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Prolactin Alters the Mammary Epithelial Hierarchy, Increasing Progenitors and Facilitating Ovarian Steroid Action

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SUMMARY

Hormones drive mammary development and function and play critical roles in breast cancer. Epidemiologic studies link prolactin (PRL) to increased risk for aggressive cancers that express estrogen receptor α (ERα). However, in contrast to ovarian steroids, PRL actions on the mammary gland outside of pregnancy are poorly understood. We employed the transgenic NRL-PRL model to examine the effects of PRL alone and with defined estrogen/progesterone exposure on stem/progenitor activity and regulatory networks that drive epithelial differentiation. PRL increased progenitors and modulated transcriptional programs, even without ovarian steroids, and with steroids further raised stem cell activity associated with elevated canonical Wnt signaling. However, despite facilitating some steroid actions, PRL opposed steroid-driven luminal maturation and increased CD61$^+$ luminal cells. Our findings demonstrate that PRL can powerfully influence the epithelial hierarchy alone and temper the actions of ovarian steroids, which may underlie its role in the development of breast cancer.

INTRODUCTION

Prolactin (PRL) is critical for mammary development and lactation (Oakes et al., 2008). Despite initial controversy, a large prospective study nested within the Nurses' Health Study has linked elevated circulating PRL to increased risk for luminal breast cancers that express estrogen receptor α (ERα$^+$) independent of estrogen exposure (Tworoger and Hankinson, 2008). More recent analyses have associated high levels of PRL 10 years prior to diagnosis with development of aggressive breast cancer in postmenopausal women (Tworoger et al., 2013). An analysis of the EPIC (European Prospective Investigation into Cancer and Nutrition) cohort found that postmenopausal women with higher circulating PRL who had used combined estrogen/progestin but not estrogen-alone hormone replacement therapy had a higher incidence of ERα$^+$ breast cancer (Tikk et al., 2014b). Reduced PRL may also play a role in the long-term protection conferred by pregnancy (Schedin, 2006). PRL levels are lower in parous than in nulliparous women, and some studies found further reduction following additional full-term pregnancies (Tikk et al., 2014a; Tworoger and Hankinson, 2008). These epidemiologic studies support a role for PRL in the development of breast cancer, and the need to understand PRL interactions with ovarian steroids in breast pathology.

Most studies of PRL actions on the mammary gland have concentrated on its role in alveolar development during pregnancy and lactogenesis. During these physiologic states, PRL acts in a tightly regulated hormonal milieu to direct lobuloalveolar development and increase expression of milk components. However, pituitary PRL secretion is influenced by many factors, and levels in nonpregnant women vary considerably (Ben Jonathan et al., 2008; Tikk et al., 2014a; Tworoger and Hankinson, 2008). Moreover, a second promoter drives PRL expression in multiple tissues other than the pituitary in women, including breast tissue, permitting local exposure (Marano and Ben-Jonathan, 2014).

A rich literature has elucidated the actions of estrogen and progesterone on mammary development and gene expression (reviewed in Arendt and Kuperwasser, 2015; Joshi et al., 2012; Stingl, 2011; Tanos et al., 2012; Tarulli et al., 2015). Identification of surface markers for human and mouse mammary epithelial cell (MEC) subsets and comparison of the transcriptomes between these species have validated the utility of mouse models to investigate hormone actions in MEC differentiation (Lim et al., 2010; Shehata et al., 2012; Tornillo and Smalley, 2015; Visvader and Stingl, 2014). Elegant use of lineage tracing is revealing the relationships among MEC subpopulations (Giraddi et al., 2015; Rios et al., 2014; van Amerongen et al., 2012; Van Keymeulen et al., 2011).

To investigate the actions of PRL in development and progression of breast cancer, we generated the NRL-PRL transgenic mouse (O'Leary et al., 2015; Rose-Hellekant et al., 2003). In this model, mammary epithelia express PRL, mimicking the mammary production of PRL in women (Marano and Ben-Jonathan, 2014; McHale et al., 2008). The locally elevated PRL does not alter estrous cycling, permitting study of PRL actions independent from ovarian steroids (Stocco et al., 2007). Nulliparous NRL-PRL females develop diverse aggressive carcinomas, many of which express ERα and resemble the luminal subtype of clinical breast cancer (Arendt et al., 2011).

Mirroring the epidemiologic data establishing that the relationship of PRL levels to breast cancer in women is independent from estrogen (Tworoger and Hankinson, 2008), cancers in NRL-PRL females can develop without postpubertal ovarian steroids. Indeed, ovariectomy after ductal elongation is complete does not alter the incidence or latency of tumor development, although supplemental 17β-estradiol decreases tumor latency (Arendt et al., 2009).

Using the NRL-PRL mouse model, we examined the effects of PRL and its crosstalk with ovarian steroids on mammary epithelial subpopulations. We demonstrate that local PRL augments epithelial progenitor subpopulations independently from ovarian steroids, associated with modification of transcriptional programs that govern the mammary hierarchy. Furthermore, PRL increases mRNAs for transcriptional partners of ERα and the progesterone receptor A (PR-A) isoform. However, despite this facilitation of the activity of estrogen and progesterone, PRL also tempers their effects, augmenting progenitor populations and perturbing differentiation pathways. Defining the actions of PRL and mechanisms of crosstalk with ovarian steroids to modulate mammary epithelial subpopulations has important implications for understanding the role of this poorly understood mammotropic hormone apart from pregnancy, and its role in the risk for development of breast cancer.

RESULTS

PRL Induces ERα$^+$/PR$^+$ Mammary Lesions and Metastatic ERα$^+$ Carcinomas in Aging Females, but No Overt Lesions Are Evident at 3 Months of Age

Elevated local transgenic PRL in the NRL-PRL model results in histologically diverse mammary metastatic carcinomas after a relatively long latency (Rose-Hellekant et al., 2003). Although these carcinomas develop without postpubertal ovarian hormones, supplemental 17β-estradiol reduces tumor latency (Arendt et al., 2009; O'Leary et al., 2015), and the majority of adenocarcinomas are ERα$^+$, with low progesterone receptor (PR) expression (Figure 1A). However, many preneoplastic lesions are strongly ERα$^+$/PR$^+$ (Figure 1A). Lung metastases are readily detectable in about 85% of tumor-bearing females (Figure 1Bi, ii), and many contain nests of ERα$^+$ cells (Figure 1Biii). These features permit the study of PRL action in the development of metastatic ERα$^+$ breast cancer.

