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Challenges to defining a role for progesterone in breast cancer

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ABSTRACT

Progesterone is an ovarian steroid hormone that is essential for normal breast development during puberty and in preparation for lactation. The actions of progesterone are primarily mediated by its high affinity receptors, including the classical progesterone receptor (PR) -A and -B isoforms, located in diverse tissues such as the brain where progesterone controls reproductive behavior, and the breast and reproductive organs. Progestins are frequently prescribed as contraceptives or to alleviate menopausal symptoms, wherein progestin is combined with estrogen as a means to block estrogen-induced endometrial growth. Estrogen is undisputed as a potent breast mitogen, and inhibitors of the estrogen receptor (ER) and estrogen producing enzymes (aromatases) are effective first-line cancer therapies. However, PR action in breast cancer remains controversial. Herein, we review existing evidence from *in vitro* and *in vivo* models, and discuss the challenges to defining a role for progesterone in breast cancer.

## 1.   Introduction: alterations between the normal and neoplastic breast

Complex factors contribute to the challenge of demonstrating a clear role for progesterone in breast cancer. First, progesterone is difficult to study in isolation from other hormones (i.e. growth factors, prolactin) that also contribute to breast cancer biology. Second, progesterone receptor (PR) isoforms are expressed in response to estrogen receptor-alpha (ER) mediated transcriptional events, but can also occur independently of ER [1]. The subset of mammary epithelial cells (MECs) in the breast that express both PR-A and PR-B also express ER, and estrogen is usually required in order to induce the robust expression of PR in these ER+ cells. As estrogen is also a potent breast mitogen, this makes it difficult to separate the effects of progesterone alone from those of estrogen. Indeed,

PR isoforms are grossly understudied relative to ER in both the normal and neoplastic breast.

Studies in steroid hormone receptor knock-out mice have revealed that the concerted actions of estrogen and progesterone are required for normal mammary gland development [2,3]; estrogen/ER promotes the growth of ducts that invade the mammary fat pad emanating from the nipple, while estrogen/ER and progesterone/PR are required for the development of the terminal end-buds (TEBs) or acini located at the ends of ducts that later become the milk producing structures in the lactating mammary gland (Fig. 1). Additional required hormones, known as epidermal growth factor (EGF) and insulin-like growth factor (IGF-1) augment the proliferation of terminal end-buds during normal breast development, and promote ductal outgrowth and side branching induced by estrogen plus progesterone [4,5]. In fact, PR isoform expression in response to estrogen requires the presence of EGF
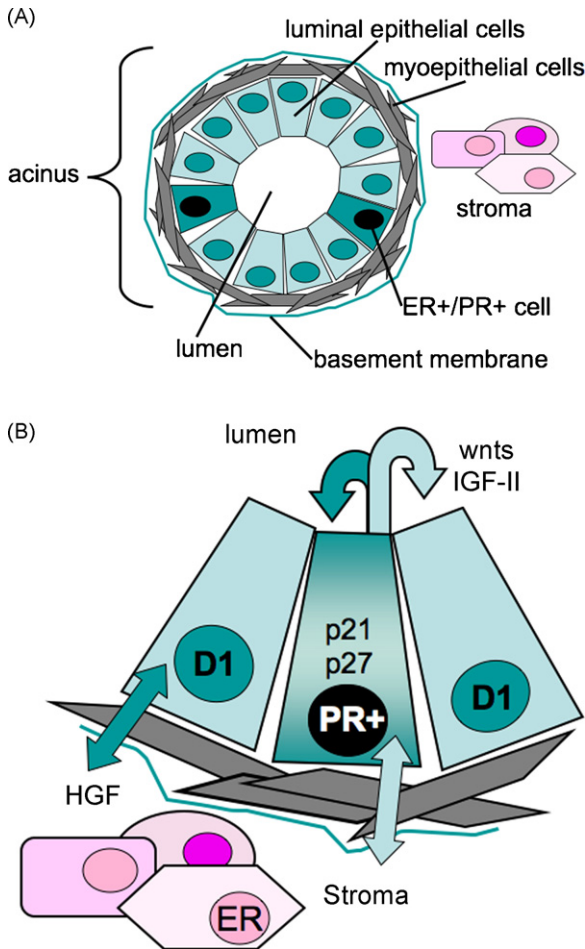
Fig. 1 – Mammary gland structure. (A) Acini, located at the ends of ducts in the adult mammary gland, are the functional units of the lactating mammary gland. Luminal epithelial cells (apical) exist as polar cells in contact with myoepithelial cells (basal). Epithelial cell populations are separated from the stroma by a basement membrane. (B) Steroid hormone receptor positive (ER+/PR+) cells occur adjacent to proliferating cells in the normal mammary gland. Communication (paracrine signaling) between the epithelial and stromal compartments mediates proliferation of ER/PR negative cells. Early events during breast cancer development may mediate switching from paracrine to autocrine mechanisms of proliferation in ER+/PR+ cells.

