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Modeling Prolactin Actions in Breast Cancer in vivo: Insights from the NRL-PRL Mouse

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Abstract

Elevated exposure to prolactin is epidemiologically associated with an increased risk of aggressive ER+ breast cancer. To understand the underlying mechanisms and crosstalk with other oncogenic factors, we developed the NRL-PRL mouse. In this model, mammary expression of a rat prolactin transgene raises local exposure to prolactin without altering estrous cycling. Nulliparous females develop metastatic, histotypically diverse mammary carcinomas independent from ovarian steroids, and most are ER+. These characteristics resemble the human clinical disease, facilitating study of tumorigenesis, and identification of novel preventive and therapeutic approaches.

9.1 Prolactin and Breast Cancer

Prolactin (PRL) plays critical roles in the proliferation and differentiation of lobuloalveoli during pregnancy (for review, [1]). Its ability to drive growth and differentiation in concert with ovarian hormones and other established players in breast cancer suggests that PRL, too, contributes to this disease. However, this idea remained controversial for some time, in part because of conflicting data from small epidemiologic studies, failure to appreciate the multiple agonists for the PRL receptor (PRLR) and the extensive PRL expression in extrapituitary sites in humans compared to experimental models. However, a myriad of studies over the last decade now support the importance of PRL in breast cancer.

Two lines of evidence link PRL action to the development of breast cancer, which are discussed in recent excellent reviews [2-4]. First, in a large prospective epidemiologic study nested within the Nurse's Health Study, elevated circulating PRL levels were found to significantly raise the risk of cancers that express estrogen receptor alpha (ER α), independent of circulating estrogen levels [2]. A recent follow-up study found that elevated circulating PRL ten years prior to diagnosis was associated with risk for metastatic ER+ disease [5]. Second, recent studies have identified polymorphisms in the *PRLR* and *PRL* genes associated with breast disease [6-8].

A link between PRL action (elevated circulating PRL or high expression of PRLR in tumors) and tumor progression, metastasis and/or therapeutic resistance has also been observed, although the supporting epidemiologic evidence is more sparse than that

correlating PRL and tumorigenesis (for review, [2]). PRLR is expressed in the vast majority of breast cancers, including both ER α + and ER α - tumors [9], and PRLR mRNA is higher in tumors than adjacent normal tissue [10]. In seeming contrast to those reports, however, activation of STAT5A, the best understood mediator of PRL signals in normal mammary physiology, is associated with a better prognosis [11,12]. However, PRL initiates activation of multiple other signaling cascades (Fig. 9.1; for reviews, [13-15]), which may in part account for the apparent disparity in these observations (see below). Data from many *in vitro* studies also demonstrate that PRL can drive processes underlying tumor progression. PRL increases proliferation and survival (for review, [15]), and increases chemotherapeutic resistance of breast cancer cells in culture [16,17]. However, similar to the clinical findings, PRL-activated STAT5A promotes differentiation in breast cancer cells *in vitro* [18,19]. Interestingly, PRL signals to STAT5 are inversely related to its ability activate an AP-1 enhancer in a panel of breast cancer cell lines [20], and the stiffness of the matrix in 3D cultures inversely modulates PRL-activated STAT5 and ERK1/2 [21]. Together, these observations suggest that features of both the cancer cell itself and the surrounding microenvironment can alter the signaling repertoire of PRL, which may have profound effects on the biological outcome.

The growing strength of the epidemiologic data suggests preventive and therapeutic opportunities directed at the actions of PRL in breast cancer. However, in order to exploit the clues from the clinical data and studies in individual breast cancer cell lines, we need to understand the signals and gene targets of PRL in diverse mammary cell contexts, and the interplay of its actions and the microenvironment in breast pathology over time. Genetically modified mouse models enable examination of the dynamic processes of tumor development and progression and interactions among oncogenic players. They permit investigation of the underlying mechanisms which can reveal potential targeted strategies, and evaluation of the likelihood of their success. Here we describe the mammary pathology that develops in the NRL-PRL transgenic mouse. This model recapitulates many features of clinical breast cancer, especially aggressive ER α + /PR- cancers, which account for the majority of breast cancer deaths [22].

