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The molecular basis of progesterone receptor action in breast carcinogenesis

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Abstract

Progesterone plays an essential role in the regulation of cell proliferation and differentiation in the mammary gland. In addition, experimental and clinical evidence points to a critical role of progesterone and the nuclear progesterone receptor (PR) in controlling mammary gland tumorigenesis. However, the molecular mechanisms of progesterone action in breast cancer still remain elusive. In its classical mechanism of action, PR acts as a ligand-induced transcription factor (TF) interacting directly with specific progesterone response elements (PREs) in the promoter of target genes. In addition to its transcriptional effects, PR activates signal transduction pathways through a rapid or non-genomic mechanism. Interestingly, progestin induces the expression of key genes involved in breast cancer growth, which lack PREs in their promoters, via a non-classical PR transcriptional mechanism through PR tethering to other TFs. Recent findings on steroid hormone receptor modulation of target genes raise the most exciting possibility that progestin may also induce long-range transcriptional control of gene expression via PR binding to cis-regulatory elements (PREs or half PREs) located far upstream or downstream from the trascriptional start site. This review will focus on the involvement and interplay of the different PR actions in breast cancer.

Keywords: breast cancer; ErbB receptors; progesterone; progesterone receptor.

Introduction

The ovarian steroid hormone progesterone (4-pregnene-3, 20-dione, P4) plays an essential role in the regulation of cell proliferation and differentiation in the female reproductive tract [1, 2]. Progestogen defines the category of hormone molecules (natural and synthetic) that act like progesterone in the uterus. The term progestin has been generally used to refer both to progesterone and to a series of synthetic hormone molecules. Progesterone also regulates diverse biological effects in a broad range of tissues, including those of the cardiovascular [3] and the central nervous systems [4]. In addition, compelling experimental and clinical evidence points to a critical role for progesterone and the nuclear progesterone receptor (PR) in controlling mammary gland tumorigenesis [5–25]. However, the molecular mechanisms through which progesterone controls breast cancer growth, resistance to endocrine treatments, and metastasis are not yet fully understood. Undoubtedly, the complexity of progesterone action in breast cancer cells has hampered the identification of the PR as both a major hormonal player in the breast cancer scenario and as a molecular target for a first-line therapy in PR- and estrogen receptor (ER)-positive breast tumors, and for a second-line strategy in cases of acquired resistance to endocrine treatments, which at present only target ER-induced breast cancer growth. We will use here the generic term progestin to refer to both progesterone and synthetic progestins.

Structure and function of progesterone receptor isoforms

The biological effects of progestins are mediated by interaction with the PR, a member of the steroid receptor superfamily of ligand-activated nuclear transcription factors (TFs). The PR consists of two isoforms, PR-A and PR-B, expressed from a single gene by the use of alternative promoters in both humans and rodents. PR-B differs from PR-A in that PR-B contains an amino terminal extension of 128-65 amino acids, depending on the species, known as the B-upstream segment (BUS). Both PR isoforms contain a C-terminal hormone-binding domain, a DNA-binding domain (DBD), a hinge region, and two transcriptional activation function (AF) domains located at the amino terminus (AF-1) and at the ligand-binding domain (AF-2). The PR-B isoform contains a third transactivation function (AF-3) within its amino terminal extension, absent in PR-A [26–28] (Figure 1). The two PRs exhibit different transcriptional properties that depend on the cell type and on the target gene promoter, PR-B being a much stronger transactivator for PR target genes [28–30]. On the other hand, PR-A is a more potent dominant-negative transcriptional inhibitor of PR-B and other steroid hormone receptors, including the ER [30, 31]. Recent screens for differential regulation of gene expression by PR-A and PR-B in breast cancer revealed that although some genes are

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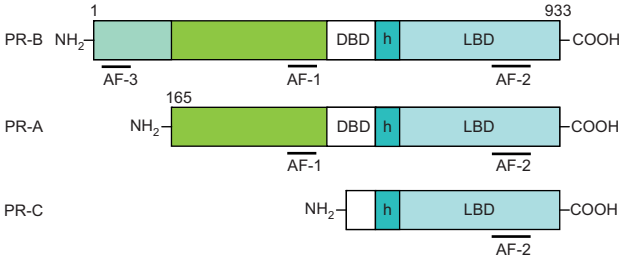

Figure 1 Schematic representation of PR domain organization. All three isoforms, PR-A, PR-B, and PR-C are expressed from a single gene by the use of alternative promoters. Both PR-B and PR-A isoforms contain a C-terminal hormone-binding domain, a DBD, a hinge region (h), and two transcriptional AF domains located at the amino terminus (AF-1) and at the ligand-binding domain (AF-2). The PR-B isoform contains a third transactivation function (AF-3) within its amino terminal extension. PR-C lacks the first zinc finger of the DBD.

modulated by progesterone through both PR isoforms, the majority of the target genes are exclusively regulated through one isoform or the other, principally through PR-B [32]. PR-A and PR-B ratios vary during the menstrual cycle in the uterus, indicating the physiological importance of these variations [33]. Studies in the knockout mice showed that PR-B, but not PR-A, is required for normal development and function of the mammary gland [34, 35]. On the contrary, PR-A is more important for ovarian and uterine development [34, 35]. Different ratios of PR isoform expression have been found in several cancers. Overexpression of PR-B has been found in ovarian, endometrial, and cervical tumors [36]. On the other hand, PR-A-rich breast tumors have poorer disease-free survival rates and exhibit resistance to endocrine therapy [37]. The PR is also expressed as a 60-kDa N-terminally truncated isoform, named PR-C, which is transcriptionally inactive and contains the hormone-binding domain and the dimerization and nuclear localization sequences, but lacks the first zinc finger of the DBD [38–40] (Figure 1). Expression of PR-C was found in breast cancer cells where it increases the transcriptional activity of PR-B and PR-A [38, 40]. In contrast, PR-C inhibits the transcriptional activity of PR-B in the uterus [41]. At present, the role of PR-C in the mammary gland development and in breast cancer remains poorly studied.

