Chapter 7

Progesterone Receptor Action:
Translating Studies in Breast Cancer Models to Clinical Insights

Carol A. Lange,* Carol A. Sartorius, Hany Abdel-Hafiz, Monique A. Spillman, Kathryn B. Horwitz and Britta M. Jacobsen

Abstract

Progesterone receptors (PR) are useful prognostic indicators of breast cancers likely to respond to anti-estrogen receptor (ER) therapies. However, the role of progesterone, therapeutic progestins, or unliganded or liganded PR in breast cancer development or progression remains controversial. PR are ligand-activated transcription factors that act in concert with intracellular signaling pathways as "sensors" of multiple growth factor inputs to hormonally regulated tissues, such as the breast. The recently defined induction of rapid signaling events upon progestin-binding to PR-B provides a means to ensure that receptors and coregulators are appropriately phosphorylated as part of optimal transcription complexes. PR-activated kinase cascades may provide additional avenues for progestin-regulated gene expression independent of PR nuclear action. Herein, we present an overview of progesterone/PR and signaling cross-talk in breast cancer models and discuss the potential significance of progestin/PR action in breast cancer biology using examples from both in vitro and in vivo models, as well as limited clinical data. Kinases are emerging as key mediators of PR action. Cross-talk between PR and membrane-initiated signaling events suggests a mechanism for coordinated regulation of gene subsets by mitogenic stimuli in hormonally responsive normal tissues. Dysregulation of this cross-talk mechanism may contribute to breast cancer biology; further studies are needed to address the potential for targeting PR in addition to ER and selected protein kinases as part of more effective breast cancer therapies.

Introduction

Normal breast development requires estrogen receptor (ERα), progesterone receptor (PR) and peptide growth factors. Estrogen stimulates ductal elongation and progestins induce ductal sidebranching and alveologenesis.[1] Epidermal growth factor (EGF), in addition to promoting the proliferation of terminal end-buds, augments estrogen-induced ductal outgrowth and progesterone-induced sidebranching.[2] Indeed, estrogen induces PR isoform expression only in the presence of EGF,[3] suggesting the existence of important cross-talk between EGFRs and both steroid receptors (SRs). Ligand-activated PRs and ERs are potent mitogens in the developing breast and mammary epithelial cells express PR as well as ERα. Moreover, estrogen is usually required to induce the expression of PR. PR and ER are normally expressed by only ~7-10% of nondividing epithelial cells in the lumen of the mature mammary gland. This nonproliferative condition

*Corresponding Author: Carol A. Lange—Departments of Medicine (Division of Hematology, Oncology and Transplant) and Pharmacology, University of Minnesota Cancer Center, 420 Delaware Street SE, MMC 806, Minneapolis, Minnesota 55455 USA.
Email: Lange047@umn.edu

Innovative Endocrinology of Cancer, edited by Lev M. Berstein and Richard J. Santen.

appears to be sustained by such inhibitory molecules as TGF-beta or high levels of p27, a CDK inhibitor (reviewed in G.W. Robinson et al [4]). In response to communication between stromal and epithelial compartments, SR-positive epithelial cells express and secrete pro-proliferative molecules, such as Wnts or IGF-II, thereby inducing the proliferation of adjacent SR-negative epithelial cells.[4,5] Recent data indicate that SR-positive cells in the breast may support the activity of nearby stem-like progenitor cells via the expression of secreted factors.[6]

In contrast to the normal breast, where proliferating cells are devoid of SRs, the majority of newly diagnosed breast cancers (~70-80%) express ER and PR. The existence of SR-positive proliferating cells in breast cancer indicates that SR-positive cells may undergo an early switch to autocrine stimulation and/or continue to divide. Breast cancer is not the only setting where PR-containing cells divide. In an in vivo model of the mammary gland during pregnancy, the PR-B isoform colocalizes with cyclin D1 in BrdU-stained (dividing) cells.[7] Thus, signaling pathways involved in normal mammary gland growth and development are likely reactivated during breast cancer progression.

Progestins are recognized as mediators of increased post-menopausal breast cancer risk when taken as part of combined hormone replacement therapy relative to estrogen alone or placebo.[8] Experimental animal models of the effects of hormones on the postmenopausal mammary gland indicate that progestins stimulate proliferation.[9,10] While progestins are not carcinogens, progesterone might induce recently initiated precancerous breast cell populations to inappropriately reenter the cell cycle or stimulate dormant stem cells to undergo self-renewal (discussed below). Breast tumors develop resistance to endocrine-based treatments (anti-estrogens and/or aromatase inhibitors) as they progress. However, the majority (65%) of resistant breast cancers retain high levels of SRs (ERα and PRs). In these resistant, SR-positive cancers, the rapid action of SRs at the membrane might begin to inappropriately trigger the classical transcriptional activities of SRs. In this way, PRs activated by extremely low or sub-threshold concentrations of hormone or PRs phosphorylated in the absence of hormone can activate membrane-associated signaling pathways, including c-Src kinase, EGFR and the p42/p44 MAPK pathway. Elevation of MAPK activity and downstream signaling frequently occurs in breast cancer, providing a strong survival and proliferative stimulus to breast cancer cells. MAPK signaling downstream of EGFR or Her2 (erbB2) is also associated with resistance to endocrine therapies.[11]

This chapter focuses specifically on the role of progesterone and progesterone receptors (PR) in the pathophysiology of breast cancer. We review the literature describing PR-initiated genomic and nongenomic signaling pathways in breast cancer progression with the purpose of highlighting key kinases involved in the integration of rapid cytoplasmic signaling events and PR nuclear actions. We also discuss the clinical findings relevant to the use of PR status in the prediction of breast cancer behavior, evidence for PR action in breast cancer and the potential for PR ligands as therapeutic agents.

Classical Actions of PRs

PRs are activated through binding with the ovarian steroid ligand, progesterone. PRs are classically defined as ligand-activated transcription factors that regulate gene expression by binding directly or indirectly to DNA. Three PR isoforms are the product of a single gene located on chromosome 11 at q22-23 that undergoes transcription via the use of alternate promoters and internal translational start sites.[12] PR isoforms consist of the full length PR-B (116 kDa), N-terminally-truncated PR-A (94 kDa) and PR-C-isoforms (60 kDa). PR-positive cells usually co-express PR-A and PR-B isoforms; these receptors have different transcriptional activities within the same promoter context, but can also recognize entirely different promoters.[13,14] PR-B is required for normal mammary gland development,[15] while PR-A is essential for uterine development and reproductive function.[16] PR-C is devoid of classical transcriptional activity and instead functions as a dominant inhibitor of uterine PR-B in the fundal myometrium during labor.[17] In the absence of progesterone, PRs are complexed with several chaperone molecules including heat shock protein (hsp) 90, hsp70, hsp40, Hop and p23; these interactions are requisite for proper protein folding

and assembly of stable PR-hsp90 heterocomplexes that are competent to bind ligand.[18] Hsps also function to connect PRs to protein trafficking systems. After binding to progesterone, the receptors undergo restructuring, dimerization and hsp dissociation. Activated receptors bind directly to specific progesterone response elements (PREs) and PRE-like sequences in the promoter regions of such target genes as *c-myc*,[19] *fatty acid synthetase*,[20] and *MMTV*.[21] Treatment with progestin also results in an upregulation of regulatory molecules without classical PREs in their proximal promoter regions, such as EGFR[22,23], *c-fos*[24,25] and *cyclin D1*.[26,27] PR regulation of genes without canonical PREs can occur through indirect DNA-binding mechanisms, as in the example of PR binding to Specificity protein 1 to promote p21 transcription in the presence of progestin.[28] PRs can also regulate genes by tethering to activating protein 1[29] or signal transducers and activators of transcription (STATs).[25,30]

When either directly or indirectly bound to DNA, PRs regulate the basal transcription machinery in conjunction with nuclear receptor coregulatory molecules. Coregulators modulate transcription through chromatin remodeling and recruitment of transcriptional machinery (e.g., RNA Polymerase-II). Histone acetyl transferases (HATs) and histone deacetylases (HDACs) function as coactivators and corepressors, respectively. Both HATs and HDACs coordinate transcriptional activity with other regulator proteins, including the ATP-dependent chromatin remodeling complexes (SWI/SNF), arginine methyltransferases (CARM1 and PRMT1) and histone kinases (reviewed in N.J. McKenna, B.W. O'Malley[31]).

