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Progesterone Receptors (PR) Mediate STAT Actions: PR and Prolactin Receptor Signaling Crosstalk in Breast Cancer Models

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Abstract

Estrogen is the major mitogenic stimulus of mammary gland development during puberty wherein ER signaling acts to induce abundant PR expression. PR signaling, in contrast, is the primary driver of mammary epithelial cell proliferation in adulthood. The high circulating levels of progesterone during pregnancy signal through PR, inducing expression of the prolactin receptor (PRLR). Cooperation between PR and prolactin (PRL) signaling, via regulation of downstream components in the PRL signaling pathway including JAKs and STATs, facilitates the alveolar morphogenesis observed during pregnancy. Indeed, these pathways are fully integrated via activation of shared signaling pathways (i.e. JAKs, MAPKs) as well as by the convergence of PRs and STATs at target genes relevant to both mammary gland biology and breast cancer progression (i.e. proliferation, stem cell outgrowth, tissue cell type heterogeneity). Thus, rather than a single mediator such as ER, transcription factor cascades (ER>PR>STATs) are responsible for rapid proliferative and developmental programming in the normal mammary gland. It is not surprising that these same mediators typify uncontrolled proliferation in a majority of breast cancers, where ER and PR are most often co-expressed and may cooperate to drive malignant tumor progression. This review will primarily focus on the integration of PR and PRL signaling in breast cancer models and the importance of this cross-talk in cancer progression in the context of mammographic density. Components of these PR/PRL signaling pathways could offer alternative drug targets and logical complements to anti-ER or anti-estrogen-based endocrine therapies.

Keywords

estrogen; progesterone; breast; cancer; prolactin; receptor; kinase

Introduction

Transcription factor cascades orchestrate mammary gland development

The mammary gland is unique among organ systems in that it develops primarily after birth, undergoing extensive postnatal development characterized by massive epithelial cell

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proliferation that occurs over a relatively short time interval. The ovarian steroid hormones, estrogen and progesterone, and the pituitary peptide hormone, prolactin (PRL), acting through their specific receptors, are critical for this process. Extensive genetic and tissue recombination studies in mouse models have revealed that estrogen receptor alpha (ER) signaling in mammary epithelial cells (MEC) is required for pubertal ductal elongation, while progesterone receptor (PR) actions in MEC are essential for ductal side branching and alveologenesis. During pregnancy, prolactin receptor (PRLR) signaling drives further mammary alveologenesis and is required for the ultimate goal of milk production and secretion. Although these hormone receptor-dependent signaling pathways dominate distinct developmental stages, the mammary gland also exhibits stage-dependent sensitivity to each hormone. Notably, although ER and PR are segregated, that is, expressed in separate normal mammary epithelial cell populations [1, 2], extensive paracrine crosstalk occurs between ER and PR signaling and between PR and PRL signaling, creating a continuum of overlapping and highly integrated signaling pathways. These same pathways are known to contribute to breast cancer biology and form the basis of approaches that employ molecular targeted therapies (i.e. endocrine therapies and combinations thereof with kinase inhibitors).

Hormones are drivers of luminal breast cancer

A majority (~70%) of breast tumors co-express ER and PR; these steroid hormone receptors (SRs) classically function as ligand activated transcription factors. SRs rapidly and dynamically shuttle between the nucleus and the cytoplasm, where they also bind membrane-associated and/or cytoplasmic protein kinases. Hormone-bound receptors rapidly activate signaling cascades that, in turn, function to regulate transcriptional activity and promoter selection via phosphorylation events [3]. The complexities regarding SR activation and regulation are discussed in other select reviews [4–8]. A growing list of selective estrogen receptor modulators, or SERMS (i.e. tamoxifen [Tam]), and aromatase inhibitors are used clinically to target ER in breast cancers [9]. Women with relatively high levels of endogenous hormones, or whose lifetime exposure to hormones is increased as a consequence of early menarche and/or late menopause, are at increased risk of developing breast cancer. Similarly, large-scale clinical trials showed that women taking hormone replacement therapies (HRT) that included estrogen and a progestin exhibited an increased risk of breast cancer [10–12]. Although estrogen treatment alone given to women who underwent a prior hysterectomy was protective, classical HRT typically contained equine estrogen and a synthetic progestin known as medroxyprogesterone acetate (MPA). The increased risk of developing breast cancer while taking HRT was fully reversible upon cessation of therapy, suggesting that combined HRT acts as a tumor-promoter (i.e. a stimulator of cell survival and/or proliferation) rather than as a mutagenic carcinogen. It is unclear whether MPA or other synthetic progestins (i.e. relative to progesterone) are the causative agents in HRT. While other large scale population studies support a role for synthetic progestins in increased breast cancer risk [13], the topic remains quite controversial [14].