[6], suggesting the existence of important cross talk between EGF receptors (EGFR) and/or family members (erbB2) and both steroid hormone receptors.

Another limitation to deciphering a role for progesterone/PR action in breast cancer development is that normal proliferating breast epithelial cells are steroid hormone receptor negative [7]. In the normal adult mammary gland, ER+/PR+ cells represent only about 7–10% of the luminal epithelial cell population; these cells are most often non-dividing, but usually lie adjacent to proliferating cells (Fig. 1). The most current information suggests that ER+/PR+ cells are capable of proliferating, but are growth-arrested by the expression of inhibitory molecules, such as TGF-beta or high levels

of p21 and p27, the endogenous inhibitors of cell-cycle-dependent protein kinases (CDKs). Communication between the breast epithelial and stromal compartments mediates the proliferation of nearby or adjacent cells by expression and secretion of locally active pro-proliferative molecules such as wnts, IGF-II [7], or stroma-derived hepatocyte growth factor (HGF) [8]. Recent evidence suggests that ER+/PR+ cells may act as "feeder cells" by providing growth-promoting substances (i.e. wnts) to nearby progenitor or stem cell populations [9].

In contrast to the normal breast, where proliferating cells are most often devoid of steroid hormone receptors, the majority of breast cancers (~70%) express ER and PR at the time of diagnosis. Although steroid hormone receptor-positive tumors are most often slower growing relative to receptor-negative tumors [10], ER+/PR+ breast epithelial cells may undergo an early switch to autocrine or paracrine signaling mechanisms whereby negative controls on proliferation are somehow lifted. Another setting where PR-containing cells clearly divide is in the pregnant mammary gland, where PR-B colocalizes with cyclin D1 in BrdU-stained (dividing) cells [11]. Thus, pathways involved in normal mammary gland growth and development may inappropriately "re-assert" themselves during breast cancer progression. Experimental evidence in model organisms (primates, mice, rats) and humans suggests a pro-proliferative role for progestins [12–14]. Herein, we review the status of progesterone/PR action in breast cancer models, and suggest a potential for future development of PR antagonists as part of combined breast cancer therapies.