Progesterone action in the mammary gland

The two most studied progesterone target tissues are the uterus and the mammary gland. In the uterine epithelium, progesterone antagonizes the capacity of estrogen, the other ovarian steroid, to induce cell proliferation and acts as the major differentiating hormone. These two ovarian steroid hormones are generally present jointly in physiological conditions and so are their specific receptors coexpressed in the same tissues. Notably, a series of findings have shown that estrogen and progesterone counterbalance each other's effects in target tissues [1, 42]. Progesterone also plays a key role in mammary gland development and function. Studies in PR knockout mice (PRKO) have shown a series of reproductive deficiencies including the inability to ovulate, uterine dysplasia and inflammation, and impaired sexual behavior [43, 44]. These animals also display incomplete mammary gland ductal branching and failure of lobuloalveolar development upon exposure to estrogens and progesterone, disclosing that indeed progesterone exerts proliferative effects in the normal breast [43]. PR-B knockout mice exhibit reduced mammary ductal morphogenesis but have conserved ovarian, uterine, and thymic responses to progesterone [35]. Interestingly, in contrast with progesterone antagonistic effect on estrogen-driven proliferation in the uterus, proliferation in the mammary epithelium reaches its peak during the progesterone-dominant luteal phase of the human menstrual cycle, further evidencing a proliferative role for progesterone in the normal breast [1]. Moreover, progestins promote proliferation in the postmenopausal breast. Findings in normal mammary tissue from postmenopausal women indicated that breast epithelial cell proliferation is significantly higher in women receiving estrogen plus the synthetic progestin medroxyprogesterone acetate (MPA) in the hormone replacement therapy (HRT) compared to women receiving no HRT or receiving estrogen alone [25].

The mammary gland is composed of epithelium and stroma. Two different cell types are present in the epithelium: an inner layer of mammary luminal epithelial cells (MECs) surrounded by an outer layer of myoepithelial cells. PR is expressed only in the MECs of human, mouse, and rat mammary glands [45–48]. ER and PR are present only in up to 30% of the luminal MECs where they colocalize [49]. Interestingly, these receptors have been found in non-proliferating cells [47, 49] giving rise to the proposed paracrine role of PR in the mammary gland, where PR-positive breast cells express mitogenic factors that induce the proliferation of the surrounding PR-negative cells [50]. The receptor activator of nuclear factor κ B ligand (RANKL) [51] and Wnt-4 [52] have both been identified as paracrine mitogens mediating progesterone action in PR-negative MECs. In addition, a progesterone-responsive mouse mammary gland transcriptome, studied in conditions in which progestins stimulate proliferation, identified the inhibitor of differentiation 4 (Id4) as a new molecular target induced by progesterone in PR-positive cells [53]. Interestingly, a recent work has revealed two waves of progesterone-promoted proliferation in the adult mouse mammary gland. In the first, progesterone induces the proliferation of PR-positive MECs by a cyclin D1-dependent mechanism. In the second and larger wave, PR-negative MECs proliferate through paracrine, RANKL-mediated mechanisms [54].

Progesterone has also been found to mediate the expansion of adult mammary stem cells (MaSCs). A recent study by Joshi et al. demonstrated a remarkable increase in the basal ($CD49f^{hi}$) MaSC pool at the diestrus phase of the mouse reproductive cycle, when progesterone levels are maximal [55]. The said study reported similar results by exogenous administration of a hormone regimen combining 17β-estradiol and progesterone to ovariectomized mice [55]. As estradiol is merely permissive in the adult gland, where it induces PR expression

[54, 56], Joshi et al. [55] propose that estradiol plus progesterone effects on the basal MaSC pool indeed represent progesterone-directed effects. A basal cell population enriched in MaSCs, capable of generating a functional mammary gland [57], correlated with the elevated mammary repopulating units observed at diestrus [55]. Interestingly, RANKL and Wnt-4 expressions were significantly elevated in luminal cells from 17β-estradiol and progesterone-treated glands, suggesting that both mitogens may act in a paracrine fashion to enrich the basal MaSCs [55]. These findings clearly demonstrated a key role of progesterone in stimulating MaSC expansion during the reproductive cycle. Similarly, Asselin-Labat et al. [58] revealed that the number of MaSCs transiently increased at mid-pregnancy in mice, when serum levels of progesterone are at their peak, while ovariectomy diminished it. A paracrine role of RANKL in mediating progesterone-induced MaSC expansion was also suggested in this study. In addition, progesterone has been found to stimulate proliferation of normal human breast cells by activation of DNA replication licensing and increasing bipotent progenitor numbers [29].

Defining the role of the progesterone receptor: a major challenge in the management of breast cancer