Estrogen and progestins may act synergistically to promote the growth of early lesions [15–17]. Progesterone (and synthetic progestins) are proliferative in normal breast tissue and in breast cancer models [18] and progesterone, via PR-dependent paracrine signaling, is a

mediator of mammary gland stem cell self-renewal and expansion [19–22]. These studies have been extended to include breast cancer stem cells [21, 22]. In addition, recent studies suggest that Her2 signaling, in cooperation with progesterone (via the action of progesterone-induced paracrine signals RANKL and Wnt), may drive the dissemination of cells from microscopic breast tumors to distant metastatic sites very early in tumor progression [23, 24]. Notably, ER and PR directly interact [25, 26] and participate in the same rapid signaling and transcriptional complexes at target genes relevant to breast cancer biology [25–27]. The presence of the B isoform of PR (PR-B) conferred estrogen-dependent gene regulation to numerous ER-target genes that required the scaffolding actions of both PR-B and PELP1 in ER-containing transcriptional complexes [26]. In estrogen treated cells, progestins dramatically altered global ER binding sites [27, 28]. These receptors also regulated up to 80% identical sets of target genes when cells were stimulated with estrogen or progestins alone [27]. While targeting PR in breast cancer is not currently standard of care, these studies and other emerging literature suggests that PR antagonists or selective PR modulators (SPRMs) that can disrupt ER signaling, may provide a viable second-line therapy for women who fail on ER targeted therapies. Such molecules could also be utilized in conjunction with current ER-directed strategies to delay or prevent endocrine resistance [29–31]. While high dose PR agonists that disrupt ER actions (i.e. including progesterone) have been suggested as useful breast cancer therapies [14], the effect of these agents on tumor cell fate (i.e. stem cell outgrowth, early dissemination, tumor heterogeneity) must be fully understood before they enter routine clinical use.

Prolactin (PRL) signaling is implicated in breast cancer

Prolactin (PRL) is a polypeptide hormone produced primarily by cells known as lactotrophs, which are located in the anterior pituitary gland of all vertebrates. In humans, PRL is also produced at multiple extra-pituitary sites and functions as a circulating cytokine hormone with both autocrine and paracrine actions [32]. The biological activities of PRL are mediated by membrane PRL receptors (PRLR), members of the cytokine receptor superfamily characterized by a non-tyrosine kinase single-pass transmembrane domain with conserved features of cytokine receptors within the extracellular domain [33, 34]. Ligand binding and activation of PRLR leads to downstream induction of the canonical Jak2/STAT5 or Jak1/STAT3 pathways, feeding into multiple signaling cascades including phosphoinositide3-kinase (PI3K)/Akt and Raf/Mek/Erk [35, 36]. Jak/Stat independent PRLR signaling can also be mediated through the Src kinases and focal adhesion kinases (FAK) [37, 38]. In the mammary gland, PRL plays a decisive role in epithelial cell proliferation and milk production [39]. The generation of PRL [39] and PRLR [40] gene knockout (KO) mice demonstrated that PRL and PRLR pathways are key regulators in mammary gland development (discussed above). In addition to its essential function in mammary gland biology, PRL has reproductive, metabolic, osmoregulatory, and immunoregulatory actions in diverse tissues [32].