2. Integration of PR classical and membrane-associated rapid signaling

PR isoforms are classically defined as ligand-activated transcription factors and members of a large family of related steroid hormone receptors (that includes ER, androgen receptor (AR), glucocorticoid receptor (GR), and mineralocorticoid receptor). PRs are activated upon binding of the naturally occurring ovarian steroid hormone, progesterone, or via binding to synthetic ligands (progestins) and regulate gene expression by binding directly or indirectly to specific sites in DNA (Fig. 2). Three PR isoforms (Fig. 2A) are the distinct protein products of a single gene located on chromosome 11 at q22-23. Transcription of PR isoforms is governed by the use of "distal" and "proximal" promoter regions [15]. The presence of internal translational start sites within common mRNAs results in the creation of three protein isoforms that 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 most often co-express PR-A and PR-B isoforms; these receptors exhibit different transcriptional activities within the same promoter context, but can also recognize entirely different gene promoters [16,17]. PR-B is essential for normal mammary gland development [18], while PR-A is required for uterine development and reproductive function [19]. PR-C is devoid of transcriptional activity, but when expressed, can enhance PR activity in breast cancer cells [20] or function as a dominant inhibitor of PR-B in the uterus [21].
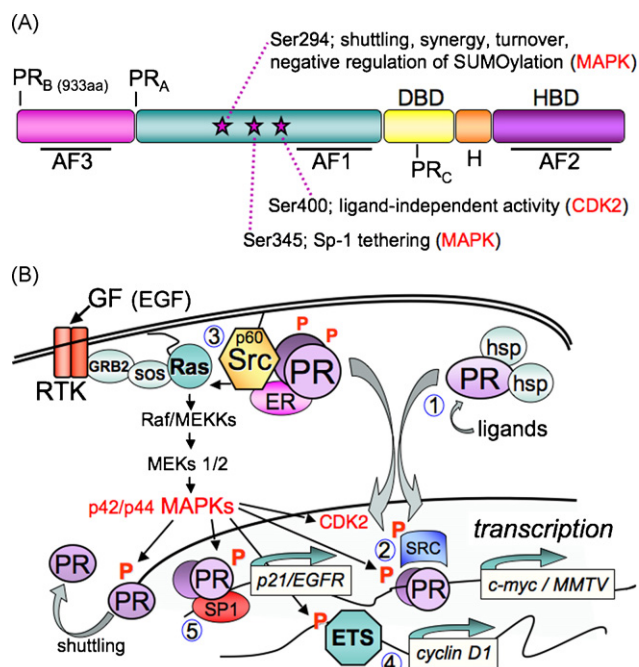
Fig. 2 – Progesterone receptor-dependent integrated actions. (A) Ligand activated PR-B and PR-A transcription factors contain a hormone binding domain (HBD), hinge region (H), DNA-binding domain (DBD), and amino terminus. Activation functions (AFs) represent the sites of co-regulator interaction required for transcription. Serines 294, 345, and 400 are regulatory sites that are phosphorlyated in response to progestins and/or mitogenic signaling pathways that modify PR function. **(B)** Phosphorylation (P) of specific sites in PR couples multiple receptor functions. **(1)** Ligand-binding mediates dissociation of heat-shock proteins (hsps) and nuclear accumulation of PR. **(2)** Nuclear PRs regulate gene expression via the classical (PRE-dependent) pathway; phosphorylated PR recruit regulatory molecules that are phospho-proteins, and may function in one or more inter-connected processes (transcription, localization, and turnover). **(3)** PR and growth factors activate MAPKs via a c-Src kinase-dependent pathway, and this may result in positive regulation of PR transcriptional activity via "feed-back" regulation (i.e. direct phosphorylation of liganded PR or co-activators), occurring in both the absence and presence of ligands and on PRE-containing or other PR-regulated gene promoters. **(4)** Activation of MAPKs by PR provides for regulation of genes whose promoters do not contain PREs and are otherwise independent of PR-transcriptional activities but utilize PR-activated MAPKs. **(5)** In response to progestins, c-Src and MAPK-dependent phosphorylation of PR Ser345 mediates tethering to Sp1 and selective regulation of growth promoting genes via Sp1 sites (p21, EGFR).

Unliganded PRs are complexed with chaperone molecules including heat shock proteins (hsps); these interactions allow proper protein folding and assembly of stable PR molecules competent to bind hormone [22]. Hsps also mediate important aspects of PR protein trafficking. After binding to proges-

terone, receptor conformational changes induce dimerization and hsp dissociation (Fig. 2). Activated receptors associate with co-regulators, including steroid receptor coactivators (SRCs 1–3), are withheld in the nucleus, and bind directly to specific progesterone response elements (PREs) and PRE-like sequences in the promoter regions of target genes such as *c-myc* [23], *fatty acid synthetase* [24], and *MMTV* [25]. Treatment with progestin also results in the upregulation of genes without canonical PREs in their proximal promoter regions, such as *epidermal growth factor receptor* [26], *c-fos* [27], *p21* [28], *IRS-2* [29], and *cyclin D1* [30]. Regulation of genes without PREs, PRE half-sites, or PRE-like sequences can occur through PR tethering to other DNA-binding transcription factors, such as specificity protein 1 [28], activating protein 1 [31] or signal transducers and activators of transcription (Stats) [32,33].