9.2 Mammary Prolactin Synthesis

Expression of the PRL gene in pituitary lactotrophs is driven from a proximal promoter that is conserved across mammals (for review, [23]). In humans, transcription of the PRL gene can also be driven from a second upstream promoter that drives expression in many tissues apart from the pituitary, including the mammary gland [24-27]. Local PRL expression is elevated in many breast cancers [28,29]. Although incompletely understood, the potential for different regulation conferred by usage of the distal promoter [25,30] would explain the independence of expression in nonpituitary tissues from dopamine, and subsequent insensitivity of these breast cancers to treatment with dopamine agonists [31].

Despite the restrictions imposed by a single promoter, rodents also express detectable PRL in their mammary glands, particularly during late pregnancy, as well as in mammary tumors [32-34]. Indeed, local PRL is necessary for epithelial differentiation and milk synthesis in mice [34,35]. This requirement for endogenous PRL at the end of pregnancy (also discussed

in [36]) suggests the intriguing possibility that mammary PRL initiates distinct signals. Interestingly, MCF7 breast cancer cells also responded differently to inducibly overexpressed PRL than to exogenous PRL [37]. Whether these observations simply reflect locally elevated ligand concentrations, or preferential signals initiated by autocrine action, remains to be determined.

Regardless, locally produced PRL also would add to the mammary milieu. In combination with the other lactogenic hormones (placental lactogens, primate growth hormone) [38,39], it is clear that examination of circulating PRL alone underestimates the exposure of mammary tissue to PRLR agonists.

9.3 Mouse Models of Elevated Prolactin

Rodents have been used extensively to model PRL actions in breast cancer. Pregnancy and pseudopregnancy elevate PRL activity in concert with the other hormonal changes of pregnancy [38]. A rich literature describes use of pituitaries transplanted under the kidney capsule to raise circulating PRL for prolonged periods by protection from inhibitory hypothalamic dopamine (for reviews, [14,40-42]). Like the hormonally complex physiologic states, the latter technique also raises ovarian steroids, particularly progesterone [41,42], via effects of the high circulating PRL on corpus lutea [43]. While PRL and progesterone physiologically cooperate in mammary function [1,44] and may also crosstalk in breast cancer [45], manipulations that increase circulating PRL levels preclude study of actions of the individual hormones.

More recently, transgenic PRL has been employed to augment PRL exposure, as we have previously reviewed [46]. In addition to widespread epithelial PRL expression driven by the metallothionein promoter [47], local mammary PRL concentrations have been raised by mammary selective promoters. These include the whey acidic protein promoter [48] and the rat neu-related lipocalin (NRL) promoter ([49], described in detail below). These transgenic models overexpress the secreted PRL ligand, revealing the outcome of direct and indirect actions of PRL on the many different mammary cell types that may contribute to breast disease, including not only epithelial subpopulations, but also immune cells, endothelial cells and fibroblasts, and other stromal cells.

Interestingly, genetic ablation of Stat1 also results in ER α + tumors in multiparous 129S6/SvEV and Balb/c mice [50,51]. Like the secreted PRL transgene in the NRL-PRL model, germline silencing of Stat1 reveals the outcome of potential effects on multiple cell types. Recent mechanistic studies have demonstrated that failure to downregulate PRLR in mammary epithelial cells (MECs) via *Socs1* is a contributor to the mammary pathology [51]. Stat1 and *Socs1* modulate signaling of multiple cytokines in addition to PRL, which are produced by the mammary stroma as well as MECs (for reviews, [50,52]). Stat1 can act as a tumor suppressor by promoting cytotoxic T lymphocyte activity [50], and SOCS1 modulates inflammation, thereby altering tumorigenesis and therapeutic responses (for review, [53]). Nonetheless, the well-differentiated nonmetastatic ER+/PR+ mammary tumors that develop in *Stat1*^{-/-} 129S6/SvEV multiparous females provide an interesting

contrast to the aggressive ER+/PR- carcinomas that develop in NRL-PRL females (Table 9.1 and discussed further below).