To identify changes that occur prior to tumor formation, we examined younger animals. Although epithelial hyperplasias and ductal atypia were frequent in mammary glands of nulliparous NRL-PRL females at 6 months of age (Figure 1C), glands appeared grossly normal at 3 months (Figure 1D), and we therefore continued our investigation at this age. Examination of the relative proportions of luminal and basal MEC subpopulations in 3-month-old cycling females using established surface markers (CD24$^+$CD49f^{lo}, luminal; CD24$^+$CD49f^{hi}, basal) did not reveal any gross differences between wild-type (WT) and NRL-PRL glands (Figure 1E).

PRL Increases Lateral Ductal Budding Regardless of the Ovarian Steroid Environment

Ovarian steroids significantly affect MEC subpopulations and function, even over the course of the estrous cycle (Asselin-Labat et al., 2010; Joshi et al., 2010). PRL has been shown to interact with both estrogen and progesterone at multiple levels in various experimental mammary systems *in vitro* and *in vivo* (Carver et al., 2009; Lee and Ormandy, 2012; O'Leary et al., 2013). To investigate effect of local PRL in the context of defined exposures to ovarian steroids, WT and NRL-PRL females were ovariectomized (Ovx) after ductal elongation was complete and then left as untreated controls, or treated with ICI 182,780 (ICI), 17β-estradiol (E), or 17β-estradiol plus progesterone (E + P) for 4 additional weeks (Figure 2A). As reported previously (Asselin-Labat et al., 2010; Joshi et al., 2010), mammary epithelial structures in WT females were strongly responsive to these hormones (Figures 2Bi–iv and S1). After postpubertal ovariectomy, glands contained only small ducts with flattened luminal epithelia and sparse secretions, and this was more pronounced when all ER-mediated signals were inhibited with ICI (Figure 2Bi, ii). In these WT females estrogen fostered ductal dilation, while estrogen and progesterone together stimulated extensive lobuloalveologenesis (Figure 2Biii, iv). PRL did not markedly alter these steroid-induced effects on epithelial structures (Figures 2Bv–viii and S1). However, ducts of NRL-PRL females exhibited extensive lateral budding, regardless of ovarian steroid hormone activity (Figure 2Bv–viii, arrowheads), compared with WT females under the same steroid treatment.

PRL Increases Expression of Progesterone Receptors, Especially the PR-A Isoform, by an ER-Dependent Mechanism

One mechanism by which PRL may influence actions of the ovarian steroids is by altering expression of their receptors. As expected, ovarian hormone activity influenced mammary transcripts for ERα and the proportion of ductal epithelia that expressed detectable receptor (Figures 3A and 3B). In WT females, reduced estrogen following ovariectomy prevented ligand-induced ERα downregulation, while ICI increased downregulation. This pattern was not altered in NRL-PRL females. In contrast, PRL increased PR expression in the absence of ovarian steroids and in combination with E + P (Figures 3A–3C). The ability of ICI to reduce this increase suggests that it is mediated by PRL-induced ligand-independent activation of ERα (O'Leary

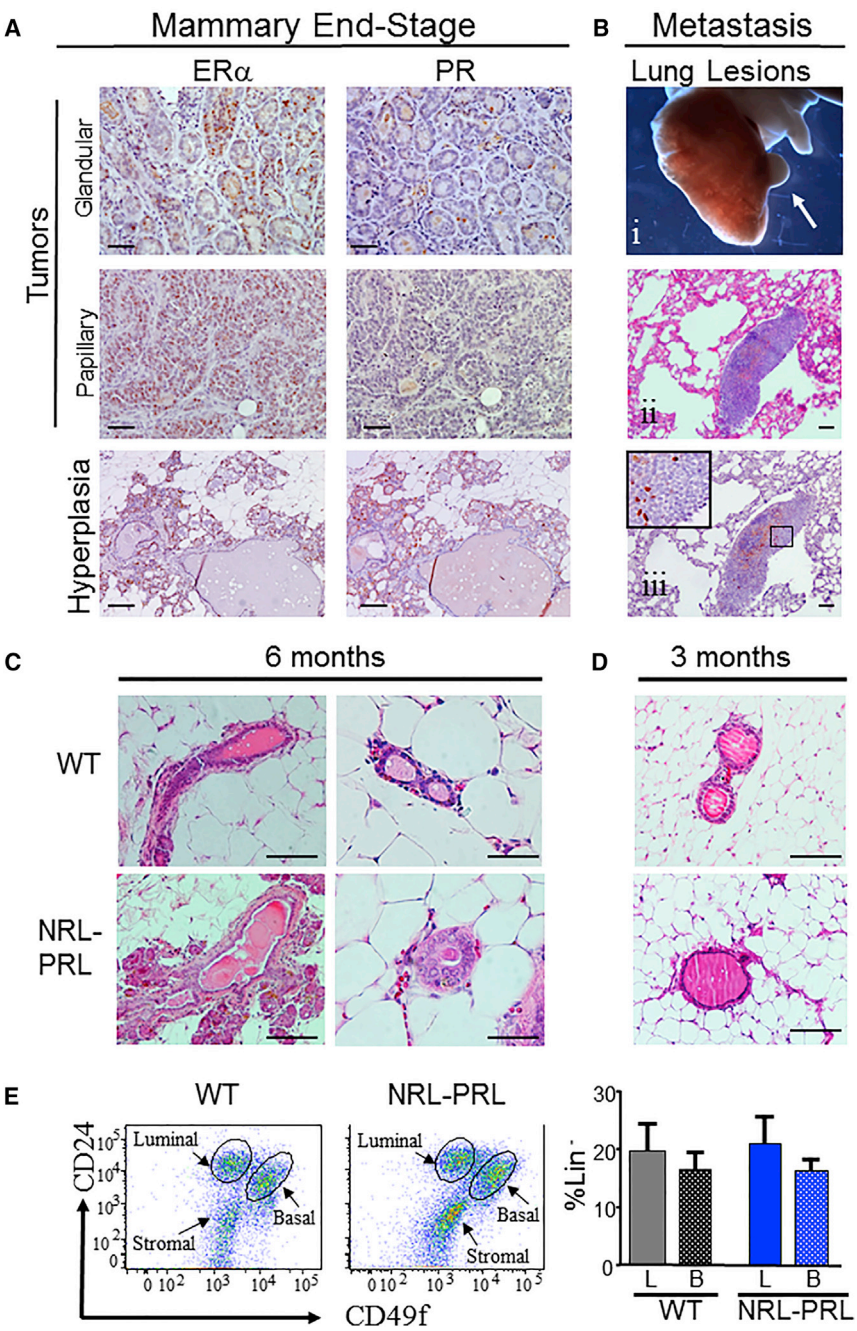


Figure 1. Nulliparous Aging NRL-PRL Females Develop Histologically Diverse Metastatic ERα$^+$ Tumors, but Mammary Glands of WT and NRL-PRL Animals Display No Major Histopathologic Differences at 3 Months of Age

(A) Independent mammary adenocarcinomas of differing histotypes and an epithelial hyperplasia from an end-stage gland were immunostained for ERα and PR.