The importance of PRL in breast cancer development and risk is less well-defined as compared to ovarian steroids. Similar to estrogen and progesterone, high PRL levels can augment mammary tumor development in mice [41, 42] and in women, elevated PRL levels are correlated with increased breast cancer risk and metastasis [43, 44]. *In vitro* studies have

indicated a role for PRL in breast cancer proliferation and survival [45–49]. PRL also appears to significantly enhance the directed motility of breast cancer cells [47], in part through its activation of downstream effectors such as the NEK3 kinase, leading to cytoskeletal and focal adhesion reorganization [50, 51]. Interestingly, PRL and nuclear PRLR can enhance the expression of the estrogen (ERα) and progesterone (PR) receptors [52–54]. In contrast, studies have also shown that activation of PRLR can suppress the mesenchymal phenotype and reduce invasive behavior [55]. Loss of PRLR expression in breast cancer can be associated with poor differentiation and larger tumors [56] whereas the gene expression signatures of an activated PRL/PRLR pathway are associated with well-differentiated tumors, reduced metastasis and higher overall survival [57]. Such results indicate that PRL may have both a pro-oncogenic as well as a metastasis suppressor role in breast cancer [55]. They also support the idea of cross-talk between the actions of PRL and the steroid hormones [52].

Signaling interactions between progesterone, prolactin and STATs

As mentioned above, major downstream effectors of PR and PRLR signaling include the STAT (signal transducer and activator of transcription) family of latent cytoplasmic proteins. All members of this family possess similar protein structures including a DNA binding domain, SH2/SH3 domains and a C-terminal transactivating domain which confer functional properties [58]. Activation of STAT proteins results in phosphorylation on tyrosine and serine residues via signaling from upstream regulators like Janus Associated Kinases (JAKs; [58]) and Mitogen Activated Protein Kinases (MAPKs; [59, 60]). Tyrosine phosphorylation induces dimerization between two STAT molecules via their SH2 domains. Activated STAT dimers then translocate to the nucleus and bind to consensus promoter sequences to initiate transcription of their specific target genes.

The STAT proteins STAT1, STAT3, and STAT5 are involved in all stages of mammary gland development [61]. Genetic deletion experiments suggest that these proteins are most important in postnatal development where distinct expression and phosphorylation events are observed during gestation, lactation and involution. STAT1 has the most unique pattern of phosphorylation; highest in virgin mice and in late involuting glands. STAT1 may have a compensatory role, working in concert with STAT3, wherein STAT3 activation is essential for the regulation of cell death and inflammatory signaling during involution [62, 63]. The STAT5 protein isoforms, encoded by the genes *STAT5A* and *STAT5B*, are necessary and sufficient for alveologenesis and expression of milk protein genes during late pregnancy and lactation [64–66]. Both progesterone and PRL are important regulators of STAT proteins (Fig. 1). Activation of STAT5 through the PRLR-Jak2 pathway is critical for its functions [65, 67, 68]. Progesterone signaling activates JAK2 and increases STAT1, 3 and 5 at both the mRNA and protein level [69, 70]. PRL transcriptionally activates STAT 1, 3 and 5 (a and b) and progesterone enhances prolactin-mediated stimulation of STAT5 activation in part via amplification of convergent signaling pathway inputs to STAT phosphorylation [69, 70].

The functions of specific STAT proteins have important implications for their potential role in breast cancer progression. For example, STAT1 is believed to be a tumor suppressor that is often lost in ER+ tumors [61] although STAT1 knock-out mice spontaneously develop ER

+ luminal mammary tumors [71]. Activation of STAT1, presumably through interferon signaling, inhibits tumor growth via upregulation of p27 [72] and via interaction with the DNA damage machinery p53 [73] and BRCA1 [74]. High levels of pSTAT1 in ER+ breast cancers from postmenopausal women are associated with greater disease-free survival [75, 76]. This suppressive role may be dependent on menopausal status since some clinical studies have observed poorer overall and disease-free survival in premenopausal women with elevated STAT1 [76, 77].