9.4 NRL-PRL Model

9.4.1 Model

In the NRL-PRL model, transgene expression is driven by a 3015bp fragment of the rat NRL proximal promoter that confers selective expression in mammary epithelia beginning at a young age [49,54]. Transplanted transgenic MECs to a cycling syngeneic female develop adenocarcinomas [46], confirming that PRL expression by these cells is sufficient to drive mammary tumorigenesis. The identity of the MEC subpopulation targeted by the NRL promoter is not well defined. However, as noted above, the secreted transgenic PRL exposes multiple mammary cell types, of which many may contribute to tumorigenesis. This promoter fragment is not sensitive to estrogen or PRL [49], but unlike the endogenous gene [55], it is highly responsive to progesterone [54].

Two independently derived NRL-PRL lines in the FVB/N strain background have been extensively characterized [TgN(Nrl-Prl)23EPS, NRL-PRL 1655-8 and TgN(Nrl-Prl)24EPS, NRL-PRL 1647-13]. The NRL-PRL 1655-8 line exhibits modestly elevated circulating lactogenic activity, while the NRL-PRL 1647-13 line has normal levels [49]. Females of both lines cycle similarly to nontransgenic controls, indicating that effects on the corpus luteum are not a component of the subsequent pathology. Although some differences in early pathology were observed [49], both lines develop similar histologic spectra of carcinomas with comparable latency. To reduce the likelihood that transgenic PRL influences the mammary gland via secondary effects on another tissue, many of our studies have focused on NRL-PRL 1647-13.

9.4.2 Early pathology

Ductal and alveolar epithelia of nulliparous NRL-PRL females exhibit increased proliferation and slightly increased apoptosis [49]. The increased turnover of morphologically normal mammary epithelium *in vivo* contrasts with PRL-augmented survival of human breast cancer cell lines *in vitro* (reviewed in [15]). Mammary pathology is detectable at 12 weeks of age. We observe focal dilated ducts with irregular epithelium, and later mammary intraepithelial neoplasms (MINs), resembling ductal carcinoma in situ (DCIS). Diffuse alveolar development is evident early in some females, and with age, all nulliparous females show extensive epithelial hyperplasias [49]. In aged females, the myoepithelial layer, associated with tumor suppressive properties, is markedly reduced in the presence of transgenic PRL [56]. Generally about 70% of transgenic females develop single carcinomas after a long latency (mean, about 17 months) [49,57].

An essential role for PRL signals mediated by Jak2 in tumorigenesis was demonstrated by MMTV-Cre-mediated conditional deletion of the *Jak2* gene in the NRL-PRL model [58]. However, the scope of the downstream effectors is unclear. Stat5 isoforms are likely to be one set of mediators, consistent with the importance of the Jak2-Stat5a pathway in the physiologic actions of PRL (for reviews, [1,59]), and the modest but significant increase in the number of cells with nuclear pStat5 in epithelial hyperplasias of aged NRL-PRL females

[56]. Interestingly, however, mammary glands of 12 week old NRL-PRL females displayed dramatic increases in Thr202/Tyr204-pErk1/2 and Ser473-pAkt, compared to age-matched nontransgenic controls [56], suggesting that other signaling cascades contribute to PRL-induced oncogenesis. PRL can also drive signaling pathways via src family kinases (SFK), [60,61], which may be important in the observed pathology.

Jak2 is also critical for mammary cancer in *Stat1*^{-/-} mice [51]. With the caveat that this model may reflect the actions of multiple cytokines in addition to PRL, these data support a role for Jak2/ Stat3/ Stat5 in ER+ carcinogenesis.

The cell cycle regulator, cyclin D1, is the major D cyclin in the mammary gland and is overexpressed in many breast cancers (for review, [62]). This D cyclin is a mediator of the PRL-driven alveolar proliferation that occurs during pregnancy [63]. In order to understand its role in the pathogenesis induced by PRL, we compared nulliparous NRL-PRL females with wildtype *Ccdn1* to those with genetic ablation of this gene [64]. Although the absence of cyclin D1 dramatically reduced PRL-stimulated epithelial proliferation and the incidence of PRL-induced carcinomas, PRL none-the-less was able to augment epithelial proliferation to levels in females with wildtype cyclin D1 and induce epithelial hyperplasias and MINs. A partial compensatory mechanism was suggested by the increased nuclear cyclin D3 in luminal epithelia in NRL-PRL/D1^{-/-} glands in the absence of effects on mRNA levels, indicating that PRL may stabilize cyclin D3 post-translationally by mechanisms similar to those described for cyclin D1 [64,65]. Cyclin D3 is high in some clinical cancers, frequently associated with elevated cyclin D1 [66-68]. Our data suggest that PRL is able to employ both D cyclins to augment MEC proliferation, in concert with its effects on other cell cycle regulators [69].

