

Recently, PRs have been implicated in mechanisms of early luminal breast cancer dissemination; progesterone induced migration of mammary cells from early lesion, but not primary tumor samples from BALB-NeuT mice (Hosseini et al. 2016). Breast tumor cells co-associate with local and circulating carcinoma-associated fibroblasts (c-CAF) that can be detected as cancer stem cell/c-CAF cell co-clusters in both mouse models of breast cancer and the blood of patients with metastatic disease (Ao et al. 2015). Related to these studies, fibroblast growth factor 2 (FGF-2), produced by CAFs, has been shown to activate PR in breast cancer models (Giulianelli et al. 2008). This ultimately leads to transcription of known PR target genes MYC and CCND1, resulting in increased hormone-independent cell proliferation in mouse mammary tumor models. Follow-up studies further defined that FGF-2 promotes interaction between ER and PR at the MYC enhancer and proximal promoter regions (Giulianelli et al. 2019). Notably, over 700 canonical proteins were recruited to MYC regulatory sequences following FGF-2 induction, which included both PR isoforms (PR-A and PR-B) as well as the expression of a novel PR splice variant protein product PR-BΔ4. These studies suggest that the PR-BΔ4 variant, which has an impaired ligand-binding domain and lacks the nuclear localization signal, may play a significant role in ligand-independent PR actions, given that it is induced and activated by FGF-2 but not progestins. The inclusion of non-canonical PR isoforms such as PR-BΔ4 in studies of ER+ breast cancer may reveal alternate avenues to prevent or treat endocrine resistance and effectively block luminal breast cancer progression and metastasis. Namely, CAF-derived FGF-2 represents a potent second ligand that is sensed by PRs in order to sustain ligand-independent PR activation in disseminated cancer cell/CAF clusters and reveals a novel strategy to target phospho-PR actions in otherwise hormone-ablated breast cancer patients.

**Stress signaling inputs to GR phosphorylation and transcriptional activity**

Glucocorticoids (i.e. corticosteroid GR agonists) are one of the most commonly prescribed and effective treatment regimens for acute inflammatory conditions as well as many chronic inflammatory diseases. However, some patients become unresponsive to these treatments due to the development of glucocorticoid resistance. One of the mechanisms that accounts for this resistance is decreased glucocorticoid signaling, which is correlated with changes in GR phosphorylation (Bloom 2004, Tsitoura & Rothman 2004). GR phosphorylation by ubiquitous protein kinases has been shown to lead to altered GR transcriptional
activity and interaction with other coregulatory proteins (Krstic et al. 1997, Galliher-Beckley et al. 2011). GR is phosphorylated on serine residues Ser113, Ser134, Ser141, Ser203, Ser211, and Ser226 by cyclin-dependent kinases, MAPK, and casein kinase II (Ismaili & Garabedian 2004) (Fig. 1). Three of these GR residues (Ser204, Ser211, and Ser226) are located within the AF1 domain, while the remainder are found in the NTD (Mason & Housley 1993, Ismaili & Garabedian 2004).

Phosphorylation of Ser134 by p38 MAPK is the most well-studied GR PTM event. Ligand-independent phosphorylation of Ser134 in response to stress-activating stimuli requires p38 MAPK in U2OS osteosarcoma cells (Galliher-Beckley et al. 2011) and TNBC models (Regan Anderson et al. 2016). In TNBC cells, p38 MAPK inhibition blocked Ser134 GR phosphorylation and abrogated the ability of GR to associate with a GRE-containing region of the BRK (breast tumor kinase) promoter (Regan Anderson et al. 2016). Ligand-independent regulation of GR transcriptional activity is further supported by studies showing p38 MAPK-induced phosphorylation of Ser134 increased the association of GR with the 14-3-3zeta signaling proteins on gene regulatory regions in chromatin, which simultaneously resulted in reduced hormone-dependent transcriptional responses (Galliher-Beckley et al. 2011). Interestingly, p38 MAPK has been shown to be active in alveolar macrophages of asthmatic patients who exhibited poor response to glucocorticoids when compared to patients with a normal response (Bhavsar et al. 2008). In contrast, p38 MAPK has also been shown to phosphorylate Ser211 in human leukemic cells, leading to increased dexamethasone-driven apoptosis (Khan et al. 2017). This suggests that p38 MAPK kinase phosphorylation of GR impacts ligand-independent GR signaling in a cell-specific manner. In breast cancer models, p38 MAPK signaling is emerging as an important mediator of stem cell biology (Lu et al. 2018, Xu et al. 2018, Grun et al. 2019) and other non-proliferative states that are relevant to cancer biology (discussed subsequently).