(B) NRL-PRL mice can develop metastatic lung lesions (i, ii), many of which also contain nests of ERα$^+$ cells (iii). Arrow indicates large lung metastasis (i).

(C) Ductal structures in mammary glands of 6-month-old WT or NRL-PRL intact females stained with H&E.

(D) H&E-stained cross-sections of mammary ducts from 3-month-old intact WT and NRL-PRL females.

(E) Left: representative flow-cytometry dot plot showing basal (CD24$^+$CD49f^{hi}) and luminal (CD24$^+$CD49f^{lo}) cells present in Lin$^-$ MECs isolated from the caudal glands of 3-month-old intact cycling females. Right: bar graph shows the mean ± SEM proportions of luminal (L) and basal (B) MEC subpopulations in these mice (n = 3).

Scale bars, 50 μm.

et al., 2013). Interestingly, PRL selectively increased mRNA for the PR-A isoform, compared with WT females (Figure 3A). This alternative transcript of the *Pgr* gene encodes an isoform that lacks the amino-terminal region of PR-B, resulting in overlapping but distinct sets of target genes (reviewed in Diep et al., 2015; Grimm et al., 2016). PR-A is preferentially induced by estrogen and is higher in ducts of nonparous females. However, E + P reduces PR-A, and instead increases expression of PR-B, which is essential for lobuloalveolar development during pregnancy (Aupperlee et al., 2005; Mulac-Jericevic et al., 2003).

PRL Upregulates Transcripts Associated with Estrogen Action, Reduces Transcripts Encoding Drivers of Luminal Maturation, and Modulates Transcripts of Enhancers of Canonical Wnt Signals

PRL and estrogen cooperate in physiologic states such as early pregnancy (reviewed in Tarulli et al., 2015), and

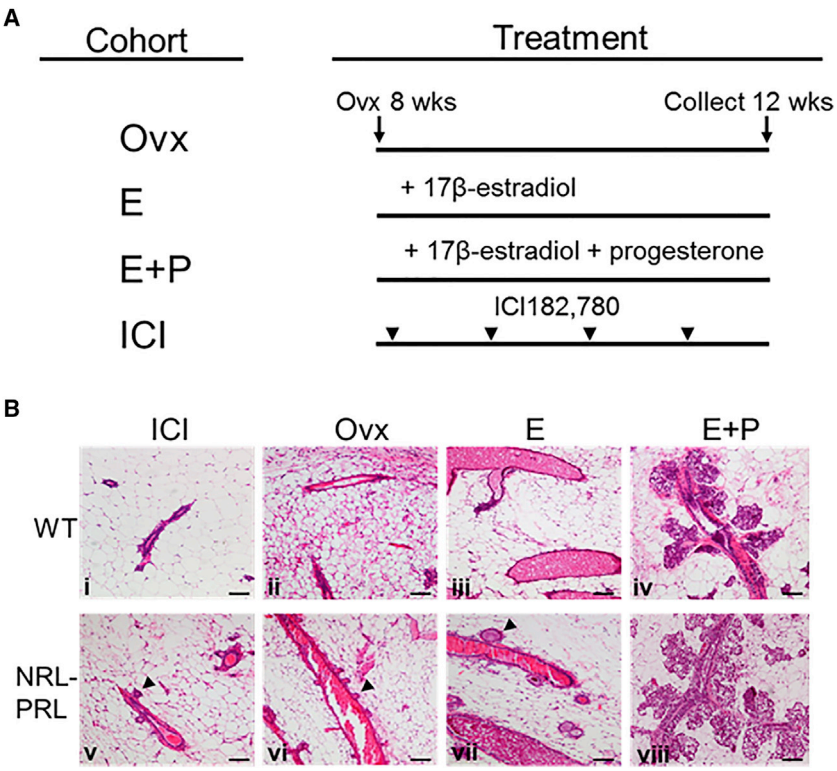


Figure 2. Ovarian Hormones Significantly Affect Mammary Epithelial Structures, and Glands from NRL-PRL Mice Display Increased Lateral Ductal Budding

(A) Schematic of experimental groups. After pubertal ductal elongation was complete, young adult female mice were ovariectomized and then left as controls (Ovx), administered supplemental 17β-estradiol (E), administered supplemental 17β-estradiol together with progesterone (E + P), or treated with the estrogen receptor antagonist, ICI 182,780 (ICI) as described in Experimental Procedures. After 4 weeks of treatment, cranial glands were processed for histology and MECs were isolated from the caudal glands for further characterization.

(B) Representative H&E-stained sections from the cranial mammary glands of the experimental cohorts in WT (i–iv) and NRL-PRL (v–viii) females. Arrowheads show budding present along ducts in NRL-PRL females. Scale bars, 50 μm.

See also Figure S1.

estrogen and PRL synergistically regulate some target genes in breast cancer cells (Fiorillo et al., 2013; Rasmussen et al., 2010; Sato et al., 2013). In light of PRL's insignificant effect on ERα expression but collaboration with ER-mediated signals to increase PR, we examined transcripts of key co-factors of ERα activity in isolated MECs by qRT-PCR. In the absence of ovarian steroid activity, PRL strongly upregulated transcripts for various partners of ERα, including the pioneering factor for ERα, FOXA1 (Bernardo et al., 2010), a selective co-activator for estrogen-dependent transcription, CITED1 (McBryan et al., 2007; Yahata et al., 2001), and a regulator of ERα$^+$ luminal cells, RUNX1 (van Bragt et al., 2014) (Figures 4A and S2). However, PRL reduced mRNA for GATA3, which promotes differentiation of committed luminal progenitors (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006) (Figure 4B). This suggests that interplay between PRL and estrogen regulates transcriptional programs that orchestrate the MEC luminal hierarchy. Indeed, in combination with E, PRL increased mRNA for SOX9, which helps to maintain mammary stem cells and luminal progenitor function (Malhotra et al., 2014), but reduced mRNA for C/EBPβ, which is important in luminal differentiation and determines patterning of steroid and prolactin receptor expression in ductal epithelia (Grimm et al., 2002; Robinson et al., 1998; Seagroves et al., 1998).