The MEC subpopulation(s) that gives rise to PRL-induced tumors and the target cells of PRL that contribute to this outcome are not known. Despite the clear role for PRL in physiologic alveologenesis but not ductal development, transgenic PRL increases ductal as well as alveolar proliferation and results in preneoplastic lesions attributable to both populations [49]. Transgenic PRL in the presence of ovarian steroids does not alter the ratio of luminal to basal MECs (O'Leary, unpublished observations). However, although PRLR mRNA is restricted to luminal MECs, transgenic PRL alters gene expression in both subpopulations, indicating paracrine effectors. Ongoing studies will dissect the crosstalk of PRL with ovarian steroids on progenitor activity, permitted by the NRL-PRL model. Progesterone is of particular interest, since it augments stem cell activity [70,71]. These studies will illuminate the direct and indirect actions of PRL that contribute to the development of ERα+ breast cancer, and also the ERα- carcinomas that develop in the NRL-PRL model [49,57,72]. In addition, they will shed light on the roles of established lineage regulators in PRL activity, such as Stat5a, Gata3 and Elf5 (for review, [73]). In light of the emerging significance of Elf5 as a suppressor of ER expression and antiestrogen sensitivity in luminal cancers [74] and suppressor of the epithelial-mesenchymal transition [75], understanding these networks is particularly important.

9.4.3 Carcinomas

Nulliparous females of both NRL-PRL transgenic lines develop aggressive, histologically diverse carcinomas with a range of latency from 14-21 months. These carcinomas aggressively invade nearby tissue, and metastasize to local lymph nodes and the lungs. They demonstrate wide histological diversity, including adenocarcinomas of several subtypes, adenosquamous carcinomas, and spindle-cell carcinomas. The majority are ER α + (defined as more than 10% positive cells). However, negligible progesterone receptor (PR) expression is detectable in most tumors. In contrast to the responsiveness of morphologically normal MECs in NRL-PRL females to estrogen [76] and the ability of supplemental 17 β -estradiol to reduce tumor latency [56], growth of established carcinomas is not sensitive to estrogen despite continued ER α expression [57]. Although tumor pStat5 levels were not correlated with ER expression [57], and ablating Jak2 did not reduce ER in established tumors [58], reduction of Stat5 expression in cell lines established from PRL-induced ER+ tumors lowers ER α expression (Brockman and Schuler, unpublished observations), suggesting that PRL is one contributor to ER expression. The aggression and lack of estrogen responsiveness of the ER+ carcinomas in NRL-PRL mice resembles clinical luminal B breast cancers [77-79]. Since few mouse models develop ER α + tumors (for reviews, [80-82]), the characteristics of the NRL-PRL tumors suggest that they are useful models of aggressive ER α + clinical disease.

Consistent with their histotypic diversity, PRL-induced carcinomas display disparate patterns of phosphorylated signaling mediators [57]. Well differentiated adenocarcinomas exhibited highest levels of pStat5, a key physiological mediator of PRL, and also lower MMP-9 mRNA. This models the correlation between high STAT5 activation and better clinical outcomes [11,83]. Many adenocarcinomas also exhibit high levels of pStat3 ([58], Arendt and Schuler, unpublished observations). Despite the requirement for Jak2 in tumor development, tumor transplantation was not dependent on Jak2, and although activation of Stat5 was dependent on Jak2, Stat3 was constitutively phosphorylated [58]. PRL also activates many MAP kinases, which may alter cell behavior [84]. The distribution of pErk1/2 differed with histotype. Stromal cells within adenocarcinomas displayed high levels of pErk1/2, whereas spindle-cell carcinomas exhibited nuclear and cytoplasmic staining of tumor epithelium [57]. One potential downstream target of Erk1/2 is the transcriptional enhancer, AP-1. AP-1 proteins, including c-Fos and c-Jun, are expressed in PRL-induced tumors at highest levels in tumors with lowest pStat5, consistent with the inverse activity of these pathways observed in breast cancer cells *in vitro* [20]. Ongoing studies are exploring the role of other upstream kinases, including SFK, in PRL-driven tumor progression. All NRL-PRL tumor histotypes displayed varying levels of pAkt within the tumor epithelium; the phosphatidylinositol 3-kinase pathway is dysregulated in many clinical cancers [85].