Direct PR Phosphorylation in Breast Cancer Models

Similar to other SR family members, phosphorylation-dephosphorylation events add multi-functionality to PR action (Fig. 1). Several protein kinases phosphorylate PR isoforms primarily on serine residues within the amino-termini and, to a lesser degree, on serine residues throughout the receptor.[12,32] PR contains a total of 14 known phosphorylation sites (reviewed in C.A. Lange[33]). Serines at positions 81, 162, 190 and 400 appear to be constitutively phosphorylated in the absence of hormone[34] (Fig. 1). One to two hours after progestin treatment, serines at positions 102, 294 and 345 are maximally phosphorylated.[35] Specific kinases have been identified that are responsible for phosphorylation of selected sites. Serines at positions 81 and 294 are phosphorylated by casein kinase II[36] and mitogen-activated protein kinase (MAPK),[37,38] respectively. Progestins can also stimulate Ser294 phosphorylation independently of MAPKs by activation of an unknown kinase(s).[39] Eight of the total 14 sites (i.e., serines 25, 162, 190, 213, 400, 554, 676 and Thr430) are phosphorylated by cyclin A/cyclin-dependent protein kinase 2 (CDK2) complexes in vitro.[34,40] Only five of these sites (i.e., serines 162, 190, 213, 400, 676) are proven in vivo phosphorylation sites.[34,36,40]

While the function of PR phosphorylation is incompletely understood, it might influence aspects of transcriptional regulation, such as interaction with coregulators, as reported for ER-α[41] and recently for PR.[42] PR phosphorylation is also involved in the regulation of ligand-dependent[38] and -independent[43,44] PR nuclear localization, receptor turnover, hormone sensitivity and transcriptional activities.[37,38,45,46] As has been reported for ERα,[47,48] phosphorylated PRs are hypersensitive relative to their underphosphorylated counterparts.[49] For example, following a brief (5-15 min) pretreatment with EGF, phosphorylated nuclear PR-B receptors are transactivated by sub-physiologic progestin levels. EGF and progestins synergistically upregulate mRNA or protein levels for a number of growth regulatory genes,[25] including cyclin D1 and cyclin E;[22] the regulation of cyclins by progestins is MAPK-dependent. Cyclins, in turn, regulate progression of cells through the cell cycle by interaction with cyclin-dependent protein kinases. Progestins activate CDK2,[27] which predominantly phosphorylates PRs at proline-directed (S/TP) sites,[34,40] perhaps allowing for the coordinate regulation of PR transcriptional activity during cell cycle progression. In support of this idea, Narayanan and coworkers[42,50] report that PR activity is highest in the S phase and lower in the G0/G1 phases of the cell cycle, but this activity is impaired during the G2/M phases, concomitant with lowered PR phosphorylation. Overexpression of Cyclin A or CDK2 enhanced PR transcriptional activity. While cyclin A interacts with the N-terminus of PR, CDK2 seems to

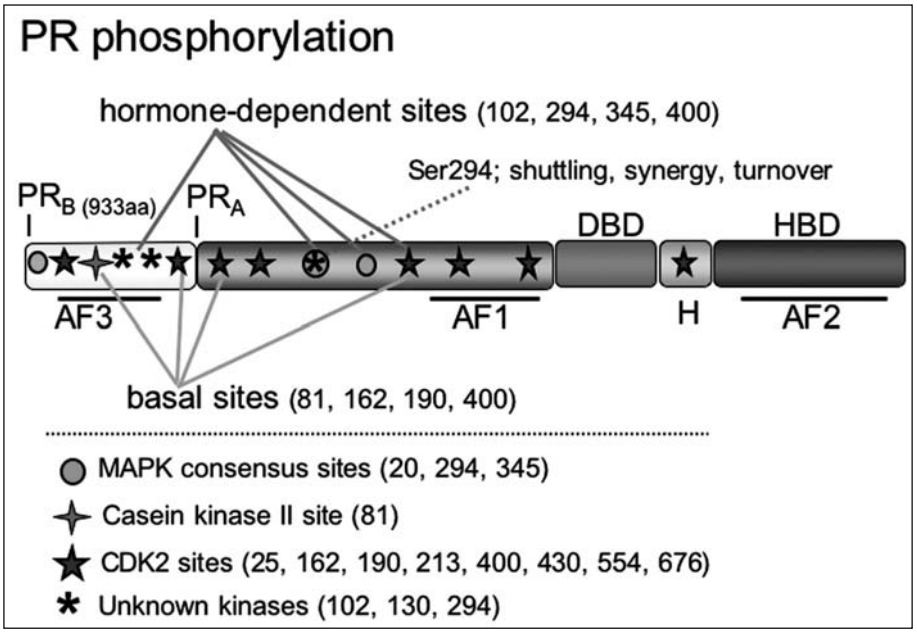

Figure 1. Phosphorylation sites in human PR. PR phosphorylation. Thirteen serine residues and one threonine residue in human PR are shown, to represent basal (constitutive) and hormone-induced phosphorylation sites[40] and may contribute to PR regulation by MAPK,[37-39] casein kinase II,[36] and CDK2.[34,40] Individual PR phosphorylation sites may be regulated by multiple protein kinases[39] and/or in a sequential manner,[143] illustrating the complexity of PR regulation by phosphorylation.

alter PR function indirectly by increasing the phosphorylation and recruitment of steroid receptor coactivator-1 (SRC-1) to liganded PR.

PR Ser294 Phosphorylation in Breast Cancer Models

PR Ser294 is rapidly phosphorylated upon exposure to ligand.[35] Ser294 is also a proline-directed or MAPK consensus site (PXXSP). Progestin-induced Ser294 phosphorylation occurs within 30-60 min independently of MAPK activation, whereas growth factor-induced Ser294 phosphorylation occurs within 3-5 mins in a MAPK-dependent manner.[39] PR Ser294 is considered a significant site for PR regulation by multiple kinases.[37-39,49] Ser294 phosphorylation appears to mediate increased PR nucleo-cytoplasmic shuttling.[39] Rapid nuclear translocation of unliganded PR and nuclear export of liganded PR requires MAPK-dependent phosphorylation of PR Ser294.[39] PR nuclear sequestration in response to MAPK activation might serve to protect inactive or active receptors from degradation in the cytoplasm or upon nuclear export.[39] Following ligand binding, PR undergoes rapid downregulation.[51] Phosphorylation of Ser294 greatly augments PR downregulation by making liganded PR a cytoplasmic target for ubiquitination and degradation by the 26S-proteosome pathway.[37,39] In several recent reports, it has been shown that reversible phosphorylation of PR Ser294 couples increased transcriptional activity to rapid down-regulation of the PR protein by the ubiquitin-proteosome pathway.[37-39,49,52] Further investigation is required to determine whether the link between these events involves regulation of transcriptional events by components of the ubiquitin pathway and/or participation of nucleo-cytoplasmic shuttling factors or chaperones.

In the absence of progestins, however, EGF-induced nuclear accumulation of PR is required for transcriptional activation. Labriola et al[43] report that exposure of T47D breast cancer cells to the EGF family member, heregulin, can stimulate PR nuclear localization, DNA binding and transcriptional activity in the absence of hormone. Heregulin exposure also resulted in activation of MAPK and PR Ser294 phosphorylation. Qiu et al[39] report that PR Ser294 phosphorylation results in similar nuclear activity. However, growth factors alone failed to stimulate PR transcriptional activity or alter PR downregulation in T47D cell variants.[38] However, in the presence of ligand, MAPK activation greatly augmented both of these events.[38,39] One explanation for these apparently conflicting results is that differential expression of EGFR family members expressed on the cell surface between T47D cell line clones might lead to differences in the activation of downstream intracellular kinases, such as CDK2.[44] Indeed, regulation of PR by alternate signaling pathways may contribute to dysregulated gene expression and changes in cell growth and/or survival. For example, PR-B regulation of IRS-2 expression in breast cancer cells requires phosphorylation of PR Ser294 and occurs in the absence of ligand.[49] In any case, these exciting data[39,43] suggest a continuum between PR hypersensitivity to extremely low ligand concentrations and complete ligand-independence, a phenomenon that is well-documented for androgen receptor (AR) and ERα.

Extranuclear Actions of PR

While the genomic effects of steroid hormone treatment are delayed by several minutes to hours (i.e., following transcription and translation), the extranuclear or nongenomic effects occur rapidly in only a few minutes. Progestin treatment of breast cancer cells causes a rapid and transient activation of MAPK signaling that is ER-dependent, but independent of PR transcriptional activity.[53,54] Migliaccio et al were the first to report that estradiol activates p60-Src kinase and MAPK in MCF-7 cells[55] and that PR and ERα interact to stimulate p60-Src kinase in T47D cells.[53] Maximal activation of p60-Src kinase is observed within 2-5 minutes and downstream activation of p42/p44 MAPKs occurs within 5-10 minutes of progestin treatment.[53,54]

Human PR contains a proline-rich (PXXP) motif that mediates direct binding to the Src-homology three (SH3) domains of signaling molecules in the p60-Src kinase family in a ligand-dependent manner.[54] In vitro experiments demonstrate that purified liganded PR-A and PR-B activate the c-Src-related protein kinase, HcK; PR-B but not PR-A activates c-Src and MAPKs in vivo. PR-B with a mutated PXXP sequence prevents c-Src/PR interaction and blocks progestin-induced activation of c-Src (or HcK) and p42/p44 MAPKs. Furthermore, mutation of the PR-B DNA-binding domain (DBD) abolished PR transcriptional activity without affecting progestin-induced c-Src or MAPK kinase activation. Therefore, nongenomic MAPK activation by progestin/PR-B/c-Src complexes probably occurs by way of a c-Src-dependent mechanism involving Ras activation via phosphorylation of the c-Src substrate adaptor proteins p190 and/or Shc and followed by Grb-2 and Sos binding (Fig. 2).