Considerable epidemiological and clinical evidence has related the cumulative and sustained exposure to ovarian steroids estrogen and progesterone to increased risk of developing breast cancer [59]. Estrogen/ER involvement in mammary cancer has long been recognized, ER being a predictive marker with associated targeted therapy. Notably, a large body of evidence highlighted that progestins/PR also play a pivotal role in mammary tumorigenesis. First, multiple findings showed that depending on the experimental culture conditions and on the presence of estrogens, progestins either support sustained in vitro growth of breast cancer cells [6–11, 17] or induce cells to progress through one or multiple rounds of cell division, followed by growth arrest at the G1/S phase [12]. Consistent with the proliferative role of PR, a series of G1/S cell cycle phase proteins are induced upon progestin stimulation of breast cancer cells including cyclins D1 and E, c-fos, and c-myc [60, 61]. Seminal work by Horwitz and coworkers provided evidence that the initial pulse of progestins primes progesterone-arrested T47D breast cancer cells for the action of secondary proliferative or differentiative signaling pathways [12, 13, 62, 63]. Hence, the commitment to one path or the other would be determined by crosstalk between growth factor/cytokine pathways and progestins/PR signaling [12, 13, 62, 63]. Second, animal models strongly implicated PR in the genesis of breast cancer. Studies in genetically modified mice revealed that: (i) the PR knockout mouse shows dramatically reduced susceptibility to carcinogenesis [14], (ii) progesterone increases genomic instability in p53 null mouse models of breast cancer [15], and (iii) treatment of breast cancer 1 (Brca-1)-deficient mice with the antiprogestin mifepristone (RU486) prevented mammary tumorigenesis [16]. In addition, progestins exert sustained proliferative response in vivo in the ER- and PR-positive C4HD model of mammary carcinogenesis induced by the synthetic progestin MPA in female BALB/c mice [17–19]. This effect is fully abrogated by antiprogestins [20]. In rats, the PR receptor modulator antagonist (CDB-4124) inhibited mammary carcinogenesis by abrogation of cell proliferation and induction of apoptosis [64]. Finally, clinical observations as well as the recent extensive, randomized, and controlled Women's Health Initiative trial revealed that postmenopausal women who undergo a combined estrogen and progestin HRT suffer a higher incidence of breast cancer than women who take estrogen alone [21, 22]. Indeed, a decreased risk of breast cancer development was observed among postmenopausal women with prior hysterectomy receiving conjugated equine estrogens as HRT [65]. Interestingly, decline in breast cancer incidence seen during the last years in developed countries appears to be linked to drops in HRT use [66].

Clinical trials have been designed to block PR action in mammary tumors. On the one hand, a phase II study of the progesterone antagonist mifepristone in patients with untreated metastatic breast carcinoma did not show promising results when used as a single agent [67]. On the other, the data obtained in a small clinical trial with another PR antagonist, onapristone, support the use of this antagonist as first-line therapy in PR- and ER-positive breast cancer [68]. Antiprogestins also show affinity for the glucocorticoid (GR) and the androgen (AR) receptors. Their lack of specificity for the PR and their toxic effects observed in early studies have hindered the investigation on the therapeutic use of antiprogestins in breast cancer (reviewed in [69, 70]). An ongoing trial has been designed to identify the subgroup of women with early stage breast cancer most likely to benefit from treatment with mifepristone. Women will be treated with mifepristone prior to surgery, and decrease in growth rate will be examined in tumor samples taken before and after exposure to this antiprogestin [71].

The series of studies described above [55, 58] on the role of progesterone in MaSC expansion in the normal mammary gland also provide a novel and exciting mechanistic explanation for progesterone involvement in mammary tumorigenesis. As proposed by Asselin-Labat et al. [58] and Joshi et al. [55], an expanded and cycling MaSC population governed by progesterone may represent a target susceptible to undergo malignant transformation. The aromatase inhibitor letrozole is currently used to inhibit ER function in the endocrine therapy of breast cancer. The fact that the MaSC pool of the mouse mammary gland was reduced by letrozole treatment [58] further indicates that suppression of MaSC function could be a valuable therapeutic approach in breast cancer. On the other hand, MPA, the most frequently used progestin in contraceptives whose use in HRT is associated with increased breast cancer risk [21, 22], was found to induce a massive expression of RANKL in mammary gland epithelial cells [72]. This finding provides another link between progestin modulation of MaSC function and mammary tumorigenesis. Indeed, as discussed above, RANKL appears to be a paracrine mediator in the mechanism of progesterone expansion of MaSCs [55, 58]. Startling findings by Horwitz et al. [73] identified a rare

population of tumor-initiating cells (CD44+) that are ER$^-$PR$^-$ CK5+ in the luminal-like ER+PR+T47D human breast tumor xenografts. The tumor-isolated CD44+ cell fraction was highly enriched for clonogenic and tumorigenic cells compared to the CD44- cell fraction. In addition, tumors originating in vivo from CD44+ cells contained a rare static ER$^-$PR$^-$ CK5+ population, an intermediate ER$^-$PR$^-$CK5$^-$ population, and an expanding ER+PR+CK5$^-$ population. These authors suggested that luminal ER+PR+ breast tumors contain a subpopulation of basal-like ER$^-$PR$^-$CK5+ that has the capacity to generate ER+PR+CK18+CK5$^-$ cells. These rare ER$^-$PR$^-$ CK5+ progenitor cells would not respond to endocrine therapies targeting the ER and would survive to repopulate the tumor, accounting for endocrine therapy resistance.

Notwithstanding all these findings, there still is no established therapeutic intervention for ER- and PR-bearing breast tumors involving the blockage of PR action. At diagnosis, over 70% of breast cancers express ER and PR. On the basis of ER presence, patients are treated with endocrine therapies that target ER or rely on estrogen deprivation. Unfortunately, at best, only two thirds of tumors expressing ER and PR are responsive to endocrine therapy, whereas the rest show de novo resistance. A significant clinical problem is that tumors initially responsive to endocrine therapy later often acquire endocrine resistance. Tumors continue growing during treatment, and once metastatic disease has developed, the cure rate is under 5%. In the light of all the findings described above pointing to a role for PR in breast cancer, it seems reasonable to propose that resistance to endocrine treatments targeting ER-induced breast cancer growth might be due to the fact that the other major hormonal player in the breast cancer scenario, the PR, remains active and takes control of tumor growth. This highlights the urgent necessity to dissect the molecular mechanisms of PR action in breast cancer and to reveal whether targeting PR may provide a first-line therapy for ER+ and PR+ tumors and/or a second-line strategy for patients with acquired endocrine resistance.