PRL-induced ER α + adenocarcinomas differ from many commonly used mouse models of breast cancer in that they are composed of heterogeneous populations of epithelial cells. These tumors display both luminal and basal epithelial subpopulations when analyzed by flow cytometry using antibodies to EPCAM and CD49f (Shea and Schuler, ms in prep). In contrast, luminal but ER- mammary carcinomas from MMTV-Her2/neu mice consist of a single population of luminal cells [86], and claudin-low carcinomas that develop from *p53*^{-/-}

mammary cells are composed of a single basal cellular subpopulation [87]. Interestingly, both subpopulations within NRL-PRL carcinomas are capable of regenerating heterogeneous tumors of the same histotype and ER α expression following transplantation, indicating tumor initiating cells (TICs) in both subpopulations. The biologic and therapeutic consequences of this heterogeneity, and characteristics of the TICs are currently under investigation. Since some PRL-induced carcinomas are ER-, similar studies will provide insight into the origin and progression of these tumors, and differences from the ER α + adenocarcinomas.

Increased PRL signals also contribute to the mammary pathology induced by genetic ablation of Stat1, yet the appearance and behavior of these tumors is substantially more benign than the carcinomas of the NRL-PRL mouse [51,88] (Table 9.1). In contrast to NRL-PRL tumors, these adenocarcinomas are generally ER+/PR+, and metastases have not been reported. Moreover, the majority are initially dependent on ovarian hormones, although this is later lost in a subset of tumors [51]. Interestingly, in contrast to NRL-PRL ER α + adenocarcinomas, tumors that develop in *Stat1*^{-/-} mice exhibit a single luminal MEC population as determined by flow cytometry. Unlike carcinomas in NRL-PRL animals, tumors in *Stat1*^{-/-} females are highly dependent on Jak2, and display high levels of Jak2-dependent pStat5 and pStat3 [51]. Comparison of the regulation of tumor behavior in the *Stat1*^{-/-} and NRL-PRL models using similar perturbations and endpoints will illuminate the underlying differences, and may reveal key regulators of the more malignant processes in NRL-PRL tumors. Such findings likely would have important implications for aggressive ER α + clinical breast cancers.

9.4.4 Crosstalk with estrogen, ER α

Similar to the clinical epidemiologic studies [2], both the incidence and latency of PRL-induced tumors are independent of postpubertal ovarian steroids [56]. However, supplemental 17 β -estradiol accelerates tumor formation in ovariectomized animals. This cooperation between transgenic PRL and estrogen is demonstrated in the proliferation of morphologically normal mammary epithelium [76], and resembles the observed interaction of these hormones in breast cancer cells *in vitro* [89-93].

Multiple mechanisms of estrogen/ PRL crosstalk have been shown in studies of breast cancer cell lines *in vitro*. PRL and estrogen can increase expression of the other's receptor [37,94]. PRL and estrogen can also cooperatively activate ER α -mediated transcriptional signals, such as activation of AP-1 enhancers [89]. Further, STAT5, one mediator of PRL actions, has been observed to positively or negatively impact estrogen-induced signals, depending on the experimental system [95-98]. Recent reports have begun to elucidate the outcomes of PRL-estrogen crosstalk on target genes in various breast cancer cell lines [91,92].