The STAT3 protein has also been found to be constitutively activated in breast cancers, especially in triple negative (ER-/PR-/HER2-) breast cancer [78], and is associated with invasive behavior and poor prognosis [79]. *In vitro* studies indicate that pSTAT3 induces the expression of genes that promote proliferation, survival, angiogenesis and stemness [80, 81]. Notably, in luminal breast cancer models, signaling via PR also drives similar functions of STAT3. For example, progestins activate STAT3 signaling and translocation to the nucleus [82]. Similar to earlier studies of PR cross talk with STAT5 [69, 70], progestin signaling was shown to induce expression of STAT3 and activate STAT3, Jak1, Jak2 and c-SRC. PR/STAT3 interaction drives expression of classical PR target genes (bcl-X) [83] and progestin-induced activation of JAK/STAT3 was required for progestin regulated growth of breast cancer cells and tumors *in vivo*. PR and STAT-containing complexes extensively cooperate with nuclear ErbB-2 receptors. An eloquent series of studies demonstrated that progestins activate an “enhanceosome” containing PR, AP1, STAT3 and ErbB-2 that translocates to the nucleus. Once there, this complex binds to DNA and induces expression of cyclin D1 to drive breast cancer cell growth *in vitro* and *in vivo* [84, 85]. In the presence of progestins, ErbB-2/PR/STAT3 transcriptional complexes also drive cell cycle progression in breast cancer cells by interaction with SP1 at Sp1 sites in the p21 promoter [83, 84].

The role of STAT5 in breast cancer is complex. Phosphorylation and nuclear localization of STAT5 in breast cancer is a positive predictor of response to endocrine therapy and patients with more activated STAT5 have decreased risk of disease recurrence and death [86]. However, STAT5 also appears to have a role in tumor formation, as TGF-in mice is delayed in the absence of the STAT5a protein [87]. This effect is also observed in an SV40-T model of mouse mammary cancer [88]. Additionally, over-expression of WT and the constitutively active form of STAT5a induced mammary tumors in mice [89] and this effect is dependent upon parity, as virgin mice do not develop tumors [90]. Interestingly, this is in contrast to the overall protective effect of parity on breast cancer prevention normally observed in mice and young (age <20) women [91]. The lack of an effect of activated STAT5a expression in virgin mice relative to parous animals may reflect the extensive cooperation between STAT5a and PR-B isoforms relative to PR-A isoforms [70, 92] (i.e. PR-A but not PR-B is primarily expressed in virgin mice). Additionally, this change is likely mediated by the increased number of luminal progenitor cells in the fully developed mammary gland due to increased PR and STAT5 signaling [93]. Similar to STAT3 (discussed above), STAT5 and PR interact and STAT5a is found with PR at PRE sites [69, 92]. PR-B but not PR-A enables STAT5a signaling. Phosphorylation of PR-B at serine 81 (a CK2 consensus site absent from PR-A) induces expression of STAT5a and in a feed forward signaling loop, STAT5a activation cooperates with pS81 PR-B to drive expression of a specific gene set co-regulated by STATs, including WNT1, thus linking this CK2-dependent PR-B signaling program to PRL/PRLR

signaling [92]. Mutation of PR-B Ser81 to Ala confers PR-Alike behavior by preventing expression of numerous pS81 PR-B target genes that require STAT5a (GAS sites are significantly located near PRE sites in numerous PR-B target genes) for their increased expression [94].

Modulation of mammographic breast density via PR/PRLR signaling

In addition to hormone exposure as a major correlate of breast cancer risk, women in the highest quartile of mammographic breast density have a 1.8–6 fold increase in breast cancer risk [95, 96]. Mammographic density (MD) refers to the fibroglandular tissue of the mammary gland, composed of fibrous stroma and the epithelial cells that line the ducts. This is in contrast to the other main component of the gland, the adipose tissue. The fibroglandular tissue appears white or bright on a mammogram, whereas the adipose tissue appears dark. A higher percent MD reflects increased collagen, stroma and epithelial cells and a relative decrease in adipose cells, compared to the total breast tissue volume.

Hormone replacement therapy (HRT) and hormone therapy for breast cancer prevention and treatment can influence mammographic breast density [97, 98]. Notably, HRT that includes both estrogen and a progestin, more than estrogen alone, was associated with increased MD and a concurrent increased risk of breast cancer in both the Women's Health Initiative (WHI) and Postmenopausal Estrogen/Progestin Intervention (PEPI) studies [99–101]. Discontinuation of HRT was associated with decreased MD [102, 103]. Similarly, endogenous hormone levels (namely, progesterone and prolactin) were reported to be associated with increased mammographic breast density [104–106]. Additionally, increased PR expression was observed in the mammary epithelial cells of women with increased MD relative to women whose MD was in the normal range [107]. Treatment with tamoxifen can cause a decrease in MD and a lower risk of breast cancer recurrence, possibly due to the loss of PR expression as an ER target gene and key downstream effector of estrogen signaling [108, 109]. Taken together, these studies suggest an important role for both PR and PRL signaling as mediators of increased MD, a reversible condition that is tightly linked to hormone action and breast cancer risk.