Ballare et al[56] report that MAPK activation by progestins is blocked by antiprogestins and antiestrogens in COS-7 cells transfected with PR and ERα. They propose that c-Src/MAPK activation by PR is mediated indirectly by the interaction of the Src-homology two (SH2) domain of c-Src with phosphotyrosine 537 of ERα.[56] In their model, activation of c-Src and the MAPK pathway by progestins depends upon the presence of unliganded ERα, which interacts constitutively with PR-B via two domains that flank the proline-rich sequence of PR. Deletion of either of these two ER-interacting domains in PR-B blocked c-Src/MAPK activation by progestins in the presence of ERα.[56] Mutation of PR-B's PXXP domain had no effect. In contrast, Boonyaratanakornkit et al[54] report that ectopic PR expression increased basal c-Src activity in COS-7 cells in the absence of progestins and independently of added ER; co-expression of both PR-B and ERα reduced basal levels of c-Src activity. Under these conditions (i.e., low basal c-Src activity), progestin binding to PR-B clearly activated c-Src. In addition, progestins activated c-Src in PR-null MCF12A cells transduced with wild-type PR but not the PXXP-mutant PR adenoviruses. Both groups found

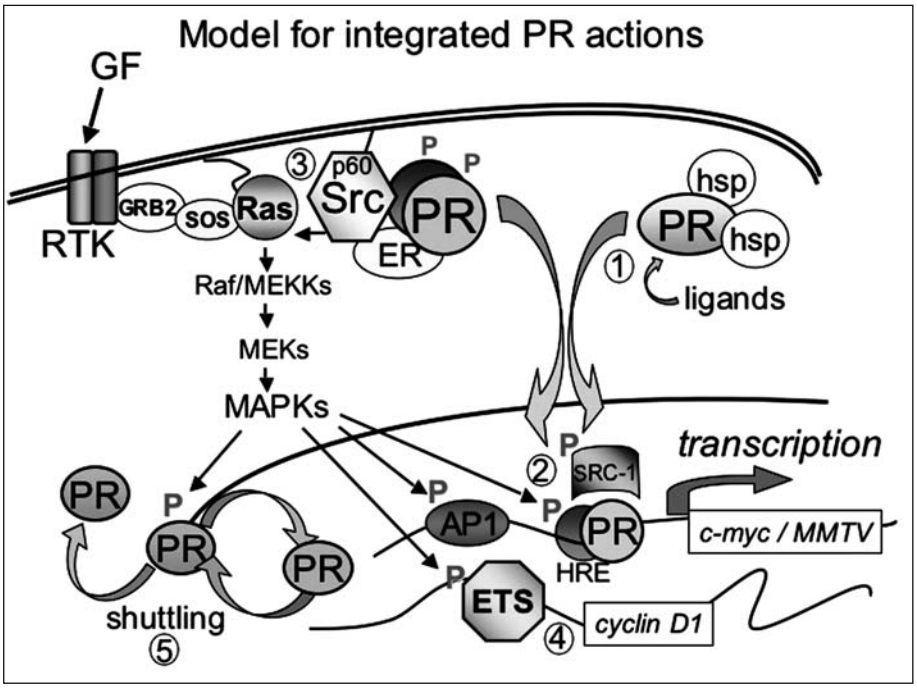

Figure 2. Functional significance of PR phosphorylation. Phosphorylation (P) of specific sites in PRs couple multiple receptor functions, including transcription, nuclear-cytoplasmic shuttling and PR downregulation. 1) Ligand-binding mediates dissociation of heat-shock proteins and nuclear accumulation of PR dimers. 2) Nuclear PRs mediate gene regulation; phosphorylated PRs recruit regulatory molecules that include phospho-proteins and likely function in inter-connected processes (transcription, elongation, localization and turnover). 3) PRs and growth factors activate MAPKs independently via a c-Src kinase-dependent pathway, resulting in positive regulation of PR action via "feed-back" regulation (i.e., direct phosphorylation of liganded PRs or coactivators). 4) Activation of MAPKs by PRs provides for regulation of gene targets whose promoters do not contain PREs and are otherwise independent of PR-transcriptional activities but utilize PR or SR-activated MAPKs. 5) MAPK regulation of PRs mediates nuclear accumulation/shuttling and nuclear export coupled to regulation of PR transcription.

that ERα interacts with the SH2-domain of c-Src, but neither group tested the effects of estrogen on the ability of progesterone to activate c-Src or MAPKs.[54,56]

Although discrepancies between these two models must be resolved, it is possible that overexpression of SRs in COS-7 cells leads to concentration-dependent effects resulting in the formation of different signaling complexes depending on the presence of other signaling and adaptor molecules. In support of this idea, Wong et al[57] identified an additional ER-interacting "adaptor" protein, termed MNAR (modulator of nongenomic activity of estrogen receptor), that contains both LXXLL (nuclear receptor binding) and PXXP (SH3-domain binding) motifs. MNAR is essential for ER-Src interaction, but it is not required for progestin/PR-dependent activation of c-Src (D.P. Edwards, personal communication). Taken together, these data indicate that multiple interactions contribute to direct protein kinase activation by SRs and suggest that at least some nongenomic signaling functions of amphibian PR have been conserved in mammals. Interestingly, a separate gene product encoding the putative mammalian homologue of membrane progesterone receptor (mPR), a progesterone-binding G-protein coupled receptor first identified in spotted sea trout oocytes,[58] has been described. Further studies are needed to determine if mPR plays a role

in progestin-induced "rapid" signaling or if mPR interacts with classical PRs. However, studies with mPR underscore the important concept that binding proteins other than classical steroid receptors may regulate some nongenomic steroid-mediated signaling events.

Integration of Rapid Signaling and Nuclear SR Actions

While its role in mammalian physiology remains unclear, SR-mediated activation of cytoplasmic signaling molecules could theoretically serve to potentiate several nuclear functions of activated SRs (Fig. 2). One mechanism by which amplification of SR nuclear functions might occur is through rapid, direct phosphorylation of SRs and/or their coregulators in response to activation of SR-induced cytoplasmic pathways that coincide with ligand binding. Clearly, such a positive feedback loop would explain the dramatic influence of activated signaling pathways on PR nuclear function. For example, several progestin-dependent functions of PR are MAPK-dependent, including upregulation of cyclins D1 and E, CDK2 activation and S-phase entry.[22,38,44,59]

Following ligand-binding, most SRs stimulate a transient (3-10 min) activation of MAPKs. However, mitogenic signaling requires sustained (hrs to days) MAPK activation in fibroblast cell models.[60] Recently, Faivre et al[61] found that in addition to rapid and transient activation of MAPK by progestin/PR-B (5-15 min), progestin-bound PR-B induced subsequent oscillations in MAPK activity that culminated in a sustained (hrs to days) phase of MAPK activation that was EGFR- and c-Src-dependent. Further studies revealed the creation of an autocrine signaling loop, in which PR-B triggered transcriptional upregulation of Wnt-1, leading to activation of frizzled-dependent MMPs and shedding of EGF ligands from the cell surface. This signaling cascade implicates Wnt-1-dependent transactivation of EGFR in response to progestins; PR induced transcriptional upregulation of Wnt-1 and EGFR mRNA was sensitive to inhibition of MAPKs. Additional experiments demonstrated that progestin-induced cyclin D1 upregulation, S-phase entry, or soft-agar growth of T47D breast cancer cells was either blocked by shRNA targeted to Wnt-1 or inhibitors of MAPK, c-Src and EGFR. Finally, progestins failed to stimulate S-phase entry in MCF-7 cells that stably express a PXXP-mutant PR-B, which is unable to bind to the SH3-domain of c-Src and activate MAPK.[59] Soft-agar growth of T47D cells stably expressing the same PR mutant (PXXP) was greatly attenuated.[61] In addition to c-Src and MAPKs, STATs are important effectors downstream of EGFR signaling. Progestins induce tyrosine phosphorylation and nuclear translocation of Stat5[25] and Stat3.[30] Proietti et al[30] demonstrate that Stat3 phosphorylation and activation by the nongenomic actions of PR is a critical event for breast cancer cell growth; T47D cell growth and tumor growth of progestin-induced mammary adenocarcinomas in BALB/c mice was dependent on PR activation of Jak1 and Jak2, c-Src and Stat3. Taken together, these data indicate that progesterone, via robust PR-B/c-Src signaling to MAPK in combination with PR-dependent transcriptional events, upregulates and activates EGFR signaling to induce cell proliferation. Dysregulation of either arm of this pathway may contribute to uncontrolled proliferation of breast cancer cells.