Studies of cancer cell biology

PR regulation of breast cancer cell proliferation vs stem cell biology

Most cancer therapies were historically designed to target rapidly dividing tumor cells. In the context of ER-positive breast cancer, tumor cells are dependent on ER transcriptional activity to promote proliferation. Thus, endocrine therapies that target ER function (i.e. selective ER modulators (SERMs), selective ER degraders (SERDs), and aromatase inhibitors (AIs)) inhibit proliferation and are very effective treatments for ER-positive breast cancer. Yet, the benefits of endocrine therapy are not sustained long-term in 10% to 41% of patients who suffer from late recurrence (Pan et al. 2017). It is hypothesized that non-proliferative cell states, such as quiescence/tumor cell dormancy, cell survival, plasticity, and epithelial to mesenchymal transition (EMT) as well as cancer stem cells (CSCs), are not well-targeted by standard cancer therapies. Indeed, a growing body of literature suggests that breast CSCs are resistant to current therapeutic approaches (Easwaran et al. 2014, Piva et al. 2014). In fact, these non-proliferative cell states are induced or maintained in response to cellular stress signaling and the selective pressure created by radiation, chemo-, and endocrine therapy that is largely cytostatic rather than cytotoxic, ultimately contributing to therapy-resistant cancer recurrence and metastasis. For example, chemotherapy has been shown to increase CD44+/CD24- CSC populations and tumorsphere formation in breast cancer patients (Li et al. 2008). In particular, CSCs tend to be non-proliferative, allowing them to readily evade standard therapies and expand over time (Fillmore & Kuperwasser 2008). Recent studies utilizing mouse models of prolactin-induced ER+ breast cancer showed that treatment with antiestrogens such as fulvestrant or tamoxifen reproducibly stimulated breast CSC self-renewal (Shea et al. 2018). These studies suggest that antiestrogen therapies may initially slow tumor growth, but also concurrently promote unwanted side effects by evoking plasticity and regenerative CSC activity in largely non-proliferative cancer cell compartments.

The proliferative effects of progestins in normal breast development are well-established. Progestins are proliferative in the breast (McGowan et al. 2007), and PR-B is the predominant isoform involved in normal mammary gland tissue expansion during breast development after puberty (Mulac-Jericevic et al. 2003). Mouse models lacking PR-B, but not PR-A, exhibited defects in mammary gland branching and alveologenesis (Mulac-Jericevic et al. 2003). Conversely, using knockout mouse models, PR-A has been shown to be required for normal uterine development and fertility (Conneely et al. 2001). More recently, PRs have been found to promote non-proliferative phenotypes that include cell survival and regulation of cancer stem/stem-like cell self-renewal. For example, progestin stimulation of PR+ mammary epithelial cells induced transcription and secretion of mitogenic factors such as Wnts, Areg, HB-EGF, and RANKL leading to proliferation of neighboring PR- cells.
(Fata et al. 2001, Tanos et al. 2013). Separate studies implicated these same PR signaling outputs (i.e. RANKL and Wnt4) in the maintenance and expansion of the normal mammary gland stem compartment (Asselin-Labat et al. 2010, Joshi et al. 2010). These studies have been extended to show that PR regulates the breast CSC compartment (Axlund et al. 2013, Cittelly et al. 2013, Hilton et al. 2014, Finlay-Schultz & Sartorius 2015, Goodman et al. 2016), and recent work has further shown that PR isoforms have divergent roles in supporting breast CSC properties using ER+ breast cancer cell line models that either lack both PRs or express only PR-A, only PR-B, or both isoforms (Truong et al. 2019). Measurement of breast CSC markers in these models indicated that although PR-A+ breast cancer cells were only weakly proliferative in soft-agar, they formed abundant tumorspheres that were enriched for both ALDH+ and CD44+/CD24- cell populations relative to PR-B+ cells (which formed fewer but larger spheres), suggesting a reversible phenotypic flip from pro-proliferative (PR-B-driven) to non-proliferative CSC (PR-A-driven) programs. Accordingly, S294A PR-A mutant models were impaired with regard to ALDH+ tumorsphere formation, yet exhibited increased proliferation in soft agar (Truong et al. 2019). These studies suggest tight linkage between highly plastic (i.e. proliferative vs CSC) regulated cell fates, and implicates PR isoform-specific modulation.