Molecular mechanisms of PR action in breast cancer

Genomic effects

The classical pathway of progesterone-inducible PR-mediated gene transcription has long been described. Upon progesterone binding, PR undergoes a conformational change and dissociates from a multiprotein chaperone complex, which includes heat shock protein (hsp) 90 and its co-chaperone p23, hsp70, and three co-chaperones: hsp40, hsp70-interacting protein (hip), and hsp70-organizing protein (hop), and immunophilins (Imp), such as FK506 binding protein 5 (FKBP51) and FK506 binding protein 4 (FKBP52) (Figure 2). PR then dimerizes, translocates to the nucleus and binds to specific progesterone response elements (PREs) in the promoter of target genes where the DNA-bound receptor recruits coactivators that facilitate transcription initiation through interaction with components of the basal transcription machinery (Figure 2). Several PR coactivators have been described. Among them, the SRC/p160 family of proteins (NcoA/SRC1, GRIP1/SRC-2, NcoA-3/SRC3/RAC3) that binds to the AF-2 region of PR has been well characterized [74]. Accumulating evidence indicates that coactivators not only participate in the initiation step of transcription but also play a role in alternative RNA splicing, revealing another mechanism through which PR controls gene expression [75]. Over the last few years, the signal transducer and activator of transcription 3 (Stat3) was identified as a downstream effector of PR, which mediates progestin proliferative effects in breast cancer [19, 62, 76]. Interestingly, Stat3 plays a key role in mammary tumorigenesis [77]. Progestins induce Stat3 expression [19, 62] and its transcriptional activation in both human and mouse breast cancer cells [19]. In addition, inhibition of Stat3 activity by the use of a dominant negative Stat3 variant resulted in abrogation of in vitro progestin-induced human and mouse breast cancer cell growth and in in vivo growth inhibition in a preclinical trial in mouse [19]. Most recently, a novel level of PR and Stat3 interaction has been identified showing that Stat3 acts as a PR coactivator at the promoters of the mouse mammary tumor virus (MMTV) and of the endogenous gene bcl-X in breast cancer cells [76].

Rapid, extranuclear, or non-genomic effects

In addition to its direct transcriptional effects, rapid, non-genomic, or extranuclear PR effects in breast cancer cells have been revealed by startling reports from Auricchio and coworkers [78–80] who demonstrated that progestin treatment of human breast cancer T47D cells activates the signal transducing c-Src/p21*ras*/p42/p44 mitogen-activated protein kinase (MAPK) pathway, which results in cell proliferation. These authors have shown that progestin ability to activate c-Src/p42/p44 MAPKs pathway depends on the presence of unliganded ER, which is the one that activates c-Src. ER and PR-B interaction occurs via two domains that flank the PR proline-rich sequence located in its amino-terminal region [80, 81]. On the other hand, Edwards and coworkers revealed a direct in vitro interaction of the polyproline motif of PR-B with the Src homology-3 (SH3) domain of c-Src, which results in c-Src activation by a SH3 displacement mechanism, in human normal breast MCF-12 cells transduced with PR-B, and in T47D cells [81]. In these two cell types, interaction is progestin-dependent. As a consequence of c-Src activation, p42/p44 MAPK phosphorylation occurs in MCF-7 cells stably expressing PR-B [81]. In these studies, expression of both ER and PR reduced basal levels of c-Src activity, facilitating the detection of progestin-induced c-Src activation [81]. To reconcile the above discrepancies between progestin direct or indirect activation of c-Src, it has recently been suggested that a plethora of signaling complexes may result depending on the presence of different signaling and adaptator molecules in a given cell type [82]. Progestins have also been found to induce the rapid activation of the PI-3K/Akt pathway, which participates in in vitro and in vivo progestin-induced breast cancer cell growth [8, 18, 83, 84]. Progestins' rapid activation of p42/p44 MAPK and PI-3K/Akt pathways also induces the development of