The RANKL/ELF5 axis is critical for alveologenesis during pregnancy, and the crosstalk between PRL and progesterone to drive alveolar commitment and differentiation has received extensive study (Lee and Ormandy, 2012). *Rankl*, *Rank*, and *Elf5* transcripts were strongly responsive to E + P in WT females (Figures 4C and S2), as expected (Fernandez-Valdivia and Lydon, 2012; Joshi et al., 2012; Lee et al., 2013; Visvader and Stingl, 2014). Although local PRL did increase *Rank* mRNA in combination with E without further elevation in E + P-treated animals, it did not significantly alter levels of *Rankl* transcripts in any steroid hormone environment. Consistent with the lack of effect on transcripts for this paracrine system in the E + P environment, PRL did not further raise *Elf5* mRNA.

Canonical Wnt signals have been implicated in progesterone-induced stem cell activity (reviewed in Joshi et al., 2012; Rajaram et al., 2015; Visvader and Stingl, 2014). *Wnt4* mRNA was strongly responsive to ovarian steroids, especially progesterone, in WT females (Figures 4D and S2), as expected (Joshi et al., 2010; Rajaram et al., 2015). PRL significantly elevated *Wnt4* transcripts over levels in WT females in the absence of ovarian steroid activity, but did not further increase these transcripts in animals treated with E + P. However, other modulators of canonical Wnt activity were significantly altered by transgenic PRL in the E + P environment. PRL significantly upregulated transcripts for RSPO1, a

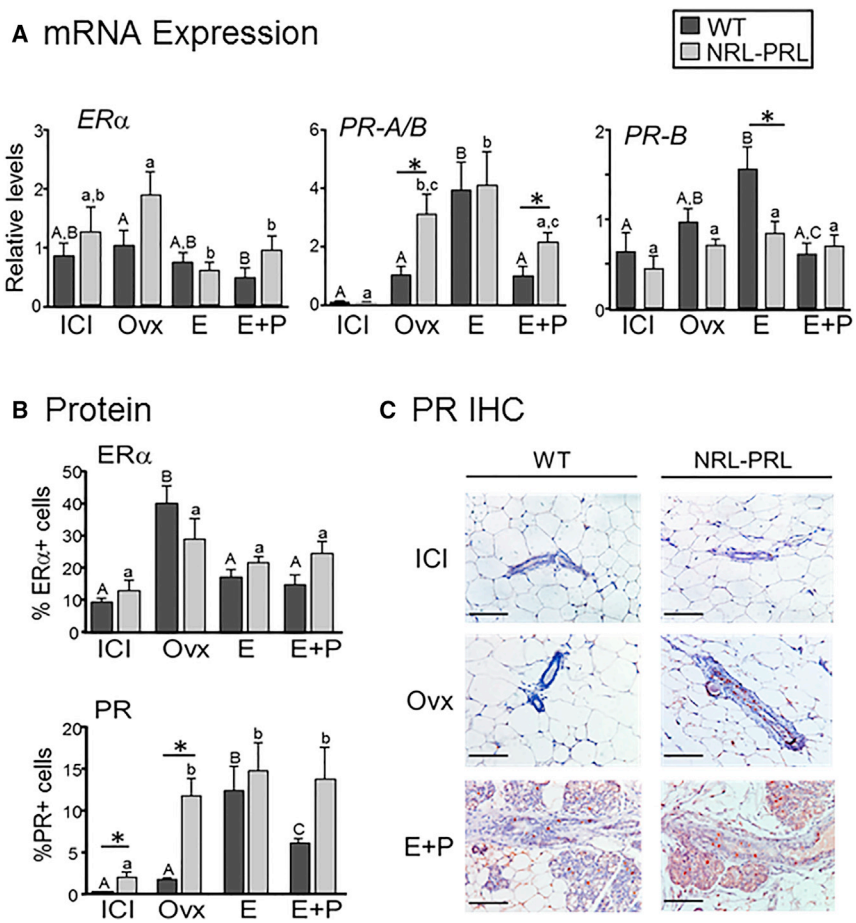


Figure 3. PRL Does Not Alter ERα Expression but Increases Progesterone Receptors, Especially the PR-A Isoform

(A) ERα, total PR-A/B mRNA, or PR-B mRNA. Transcripts in MECs from individual mice were quantified by qPCR. Transcript levels are shown relative to WT Ovx.

(B) Proportion of epithelia expressing ERα or PR detected by immunohistochemistry.

(C) Representative mammary sections from ICI-, Ovx-, and E + P-treated WT and NRL-PRL females immunostained for PR. Scale bars, 50 μm.

Error bars in (A) and (B) represent mean ± SEM; n = 3–4 mice. Significant differences were determined by the Kruskal-Wallis test followed by the Mann-Whitney post test, $p < 0.05$. Different letters represent significant differences among treatments within each genotype (WT, uppercase; PRL, lowercase). Asterisks denote statistically significant differences between WT and NRL-PRL females for the same treatment ($^*p < 0.05$).

secreted enhancer of Wnt signals (Cai et al., 2014), and reduced mRNA for WIF1, an inhibitor of Wnt activity, in E + P-treated females (Clevers and Nusse, 2012).

Together, these PRL-altered patterns of transcriptional and paracrine regulators, both alone and in combination with ovarian steroids, predict effects on MEC fate and increased stem/progenitor activity. This is of considerable interest in light of the capacity of stem/progenitor cells for growth, suggesting that they may be tumor precursors (Lim et al., 2009; Molyneux et al., 2010).