The genomic or classical actions of steroid hormone treatment are delayed by several minutes to hours, dictated by the time required for transcription and translation of target genes. Recently, however, rapidly occurring (within a few minutes) extranuclear or non-genomic effects of cell membrane-localized steroid hormone receptors have entered the forefront. For example, progestin treatment of breast cancer cells causes a rapid and transient (2–15 min) activation of cytoplasmic protein kinases, including mitogen-activated protein kinase (MAPK), PI3K, and p60-Src kinase [34–36]. Similar activities have been reported for membrane-associated ERalpha and AR [37]. These effects are mediated by direct binding of steroid hormone receptors to protein–protein interaction domains of signaling molecules located in or near the plasma membrane, in close proximity to growth factor receptors and their immediate effectors. Human PR contains an N-terminal 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 [34]. *In vitro* experiments demonstrate that progestin-bound, purified PR-A and PR-B directly activate the c-Src-related protein kinase, Hck; PR-B but not PR-A activates c-Src and MAPKs *in vivo*. Mutation of the PXXP sequence in PR-B disrupts the 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 blocking progestin-induced c-Src or MAP kinase activation. Thus, non-genomic MAPK activation by progestin/PR-B/c-Src complexes most likely occurs by way of a c-Src-dependent mechanism involving Ras activation of the Raf/MEK/MAPK module (Fig. 2B). ER in association with other signaling and adaptor molecules is suspected to reside in similar cytoplasmic signaling complexes, possibly in association with PR and c-Src [37].

In studies using human breast or prostate cancer cell lines, the rapid signaling actions of membrane-associated AR, PR, and/or ER have been shown to contribute to the regulation of cell proliferation in response to their respective hormone ligands [38–40]. While potential roles in human physiology (i.e. whole organisms) are less clear, steroid hormone receptor-mediated activation of cytoplasmic signaling molecules may primarily serve to potentiate the nuclear functions of these receptors (Fig. 2). For example, amplification

of PR nuclear functions likely occurs through rapid, direct phosphorylation of PR proteins and/or receptor co-regulators in response to activation of PR-induced cytoplasmic pathways that are mechanistically coupled to ligand binding. Thus, appropriately phosphorylated and activated receptor complexes are efficiently directed to selected target genes. Clearly, such positive feedback explains the dramatic influence of activated signaling pathways on PR nuclear function. Indeed, several progestin/PR-dependent events are MAPK or c-Src-dependent, including upregulation of cyclins D1 and E, CDK2 activation, S-phase entry, and anchorage-independent cell growth in soft-agar [26,41,42]. C-Src- and MAPK-dependent direct phosphorylation of PR Ser345 is required for PR tethering to Sp1 transcription factors bound to the *p21* and *EGFR* promoters [43]. PR/Sp1 tethering upon c-Src/MAPK pathway activation is predicted to alter PR promoter selectivity, favoring the use of Sp1-driven promoters within PR-target genes. Kinases also confer hyperactivity and ligand-independence to phosphorylated PR-B [42,44,45]. For example, MAPKs mediate PR hypersensitivity to ligand by phosphorylation of PR Ser294, an event that derepresses receptor activity by preventing PR sumoylation [46]. Activated CDK2 or loss of p27 induces PR ligand-independent activity via Ser400 phosphorylation [42]. Although more studies are needed, it is becoming clear that activation of cytoplasmic protein kinases is an integral feature of PR nuclear action (i.e. phosphorylation events are required for gene regulation leading to changes in cell biology). Thus, rapid phosphorylation events may primarily act to alter PR transcriptional activity, but clearly also mediate promoter selectivity [47].