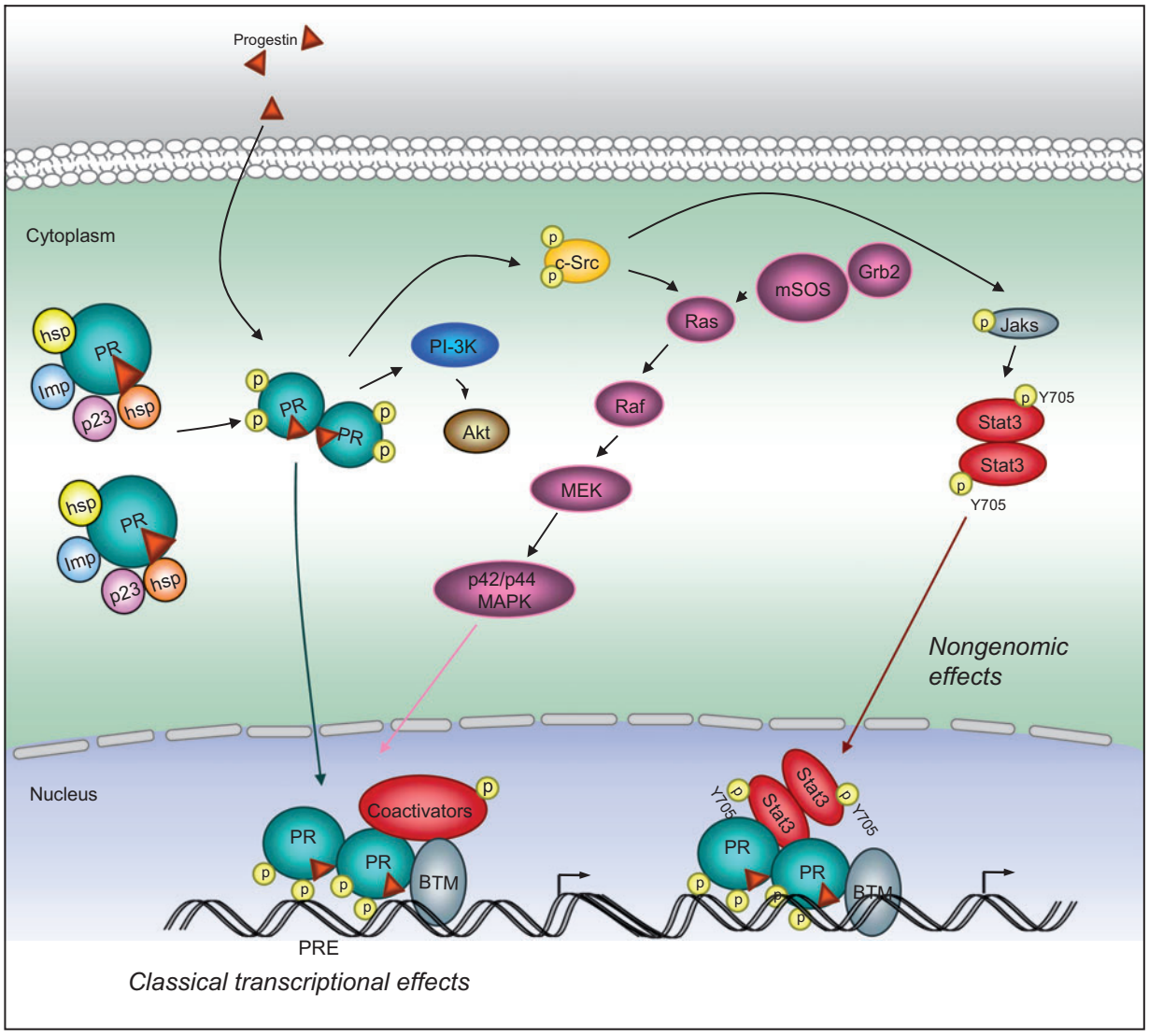

Figure 2 Genomic and non-genomic actions of PR in breast cancer cells. Left, classical transcriptional effects. Upon progesterone binding, PR undergoes a conformational change and dissociates from a multiprotein chaperone complex, which includes hsp, p23, and Imp. PR then dimerizes and translocates to the nucleus where it binds to specific PREs in the promoter of target genes. The DNA-bound PR recruits coactivators that facilitate transcription initiation through interaction with components of the basal transcription machinery (BTM). Right, PR non-genomic action occurs through PR activation of cytoplasmic signaling pathways, such as c-Src/p21ras/MAPKs and PI-3K/Akt. Shown is also PR rapid phosphorylation of Stat3 induced via c-Src and Jaks. Also illustrated is a general model proposing that phosphorylation of PR and/or its coactivators, via signaling pathways rapidly activated by progestins, integrates non-genomic and direct PR transcriptional effects. Particularly, we are here illustrating the recently described recruitment of Stat3 as PR coactivator upon its phosphorylation induced by progestin-rapidly activated c-Src and Jaks.

breast cancer metastasis [8]. In addition, non-genomic progestin activation of Jaks and c-Src has been found to induce the rapid phosphorylation of Stat3 at tyrosine (Tyr) 705 [19]. Figure 2 illustrates non-genomic PR effects. Also illustrated is a model proposing that phosphorylation of PR and/or its coactivators, via signaling pathways rapidly activated by progestins, integrates non-genomic and direct PR transcriptional effects. Coordinated non-genomic and non-classical transcriptional PR actions are discussed in detail in the "Non-classical PR tethering transcriptional mechanisms" and "Crosstalk between PR and ErbBs in breast cancer" sections below.

Non-classical PR tethering transcriptional mechanisms

Intriguingly, progestin induces the expression of an important number of genes that lack a canonical PRE in their promoters, including key regulators of cell cycle progression, such as cyclin D1 and $p21^{CIP1}$ [12, 85, 86]. This may occur via a non-classical PR transcriptional mechanism through PR tethering to other TFs in the promoter of targets genes. In the case of $p21^{CIP1}$, progestins modulate its expression via PR tethering to the TF SP1 at the third and fourth SP1 binding sites

in the $p21^{CIP1}$ promoter [86–88]. Similar interaction between PR and SP1 at the $p27^{KIP1}$ promoter has also been found [87]. The coactivators CBP/p300 and TReP-132 are corecruited along with SP-1 and PR to $p21^{CIP1}$ and $p27^{KIP1}$promoters [87]. This tethering mechanism raises an exciting question: does PR rapid stimulation of signaling pathways induce the phosphorylation of TFs that in turn participate in non-classical PR transcriptional tethering mechanisms? It is to be noted that although two SP-1 phosphorylation sites for p42/p44 MAPKs have been identified [89], no progestin modulation of SP-1 phosphorylation state has so far been reported. However, accumulating evidence indicates that phosphorylation of the proteins recruited to the non-classical transcriptional complexes, including PR itself, is a key requirement for the assembly of the said complexes. PR rapid stimulation of p42/p44 MAPKs and c-Src has already been found to mediate progestin-induced activation of the TF Elk-1 [90]. In addition, progestin's rapid phosphorylation of another TF, Stat3, via Jaks and c-Src, induces its recruitment to the SP-1 binding sites at the $p21^{CIP1}$ promoter along with PR [76]. In this multimeric SP-1/Stat3/PR transcriptional complex, Stat3 acts as a PR coactivator. Startling findings by the Lange laboratory highlighted the exciting notion that PR phosphorylation at specific residues is also required for its recruitment to non-classical transcriptional complexes. These authors showed that progestins' rapid phosphorylation of the epidermal growth factor receptor (EGF-R/ErbB-1) results in p42/p44 MAPKs activation which in turn phosphorylates PR at Ser345 by a feed-forward mechanism [88]. This phosphorylation is mandatory for PR tethering to SP-1 bound to its response elements at the $p21^{CIP1}$ promoter, EGF-R upregulation, and breast cancer cell proliferation [88]. Figure 3 illustrates the proposed model of PR action integrating non-genomic and non-classical transcriptional mechanisms.