PRL also activates ER α in the absence of estrogen ligand in the mammary glands of the NRL-PRL mouse, demonstrating that this mechanism, which is believed to contribute to anti-estrogen resistant breast cancer [99], is active *in vivo* [100]. Although PRL initiates phosphorylation of ER α in the AF-1 domain (Ser118, Ser167) [101] and induces recruitment of ER α to target genes [102] in breast cancer cells, the ability of this mechanism to exert

physiologically significant effects *in vivo* has not been clear. In order to examine this directly, we utilized the ER α (G525L) mouse, which expresses a mutant ER α that is refractory to 17 β -estradiol, but can be activated by kinases downstream of growth factors and cytokines [103]. NRL-PRL females with the ER α (G525L) mutation displayed ER-dependent ductal elongation and gene expression at puberty [100], confirming the ability of PRL to act via this mechanism. Moreover, ligand independent activation of ER α also mediates a significant portion of the crosstalk between PRL and the EGFR ligand, TGF α ([100], see section 9.4.5 below).

Together, these studies underscore the multiple mechanisms of cooperation between PRL and estrogen in carcinogenesis. Interestingly, however, established carcinomas in NRL-PRL females do not respond to estrogen [57], resembling antiestrogen resistant, luminal B clinical tumors, as discussed further below.

9.4.5 Crosstalk with growth factors

Advancing lesions can coopt other agents, such as local growth factors, to promote progression. Indeed, PRL-induced tumors express varying levels of erbB family members, including erbB2 [58]. In breast cancer cells *in vitro*, PRL strongly cooperates with EGF, TGF α , and IGF-1, prolonging phosphorylation of ERK1/2 and AKT and altering ligand-induced EGFR and IGF-1R trafficking [104], suggesting positive interactions *in vivo*.

In order to examine the effect of crosstalk between mammary PRL and EGFR ligands in the dynamic *in vivo* environment, we employed NRL-TGF α animals [54], which overexpress the EGFR ligand, TGF α , similar to many breast cancers [105-107]. Compared to single NRL-TGF α females, mammary tumor development was dramatically accelerated in bitransgenic NRL-PRL/TGF α females [108], and pErk1/2 and pAkt were further elevated in mammary tissue, as predicted by the cooperative interaction in *in vitro* studies [76,108]. Interestingly, the stroma surrounding PRL/TGF α -induced tumors exhibited significantly increased cell and collagen density, compared to that surrounding tumors induced by TGF α alone [108]. In light of the increasing recognition of the importance of the stroma on tumor progression [109-111], our data suggest that PRL actions on the microenvironment may be significant contributors to PRL-induced mammary pathology (see section 9.4.7 below).

Elevated growth factor activity is linked to resistance of ER α + cancers to anti-estrogen therapies [112-115]. These resistant clinical cancers also display lack of responsiveness to estrogens [77-79]. Similarly, we found that proliferation of mammary epithelia in NRL-PRL/TGF α females was strikingly insensitive to ovarian steroids or supplemental 17 β -estradiol despite high levels of ER α expression, in contrast to the strong response of MECs in nontransgenic and single transgenic NRL-PRL and NRL-TGF α mice [76]. Moreover, 17 β -estradiol also failed to induce progesterone receptor expression. Consistently, tumor incidence and latency in these animals were not altered by ovariectomy or additional estrogen. These findings suggest that tumors that express high levels of PRLR and erbB family members are likely to be less responsive to aromatase inhibitors, but that therapies directed at the PRLR in combination with growth factors may impact therapeutic outcome.

Although MEC proliferation and tumor latency in PRL/TGF α females were independent of estrogen, the strong crosstalk between PRL and TGF α to kinase cascades that phosphorylate the AF-1 domain of ER α suggest that ER α may none-the-less play a role in the tumorigenesis induced by these oncogenes in combination. In order to investigate the role of ligand-independent activation of ER α *in vivo*, we employed the ER α (G525L) mouse, which expresses an ER α that can be activated by phosphorylation, but is refractory to 17 β -estradiol [103]. As predicted, this model revealed that ligand-independent activation of ER α accounted for a significant portion of PRL/TGF α -stimulated MEC proliferation [100]. Interestingly, ER-dependent signals were not necessary for development of PRL/TGF α -induced ER α + tumors. ER-dependent signals did alter gene expression, however, indicating that even when ER is not a strong mediator of mitogenic signals, it may alter tumor phenotype and behavior.