The extranuclear actions of PRs may contribute to deregulated breast cancer cell growth[59] and/or increased breast cancer risk,[8] perhaps by linking steroid hormone action to the regulation of MAPK-regulated genes (i.e., transcription factor targets of MAPK). Similarly, the extranuclear actions of liganded ERα are thought to induce a state of "adaptive hypersensitivity" during endocrine therapy in which growth factor signaling pathways are co-opted by upregulated ERα.[62] In this model of ER-dependent MAPK activation, liganded ERα associated with the cell membrane interacts with the adapter protein Shc and induces its phosphorylation, leading to recruitment of Grb-2 and Sos, followed by activation of Ras and the Raf-1/MEK/MAPK module. ERα activation of MAPK may explain why many tumors respond well to aromatase inhibitors, yet fail to respond to selective estrogen receptor modulators (SERMS) designed to inhibit ER transcriptional activity. SERMs can act as partial transcriptional agonists of phosphorylated receptors and may not block ER-dependent MAPK activation.[62] In theory, PR-B or AR in SR-positive breast cancers could participate in MAPK-activating complexes, perhaps bypassing anti-estrogen therapies. Few groups have studied membrane-associated or cytoplasmic signaling complexes containing both ERα and

PR-B or AR.[63,64] However, AR is frequently (70%) expressed in metastatic breast cancer,[65] and expression of functional AR defines a sub-set of ER/PR-negative breast cancers.[66] These studies suggest that it will be important to target SRs that may substitute for ERα in the activation of c-Src-dependent mitogenic signaling cascades.

PR Action and Breast Cancer Cell Growth, Apoptosis and Aggressiveness in Vitro

Among the most controversial issues regarding the role of progestins in breast cancers is their influence, or lack thereof, on tumor cell proliferation. Complicating the interpretation of the results utilizing in vitro breast cancer models of receptor function is the use, in addition to progesterone, of a myriad of different synthetic progestins with activities unrestricted to PR. For example, while the 19-nor progestins—norgestrel and gestodene—enhance MCF-7 cell proliferation, this effect is inhibitable by antiestrogens but not antiprogestins,[67,68] indicating the lack of involvement of PR signaling. Indeed, cross-reactivity of synthetic progestins at pharmacologic doses with ER has been reported.[69,70] One explanation of these confusing results is that progestin may interact with different PR isoforms to carry out inhibitory or proliferative functions. Sumida et al demonstrate the growth inhibitory effects of progestins with either PR isoform,[71] and McGowan et al show that overexpression of PR-A sensitized breast cancer cells to progestin-mediated growth inhibition.[72] In contrast, Moore et al report prolonged proliferative and survival effects of progestins on breast cancer cells.[73,74]

Flow cytometric studies have also addressed questions of progestin-mediated proliferation by using a single physiological progestin pulse under transiently estrogen deprived conditions. These studies show biphasic effects of progestins in vitro, with cells accelerating through the first mitotic cell cycle then arresting in late G1 of the next cycle.[27,75] Cycle arrest is associated with decreases in cyclins D1, D3 and E, loss of cyclin A and B and induction of the cell cycle inhibitors p21 and p27. Pulsing with progesterone did not restart proliferation; rather it delayed p21 depletion.[27] Similarly, Lin et al [76] report decreased cell proliferation in response to progesterone in conjunction with upregulation of p21, decreased cyclins A, B1 and D1 expression and downregulation of phosphorylated p42/44 MAPK. Thus, these studies suggest that progestins tend to be anti-proliferative in vitro in mono-layer cell cultures.

Equally confusing are conflicting reports of the effects of PR and progestins on apoptosis in vitro. Several studies report pro-apoptotic effects of progestins concomitant with decreases in expression levels of the anti-apoptotic genes bcl-2 and bcl-X_L.[77-80] Antiprogestin/partial agonists, such as RU486, have also been shown to promote apoptosis,[81] but dosage effects confound the interpretation of results.[82] On the other hand, recent studies suggest that unliganded PR[83] and/or progestin-occupied PR[84] protect cells from damage and apoptosis induced by radiation[84] or chemotherapeutic agents, such as taxanes,[83] doxorubicin or 5-fluorouracil.[85] Moore et al[74] report progestin-induced protection of breast cancer cell death accompanied by upregulation of bcl-X_L, but loss of bcl-2. These contradictory in vitro data prevent a definitive conclusion regarding the apoptotic effects of progestins.

Similarly, the effects of progesterone on invasiveness of breast cancer cells in vitro are poorly understood. Many studies show that progestins increase cell invasiveness[72,83,86] with PR-A exaggerating this phenotype. Sumida et al, however, report that treatment with progestins reduce cell invasiveness.[71]

Notably, studies using human breast cancer cell line models (T47D or MCF-7) grown in soft-agar (i.e., as 3-D colonies) clearly demonstrate a proliferative role for synthetic progestins (R5020) or progesterone in response to PR-dependent transcriptional upregulation of Wnt-1.[87] These results suggest that breast epithelial cells may require a specific architecture (i.e., polarity) for the mitogenic and other "appropriate" gene expression effects of progestins to occur. This architecture is not modeled on plastic surfaces in vitro (i.e., mono-layer cultures). Differences in cell behavior when grown using plastic as mono-layer cultures vs. 3D models have clearly contributed to the controversial area of PR action as a breast cancer cell mitogen. Therefore, we recommend

that future investigations into the effects of progestins on tumor cell behavior utilize 3-D models or in vivo models of PR-positive breast cancer.

Expression Profiling in Vitro

Results from expression profiling of breast cancer cells in vitro are consistent with the results from experimental mouse models, which suggest that the two PR isoforms subserve different functions. In mice—where the PR-A to PR-B ratio is 3:1 compared to humans where it is 1:1—ablation of one or the other PR isoform leads to divergent effects on the mammary gland. PR-A knockout (leaving only PR-B) leads to normal early development,[16] while PR-B knockout (leaving only PR-A) leads to reduced pregnancy-associated lobuloalveolar development and reduced side-branching.[15] On the other hand, overexpression of PR-B causes precocious ductal arrest and inappropriate ductal development,[88] while overexpresison of PR-A causes mammary epithelial cell hyperplasia, excessive ductal branching and a disorganized basement membrane.[89] To explain these isoform-specific differences, gene profiling studies have been performed in vitro using human breast cancer cells expressing PR-A or PR-B. The first such study used 6 hrs of progesterone treatment in an attempt to identify direct PR target genes.[14,90] Of 94 genes identified, 65 were regulated only by PR-B, 4 only by PR-A and 25 by both PR isoforms. This regulatory pattern was confirmed in subsequent studies using breast cancer cells with inducible PR-A vs. PR-B treated 6 hrs with progesterone.[83] The latter studies also demonstrate that unliganded PR can regulate transcription; CDK2 mediates ligand-independent activation of PR-B via Ser400 phosphorylation (44).

More recent studies used progesterone-treated breast cancer cells that express both PR isoforms.[91-93] Analysis of the protein pathways indicate that progesterone suppresses genes involved in proliferation and metastasis,[91] supporting an anti-proliferative role for this hormone. However, a remarkable number of the genes upregulated by progestins encode proteins involved in signal transduction and cell adhesion,[83,14] lending some support to the concept that progestins/PR may contribute to the dysregulation of pathways important for breast cancer progression that are perhaps not well modeled in vitro. Additionally, the above studies address gene regulation in response to unliganded or liganded PRs (i.e., single hormone exposure). We propose that PR isoforms act as sensors for signal transduction pathways (discussed above) and thus promoter selectivity is predicted to be highly sensitive to phosphorylation events. Further studies will be needed to address alterations in the signature of PR regulated genes in the context of the high kinase activities characteristic of aggressive breast cancer.

Progestins and Antiprogestins in Breast Tumor Models

Antiprogestins

For a time, therapeutic interest in antiprogestins led to many more studies on these drugs than on the biology of progestins themselves in breast tumors. Several rodent and human tumor models have been used to study the efficacy of antiprogestins for endocrine therapy. These include carcinogen- (DMBA or MNU) induced mammary tumors in the rat, serially transplantable MXT (+) mouse and human T61 mammary tumors and MCF7 human tumor xenografts. Tumors in each of these models are ER+ and PR+. Several different antiprogestins, including mifepristone (RU 38.486; Roussel), the Schering compounds onapristone (ZK 98.299 and ZK 112.993) and the ORG compounds (31710 and 31806) effectively inhibit tumor growth 40 to >90%, depending on the drug, dose and model.[94-103] Antiprogestins were at least as effective as tamoxifen as a single-line therapy. Combination treatment of established tumors in both the rodent and human tumor models with an antiprogestin and an antiestrogen (tamoxifen or ICI164384) had an additive effect on inhibition of tumor growth.[95,100] These studies led to speculation that antiprogestins would be useful for endocrine therapies and fueled the notion that progestins induced proliferation. Indeed, several small clinical studies investigated the potential of mifepristone and onapristone as first- or third-line therapies (reviewed in J.G. Klijn et al[104]). However, because of apparent liver toxicity

(onapristone), discrepancies among results and the abortifacient properties of these hormones, the testing of antiprogestins for breast cancer therapy has generally been discontinued.

Only two of the above studies examined progestins alone. Megestrol acetate or MPA had no effect on MXT mouse tumors or slightly inhibited DMBA rat tumor growth.[94,101] This suggests that antiprogestins do not directly antagonize progesterone-mediated tumor growth, even though PR expression was required for inhibition.[102] It is possible that they exert a PR-dependent antiestrogenic effect through ER transrepression[105] or that they suppress effects of unliganded PR.[83]

Progestins

Human tumor models utilize immune-compromised mice as hosts for "xenografted" breast cancer cell lines. Several ER+ and PR+ human breast cancer cell lines (MCF7, ZR-75, T47D) are grown as solid tumors in this manner.[106] Tumors derived from each of these cell lines are estrogen-dependent and require continuous estradiol administration for growth. They have been widely used as models for studying estrogen-suppression based therapies, such as antiestrogens and aromatase inhibitors.[107,108] Only a few studies, however, have assessed effects of progestins in these models. Neither MCF7 nor T47D cells grow in response to progesterone in ovariectomized female mice[109-111] in the absence or presence of estradiol.