PRL Cooperates with Progesterone to Further Increase Stem Cells and Increases MEC Progenitor Activity Independent of Ovarian Steroids

To directly assess PRL interactions with ovarian steroids on stem cell activity, we transplanted limiting dilutions of mammary epithelia from ICI-treated and E + P-treated females into cleared fat pads of prepubertal WT females, and evaluated their ability to generate ductal trees (Stingl, 2009). In the absence of estrogen activity, PRL displayed a trend to increase stem cell frequency compared with similarly treated WT animals, although this did not reach statistical significance (Figure 5A). E + P and the hormonal alterations of pregnancy increase mammary stem cell activity (Asselin-Labat et al., 2010; Joshi et al., 2010), confirmed here in WT animals. In the E + P environment, transgenic PRL doubled stem cell frequency over that observed in similarly treated WT females ($p = 0.033$), demonstrating functional cooperation between PRL and progesterone. To complement these studies, we examined functional progenitor activity using an *in vitro* assay for colony-forming cells (CFCs), which detects additional epithelial progenitor subpopulations, including luminal progenitors (Lim et al., 2010). Previous reports have shown that estrogen has relatively little effect on activity detected by this assay in WT females (Asselin-Labat et al., 2006, 2010; Joshi et al., 2010) and, as expected, we observed no significant differences in numbers of colonies from WT Ovx-, ICI-, and E-treated females (Figures 5B and S3). As previously reported, E + P increased CFC activity in WT females about 2-fold (Joshi et al., 2010). Interestingly, PRL significantly increased CFCs independent of ER activity and with E

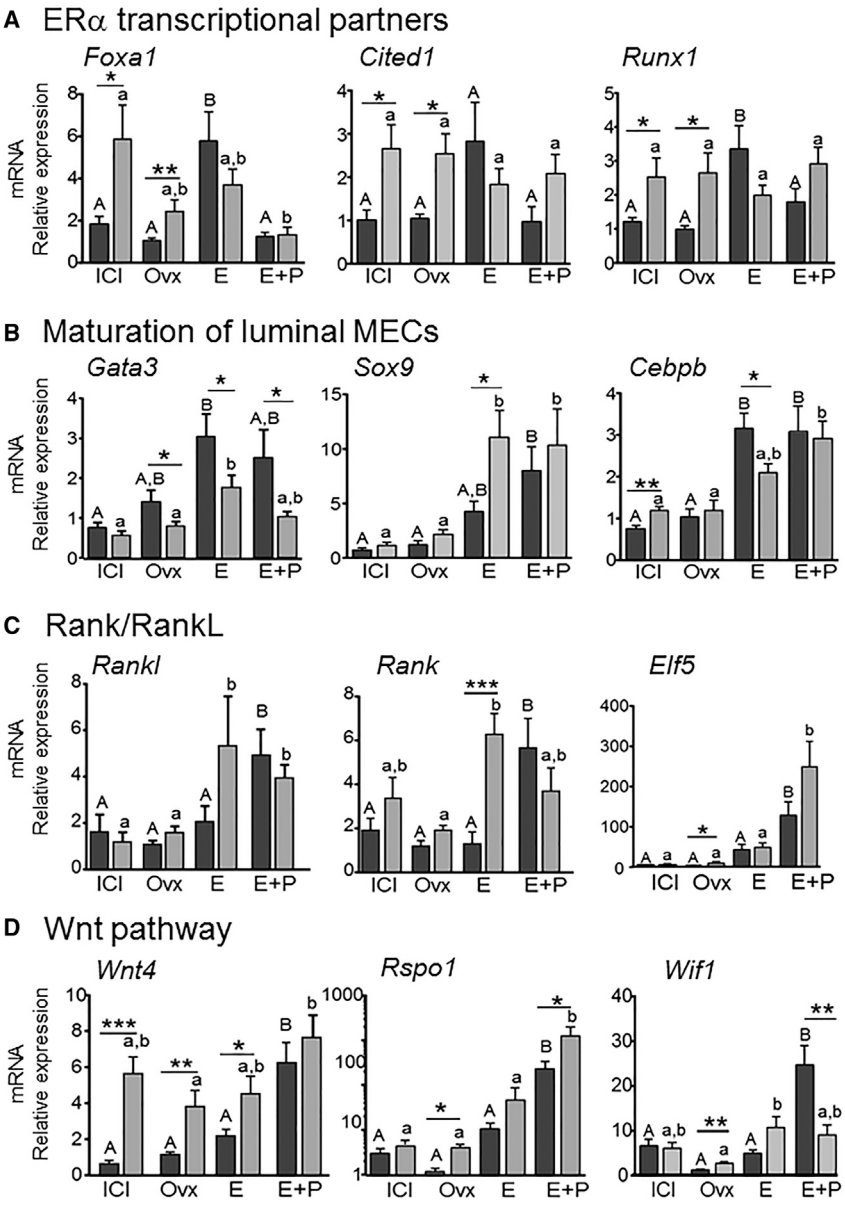


Figure 4. PRL Upregulates Transcripts Associated with Estrogen Action, Reduces Transcripts Encoding Drivers of Luminal Maturation, and Modulates Transcripts of Enhancers of Canonical Wnt Signals

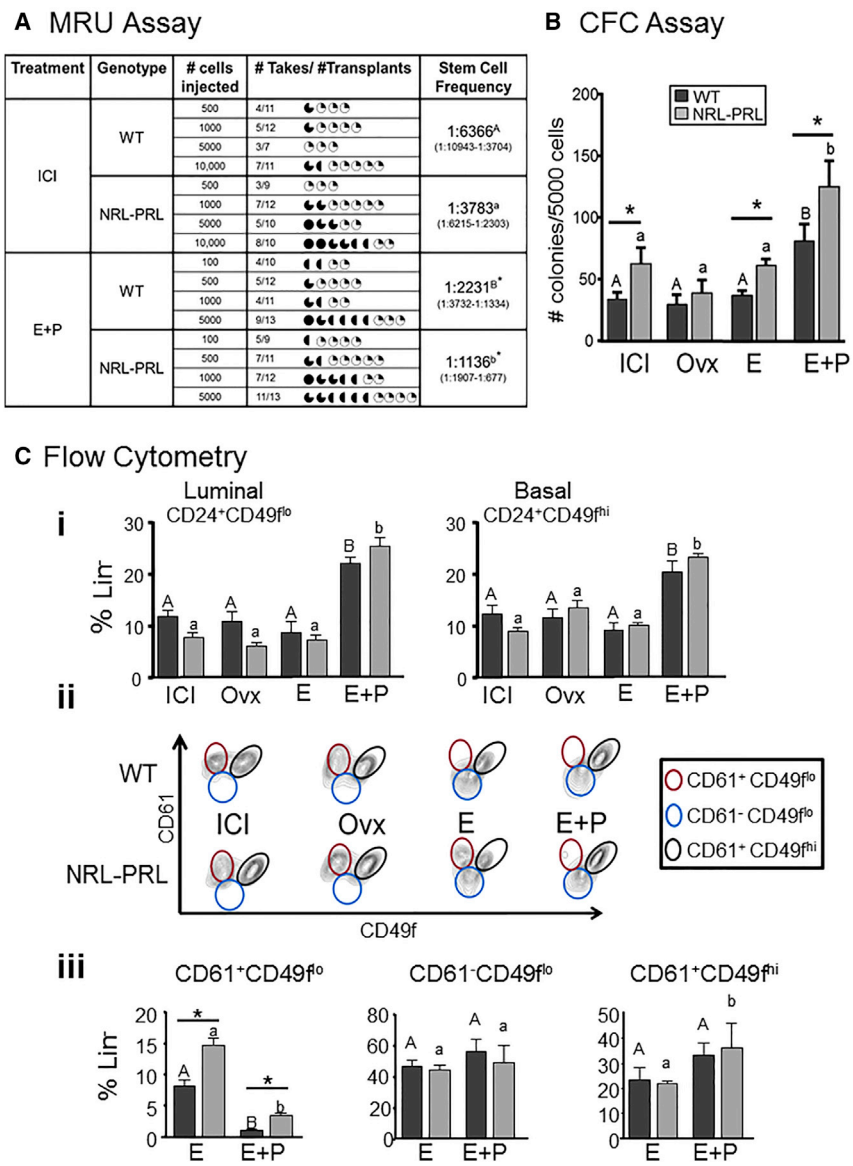
(A–D) Transcripts in MECs from individual mice were quantified by qPCR. Error bars represent mean ± SEM; n = 3–4 mice. Significant differences were determined by the Kruskal-Wallis test followed by the Mann-Whitney post test. Different letters represent significant differences among treatments within each genotype (WT, uppercase; PRL, lowercase), $p < 0.05$. Asterisks denote statistically significant differences between WT and NRL-PRL females for the same treatment (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figure S2.