How might the membrane-associated signaling actions of steroid hormone receptors, including PR, contribute to deregulated breast cancer cell growth and/or increased breast cancer risk? Perhaps by linking steroid hormone action to the expression of MAPK-regulated genes (i.e. the endpoint of MAPK signaling is the phosphorylation of transcription factors). In support of this concept, the extranuclear actions of liganded ERs induce a state of "adaptive hypersensitivity" during endocrine therapy in which growth factor signaling pathways are co-opted by upregulated ERs [48]. In this model of ER-dependent MAPK activation, liganded ERs localized at the cell membrane interact with the adapter protein Shc and induce its phosphorylation, leading to recruitment of adaptor molecules and activation of Ras and the Raf-1/MEK/MAPK module. MAPK then regulates genes via direct phosphorylation of Ets factors and/or AP1 components (i.e. independently of ER transcriptional activity). ER activation of MAPK explains why many tumors respond well to aromatase inhibitors, yet fail to respond to selective estrogen receptor modulators (SERMS) designed to inhibit ER transcriptional activity in the nucleus, but not ER-dependent MAPK activation in the cytoplasm. Breast cancers often exhibit heightened c-Src and MAPK activities [49,50] and elevated cyclin D1, an AP1 target gene whose expression is sensitive to multiple kinase inputs [51–53]. Steroid hormone receptors including PRs may contribute to the constitutive signaling of cytoplasmic mitogenic protein kinases via their membrane-associated activities, thereby circumventing endocrine-based (i.e. antiestrogen) therapies (Fig. 2).

3. Probing PR action in animal models

Studies in rodents demonstrate that PR-A and PR-B are differentially expressed during mammary gland development, with PR-A predominantly expressed during ductal sidebranching, while PR-B expression coincides with the formation of alveoli [54,55]. PR-B but not PR-A is expressed in proliferating cells. Some but not all proliferating cells in both compartments are PR-B+, suggesting that progesterone may induce proliferation through either direct or paracrine mechanisms. In contrast, cells in adult virgin glands are PR and cyclin D1 positive, but fail to proliferate, possibly due to high levels of the cyclin-dependent protein kinase inhibitors, p21 and p27 [55]. During the menstrual cycle, MEC undergo sequential waves of proliferation and apoptosis. Notably, in primates (macaques and humans) increased terminal duct lobular unit cell proliferation coincides with the peak of serum progesterone that occurs during the luteal phase [13,56–58], again suggesting a paracrine mechanism for this hormone in adult tissues. Upregulation of local IGF-1 may be a cooperating factor in this regard [59]. In animal models of postmenopausal hormone replacement therapy, both parous and nulliparous early and late postmenopausal mice were subjected to estrogen alone (E) or estrogen plus progestin (E + P); E + P produced a greater proliferative response relative to E alone regardless of parity or treatment time. E + P was also shown to act directly on the mammary gland, rather than via systemic effects [60]. Similar results occurred in surgically postmenopausal macaques [61] and in postmenopausal humans [62]. Although breast cancer development was not modeled in the above animal studies, the results (i.e. increased proliferation) are consistent with human clinical data [63,64], which revealed increased tumor number and size in women taking E + P, while E alone did not significantly alter breast cancer risk or tumor size.

Progesterone may act via proto-oncogenes and growth factors to affect breast cell proliferation and breast cancer etiology. As the majority of early breast cancer lesions express both ER and PR and these receptors remain high in at least 60% of advanced decease, early events may include a switch in the ability of normally quiescent ER+/PR+ cells to respond directly to steroid hormones and proliferate (Fig. 1). Notably, deregulation of the cell cycle is a hallmark of breast cancer. Up to 40% of breast cancers overexpress cyclin D1, while at least 30% have lost p27 or p21 and/or contain activated CDK2 [65,66]. Mutation or loss of p53 is also a frequent occurrence [67]. Numerous *in vitro* studies have demonstrated linkage of PR action to cell cycle regulation [42,68–70]. Namely, PR interacts directly with cyclins A or E and CDK2 [69,70]. PR activity is highest in the DNA synthesis (S) phase of the cell cycle, when CDK2 activity peaks [68,69]. Furthermore, PR transcriptional activity becomes ligand-independent and CDK2-driven upon loss of p27 [42]. Progestins, acting through PR-B-dependent transcription, induce cyclin D1 expression and cell cycle re-entry in antiestrogen-arrested breast cancer cells [71]. This suggests that in the breast, progesterone/PR action is tightly coupled to mechanisms of cell cycle control. In breast cancer, the mitogenic potential of activated PRs may manifest particularly during loss of checkpoint control and/or elevation of CDK or other mitogenic kinase activities. For example,