The mechanistic role of increased mammographic density and hormone signaling in breast cancer development have been investigated in *in vivo* and *in vitro* studies. Mice with defective collagen breakdown (ie. PyV MT. Col1a1tmJae) in the mammary gland have increased collagen density and three times the risk of tumor formation and metastasis [110]. *In vitro* modeling of mammographic density using differing concentrations of rat tail collagen I in free floating gels has been used to study the mechanisms contributing to increased tumorigenesis [110, 111]. Enhanced activation and use of distinct effector pathways downstream of PRL signaling was observed in breast cancer cell models in the presence or absence of stiff matrices [112]. For example, PRL signaling in a compliant matrix activated STAT5. However, if the same cells were instead maintained in a stiff matrix, PRL preferentially activated Src/FAK and ERK1/2 signaling. Interestingly, PRL signaling to STAT5 was inverse to its ability to activate AP1, a less well-studied PRL-induced pathway. Consistent with this finding, signaling through STAT5 reduced PRL signals to the

transcription factor, AP-1; a protein complex made up of MAPK substrates (i.e. Fos/Jun) which is also known to interact with and mediate both ER and PR signaling [113].

Prolactin has also been implicated in cell migration in the context of varying matrix microenvironments (Fig. 2A). In stiff matrices, PRL activates the expression of MMPs including MMP9 in T47D ATCC cells that express PRLR, ER and PR [112]. MMP9 is a type IV collagenase that contributes to tumor invasiveness *in vitro* by degradation of basement membrane proteins, and elevated MMP9 expression is associated with breast cancer tumor invasiveness and metastasis [114–116]. Other studies have shown that MMP9 levels are inversely correlated with pSTAT5 levels [117]. In breast cancer cells, we discovered that PRL-induced expression of MMP9 is dependent on co-expression of PR-B. Notably, when T47D ATTC cells expressing PR-B were grown in high density collagen (HD; stiff matrix) relative to low density collagen (LD; compliant matrix), PRL treatment induced increased MMP9 expression (Fig. 2B; left). In contrast, T47D ATTC cells naturally lacking PR expression (T47D Y cells) failed to induce MMP9 expression upon exposure to PRL in stiff matrix (Fig. 2B; right). This finding suggests that PR-B (but not progesterone) is required to enable an alternative signaling pathway specifically activated by PRL in stiff matrices, such as ERK1/2 MAPKs [112]. Notably, HB-EGF is a PR-B target gene product whose progesterone-induced expression requires rapid activation of ERK1/2 MAPKs [118]. In the presence of the synthetic progestin, R5020, T47D ATCC cells cultured in LD compliant matrix showed increased expression of HB-EGF mRNA levels relative to vehicle treated controls. However, progestin-induced HB-EGF expression was further increased by exposure to a stiff matrix (Fig. 2C), consistent with the concept that ERK1/2 MAPK signaling is elevated in the context of HD stiff matrix [110]. These data illustrate a cooperative role for progesterone and prolactin in pro-tumorigenic signaling relevant to altered hormone action in a model of mammographically dense breast tissue. Understanding the details of cross-talk between these two signaling pathways may provide further insight into the role of hormonal regulation in the context of increased MD, thereby opening new avenues of research into a potential means of blocking the relevant interactions and signaling pathways in order to prevent breast cancer development in high risk women.