New avenues in the field of steroid hormone receptor modulation of target genes have been opened by a series of seminal findings on the ER and the AR [91–94] and by the availability of techniques, such as chromosome conformation capture (from 3C to 5C) and its chromatin immunoprecipitation (ChIP)-based modifications. Data from genome-wide studies showed that a significant number of ER-binding sites are located in distal intergenic regions, upstream or downstream from the transcriptional start sites [91, 94]. Pioneering studies also revealed that ER modulates cyclin D1 expression in breast cancer cells through the combinatorial recruitment of a network of TFs to their closely spaced response elements at a downstream enhancer region [95]. In addition,

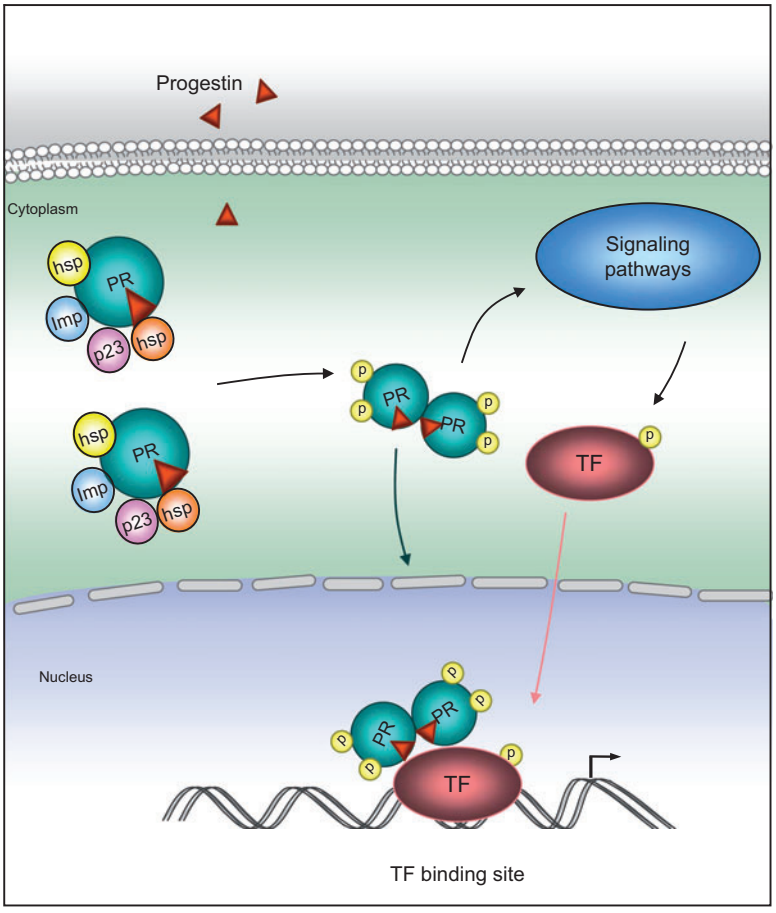

Figure 3 Model of PR action integrating non-genomic and non-classical transcriptional mechanisms. PR induces the rapid stimulation of signaling pathways that in turn phosphorylate TFs. Upon progestin-induced phosphorylation, the TF binds to its response elements in the promoter/enhancer regions of target genes. PR is recruited along with the TF to the chromatin target, a non-classical PR-tethering mechanism.

ER has been found to modulate c-myc, another key gene in the breast cancer scenario, via binding to a distal enhancer containing a half-estrogen response element and an activator protein 1 (AP-1) site [96]. Similarly, in AR-dependent prostate cancer, AR controls the expression of the prostate-specific antigen (PSA) and the transmembrane protease, serine 2 (TMPRSS2)-ETS fusion genes through a long-range mechanism involving the combinatorial recruitment of AR, several TFs, and co-regulators that do not bind DNA directly [92, 93, 97]. Comparable long-range transcriptional control of the expression of a specific gene or a set of genes via PR binding to cis-regulatory elements (PREs) located far upstream or downstream from the promoters has not yet been revealed in any cell model. However, in the light of the findings on ER and AR described above, it seems reasonable to postulate that this mechanism may be involved in progestin control of key target genes lacking canonical PREs or half PREs in their promoters, which mediate progestin-induced breast cancer growth. Indeed, a curated database of nuclear receptor cistrome data sets and other associated data sets, including collaborating factor cistromes, epigenomes, and transcriptomes, has most recently been developed by Tang et al. [98]. These authors analyzed the cistromes of several nuclear receptors and found that for most of them, including PR, the median distance between a binding site and its nearest gene was more than 10 kb. Furthermore, they integrated cistrome and transcriptome data and made probabilistic predictions on PR and other nuclear receptor target genes in various cancer cell line models. Particularly for PR, predictions of target genes were made in T47D breast cancer cells treated with progesterone for 3 and 24 h [99]. It is to be noted that DNA looping constitutes a requisite for the interaction between remote cis-acting elements and gene promoters. Interestingly, it has been found to take place in the mechanism of progesterone regulation of RUSH/SMARCA3, a progesterone target gene, encoding a member of the switch/sucrose nonfermenting (SWI/SNF) family, in rabbit endometrium [100].

Crosstalk between PR and ErbBs in breast cancer

Crosstalks between progestins/PR and the ErbB family of receptor tyrosine kinases play a key role in breast cancer. The ErbB family is composed of four members: EGF-R/ErbB-1, ErbB-2, ErbB-3, and ErbB-4. ErbB ligands include all isoforms of heregulins (HRG), which bind to ErbB-3 and ErbB-4 and recognize EGF-R and ErbB-2 as co-receptors, and the EGF, which binds to EGF-R [101]. Upon ligand binding, ErbBs dimerize, and their intrinsic tyrosine kinase is stimulated leading to the activation of signal transduction pathways that mediate ErbB proliferative effects in mammary tumor cells. Interestingly, the interaction between PR and ErbBs in breast cancer is of bidirectional nature, where PR modulates the expression of ErbB ligands and induces ErbB phosphorylation [7, 12, 63, 88, 102, 103], and conversely, ErbB signaling pathways modulate PR transcriptional function, mostly by regulation of the PR phosphorylation state [88, 104] (Figure 4). It is to be noted that PR is a heavily phosphorylated protein in multiple serine residues (reviewed in [105, 106]).