TGF-α (an EGFR ligand) transgenic mice develop ER+/PR+ proliferative hyperplasias (early lesions) that rapidly progress to ER+/PR- tumors [72]. Recent *in vitro* studies demonstrate that BRCA-1 knock-down enhances progestin-induced PR transcriptional activity, while progestin-induced MEC proliferation is increased in genetically engineered mice lacking BRCA-1 in the mammary gland [73]. Related to this finding, in recent studies using mice lacking mammary gland expression of both BRCA-1 and p53, PR protein levels were dramatically increased, and the development of aggressive ER+/PR+ tumors in virgin mice was completely blocked by antiprogestins [74]. In future studies, it will be important to define how negative regulation of cell proliferation in ER+/PR+ normal MECs is somehow lifted to allow progression of early lesions to malignant cancer, and if early events include loss of checkpoint control or alteration of DNA damage and repair pathways in PR+ cells.

4. PR action in human breast cancer cell models

The biochemistry of PR action is well characterized, having been largely defined using PR+ human breast cancer cell lines, or PR-null cells into which wild-type or modified PRs has been re-expressed. Numerous studies have focused on PR interactions with regulatory proteins, changes in PR subcellular localization, or post-translational modifications of PRs (i.e. phosphorylation, ubiquitinylation, or sumoylation) or other conditions that affect PR transcriptional activities, usually measured on artificial gene promoters (reporter genes) containing one or more tandem PRE sites [75]. Growth factors, including EGF or heregulin, promote transcriptional synergy in the presence of progestins on PR-target genes [45,76,77]. As discussed above, phosphorylation events primarily serve to augment PR action in a promoter selective manner [46]. Despite this depth of basic understanding, the details of gene regulation and the associated changes in cell biology in response to PR activation remain elusive. Only a handful of endogenous progesterone-responsive genes have been described in detail [23,29,78]. The majority of genes regulated in response to progesterone lack PR-binding consensus sequences or progesterone responsive elements (PREs), and the presence of one or more PREs or PRE half-sites fails to accurately predict progesterone-responsive regulation [17]. Many genes are regulated upon PR expression, but independently of progesterone [16,79]. Furthermore, several genes are downregulated in response to progesterone/PR-dependent transcriptional repression, largely by unknown mechanisms [16,17]. In most cases, the regulation of specific genes in response to progesterone/PR is only loosely tied (by correlation) to changes in cell or tumor biology. For example, many PR-regulated genes are associated with aspects of tumor progression towards aggressive tumor phenotype. In addition, the PR-A to PR-B ratio is frequently altered (i.e. away from 1:1) in breast tumors relative to normal tissue [80], a condition predicted to dramatically alter the genetic program [16,81].

Confounding the role of progesterone in breast cancer is the finding that progesterone has biphasic effects on the proliferation of breast cancer cell lines grown *in vitro* (cells grown in plastic culture dishes supplemented with progestin-

containing media). Cultured cell lines undergo an initial burst of proliferation characterized by increased S-phase entry that peaks at 18 h of progesterone treatment [30,82,83]. Cell cycle progression is driven by successive upregulation of G1/S and G2 cyclins, p21, and elevated CDK2 activity. This is followed 24–48 h (one to three cell cycles) later by cell growth inhibition in which p27 is upregulated and the cells ultimately arrest at the G1/S boundary. Thus, the response of cultured breast cancer cells to progesterone is both proliferative and inhibitory, in contrast to the clear mitogenic effects of estrogens in the same cell line models. For this reason, it has been suggested that progesterone acts primarily as a priming agent, with growth promoting activity dependent upon cellular context and/or the presence of secondary agents [84]. For example, progestins upregulate selected components of growth factor-initiated signaling pathways, including IRS-2 [29], and EGFR family members and their ligands; progestin-treated breast cancer cells are more responsive to EGF-induced proliferation than are progestin-naïve cells [30]. Thus, progesterone may act in part by sensitizing breast cancer cells to growth factor and cytokine signals [85].