Conclusions

Herein we have focused on PRLR and PR as inter-dependent signaling pathways relevant to both mammary gland development and breast cancer biology, whose downstream biological effects can be modulated by major determinants of increased breast cancer risk (i.e. lifetime hormone exposure, mammographic breast density, etc.). Both PR and STAT5a are key transcription factors in these pathways and have been shown to be mediators of breast cancer stem cell outgrowth [119]. This evidence, coupled with their established function in the same transcriptional complexes at phospho-PR-target genes with high cancer relevance, supports the importance of this PR-PRLR cross-talk [5]. Targeting this cooperative PRLR and PR signaling (i.e. at the level of STATs or as part of combination therapies) may provide an effective means of blocking early tumor progression by limiting the breast cancer stem cell compartment. Additionally, a deeper understanding of the details of cross-talk between these mediators and the modulation of this by matrix interactions will be critical to the

development of breast cancer prevention strategies for high risk women, such as BRCA1/2 mutation carriers, where mammographic breast density is a relevant risk factor.

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Highlights

- PR and PRLR signaling cooperate in breast cancer
- PR/PRLR signaling may play a role in modulating mammographic breast density.
- PR/PRLR pathways could offer alternative targets for breast cancer prevention.

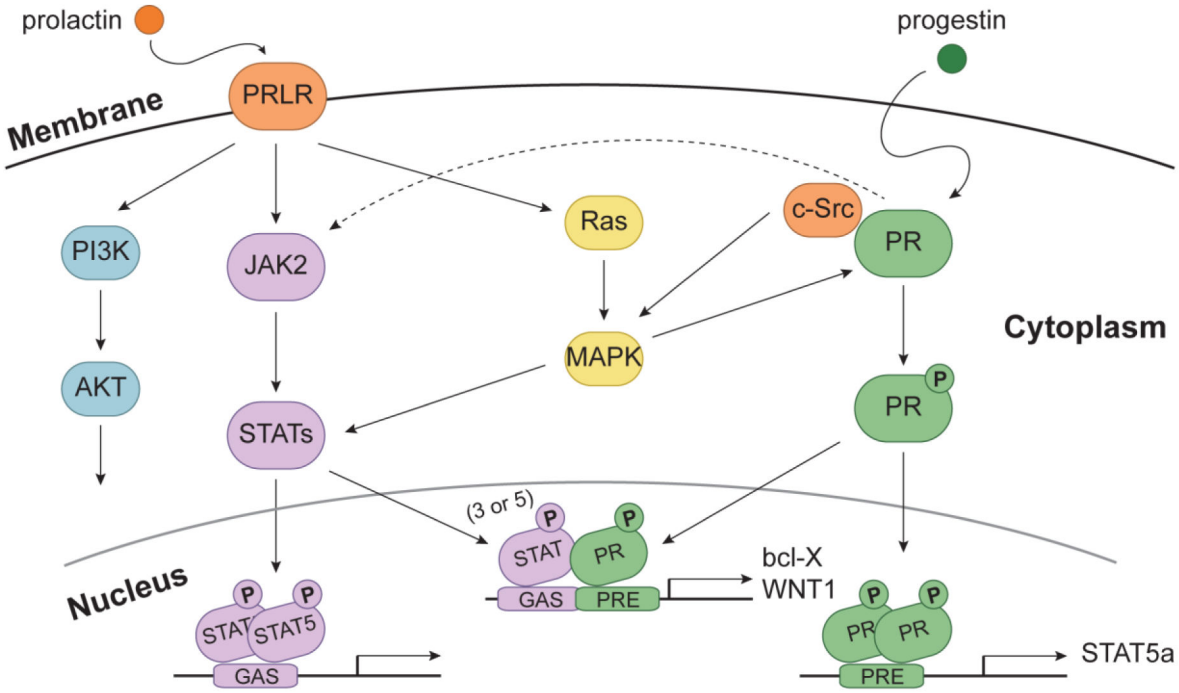

Figure 1. Convergence of PR and Prolactin Receptor (PRLR) Signaling Interactions

Prolactin and progesterone are important regulators of STAT proteins. Prolactin-induced activation of PRLR triggers several signaling cascades. For example, signaling to JAK2 kinase activates STAT5. Activation of the MAPK pathway can also lead to activation of STATs through PRLR. Progestin-induced activation of PR leads to activation of PR genes that include the downstream components of the PRLR signaling pathway (e.g. STAT5a). In addition, progesterone enhances prolactin-mediated stimulation of STAT activation in part through the JAK2 and MAPK pathways. Progesterone-induced activation of c-Src also provides a hormone-induced input to PRLR (i.e. via STAT phosphorylation) signaling.