Divergent roles of PR isoforms may be explained by isoform-specific transcriptional control of overlapping but distinct gene programs which, in part, is directed by PTMs. Notably, phospho-Ser294 PR species are required for both PR-A- and PR-B-induced tumorsphere formation (Knutson et al. 2017, Truong et al. 2019). However, phospho-PR-A is preferentially recruited to promoters of CSC-associated genes (e.g. Wnt4, KLF4, and NOTCH2) relative to phospho-PR-B; PR isoform recruitment was significantly reduced in phospho-mutant models (i.e. S294A). Of note, the effect of PR isoforms on CSC markers and tumorsphere formation was independent of exogenously added progesterin (i.e. ligand-independent in defined media), and the addition of progesterin did not have a significant effect on tumorsphere number or size. In the context of endocrine therapy resistance, SRs may promote tumor progression via ligand-independent mechanisms, resulting from the upregulation of growth factor and/or stress signaling pathways that occurs following hormone withdrawal (Nicholson et al. 2004, Need et al. 2015). Relevant to this concept, EGF induces MAPK-dependent and ligand-independent PR Ser294 phosphorylation, which greatly enhanced PR-A and PR-B driven secondary tumorsphere formation, an in vitro readout of CSC self-renewal (Truong et al. 2019); mutation of PR Ser294 to Ala blocked these effects. These findings indicate that PR Ser294 phosphorylation is a crucial event that defines both PR-A and PR-B actions in the regulation of CSC populations; the increased potency of PR-A over PR-B as a driver of breast cancer stemness may be explained by its greater stability (Faire et al. 2008) and resistance to deSUMOylation relative to PR-B (Daniel et al. 2007). Understanding how SRs contribute to cancer cell fate transitions, plasticity, heterogeneity, and other non-proliferative cancer cell biologies is important for preventing and targeting therapy resistance.

**PR signaling impacts the cell cycle**

One functional characteristic of CSCs is the ability to maintain a cellular quiescence, reversibly existing in the G0 phase of the cell cycle (Bighetti-Trevisan et al. 2019). This quiescent state allows for long-term survival in response to cellular or microenvironmental stress (i.e. chemotherapy, hypoxia, etc.). Signaling molecules that regulate quiescence include cyclin-dependent kinase (CDK) inhibitors (i.e. p21, p27, and p57), as well as the members of the FOXO family of transcription factors. Our prior work showed that PRs regulate the cyclin dependent kinase inhibitor p21 and FOXO1 to promote cellular senescence, the irreversible exit from the cell cycle (i.e. permanent accumulation in G0), in ovarian cancer models (Diep et al. 2013, 2016). Senescence, FOXO1 and p21 gene expression, and PR Ser294 phosphorylation were induced by progesterin (ligand-dependent). Chemical inhibition of FOXO1 inhibited PR Ser294 phosphorylation and the PR-dependent senescence phenotype. In these studies, PR-B was the dominant isoform required for induction of both FOXO1 and p21 expression as well as progesterin-induced cellular senescence, while PR-A repressed these genes and failed to induce a senescent state upon treatment of ovarian cancer cells with progestins.

Many of the same signaling molecules and transcription factors regulate both senescence and quiescence. While a direct link between PR and CSC quiescence has not yet been established in ER+ PR+ breast cancer models, it is possible that context-dependent signals, such as breast vs ovarian cells of origin, cancer type-specific or MAPK/CDK signaling pathway activation, and/or ligand-independent PR phosphorylation, will promote a PR-dependent cellular quiescence that contributes to CSC survival. In support of this, FOXO1 expression increased PR Ser294 phosphorylation and promoted ALDH+ tumorsphere
formation in both PR-A and PR-B expressing breast cancer models (Truong et al. 2019). Accordingly, inhibition of PR and FOXO1 with onapristone and AS1842856, respectively, had a synergistic inhibitory effect on CSC self-renewal in multiple breast cancer models. Future studies will examine the cooperative effect of PR isoforms, FOXO1, and p21 on CSC quiescence/tumor cell dormancy in ER+ breast cancer models.

**GR promotes triple negative breast cancer cell survival, migration, and invasion**

As with PR isoforms, the effects of GR on breast cancer biology are also highly context dependent and most relevant to non-proliferative cancer cell fates. Like PR expression, GR expression in ER+ luminal breast cancer is associated with a better prognosis. In sharp contrast, however, high GR expression is associated with more aggressive triple negative breast cancer (TNBC) biology and poor prognosis when compared to patients with low GR expression (Pan et al. 2011). In TNBC models, expression of GR and/or GR-associated target genes (i.e. Brk/PTK6) have been associated with pro-survival signaling, EMT, and cellular migration/invasion in vitro as well as metastasis in vivo (Lofgren et al. 2011, Regan Anderson et al. 2013, 2016, 2018). Recently, activation of GR with glucocorticoids was shown to drive breast cancer metastasis in vivo (Obradovic et al. 2019).