Notably, the potent crosstalk of local transgenic PRL and TGF α also resulted in mammary tumors in male mice, which did not occur in the single transgenic animals [101]. Although breast cancer is relatively rare in men compared to women, the incidence is rising and the disease is less responsive to treatment [116-118]. Interestingly, hyperprolactinemia secondary to medications or prolactinomas is a significant risk factor [119-122], suggesting that the NRL-PRL mouse may be a useful tool for male breast cancer.

9.4.6 Crosstalk with p53

TP53 is mutated in many breast cancers across subtypes, and is associated with poor outcomes [85]. In order to examine the effect of PRL exposure on the cancers that develop in this context, we transplanted NRL-PRL $\times$ *p53*^{-/-} mammary cells to mammary glands of syngeneic wildtype mice [72]. PRL decreased the latency of the resulting carcinomas compared to *p53*^{-/-} cells alone, and increased their proliferation and invasiveness. Strikingly, PRL also increased the proportion of triple negative claudin-low carcinomas. PRL/*p53*^{-/-} tumors displayed elevated levels of AP-1 components associated with aggression of clinical tumors [123,124], compared to *p53*^{-/-} tumors, and altered levels of transcripts associated with cell cycle progression, invasion and stromal reactivity. The latter included those for *Colla1* and *Vegfa*, supporting a role for PRL in modulation of the tumor microenvironment. Interestingly, imaging of collagen surrounding PRL/*p53*^{-/-} tumors using polarized microscopy of picrosirius red-stained sections demonstrated that many carcinomas displayed straight collagen fibers oriented perpendicularly to the tumor edge, which is associated with more aggressive behavior clinically [125], compared to *p53*^{-/-} tumors (see section 9.4.7 below). Together, these studies suggest that PRL can promote development of more breast cancer subtypes than just luminal tumors, which may have been overlooked in epidemiologic studies that pooled all ER- cancers, and that actions on non-tumor cells in the microenvironment may mediate some of its oncogenic effects.

9.4.7 Interaction with the extracellular matrix

Accumulating evidence indicates that the development and progression of breast cancer can be strongly influenced by the density and composition of the extracellular matrix (ECM; for reviews, [126,127]). Hormones, including PRL, are associated with elevated mammographic density (increased stromal cells and/or ECM) in some clinical studies [128-130], which is

also linked to increased risk of breast cancer [131,132]. In established carcinomas, the density of the surrounding ECM is frequently elevated (desmoplasia) [109,133]. Moreover, aligned collagen fibers that are oriented perpendicularly to the tumor predict a 3-fold relative increased risk for relapse, particularly in patients with ER+ cancers [125]. These epidemiologic observations are supported by experimental evidence that increased mechanical stiffness of the ECM promotes tumorigenesis [134,135].

PRL increases synthesis of mammary ECM components in mouse models. Mammary glands of *Prlr*^{-/-} females contain reduced transcripts for many ECM proteins [136]. Consistently, mammary glands of 3 month old ovariectomized NRL-PRL females contain increased transcripts for collagens 1a and 5 (Rugowski and Schuler, unpublished data). Furthermore, mammary glands of aged NRL-PRL females also exhibit increased tenascin C mRNA compared to age-matched nontransgenic females [56], which is also associated with progression of clinical disease [137,138]. These observations indicate that PRL potentially can augment ECM density and alter its composition, with consequences for breast disease.

The NRL-PRL model in the context of a second oncogene has permitted us to examine the effect of PRL on the ECM surrounding tumors of similar histotypes. Mammary tumors in NRL-PRL/TGF α females displayed increased surrounding stromal density, including increased cellularity, compared to those in NRL-TGF α females tumors [108]. PRL action in the absence of p53 revealed the ability of PRL to modify the alignment of collagen fibers in addition to increase collagen synthesis [72], associated with increased metastases and poor outcomes [125].