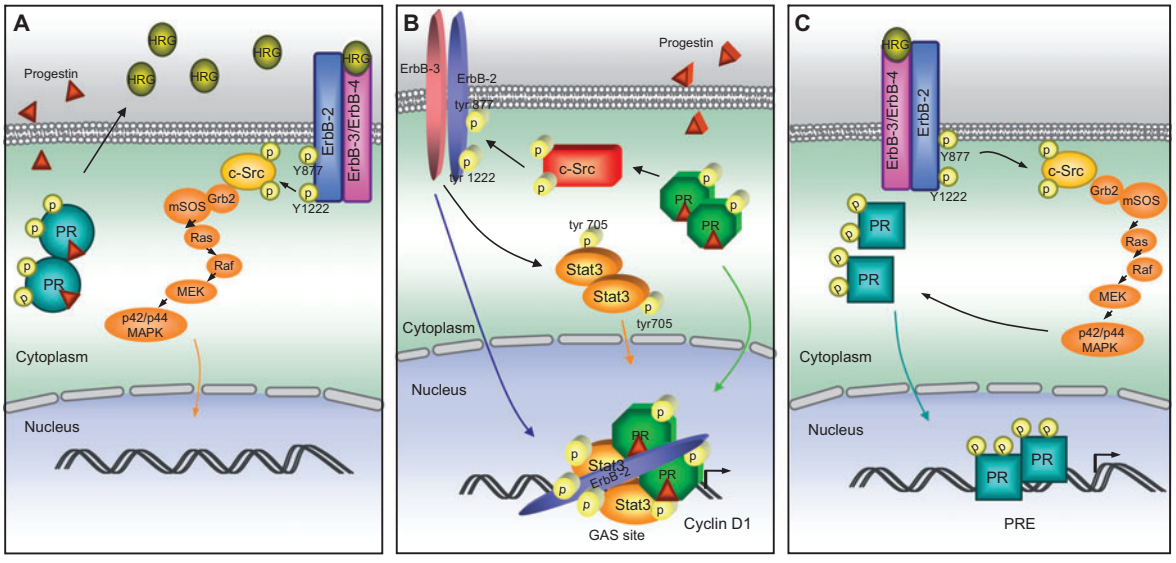

Figure 4 Bidirectional cross-talk between PR and ErbB signaling in breast cancer. (A) Progestins upregulate the expression of ErbB ligands, such as HRG, which binds to ErbB-3 and ErbB-4 and induces ErbB dimerization. Heterodimers containing ErbB-2 as the preferred partner are illustrated. Dimerization results in the stimulation of ErbB intrinsic tyrosine kinase activity leading to the activation of cytoplasmic signaling cascades, such as the c-Src/p42/p44 MAPKs, which stimulate mammary tumor cell growth. (B) Progestins induce the rapid phosphorylation of ErbB-2 via c-Src, its nuclear localization, and the assembly of a non-classical transcriptional complex between Stat3 and ErbB-2 at the Stat3 response element (GAS site) of cyclin D1 promoter. In this complex, the TF (Stat3) is first phosphorylated at the cytoplasmic level via its coactivator (ErbB-2) function as an upstream effector. PR is also loaded onto the Stat3/ErbB-2 complex, unraveling a new non-classical PR genomic mechanism. (C) Ligand-independent activation of the PR. The figure depicts HRG transcriptional activation of PR via ErbB-2 and p42/p44 MAPKs.

Phosphorylation of PR is mediated by several kinases [88, 102, 105–115]. Startling findings by the Weigel laboratory revealed that cyclin A/cdk2 phosphorylates PR at multiple Ser residues including Ser162, Ser190, and Ser400 in vitro, which are also authentic in vivo targets [115]. Cdk2-induced phosphorylation of PR has been found to modulate agonist-dependent PR activity [110, 111, 113]. Undoubtedly, the vast majority of the studies on kinase regulation of PR activity have focused on p42/p44 MAPKs, which have been found to modulate different aspects of PR function including PR turnover, subcellular localization, and transcriptional activity [88, 102, 107–112]. In addition, accumulated evidence has identified phosphorylation as one of the molecular mechanisms by which PR and ErbB signaling converge [88, 102, 104, 116]. Progestins have been found to upregulate EGF-R, ErbB-2, and ErbB-3 in breast cancer cells [7, 12, 63]. In addition, PR induces the rapid phosphorylation of EGF-R at the Tyr 1173 [88], an autophosphorylation site, and of ErbB-2 at Tyr 1222, a major autophosphorylation site, as well as at Tyr 877, a site different from the autophosphorylation ones and located in the activation loop of ErbB-2 kinase domain [117, 118].

Progestins have also been found to induce the expression of HRG, one of the ErbB ligands, in mammary tumor cells [7]. Inhibition of HRG expression abolished progestin-induced breast cancer cell growth indicating that HRG acts as a mediator of progestin-induced growth [7]. Mammary tumor progression to a progestin-independent phenotype was accompanied by a high constitutive expression of HRG [7]. Notably, the dogma of ErbB-2 mechanism of action has been challenged by the most exciting findings of Hung and coworkers demonstrating that ErbB-2 migrates to the nuclear compartment where it binds DNA at specific sequences, which they named human epidermal growth factor receptor-2 (HER-2)-associated sequences (HAS) [119]. Recently, it has been demonstrated that progestins induce the nuclear migration of ErbB-2 in breast cancer cells and the assembly of a transcriptional complex where ErbB-2 acts as a coactivator of Stat3 at the promoter of cyclin D1 [120]. This complex is directly involved in progestin-induced in vitro and in vivo mammary tumor growth. Notably, PR is also loaded onto the Stat3/ErbB-2 complex, and the corecruitment of ErbB-2 is an absolute requirement for PR tethering to Stat3, unraveling a new and unexpected feature of the non-classical PR genomic mechanisms [120].