Cancer cell survival or resisting cell death is an original hallmark of cancer (Hanahan & Weinberg 2000). Cellular mechanisms associated with cell survival include the regulation of pro- and anti-apoptotic BH3 family proteins, the extrinsic death receptor signaling molecules, and the intrinsic caspase and apoptosome proteins that ultimately lead to cell death. Intrinsic activation of cell death pathways is often initiated by cell stress caused by DNA damage, ROS production, or metabolic/nutrient stress. GR is a long-established sensor of physiologic (i.e. hormonal) stress, and more recent data indicate that GR is also an exquisite sensor of cellular stress and stress signaling within the TME. In response to systemic glucocorticoid exposure, GR is known to promote cell death in lymphocytes and monocytes in order to reduce inflammation (Strehl et al. 2019). However, activation of GR also promotes cell survival in a variety of epithelial cell types, including in breast cancer models. In studies of TNBC, ligand-dependent and ligand-independent GR activation have been shown to promote expression of pro-survival proteins (Wu et al. 2004, Regan Anderson et al. 2018). For example, GR promotes breast tumor kinase (Brk/PTK6) expression in response to dexamethasone (ligand-dependent), and also in response to hypoxia, cell stress induced by chemotherapy, and anoikis (ligand-independent). In TNBC models, hypoxia, paclitaxel, 5-FU, and non-adherent culture all led to p38-MAPK-dependent GR Ser134 phosphorylation and ligand-independent activation of gene programs implicated in cellular stress-response (Regan Anderson et al. 2016, 2018). GR-dependent Brk/PTK6 induction protected cells from chemotherapy-induced cell death and promoted cell migration (Regan Anderson et al. 2018). These studies further our mechanistic understanding of how GR and glucocorticoids promote plasticity in order to protect TNBC cells from cell stress and chemotherapy-induced cell death and may have clinical implications for the routine use of high-dose steroids prior to chemotherapy.

Cells sense both local external (TME) and internal stress (hypoxia) via activation of JNKs and p38 MAPKs, also historically known as stress-activated protein kinases or SAPKs (Laderoute et al. 1999, Sumbayev & Yasinska 2005). Notably, upregulation of the p38 MAPK signaling pathway has emerged as a mechanism by which tumor cells increase their metastatic capacity. Mechanistic studies have shown that p38 MAPK signaling promotes the invasive and metastatic capacities of breast tumor cells (Limoge et al. 2017). As discussed previously, phosphorylation of Ser134 GR is p38 MAPK-dependent in osteosarcoma (Gallilher-Beckley et al. 2011) and breast cancer models (Regan Anderson et al. 2016). The role of hypoxia/HIFs in promoting EMT and increased migration of TNBC cell models is well-established (Brooks et al. 2016, Tirpe et al. 2019); our most recent studies indicate that GR is a principle driver of TME- (i.e. cytokine) and stress-induced TNBC migration, specifically via p38 MAPK-dependent GR Ser134 phosphorylation (C Perez Kerkvliet and CA Lange, unpublished results). While these effects in TNBC cell models were ligand-independent, others have reported that glucocorticoid treatment promoted metastasis in vivo using patient-derived xenograft (PDX) models (Obradovic et al. 2019). It is important to note that GR activation by glucocorticoids has been shown to suppress the migration of immune cells, highlighting the tissue- and cell-specific roles of GR/phospho-GR (Stahn & Buttgeriet 2008) and underscoring the need to select patients using appropriate biomarkers of phospho-GR signaling in GR+ TNBC patients.
Ligand-independent SRs are important therapeutic targets

Phospho-SR species are predicted to be valuable biomarkers of aggressive breast cancer behavior. Detection of phosphorylated receptors or their respective target gene signatures may provide a useful means by which to further classify more aggressive breast tumors and thus estimate risk of therapy-resistant recurrence. An important caveat to targeting phospho-SR species is that these species may be insensitive to existing receptor antagonists; phosphorylation events confer partial agonist activity to SR antagonists (Wardell et al. 2010) and phospho-ER species are strongly implicated in the development of tamoxifen resistance (Likhite et al. 2006, Kastrati et al. 2019). Notably, RU486 (an antagonist for both PR and GR) does not significantly reduce ligand-independent phosphorylation of PR or GR. However, at least in vitro, ligand-independent actions of phospho-SRs that are not further stimulated by agonists are often sensitive to selected antagonists. For example, ER-scaffolding actions of PR-B appear to be insensitive to type I antagonists (i.e. RU486) that induce PR Ser294 phosphorylation similar to that of PR agonists (Daniel et al. 2015), but are blocked by type II antagonists (i.e. onapristone) that also selected antagonists. For example, ER-scaffolding actions of phospho-SRs that are not further stimulated by agonists are often sensitive to selected antagonists. For example, ER-scaffolding actions of PR-B appear to be insensitive to type I antagonists (i.e. RU486) that induce PR Ser294 phosphorylation similar to that of PR agonists (Daniel et al. 2015), but are blocked by type II antagonists (i.e. onapristone) that also