Conversely, the ECM also modulates PRL actions, providing insight into apparent disparity between the association of PRL exposure with poor outcomes in breast cancer and positive prognosis associated with activated STAT5a [2,11]. In ER+ breast cancer cells *in vitro*, we have observed that matrix stiffness modulates the spectrum of PRL-induced signals and subsequent cell behavior [21]: stiff collagen matrices prolong PRL signals via SFK-FAK-ERK1/2 and reduces those via STAT5 compared to compliant matrices, associated with increased MMP-2 synthesis and activity and MMP-dependent invasion. Moreover, PRL aligns stiff matrices, increasing radially oriented collagen fibers [21], consistent with invasive carcinomas [125]. Matrix stiffness also increases pro-tumorigenic outcomes of crosstalk between PRL and 17 β -estradiol (Barcus and Schuler, ms in prep). We are extending these *in vitro* studies to the more complex *in vivo* environment, taking advantage of a mouse model with mutated collagen 1a (*coll1a1*^{tmJae}), which is resistant to degradation [139]. In combination with the NRL-PRL mouse, these studies will shed light on the interplay of PRL and estrogen signals with stiffness of the ECM in breast cancer development, progression and therapeutic responsiveness.

9.5 Summary and Future Directions

The NRL-PRL model recapitulates the epidemiologic link between PRL exposure and aggressive ER+ breast cancer in women, and has confirmed the potency of PRL cooperation with multiple other factors implicated disease progression. Indeed, PRL in this model cooperates with all of the oncogenes that we have examined (TGF α , absence of p53,

estrogen, as well as elevated β -catenin ($APC^{min/+}$) ([56,72,76,101,108], O'Leary and Schuler, ms in prep).

As described herein, NRL-PRL mice enable discovery of PRL actions in mammary tumorigenesis, and dissection of crosstalk with ovarian steroids, apart from the complex hormonal milieu of pregnancy. Our studies to date provide the foundation to identify the role(s) of PRL on the dynamics of MEC subpopulations, and illuminate the position of PRL in paracrine networks. These studies will point to origin of ER+ tumors, and the contributions of PRL to other breast cancer subtypes, which may not have been distinguished in the epidemiologic studies.

The estrogen-insensitive metastatic carcinomas that develop in this model provide the tools to investigate the progression, metastasis and therapeutic responsiveness of ER+ cancers, and hormonal contributions to these processes. These studies are likely to reveal significant influences of PRL on the tumor microenvironment, including the ECM, and other mammary cell types. For example, PRL promotes endothelial migration and VegfA transcription [140] (Friedl, this book), and increases GM-CSF expression [141], indicating potential roles in angiogenesis and immune responses. They will also elucidate the importance and determination of the components of the repertoire of PRL-initiated signals in oncogenesis and tumor progression, including the roles of Stat5 isoforms.

The ability of the mammary phenotype of the NRL-PRL mouse to mimic clinical breast cancer underscores the value of characterized mouse models to investigate this disease. Building on our extensive knowledge of murine mammary biology, the immunocompetent NRL-PRL model will illuminate hormone actions in the dynamic processes of breast cancer development and progression.

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Figure 9.1.

Prolactin (PRL) actions on mammary epithelia may contribute to breast cancer by multiple pathways. PRL binds to its receptor (PRLR), stabilizing a homodimer, and altering receptor conformation to activate associated kinases (JAK2 and Src Family Kinases, SFK). Subsequent phosphorylation cascades activate multiple signaling mediators, which may vary in strength depending on features of the tumor cell (e.g., levels of mediators, p53 status), as well as features of the microenvironment (e.g., ECM composition and stiffness, growth factors).

Table 9.1
Differences in the NRL-PRL and *Stat1*^{-/-} models of mammary cancer

	NRL-PRL	<i>Stat1</i> ^{-/-}
Model		
mouse strain	FVB/N	129S6/SvEV, Balb/c
PRL specificity	yes	multiple cytokines
parity	nulliparous	multiparous
Tumor phenotype		
tumor incidence/ mean latency	70%/ 17 months	65%/ 23 months
spectrum of tumor histotypes	broad	narrow
steroid receptor expression	ER α + /PR-; ER α - /PR-	ER α + /PR+
metastasis	yes	no
estrogen sensitive growth	no	initially, then lost
dependence on Jak2	little	much
cooperation with other oncogenes	yesNRL-TGF α , <i>p53</i> ^{-/-}	yesMMTV-neu, <i>p53</i> ^{-/-}

Although both NRL-PRL and *Stat1*^{-/-} females exhibit similar early mammary pathology and develop ER α + tumors, the established tumors display marked differences in appearance and behavior. *Stat1*^{-/-} mammary phenotype from [50,51,88].