The other scenario of ErbB and progestin interaction, i.e., the capacity of ErbBs to transactivate PR, has also been explored. Thus, it was demonstrated that HRG induces PR transactivation through a mechanism that requires both a functional ErbB-2 and p42/p44 MAPK activation [104]. The said study also showed that HRG-activated p42/p44 MAPKs phosphorylate both human and mouse PR at the residue Ser294 [104]. Ligand-independent activation of PR by modulators of kinases and phosphatases has also been found in the course of studies of molecular mechanisms involved in PR activation. Indeed, okadaic acid, an inhibitor of protein phosphatases 1 and 2, and 8-bromo-cyclic AMP (cAMP), an activator of cAMP-dependent protein kinase A, have been shown to potently stimulate chicken PR (cPR)-mediated transcription in the absence of progesterone [121, 122]. Vanadate, an inhibitor of phosphotyrosine phosphatases, induced cPR-mediated transcription in the absence of progestins [121]. Human PR appears to be less susceptible than cPR to ligand-independent activation by modulators of protein phosphorylation. Thus, treatment of T47D cells with 8-bromo-cAMP or okadaic acid alone had no measurable effect on PR expression at protein level or in PR number and did not stimulate hPR transcriptional activity [123]. However, both compounds augmented hPR-mediated target gene transcription when added together with progestins [123, 124]. In human breast cancer cells, EGF was found to induce the rapid and transient phosphorylation of PR-B at Ser294, PR nuclear translocation, and DNA binding of the wild-type PR-B, but not of a mutant PR containing an Ala at position 294 [108]. Although EGF alone induced a small increase in PR-B transcriptional activation, it significantly increased the transcriptional activity of wild-type PR-B upon progestin stimulation [108]. Phosphorylation of PR-B at Ser 294 by progestins or growth factor-activated p42/p44 MAPKs was identified as key for PR-B transcriptional activity [104, 107–109]. On the other hand, sumoylation has been found to repress PR transcriptional activity on several PRE-driven and endogenous promoters [125]. EGF treatment or transient expression of MAPK kinase or cdk2, which induce PR-B Ser294 phosphorylation, in turn, block PR-B sumoylation, derepressing receptor activity [125]. T47D cells stably expressing a sumoylation-deficient mutant PR-B (K388R) exhibit increased ligand-independent proliferation compared to cells expressing wild-type PR-B or the phospho-mutant S294A PR-B, which is in turn heavily sumoylated [126]. In addition, basal, ligand-independent expressions of a series of PR target genes involved in breast cancer growth, such as insulin receptor substrate-1 (IRS-1) and stanniocalcin1 (STC1), were significantly elevated in cells containing desumoylated PR-B (K388R) [126]. These findings highlight the regulation of gene expression by phosphorylated and undersumoylated PRs as a novel mechanism of hormone-independent PR function in breast cancer [126]. Furthermore, the serine-threonine protein kinase ck2 phosphorylates PR-B at the residue Ser 81 located in the BUS region, unique to PR-B. Notably, PR-B Ser 81 phosphorylation is modulated by progestins in intact cells but occurs also in a PR-independent manner when cells enter the G_1/S phase of the cell cycle [127]. This specific phosphorylation was found to be a requisite for basal and/or progestin regulation of the expression of a series of selected genes by PR-B [127]. Recently, it has been demonstrated that HRG induces the transcriptional activation of Stat3 in breast cancer through the co-option of unliganded PR function as a signaling molecule. The presence of a PR specifically phosphorylated at Ser294 by HRG-activated p42/p44 MAPKs was mandatory for HRG induction of Stat3 transcriptional activity [116].

Progestin modulation of microRNAs in breast cancer

MicroRNAs (miRNAs) are a recently discovered class of non-coding endogenous RNAs with regulatory functions.

Accumulating evidence has clearly pointed to an important role for miRNAs in breast cancer development and metastasis [128–131]. Estrogen has been found to induce a series of miRNAs, including the let-7 and the miR-200 families and to downregulate the expression of others, among them the well characterized miR-21, which functions as an oncomir promoting cell growth [132]. Correlation between these miRNA levels of expression and breast cancer molecular phenotypes has already been reported [128]. In contrast to the findings already described with estradiol, to the best of our knowledge, there is only one report in literature demonstrating progestin modulation of miRNA expression in breast cancer cells. The said study revealed that progestins modulate the expression of a set of miRNAs. miR-16, whose function as a tumor suppressor in leukemia has previously been shown, was identified as one of the downregulated miRNAs in murine and human breast cancer cells [133]. Progestin modulation of miR-16 expression directly correlated to its ability to induce mammary tumor growth [133]. Regarding the interplay between miRNAs and PR, it has been reported that two miRNAs inhibited by estradiol (miR-26a and miR-181a) directly target the *PGR* 3′UTR and reduce PR mRNA and protein [134].

Summary and conclusion

Accumulating experimental and clinical evidence indicates that PR is a major hormonal player in the breast cancer scenario, together with ER. However, for reasons that are likely related to the complexity of PR function, most studies have focused only on ER role in mammary tumorigenesis. Indeed, endocrine treatments available at present only target ER-induced breast cancer growth. Several unique features of PR biology hindered it from being held as a target for therapy. First, PR is an ER-dependent gene, total PR levels being regulated by estrogen in the breast. In addition, PR-A and PR-B isoforms bear distinct transcriptional activities under different cellular contexts and modulate differential sets of genes in breast cancer cells. Most interestingly, unliganded PR-A and PR-B also regulate the expression of genes involved in breast cancer growth and metastasis. Moreover, PR is a heavily phosphorylated protein, critical in regulating PR transcriptional activity. PR phosphorylation is not only induced by progestins but also by growth factors. Finally, PR participates in an extensive and bidirectional crosstalk with growth factor signaling pathways. This interplay is directly involved in breast cancer growth. Only a deeper understanding of the molecular mechanisms of PR action and interaction with ER and growth factor signaling in breast tumors will definitely reveal the enormous potential value of establishing PR-targeted therapies.

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