supplementation over that observed in similarly treated WT females. Moreover, PRL further raised CFCs in combination with E + P. Together, these results indicate that PRL can functionally enhance MEC stem/progenitor activity, even in the absence of ovarian steroids, and cooperates with estrogen/progesterone to further expand these subpopulations.

PRL Alters MEC Subpopulations Independently of Ovarian Steroids and Increases CD61^{+}CD49f^{lo} MECs in Combination with Estrogen and Progesterone

To extend these studies, we performed flow cytometry with commonly utilized surface markers (CD24; CD49f, α6-integrin). As shown in Figure 5Ci, PRL did not significantly alter the proportions of luminal (CD24^{+}CD49f^{lo}) and basal (CD24^{+}CD49f^{hi}) MECs in any steroid hormone milieu. CD61 (β3-integrin) has been widely employed to further resolve MEC subpopulations. This surface marker enriches for luminal progenitors in FVB/N mice (Visvader and Stingl, 2014), although not in the C57Bl6/J strain background, and its widespread use permits comparison with reports examining related pathways (Asselin-Labat et al., 2007; Bernardo et al., 2010; Chakrabarti et al., 2012; Forster et al., 2014; Lee et al., 2013; Michalak et al., 2013; Yamaji et al., 2009). In the absence of estrogen activity, mammary glands of WT females contained populations with basal



Treatment	Genotype	# cells injected	# Takes/ #Transplants	Stem Cell Frequency
ICI	WT	500	4/11	1:6366[A] (1:10943-1:3704)
		1000	5/12	
		5000	3/7	
		10,000	7/11	
	NRL-PRL	500	3/9	1:3783[a] (1:6215-1:2303)
		1000	7/12	
		5000	5/10	
		10,000	8/10	
E+P	WT	100	4/10	1:2231[B*] (1:3732-1:1334)
		500	5/12	
		1000	4/11	
		5000	9/13	
	NRL-PRL	100	5/9	1:1136[b*] (1:1907-1:677)
		500	7/11	
		1000	7/12	
		5000	11/13	

Figure 5. MECs from NRL-PRL Mice Contain Higher Stem/Progenitor Activity, Particularly in Combination with Estrogen/Progesterone Treatment

(A) PRL augments the progesterone-induced increase in stem cell activity. Limiting dilutions of single MEC preparations from ICI-treated or E + P-treated WT and NRL-PRL females were transplanted to cleared fat pads of 3-week-old WT females, and after 6 weeks the ability to generate a ductal tree was assessed. The frequency of MRUs was calculated by the method of maximum likelihood (http://bioinf.wehi.edu.au/software/limdil) (Hu and Smyth, 2009).

(B) Frequency of colony-forming cells (CFC) in MECs from WT and NRL-PRL females treated as shown. Error bars represent the mean ± SEM; n = 4–6 individual mice. See also Figure S3.

(C) (i) Proportions of luminal (L, $CD24^+CD49f^{lo}$) or basal (B, $CD24^+CD49f^{hi}$) MECs, determined by flow cytometry. (mean ± SEM; n = 4–6 mice). (ii) Representative flow-cytometry contour maps of CD61 expression in Lin^- MECs. (iii) Quantitation of MEC subpopulations distinguished by CD61 expression. Mean ± SEM; n = 3–4 mice.

In (B) and (C), significant differences were determined by the Kruskal-Wallis test followed by the Mann-Whitney post test, $p < 0.05$. Different letters represent significant differences among treatments within each genotype (WT, uppercase; PRL, lowercase). Asterisks denote statistically significant differences between WT and NRL-PRL females for the same treatment (*$p < 0.05$).

and progenitor surface markers, but few mature luminal cells, as previously observed (Asselin-Labat et al., 2010; Joshi et al., 2010; Shehata et al., 2012), and PRL did not substantially alter this pattern. However, in animals treated with steroid hormones, PRL shifted the distribution of MECs expressing these surface markers (Figure 5Cii). WT females treated with E displayed an apparent shift from $CD61^+CD49f^{lo}$ progenitors to $CD61^-CD49f^{lo}$ mature luminal cells, which was obviated in similarly treated PRL females (Figure 5Cii, iii). Although addition of progesterone further reduced $CD61^+CD49f^{lo}$ cells in WT females as reported (Lee et al., 2013), transgenic PRL significantly augmented this subpopulation (Figure 5Cii, iii). Notably, PRL did not alter the proportions of $CD61^-CD49f^{lo}$ or $CD61^+CD49f^{hi}$ cells (Figure 5Ciii).