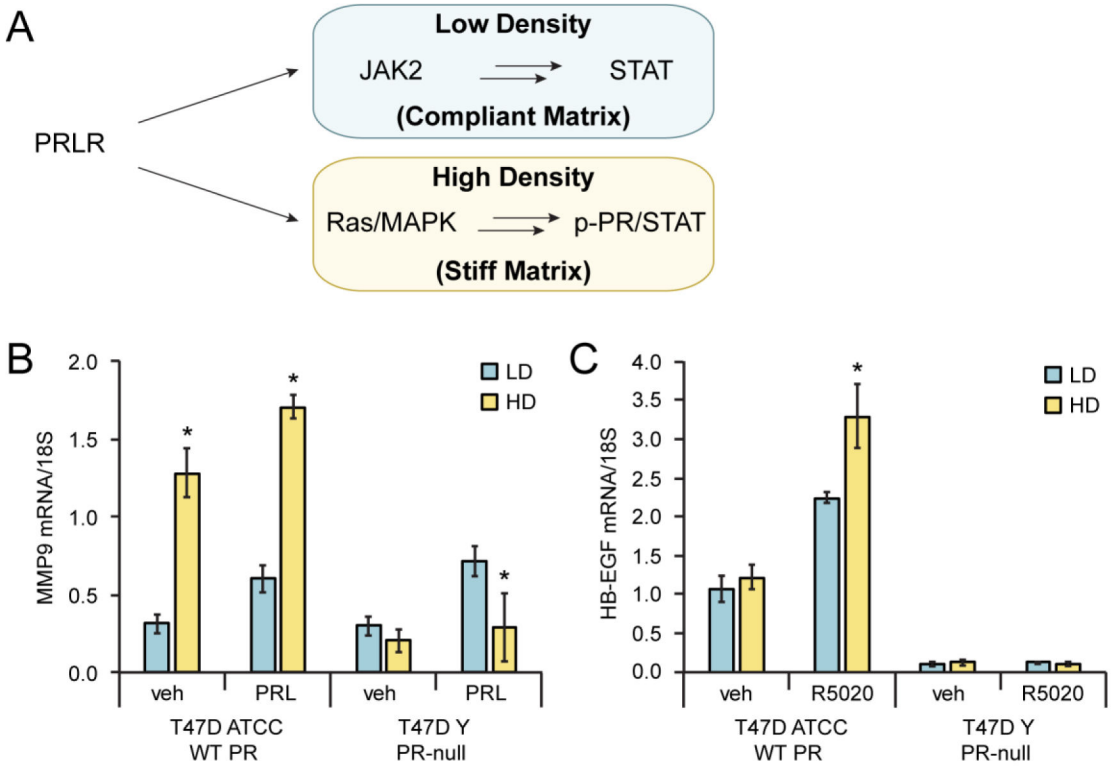

Figure 2. Progestin- and prolactin-induced signaling is dependent on PR expression and matrix microenvironment

(**A**) PRLR signaling favors JAK2/STAT5 signaling in low density (compliant matrix), whereas high density (stiff matrix) enhances PR/STAT mediated signaling through c-SRC and MAPK pathways [112]. mRNA expression levels of matrix metalloproteinase-9 (**B**; MMP-9) and heparin-binding epidermal growth factor-like (**C;** HB-EGF) are pictured. T47D ATCC (WT; PR expressing) and T47D-Y (PR-null) cells were grown in MEM supplemented with 5% FBS, Pen/Strep, NEAA, and insulin (6 ng/ml). Cells were plated in low density (LD; 1.2 mg/ml) or high density (HD; 2.8 mg/ml) type I rat tail collagen as previously described [110]. Collagen gels were released from wells 24h after plating and serum-starved for an additional 24h. Cells were then treated with vehicle (veh), R5020 (10 nM) or PRL (8 nM) for 24h. RNA was extracted from collagen gels using Trizol and qRT-PCR analysis was performed to determine mRNA expression of MMP-9 and HB-EGF. Data shown is representative of three individual experiments (performed in triplicate).