In our experience, progesterone or MPA had negligible, nonsignificant growth inhibitory effects in ovariectomized mice bearing T47D xenografts in an estrogenized background.[111] These data suggest that in hormone-dependent models of human breast cancer, progestins are neither mitogenic nor effective at suppressing estrogen-dependent growth. ER^{neg} and PR^{neg} MDA-231 human breast cancer cells form hormone-independent tumors in vivo. If PR was expressed in these cells, progesterone treatment reduced tumor formation.[86]

There is one example of progestin-dependent murine mammary tumor growth. Long-term (10-12 months) chronic treatment of female BALB/c mice with MPA leads to the formation ER+ and PR+ mammary tumors.[112,113] They are maintained by serial transplantation and have a growth requirement for progestins (either progesterone or MPA) rather than estrogens.[114] After serial passage, some tumors acquire progestin independence. Both progestin-dependent and -independent tumors can be inhibited by antiprogestins and antisense oligonucleotides to PR.[115,116] Whereas most clinical human tumors are ER+ and PR+ and respond to antiestrogen therapies, it is possible that some tumors that originate during long-term HRT or in association with pregnancy may have developed in response to progestins. The BALB/c mice would serve as potential models for these rare tumors.

Progesterone Regulation of BRCA1

Carriers of mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 exhibit a 10-fold higher risk of developing tumors in hormonally responsive tissues, such as the breast and ovaries (cumulative risk of 85-90% by age 70) compared to the general population.[117] BRCA1 mutant breast tumors have a poor nuclear grade, high frequency of p53 mutations and are more often ER- and PR-negative compared to sporadic cancers. Because oophorectomy of premenopausal women reduces breast cancer risk substantially (>40%),[118] linkages between steroid hormones and BRCA1 tumor types have been sought since their discovery.[119-121] Fibroblasts from $Brca^{-/-}$ knockout mice that are also $p53^{-/-}$ exhibit ligand-independent activation of ER and PR-dependent transcription;[122] see also Rosen et al.[117] Haploinsufficiency of BRCA1 may be a deleterious state that initiates alterations in steroid hormone receptor expression and tumor mitogenic response.[123] Poole et al[124] report the accumulation of lateral branching and extensive alveologenesis in the mammary glands of nulliparous BRCA1/p53-deficient mice. PR, but not ER, were overexpressed due to a defect in their proteasome-dependent degradation. Notably, treatment of these mice with the PR antagonist mifepristone (RU486) blocked mammary tumorigenesis. These provocative studies suggest that antiprogestin therapy may help prevent the development of breast cancer in individuals with BRCA1 mutations.

General Steroid Receptors and Breast Cancer

A recent study[125] described the steroid receptor assay results of 54,865 patients with stage I–IIIA breast cancers. Their biopsy or mastectomy specimens were sent to two central laboratories that performed identical assays, monitored with tightly controlled quality control procedures. The authors report that ~82% of breast cancers were ER+ and of these ~71% were also PR+. Thus among all breast cancers, ~58% expressed both ER and PR. It is now well established that independent of treatment modalities, women with steroid receptor positive tumors live longer than their receptor negative counterparts. Large studies with long-term follow-up, such as those from San Antonio or the NSABP, indicate a 10% survival advantage for patients with receptor positive disease.[126] Positive hormone receptor status is an independent predictor of outcome and augurs a more favorable prognosis even after controlling for patient age, disease stage, tumor grade, histology, race/ethnicity and US geographical distribution.

Progesterone Receptors and Tamoxifen Responsiveness

The independent role of ER-positivity as a marker of good prognosis and responsiveness to endocrine therapies has been appreciated since the early 1970s. Resistance of a subset of ER+ tumors to endocrine therapies may be due to aberrant estrogen signaling in ER+ tumors that lack PR.[127] Indeed, compared to ER+ and PR− tumors, pretreatment PR-positivity in ER+ tumors is associated with improved outcome prediction as shown by 5 year disease survival rates[128] and by improved response to such adjuvant endocrine therapies as tamoxifen.[128-133] However, not all studies have demonstrated a value for PR, due perhaps to assay variability.[134] The presence of both ER and PR in metastatic disease has also been shown to predict improved response to tamoxifen treatment.[131,135]

Clinical Significance of PR-A vs. PR-B: Two Subsets of ER+, PR+ Tumors?

We first showed that human breast cancer cells express two forms of PR, the PR-A and PR-B isoforms.[136] Despite having a similar primary amino acid structure over the majority of their length, these receptors regulate entirely different gene subsets.[83,14] The clinical implications of this remain under investigation. Studies using monoclonal antibodies show that PR-A and PR-B colocalize in the same cells in normal endometrium[137] and breast cancers,[138] further adding to the complexity of analyzing expression ratios of the two isoforms by IHC. By immunoblotting, their ratio changes during malignant progression, with approximately equimolar levels of PR-A and PR-B in normal human tissues, but aberrant PR-A:PR-B ratios in breast cancers. An immunoblotting study by Graham et al[139] of 202 PR+ breast cancers showed a median PR-A:PR-B ratio of ~1.3 (close to equimolar), but with outliers ranging between 0.04 (essentially PR-B+) to ~180 (essentially PR-A+) in a significant number of tumors. These authors concluded that when ratios are aberrant, the PR-A isoform tends to be in excess,[140,141] and tumors tend to be less differentiated.[141] We[142] studied the association between PR-A:PR-B ratios and clinical outcome in 297 ER+, axillary node-positive patients, using MAb 1294 for immunoblotting. Eighteen percent of tumors had more than a 2-fold excess of PR-B over PR-A; 10% had more than a 2-fold excess of PR-A over PR-B. We concluded that high PR-A levels were due to loss of PR-B. Our studies also included clinical data showing that tamoxifen-treated patients with high PR-A:PR-B ratios were 2.76 times more likely to relapse. Thus, clinical studies that have addressed the issue of PR isoforms agree that an excess of PR-A is harmful. We suggest that patients with PR-A rich tumors may represent an ER+/PR+ subgroup with intrinsic insensitivity to tamoxifen and perhaps to other selective ER modulators. Growth factor signaling is tightly linked to tamoxifen resistance. Notably, Ser294 phosphorylated PR-B is hypersensitive to low progesterone concentrations and thus degrades very rapidly relative to PR-A, which is hypo-phosphorylated at this site (discussed above); hyperactive but unstable PR-B relative to PR-A may contribute to increased PR-A/PR-B ratios in a subset of breast cancers. In this setting, targeting PR-B and relevant kinases would seem appropriate, but remains untested clinically.

Concluding Remarks

Studies aimed at defining a proliferative role for progestins in breast cancer models remain controversial, but have perhaps been hindered by observations made with liganded receptors in the absence of controlled inhibition or activation of alternate signaling pathways. In the context of multiple signaling inputs, PR clearly coordinates receptor responses to growth factors and steroid hormones. The newly discovered ability of SRs to activate kinase pathways classically defined as key regulators of cell growth underscores the concept that activation of signal transduction pathways is an integral feature of SR action. This aspect of SR function is likely to play an important role in cancer progression and the development of resistance to endocrine therapies.[62] Targeting the relevant protein kinases (c-Src, MAPKs and CDKs) as an integral feature of SR (PR, ER) action should provide significant improvements over the use of traditional SR-blocking strategies for advanced or progressive breast cancers.

Acknowledgements

We thank Michael Franklin for editorial comments and Dean Edwards (Baylor College of Medicine) and Natalie Ahn (University of Colorado, Boulder) for generous gifts of reagents. Studies contributed by Carol Lange's laboratory were supported by NIH grants DK53825/CA123763 and CA116790. These studies were also supported by grants to Britta Jacobsen (Department of Defense 06-1-0503), Carol Sartorius (Susan G. Komen Foundation: BCTR0402682) and grants to Kathryn Horwitz from the NIH (CA26869), the Avon Foundation, the Breast Cancer Research Foundation and the National Foundation for Cancer Research.