Table 1  PR and GR post-translational modifications and associated ligand-dependent (LD) and ligand-independent (LI) biological outcomes.

<table>
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<th>Steroid receptor</th>
<th>PTM</th>
<th>Kinase pathway</th>
<th>Biological outcome</th>
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<tbody>
<tr>
<td>PR</td>
<td>S81</td>
<td>CK2</td>
<td>Proliferation, survival, migration, and transcriptional regulation (Hagan et al. 2011)</td>
</tr>
<tr>
<td>PR</td>
<td>S294 (Phosphorylation)</td>
<td>MAPK, CDK</td>
<td>Nuclear localization (Li; Qui &amp; Lange 2003), turnover, transcriptional regulation (LD; Daniel &amp; Lange 2009, Shen et al. 2001), proliferation (Daniel et al. 2007), stemness (Truong et al. 2019), senescence (Diep et al. 2013), and cell cycle progression (Moore et al. 2001)</td>
</tr>
<tr>
<td>PR</td>
<td>S345 (Phosphorylation)</td>
<td>MAPK, CDK</td>
<td>Proliferation, migration (Faiivre et al. 2008, Dressing et al. 2014)</td>
</tr>
<tr>
<td>PR</td>
<td>K388 (SUMOylation)</td>
<td>CK2</td>
<td>Transcriptional regulation and slows PR turnover (Abdel-Hafiz et al. 2002, Daniel et al. 2007)</td>
</tr>
<tr>
<td>PR</td>
<td>S676 (Phosphorylation)</td>
<td>CDK2</td>
<td>Disrupts nuclear translocation and delays MAPK-induced Ser345 and Ser294 phosphorylation (Daniel et al. 2010)</td>
</tr>
<tr>
<td>GR</td>
<td>S134 (Phosphorylation)</td>
<td>p38</td>
<td>Response to stress-activating stimuli/p38 MAPK, hypoxia (Regan Anderson et al. 2016), stress-induced survival, migration, and stemness (Perez Kerkvliet et al. 2020)</td>
</tr>
<tr>
<td>GR</td>
<td>S211 (Phosphorylation)</td>
<td>p38</td>
<td>Apoptosis (LD; Khan et al. 2017)</td>
</tr>
<tr>
<td>GR</td>
<td>S404 (Phosphorylation)</td>
<td>GSK-3</td>
<td>Turnover and resistance to LD apoptosis (Galliher-Beckley et al. 2008)</td>
</tr>
</tbody>
</table>
with existing therapies, the knowledge gap of the roles that PR/GR play as modulators of other SR (i.e. ER or AR) actions and the impact of phosphorylation events to SRs on hormone responsiveness and aggressive breast cancer biology must be elucidated.

Conclusions

In summary, SRs act as highly context-dependent sensors of the cellular signaling environment underpinned by PTMs that are intricately controlled by growth factors and cytokines within the tumor microenvironment and their intracellular MAPK effectors. Phospho-PR and -GR exhibit extensive functional redundancy with regard to shared ligands and binding sites in chromatin/target gene overlap. Both SRs are also capable of initiating feed-forward signaling loops, a paradigm in which their target gene products further phosphorylate the initiating SR, leading to a sustained signal that can be maintained indefinitely in the absence of steroid ligands. Understanding this mechanism is critical for the treatment of both luminal ER+ and triple negative breast cancers; sustained phospho-SR signaling may contribute to the emergence of therapy-resistant biologies in a minority population of cancer cells, including non-proliferative stem or dormant cell states, and cell fates associated with EMT, migration, survival, and therapy resistance. Therapeutically, targeting this paradigm will likely involve expanding hormonal modulation/kinase inhibitor repertoires to include agents specifically targeting phospho-SRs and their target genes.

Declaration of interest

Carol A Lange serves on the Board of Scientific Advisors for Context Therapeutics. The other authors have nothing to disclose.

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