PRL Modulates Gene Expression in Luminal and Basal MEC Subpopulations, Consistent with Increased Stem/Progenitor Activity

To further investigate the cooperation between PRL and E + P on MEC subpopulations, we examined cytokeratin expression in sorted luminal ($CD24^+CD49f^{lo}$) and basal ($CD24^+CD49f^{hi}$) MECs. In WT females, *Krt8* (K8) and *Krt18* (K18) mRNAs were restricted to luminal MECs, and *Krt5* (K5) and *Krt14* (K14) mRNAs were restricted to basal MECs. However, transgenic PRL increased transcripts for luminal cytokeratins in cells expressing basal surface markers (Figure 6A), and these glands displayed readily detectable double-positive $K8^+K14^+$ and $K8^+K5^+$ MECs in this steroid environment (Figure 6B; $K8^+K14^+$/total $K14^+$: WT 11%, PRL 25%; $K8^+K5^+$/total $K5^+$: WT 11.5%, PRL 21%), characteristics of

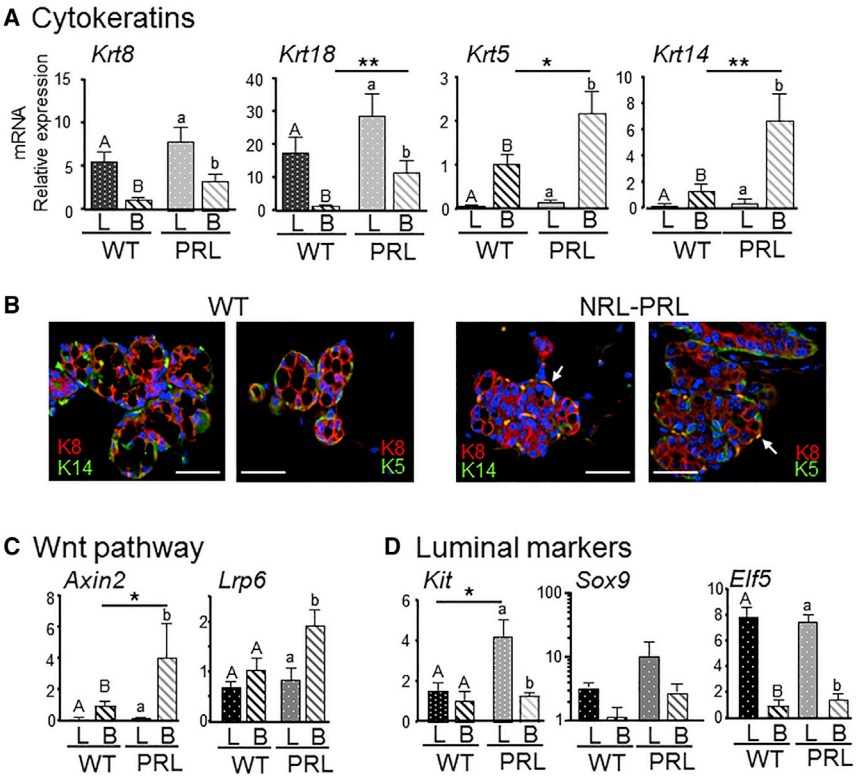


Figure 6. PRL Modulates Gene Expression in Luminal and Basal MEC Subpopulations, Consistent with Increased Progenitor Activity

(A) Cytokeratin transcripts in sorted luminal (L, $CD24^{+}CD49f^{lo}$) and basal (B, $CD24^{+}CD49f^{hi}$) MEC subpopulations from E + P-treated WT or NRL-PRL females.

(B) Representative merged immunofluorescence images of E + P-treated WT and NRL-PRL mammary glands stained for luminal (K8) and basal (K14 or K5) cytokeratins. Arrows indicate cells in the basal layer that express both luminal and basal cytokeratins. Scale bars, 25 μm.

(C) Transcript levels of canonical Wnt target genes in sorted luminal and basal cells from E + P-treated females.

(D) Levels of transcripts of associated with luminal progenitors, in sorted luminal and basal cells from E + P-treated females.

In (A), (C), and (D), data are mean ± SEM; n = 3–4 mice. Significant differences were determined by the Kruskal-Wallis test followed by the Mann-Whitney post test. Different letters represent significant differences among treatments within each genotype (WT, uppercase; PRL, lowercase), $p < 0.05$. Asterisks denote statistically significant differences between WT and NRL-PRL females for the same treatment (*$p < 0.05$, **$p < 0.01$). See also Figure S4.

bipotent progenitors (Rios et al., 2014; van Amerongen et al., 2012). Consistent with the reported elevated Wnt activity in these double-positive cells (van Amerongen et al., 2012), transcripts for the canonical Wnt target gene, *Axin2*, were higher in basal MECs from PRL females (Figure 6C), as predicted by our analysis of Wnt signaling components (Figure 4D). Moreover, luminal cells from PRL females contained higher levels of mRNA for the driver of luminal progenitor activity, *Kit* (Figure 6D), consistent with the abundance of $CD61^{+}$ luminal cells and higher CFC activity observed in these glands. However, levels of *Elf5* transcripts were not altered, confirming that this regulator of the alveolar lineage is not altered by PRL in this model. Further fractionation of the $CD24^{+}CD49f^{lo}$ subpopulations confirmed that $CD61^{+}$ luminal cells were highly enriched for mRNAs marking luminal progenitors (Figure S4) (Lim et al., 2010).

DISCUSSION

Although considerable research has illuminated the actions of estrogen and progesterone on the mammary gland and the pathways by which these steroids may influence the development of breast cancer (reviewed in Arendt and Kuperwasser, 2015; Joshi et al., 2012; Tanos et al., 2012; Tarulli et al., 2015; Visvader and Stingl, 2014), PRL has remained a hidden partner in these processes. PRL activity is generally associated with pregnancy and lactation, but many factors increase pituitary PRL secretion apart from these physiologic states (Ben Jonathan et al., 2008). Moreover, other sources of PRLR agonists in women contribute to mammary exposure, including extrapituitary PRL, which can be synthesized within the mammary gland (Marano and Ben-Jonathan, 2014), and hGH, which potently activates human, but not nonprimate PRLR (Utama et al., 2009). Local transgenic expression of PRL in the NRL-PRL mouse model permits elucidation of the role of PRL in the dynamic differentiation of mammary subpopulations.