References

1. Hovey RC, Trott JF, Vonderhaar BK. Establishing a framework for the functional mammary gland: from endocrinology to morphology. J Mammary Gland Biol Neoplasia 2002; 7(1):17-38.
2. Haslam SZ, Counterman LJ, Nummy KA. Effects of epidermal growth factor, estrogen and progestin on DNA synthesis in mammary cells in vivo are determined by the developmental state of the gland. J Cell Physiol 1993; 155(1):72-78.
3. Ankrapp DP, Bennett JM, Haslam SZ. Role of epidermal growth factor in the acquisition of ovarian steroid hormone responsiveness in the normal mouse mammary gland. J Cell Physiol 1998; 174(2):251-60.
4. Robinson GW, Hennighausen L, Johnson PF. Side-branching in the mammary gland: the progesterone-Wnt connection. Genes Dev 2000; 14(8):889-94.
5. Rosen JM. Hormone receptor patterning plays a critical role in normal lobuloalveolar development and breast cancer progression. Breast Dis 2003; 18:3-9.
6. Li Y, Rosen JM. Stem/progenitor cells in mouse mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia 2005; 10(1):17-24.
7. Aupperlee MD, Smith KT, Kariagina A et al. Progesterone receptor isoforms A and B: temporal and spatial differences in expression during murine mammary gland development. Endocrinology 2005; 146(8):3577-88.
8. Chlebowski RT, Hendrix SL, Langer RD et al. Influence of estrogen plus progestin on breast cancer and mammography in healthy postmenopausal women: the Women's Health Initiative Randomized Trial. JAMA 2003; 289(24):3243-53.
9. Haslam SZ. Experimental mouse model of hormonal therapy effects on the postmenopausal mammary gland. Breast Dis 2005; 24:71-8.
10. Haslam SZ, Osuch JR, Raafat AM et al. Postmenopausal hormone replacement therapy: effects on normal mammary gland in humans and in a mouse postmenopausal model. J Mammary Gland Biol Neoplasia 2002; 7(1):93-105.
11. Schiff R, Massarweh SA, Shou J et al. Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. Cancer Chemother Pharmacol 2005; 56 Suppl 1:10-20.
12. Horwitz KB, Sheridan PL, Wei LL et al. Human progesterone receptors: synthesis, structure and phosphorylation. Prog Clin Biol Res 1990; 322(41):41-52.
13. Jacobsen BM, Richer JK, Schittone SA et al. New human breast cancer cells to study progesterone receptor isoform ratio effects and ligand-independent gene regulation. J Biol Chem 2002; 277(31):27793-800.

14. Richer JK, Jacobsen BM, Manning NG et al. Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J Biol Chem 2002; 277(7):5209-18.
15. Mulac-Jericevic B, Lydon JP, DeMayo FJ et al. Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. Proc Natl Acad Sci USA 2003; 100(17):9744-9.
16. Mulac-Jericevic B, Mullinax RA, DeMayo FJ et al. Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. Science 2000; 289(5485):1751-4.
17. Condon JC, Hardy DB, Kovaric K et al. Up-regulation of the progesterone receptor (PR)-C isoform in laboring myometrium by activation of nuclear factor-kappaB may contribute to the onset of labor through inhibition of PR function. Mol Endocrinol 2006; 20(4):764-75.
18. Pratt WB, Toft DO. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med (Maywood) 2003; 228(2):111-33.
19. Moore MR, Zhou JL, Blankenship KA et al. A sequence in the 5' flanking region confers progestin responsiveness on the human c-myc gene. J Steroid Biochem Mol Biol 1997; 62(4):243-52.
20. Chalbos D, Chambon M, Ailhaud G et al. Fatty acid synthetase and its mRNA are induced by progestins in breast cancer cells. J Biol Chem 1987; 262(21):9923-6.
21. Krusekopf S, Chauchereau A, Milgrom E et al. Co-operation of progestational steroids with epidermal growth factor in activation of gene expression in mammary tumor cells. J Steroid Biochem Mol Biol 1991; 40(1-3):239-245.
22. Lange CA, Richer JK, Shen T et al. Convergence of progesterone and epidermal growth factor signaling in breast cancer. Potentiation of mitogen-activated protein kinase pathways. J Biol Chem 1998; 273(47):31308-16.
23. Brass AL, Barnard J, Patai BL et al. Androgen up-regulates epidermal growth factor receptor expression and binding affinity in PC3 cell lines expressing the human androgen receptor. Cancer Res 1995; 55(14):3197-203.
24. Church DR, Lee E, Thompson TA et al. Induction of AP-1 activity by androgen activation of the androgen receptor in LNCaP human prostate carcinoma cells. Prostate 2005; 63(2):155-68.
25. Richer JK, Lange CA, Manning NG et al. Convergence of progesterone with growth factor and cytokine signaling in breast cancer. Progesterone receptors regulate signal transducers and activators of transcription expression and activity. J Biol Chem 1998; 273(47):31317-26.
26. Gregory CW, Johnson RT Jr, Presnell SC et al. Androgen receptor regulation of G1 cyclin and cyclin-dependent kinase function in the CWR22 human prostate cancer xenograft. J Androl 2001; 22(4):537-48.
27. Groshong SD, Owen GI, Grimison B et al. Biphasic regulation of breast cancer cell growth by progesterone: role of the cyclin-dependent kinase inhibitors, p21 and p27(Kip1). Mol Endocrinol 1997; 11(11):1593-607.
28. Owen GI, Richer JK, Tung L et al. Progesterone regulates transcription of the p21(WAF1) cyclin-dependent kinase inhibitor gene through Sp1 and CBP/p300 J Biol Chem 1998; 273(17):10696-701.
29. Tseng L, Tang M, Wang Z et al. Progesterone receptor (hPR) upregulates the fibronectin promoter activity in human decidual fibroblasts. DNA Cell Biol 2003; 22(10):633-40.
30. Proietti C, Salatino M, Rosemblit C et al. Progestins induce transcriptional activation of signal transducer and activator of transcription 3 (Stat3) via a Jak- and Src-dependent mechanism in breast cancer cells. Mol Cell Biol 2005; 25(12):4826-40.
31. McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 2002; 108(4):465-74.
32. Takimoto G, Horwitz K. Progesterone receptor phosphorylation—Complexities in defining a functional role. Trends Endocrinol Metab 1993; 4:1-7.
33. Lange CA. Making sense of cross-talk between steroid hormone receptors and intracellular signaling pathways: who will have the last word? Mol Endocrinol 2004; 18(2):269-78.
34. Zhang Y, Beck CA, Poletti A et al. Phosphorylation of human progesterone receptor by cyclin-dependent kinase 2 on three sites that are authentic basal phosphorylation sites in vivo. Mol Endocrinol 1997; 11(6):823-32.
35. Zhang Y, Beck CA, Poletti A et al. Identification of a group of Ser-Pro motif hormone-inducible phosphorylation sites in the human progesterone receptor. Mol Endocrinol 1995; 9(8):1029-40.
36. Zhang Y, Beck CA, Poletti A et al. Identification of phosphorylation sites unique to the B form of human progesterone receptor. In vitro phosphorylation by casein kinase II. J Biol Chem 1994; 269(49):31034-40.
37. Lange CA, Shen T, Horwitz KB. Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci USA 2000; 97(3):1032-7.

38. Shen T, Horwitz KB, Lange CA. Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294. Mol Cell Biol 2001; 21(18):6122-31.
39. Qiu M, Olsen A, Faivre E. Mitogen-activated protein kinase regulates nuclear association of human progesterone receptors. Mol Endocrinol 2003; 17(4):628-42.
40. Knotts TA, Orkiszewski RS, Cook RG et al. Identification of a phosphorylation site in the hinge region of the human progesterone receptor and additional amino-terminal phosphorylation sites. J Biol Chem 2001; 276(11):8475-83.
41. Font de Mora J, Brown M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. Mol Cell Biol 2000; 20(14):5041-7.
42. Narayanan R, Adigun AA, Edwards DP et al. Cyclin-dependent kinase activity is required for progesterone receptor function: novel role for cyclin A/Cdk2 as a progesterone receptor coactivator. Mol Cell Biol 2005; 25(1):264-77.
43. Labriola L, Salatino M, Proietti CJ et al. Heregulin induces transcriptional activation of the progesterone receptor by a mechanism that requires functional ErbB-2 and mitogen-activated protein kinase activation in breast cancer cells. Mol Cell Biol 2003; 23(3):1095-111.
44. Pierson-Mullany LK, Lange CA. Phosphorylation of progesterone receptor serine 400 mediates ligand-independent transcriptional activity in response to activation of cyclin-dependent protein kinase 2. Mol Cell Biol 2004; 24(24):10542-57.
45. Takimoto GS, Hovland AR, Tasset DM et al. Role of phosphorylation on DNA binding and transcriptional functions of human progesterone receptors. J Biol Chem 1996; 271(23):13308-16.
46. Takimoto GS, Tasset DM, Eppert AC et al. Hormone-induced progesterone receptor phosphorylation consists of sequential DNA-independent and DNA-dependent stages: analysis with zinc finger mutants and the progesterone antagonist ZK98299. Proc Natl Acad Sci USA 1992; 89(7):3050-4.
47. Migliaccio A, Di Domenico M, Green S et al. Phosphorylation on tyrosine of in vitro synthesized human estrogen receptor activates its hormone binding. Mol Endocrinol 1989; 3(7):1061-1069.
48. Ali S, Metzger D, Bornert JM et al. Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. EMBO J 1993; 12(3):1150-1160.
49. Qiu M, Lange CA. MAP kinases couple multiple functions of human progesterone receptors: degradation, transcriptional synergy and nuclear association. J Steroid Biochem Mol Biol 2003; 85:147-157.
50. Narayanan R, Edwards DP, Weigel NL. Human progesterone receptor displays cell cycle-dependent changes in transcriptional activity. Mol Cell Biol 2005; 25(8):2885-98.
51. Nardulli AM, Katzenellenbogen BS. Progesterone receptor regulation in T47D human breast cancer cells: analysis by density labeling of progesterone receptor synthesis and degradation and their modulation by progestin. Endocrinology 1988; 122(4):1532-1540.
52. Daniel AR, Qiu M, Faivre EJ et al. Linkage of progestin and epidermal growth factor signaling: phosphorylation of progesterone receptors mediates transcriptional hypersensitivity and increased ligand-independent breast cancer cell growth. Steroids 2007; 72(2):188-201.
53. Migliaccio A, Piccolo D, Castoria G et al. Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. EMBO J 1998; 17(7):2008-18.
54. Boonyaratanakornkit V, Scott MP, Ribon V et al. Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol Cell 2001; 8(2):269-80.
55. Migliaccio A, Di Domenico M, Castoria G et al. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 1996; 15(6):1292-300.
56. Ballare C, Uhrig M, Bechtold T et al. Two domains of the progesterone receptor interact with the estrogen receptor and are required for progesterone activation of the c-Src/Erk pathway in mammalian cells. Mol Cell Biol 2003; 23(6):1994-2008.
57. Wong C, McNally C, Nickbarg E et al. Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. Proc Natl Acad Sci USA 2002; 99(23):14783-14788.
58. Zhu Y, Bond J, Thomas P. Identification, classification and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci USA 2003; 100(5):2237-42.
59. Skildum A, Faivre E, Lange CA. Progesterone receptors induce cell cycle progression via activation of mitogen-activated protein kinases. Mol Endocrinol 2005; 19(2):327-39.
60. Murphy LO, Blenis J. MAPK signal specificity: the right place at the right time. Trends Biochem Sci 2006; 31(5):268-75.
61. Faivre E, Lange C. Progesterone receptors upregulate Wnt-1 to induce EGFR transactivation and c-Src-dependent sustained activation of Erk1/2 MAP Kinase in breast cancer cells. Mol Cell Biol 2006; 27(2):466-80.