Of note is that progesterone treatment of PR+ breast cancer cells growing in culture has also been implicated in pro-survival (resistance to chemotherapy-induced apoptosis [86]) and tumor cell differentiation (from luminal to myoepithelial phenotype) with minimal effects on tumor growth [87]; this transition is associated with poor clinical prognosis. Similarly, epithelial to mesenchymal transition (EMT) is an early event that precedes tumor cell invasion and metastasis, and may occur independently of changes in proliferation. During EMT, stationary epithelial cells become fibroblast-like and acquire the ability to migrate and invade locally. Interestingly, many progesterone-regulated genes encode molecules involved in signal transduction and cell adhesion to extracellular matrix (ECM) or other basement membrane components [16,17]. IRS-2, a PR-B-regulated gene, is a mediator of increased cell motility [47]. The effects of progestins have recently been evaluated in soft-agar, a type of 3D culture system that allows breast cancer cells to grow as anchorage-independent colonies more similar to the organization of acini found *in vivo*. In contrast to mono-layer cultures, progestins are clearly mitogenic in this system, wherein PR-B induces transcriptional upregulation of wnt-1, leading to sustained MAPK activity, upregulation of cyclin D1, and the formation of abundant large colonies [41]. This suggests that the mitogenic actions of progesterone require the establishment of cell polarity, a property that is not supported in 2D culture systems. In addition to gaining further insight into the role of what appears to be excessive cross talk between progesterone/PR and signaling pathway components, a clear definition of the specific actions of progesterone/PR that are relevant to more advanced breast cancer cell biology (i.e. tumor progression to metastasis, including EMT) is needed. This may require study conditions that mimic or preserve breast epithelial cell architecture in which PR+ luminal epithelial cells are polarized, and in contact with basement membrane components (Fig. 1). In support of this concept, normal MECs respond differently to estrogen and progesterone when cultured in the presence of variable ECM components (i.e. collagen type I, fibronectin, laminin) [8]; ECM proteins increase the expression of EGF and IGF receptors. In

this context, estrogen plus progestin inhibit EGF/IGF-induced MEC proliferation in an ECM-dependent manner [88]. Clearly, ER and PR interactions with ECM are complex [8]. Deregulation of ECM protein expression and/or integrin signaling (i.e. early events in cancer progression) is likely to dramatically alter hormone responsiveness.

5. Future perspective

Many aspects of PR action originally discovered in animal or cell line models of breast cancer have not been established in humans. However, a direct role for PRs in breast cancer is illustrated by the clinical findings of the Women's Health Initiative (WHI) and Million Women Study, demonstrating that women taking a progestin in combination with estrogen as part of hormone replacement therapy (EPT), experienced a greater breast cancer risk relative to estrogen alone; tumors were larger and of higher grade [63,64]. The Million Women Study also found that women were more likely to die of breast cancer if they were taking EPT at the time of diagnosis. Thus, while substantial preclinical data suggest an important role for PR function in modulating breast cancer biology, validation of these findings are dependent on a clinical strategy to disrupt PR function in human breast cancers. It will then be important to decipher the contribution of both nuclear and membrane PR activities, and target them appropriately with selective PR modulators, in addition to targeting the relevant kinases (c-Src, MAPKs, and CDK2) required for steroid hormone receptor action. We suggest that PR activities be routinely targeted as part of combination therapies aimed at blocking both ER-α and PR-B, along with the associated essential protein kinases.

Acknowledgements

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