62. Santen R, Jeng MH, Wang JP et al. Adaptive hypersensitivity to estradiol: potential mechanism for secondary hormonal responses in breast cancer patients. J Steroid Biochem Mol Biol 2001; 79(1-5):115-25.
63. Migliaccio A, Castoria G, Di Domenico M et al. The progesterone receptor/estradiol receptor association and the progestin-triggered S-phase entry. Ernst Schering Res Found Workshop 2005(52):39-54.
64. Migliaccio A, Castoria G, Di Domenico M et al. Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. EMBO J 2000; 19(20):5406-17.
65. Schippinger W, Regitnig P, Dandachi N et al. Evaluation of the prognostic significance of androgen receptor expression in metastatic breast cancer. Virchows Arch 2006; 449(1):24-30.
66. Doane AS, Danso M, Lal P et al. An estrogen receptor-negative breast cancer subset characterized by a hormonally regulated transcriptional program and response to androgen. Oncogene 2006; 29; 25(28):3994-4008.
67. Catherino WH, Jeng MH, Jordan VC. Norgestrel and gestodene stimulate breast cancer cell growth through an oestrogen receptor mediated mechanism. Br J Cancer 1993; 67(5):945-52.
68. Jeng MH, Parker CJ, Jordan VC. Estrogenic potential of progestins in oral contraceptives to stimulate human breast cancer cell proliferation. Cancer Res 1992; 52(23):6539-46.
69. Kalkhoven E, Kwakkenbos-Isbrucker L, de Laat SW et al. Synthetic progestins induce proliferation of breast tumor cell lines via the progesterone or estrogen receptor. Mol Cell Endocrinol 1994; 102(1-2):45-52.
70. van der Burg B, Kalkhoven E, Isbrucker L et al. Effects of progestins on the proliferation of estrogen-dependent human breast cancer cells under growth factor-defined conditions. J Steroid Biochem Mol Biol 1992; 42(5):457-65.
71. Sumida T, Itahana Y, Hamakawa H et al. Reduction of human metastatic breast cancer cell aggressiveness on introduction of either form a or B of the progesterone receptor and then treatment with progestins. Cancer Res 2004; 64(21):7886-92.
72. McGowan EM, Clarke CL. Effect of overexpression of progesterone receptor A on endogenous progestin-sensitive endpoints in breast cancer cells. Mol Endocrinol 1999; 13(10):1657-71.
73. Moore MR, Hagley RD, Hissom JR. Progestin effects on lactate dehydrogenase and growth in the human breast cancer cell line T47D. Prog Clin Biol Res 1988; 262:161-79.
74. Moore MR, Conover JL, Franks KM. Progestin effects on long-term growth, death and Bcl-xL in breast cancer cells. Biochem Biophys Res Commun 2000; 277(3):650-4.
75. Musgrove EA, Lee CS, Sutherland RL. Progestins both stimulate and inhibit breast cancer cell cycle progression while increasing expression of transforming growth factor alpha, epidermal growth factor receptor, c-fos and c-myc genes. Mol Cell Biol 1991; 11(10):5032-43.
76. Lin VC, Woon CT, Aw SE et al. Distinct molecular pathways mediate progesterone-induced growth inhibition and focal adhesion. Endocrinology 2003; 144(12):5650-7.
77. Gompel A, Somai S, Chaouat M et al. Hormonal regulation of apoptosis in breast cells and tissues. Steroids 2000; 65(10-11):593-8.
78. Kandouz M, Lombet A, Perrot JY et al. Proapoptotic effects of antiestrogens, progestins and androgen in breast cancer cells. J Steroid Biochem Mol Biol 1999; 69(1-6):463-71.
79. Franke HR, Vermes I. Differential effects of progestogens on breast cancer cell lines. Maturitas 2003; 46(Suppl 1):S55-8.
80. Formby B, Wiley TS. Progesterone inhibits growth and induces apoptosis in breast cancer cells: inverse effects on Bcl-2 and p53 Ann Clin Lab Sci 1998; 28(6):360-9.
81. Bardon S, Vignon F, Montcourrier P et al. Steroid receptor-mediated cytotoxicity of an antiestrogen and an antiprogestin in breast cancer cells. Cancer Res 1987; 47(5):1441-8.
82. Horwitz KB. The antiprogestin RU38 486: receptor-mediated progestin versus antiprogestin actions screened in estrogen-insensitive T47Dco human breast cancer cells. Endocrinology 1985; 116(6):2236-45.
83. Jacobsen BM, Schittone SA, Richer JK et al. Progesterone-independent effects of human progesterone receptors (PRs) in estrogen receptor-positive breast cancer: PR isoform-specific gene regulation and tumor biology. Mol Endocrinol 2005; 19(3):574-87.
84. Vares G, Ory K, Lectard B et al. Progesterone prevents radiation-induced apoptosis in breast cancer cells. Oncogene 2004; 23(26):4603-13.
85. Moore MR, Spence JB, Kiningham KK et al. Progestin inhibition of cell death in human breast cancer cell lines. J Steroid Biochem Mol Biol 2006; 98(4-5):218-27.
86. Lin VC, Eng AS, Hen NE et al. Effect of progesterone on the invasive properties and tumor growth of progesterone receptor-transfected breast cancer cells MDA-MB-231. Clin Cancer Res 2001; 7(9):2880-6.

87. Faivre EJ, Lange CA. Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. Mol Cell Biol 2007; 27(2):466-80.
88. Shyamala G, Yang X, Cardiff RD et al. Impact of progesterone receptor on cell-fate decisions during mammary gland development. Proc Natl Acad Sci USA 2000; 97(7):3044-9.
89. Shyamala G, Yang X, Silberstein G et al. Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor exhibit developmental abnormalities in mammary glands. Proc Natl Acad Sci USA 1998; 95(2):696-701.
90. Jacobsen BM, Richer JK, Sartorius CA et al. Expression profiling of human breast cancers and gene regulation by progesterone receptors. J Mammary Gland Biol. Neoplasia 2003; 8(3):257-68.
91. Leo JC, Wang SM, Guo CH et al. Gene regulation profile reveals consistent anticancer properties of progesterone in hormone-independent breast cancer cells transfected with progesterone receptor. Int J Cancer 2005; 117(4):561-8.
92. Ghatge RP, Jacobsen BM, Schittone SA et al. The progestational and androgenic properties of medroxyprogesterone acetate: gene regulatory overlap with dihydrotestosterone in breast cancer cells. Breast Cancer Res 2005; 7(6):R1036-50.
93. Graham JD, Yager ML, Hill HD et al. Altered progesterone receptor isoform expression remodels progestin responsiveness of breast cancer cells. Mol Endocrinol 2005; 19(11):2713-35.
94. Bakker GH, Setyono-Han B, Henkelman MS et al. Comparison of the actions of the antiprogestin mifepristone (RU486), the progestin megestrol acetate, the LHRH analog buserelin and ovariectomy in treatment of rat mammary tumors. Cancer Treat Rep 1987; 71(11):1021-7.
95. Bakker GH, Setyono-Han B, Portengen H et al. Endocrine and antitumor effects of combined treatment with an antiprogestin and antiestrogen or luteinizing hormone-releasing hormone agonist in female rats bearing mammary tumors. Endocrinology 1989; 125(3):1593-8.
96. Bakker GH, Setyono-Han B, Portengen H et al. Treatment of breast cancer with different antiprogestins: preclinical and clinical studies. J Steroid Biochem Mol Biol 1990; 37(6):789-94.
97. El Etreby MF, Liang Y. Effect of antiprogestins and tamoxifen on growth inhibition of MCF-7 human breast cancer cells in nude mice. Breast Cancer Res Treat 1998; 49(2):109-17.
98. Michna H, Schneider MR, Nishino Y et al. Antitumor activity of the antiprogestins ZK 98.299 and RU 38.486 in hormone dependent rat and mouse mammary tumors: mechanistic studies. Breast Cancer Res Treat 1989; 14(3):275-88.
99. Michna H, Schneider MR, Nishino Y et al. The antitumor mechanism of progesterone antagonists is a receptor mediated antiproliferative effect by induction of terminal cell death. J Steroid Biochem 1989; 34(1-6):447-53.
100. Nishino Y, Schneider MR, Michna H. Enhancement of the antitumor efficacy of the antiprogestin, onapristone, by combination with the antiestrogen, ICI 164384. J Cancer Res Clin Oncol 1994; 120(5):298-302.
101. Schneider MR, Michna H, Nishino Y et al. Antitumor activity of the progesterone antagonists ZK 98.299 and RU 38.486 in the hormone-dependent MXT mammary tumor model of the mouse and the DMBA- and the MNU-induced mammary tumor models of the rat. Eur J Cancer Clin Oncol 1989; 25(4):691-701.
102. Schneider MR, Michna H, Habenicht UF et al. The tumour-inhibiting potential of the progesterone antagonist Onapristone in the human mammary carcinoma T61 in nude mice. J Cancer Res Clin Oncol 1992; 118(3):187-9.
103. Schneider MR, Michna H, Nishino Y et al. Tumor-inhibiting potential of ZK 112.993, a new progesterone antagonist, in hormone-sensitive, experimental rodent and human mammary tumors. Anticancer Res 1990; 10(3):683-7.
104. Klijn JG, Setyono-Han B, Foekens JA. Progesterone antagonists and progesterone receptor modulators in the treatment of breast cancer. Steroids 2000; 65(10-11):825-30.
105. Sathya G, Jansen MS, Nagel SC. Identification and characterization of novel estrogen receptor-beta-sparing antiprogestins. Endocrinology 2002; 143(8):3071-82.
106. Clarke R. Human breast cancer cell line xenografts as models of breast cancer. The immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines. Breast Cancer Res Treat 1996; 39(1):69-86.
107. Yue W, Wang J, Savinov A et al. Effect of aromatase inhibitors on growth of mammary tumors in a nude mouse model. Cancer Res 1995; 55(14):3073-7.
108. Osborne CK, Coronado E, Allred DC et al. Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. J Natl Cancer Inst 1991; 83(20):1477-82.
109. Shafie SM, Grantham FH. Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. J Natl Cancer Inst 1981; 67(1):51-6.

110. Osborne CK, Hobbs K, Clark GM. Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res 1985; 45(2):584-90.
111. Sartorius CA, Harvell DM, Shen T et al. Progestins initiate a luminal to myoepithelial switch in estrogen-dependent human breast tumors without altering growth. Cancer Res 2005; 65(21):9779-88.
112. Lanari C, Molinolo AA, Pasqualini CD. Induction of mammary adenocarcinomas by medroxyprogesterone acetate in BALB/c female mice. Cancer Lett 1986; 33(2):215-23.
113. Lanari C, Kordon E, Molinolo A et al. Mammary adenocarcinomas induced by medroxyprogesterone acetate: hormone dependence and EGF receptors of BALB/c in vivo sublines. Int J Cancer 1989; 43(5):845-50.
114. Kordon E, Lanari C, Meiss R et al. Hormone dependence of a mouse mammary tumor line induced in vivo by medroxyprogesterone acetate. Breast Cancer Res Treat 1990; 17(1):33-43.
115. Montecchia MF, Lamb C, Molinolo AA et al. Progesterone receptor involvement in independent tumor growth in MPA-induced murine mammary adenocarcinomas. J Steroid Biochem Mol Biol 1999; 68(1-2):11-21.
116. Lamb CA, Helguero LA, Giulianelli S et al. Antisense oligonucleotides targeting the progesterone receptor inhibit hormone-independent breast cancer growth in mice. Breast Cancer Res 2005; 7(6): R1111-21.
117. Rosen EM, Fan S, Isaacs C. BRCA1 in hormonal carcinogenesis: basic and clinical research. Endocr Relat Cancer 2005; 12(3):533-48.
118. Rebbeck TR, Levin AM, Eisen A et al. Breast cancer risk after bilateral prophylactic oophorectomy in BRCA1 mutation carriers. J Natl Cancer Inst 1999; 91(17):1475-9.
119. Gudas JM, Nguyen H, Li T et al. Hormone-dependent regulation of BRCA1 in human breast cancer cells. Cancer Res 1995; 55(20):4561-5.
120. Marks JR, Huper G, Vaughn JP et al. BRCA1 expression is not directly responsive to estrogen. Oncogene 1997; 14(1):115-21.
121. Spillman MA, Bowcock AM. BRCA1 and BRCA2 mRNA levels are coordinately elevated in human breast cancer cells in response to estrogen. Oncogene 1996; 13(8):1639-45.
122. Zheng WQ, Lu J, Zheng JM et al. Variation of ER status between primary and metastatic breast cancer and relationship to p53 expression*. Steroids 2001; 66(12):905-10.
123. King TA, Gemignani ML, Li W et al. Increased progesterone receptor expression in benign epithelium of BRCA1-related breast cancers. Cancer Res 2004; 64(15):5051-3.
124. Poole AJ, Li Y, Kim Y et al. Prevention of Brca1-mediated mammary tumorigenesis in mice by a progesterone antagonist. Science 2006; 314(5804):1467-70.
125. Arpino G, Weiss H, Lee AV et al. Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. J Natl Cancer Inst 2005; 97(17):1254-61.
126. Grann VR, Troxel AB, Zojwalla NJ et al. Hormone receptor status and survival in a population-based cohort of patients with breast carcinoma. Cancer 2005; 103(11):2241-51.
127. Horwitz KB, Costlow ME, McGuire WL. MCF-7; a human breast cancer cell line with estrogen androgen, progesterone and glucocorticoid receptors. Steroids 1975; 26(6):785-95.
128. Bardou VJ, Arpino G, Elledge RM et al. Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases. J Clin Oncol 2003; 21(10):1973-9.
129. Clark GM, McGuire WL, Hubay CA et al. Progesterone receptors as a prognostic factor in Stage II breast cancer. N Engl J Med 1983; 309(22):1343-7.
130. McGuire WL, Clark GM. The prognostic role of progesterone receptors in human breast cancer. Semin Oncol 1983; 10(4 Suppl 4):2-6.
131. Ravdin PM, Green S, Dorr TM et al. Prognostic significance of progesterone receptor levels in estrogen receptor-positive patients with metastatic breast cancer treated with tamoxifen: results of a prospective Southwest Oncology Group study. J Clin Oncol 1992; 10(8):1284-91.
132. Muss HB. Endocrine therapy for advanced breast cancer: a review. Breast Cancer Res Treat 1992; 21(1):15-26.
133. Osborne CK, Schiff R, Arpino G et al. Endocrine responsiveness: understanding how progesterone receptor can be used to select endocrine therapy. Breast 2005; 14(6):458-65.
134. Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet 1998; 351(9114):1451-67.
135. Allegra JC, Lippman ME, Thompson EB et al. Estrogen receptor status: an important variable in predicting response to endocrine therapy in metastatic breast cancer. Eur J Cancer 1980; 16(3):323-31.
136. Horwitz KB, Alexander PS. In situ photolinked nuclear progesterone receptors of human breast cancer cells: subunit molecular weights after transformation and translocation. Endocrinology 1983; 113(6):2195-201.

137. Mote PA, Balleine RL, McGowan EM et al. Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle. J Clin Endocrinol Metab 1999; 84(8):2963-71.
138. Mote PA, Bartow S, Tran N et al. Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis. Breast Cancer Res Treat 2002; 72(2):163-72.
139. Graham JD, Yeates C, Balleine RL et al. Characterization of progesterone receptor A and B expression in human breast cancer. Cancer Res 1995; 55(21):5063-8.
140. Graham JD, Yeates C, Balleine RL et al. Progesterone receptor A and B protein expression in human breast cancer. J Steroid Biochem Mol Biol 1996; 56(1-6 Spec No):93-8.
141. Bamberger A, Milde-Langosch K, Schulte H et al. Progesterone receptor isoforms, pr-b and pr-a, in breast cancer: correlations with clinicopathologic tumor parameters and expression of ap-1 factors. Horm Res 2000; 54(1):32-7.
142. Hopp TA, Weiss HL, Hilsenbeck SG et al. Progesterone Receptor (PR) A and B in Breast Cancer: PR-A Rich Tumors have poorer disease-free survival. Clin Cancer Res 2004; 10(8):2751-60.
143. Clemm DL, Sherman L, Boonyaratanakornkit V et al. Differential hormone-dependent phosphorylation of progesterone receptor A and B forms revealed by a phosphoserine site-specific monoclonal antibody. Mol Endocrinol 2000; 14(1):52-65.