Extensive recent studies are illuminating the transcriptional networks that govern the mammary epithelial hierarchy (diagrammed in Figure 7A). The PRL-induced functional changes and altered transcript levels for key regulators reported here indicate that PRL can strongly

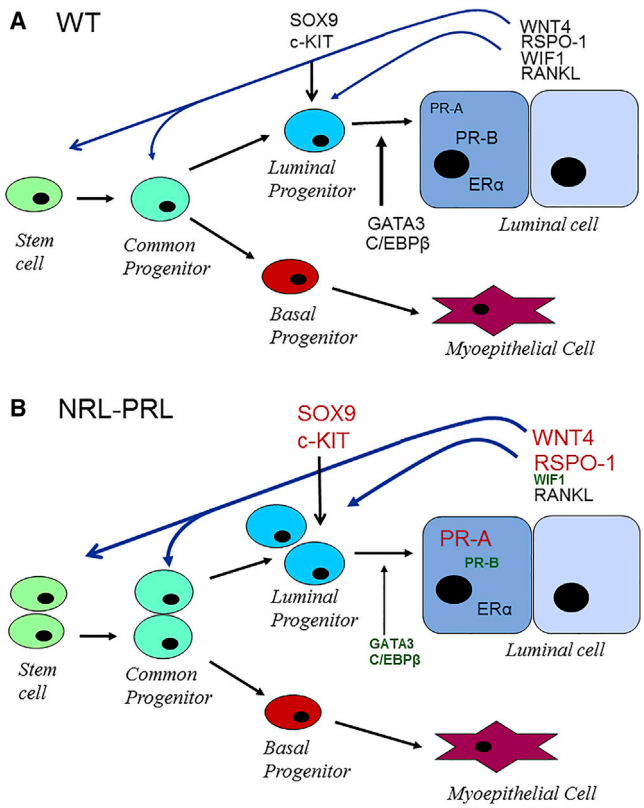


Figure 7. Schematic of the Effects of PRL on Transcriptional/Paracrine Regulators, Resulting in Augmented Progenitor/Stem Cells and Perturbed Differentiation Pathways, which May Contribute to Heightened Risk for Breast Cancer

(A) MEC hierarchy and its regulation in WT females.

(B) MEC hierarchy and altered regulators in NRL-PRL females. Transcripts elevated above WT levels are shown in larger, red font; reduced transcripts are shown in smaller, green font.

influence the orchestrated differentiation of MEC subpopulations (Figure 7B). In addition to actions independent from estrogen and progesterone, PRL can facilitate the activity of ovarian hormones but also temper some of their actions, modulating expression of well-characterized paracrine mediators and transcriptional coordinators of regulatory networks to augment stem/progenitor populations and perturb differentiation pathways, which may contribute to the development of breast cancer.

In the absence of ovarian steroids post puberty, local PRL exposure increased mammary epithelial progenitor activity, associated with increased *Wnt4* and reduced *Gata3* transcripts, indicating that PRL can influence critical MEC subpopulations independently of estrogen and progesterone. Under these conditions, PRL also activated transcriptional programs that facilitated the actions of these steroids. Although PRL did not alter the expression of ERα itself, it increased transcripts encoding FOXA1, RUNX1, and CITED1, which cooperate in ERα action (Bernardo et al., 2010; McBryan et al., 2007; van Bragt et al., 2014; Yahata et al., 2001). In contrast, PRL increased the expression of PR, particularly the PR-A isoform. This PRL-induced PR expression was inhibited by the ER antagonist, ICI, suggesting that it is likely mediated by ligand-independent activation of ERα (O'Leary et al., 2013).

Although PRL increased transcripts encoding ERα transcriptional partners in the absence of ovarian hormones, our studies revealed that PRL opposed estrogen-induced maturation of luminal MECs, increasing progenitor activity as demonstrated in the CFC assay and expanding the $CD61^+$ luminal subpopulation. This was associated with PRL-induced alterations in levels of transcripts for key regulators of these differentiation pathways, including reduced levels of *Gata3* and *Cebpb* mRNAs, which promote differentiation, and higher *Sox9*, *Rank*, and *Wnt4* mRNAs, which augment stem/progenitor subpopulations (Asselin-Labat et al., 2007; Cordero et al., 2016; Joshi et al., 2015; Kouros-Mehr et al., 2006; Malhotra et al., 2014; Robinson et al., 1998; Seagroves et al., 1998). Interestingly, our studies also revealed that PRL in combination with estrogen increased *Rank* mRNA. In light of the importance of Rankl-Rank signals and crosstalk with PRL-initiated pathways to coordinate physiologic alveologenesis and lactogenesis (Cordero et al., 2016), and their emerging role in breast cancer (Yoldi et al., 2016), PRL-estrogen interactions to this signaling pathway deserve further study.

Progesterone, acting on estrogen-induced PR, has been shown to increase mammary stem cell activity in multiple studies, and WNT4 and RANKL have been implicated as mediators in this process (Fernandez-Valdivia and Lydon, 2012; Joshi et al., 2010; Rajaram et al., 2015). Here, local PRL further raised functional stem and progenitor cell activity in combination with estrogen/progesterone. In this hormonal context, PRL reduced transcripts encoding GATA3, predicted to reduce luminal maturation, which was associated with an increase in the $CD61^+$ luminal subpopulation. PRL also altered secreted modulators of Wnt signaling to elevate canonical Wnt activity (Clevers and Nusse, 2012), without significantly altering *Wnt4* or *Rank*/*Rankl* mRNAs. These changes were reflected in increased expression of luminal cytokeratins in cells with basal surface markers, visualized as double-positive MECs in the basal layer resembling the Wnt-responsive bipotent stem cells revealed by lineage tracing (Rios et al., 2014; van Amerongen et al., 2012).

However, our experimental paradigm did not reveal cooperation between PRL and estrogen/progesterone on expression of RANKL and ELF5, drivers of alveolar commitment and differentiation that are regulated by crosstalk among these hormones during pregnancy (reviewed in Lee and Ormandy, 2012). Instead, progesterone was the

dominant driver of both genes, compatible with the described progesterone-RANKL-ELF5 cascade (Lee et al., 2013). Consistently, glands of NRL-PRL females treated with estrogen/progesterone contained a higher proportion of CD61$^+$ luminal cells compared with similarly treated WT females, in contrast to the ELF5-induced reduction in this population (Lee et al., 2013). Our studies also revealed that PRL preferentially increased expression of the shorter PR-A isoform. The PR-A and PR-B isoforms are differentially expressed during physiologic states (Aupperlee et al., 2005; Mulac-Jericevic et al., 2003). Phosphorylation of sites unique to PR-B or near the N-terminal region common to both isoforms modify the scope of progestin target genes (reviewed in Grimm et al., 2016), and mice lacking PR-B were unable to activate RANKL signaling (Mulac-Jericevic et al., 2003), suggesting the intriguing possibility that PRL-induced changes in the balance of PR isoforms may play a role in the failure of PRL and estrogen/progesterone to cooperatively alter RANKL/ELF5 expression. Taken together, these results suggest that PRL acts in this model to modulate the responsiveness of the "hormone-sensing" luminal MEC compartment, rather than the ER-alveolar lineage promoted by ELF5.

Our findings offer clues to the association between PRL exposure and higher risk for development of ERα^+ breast cancers (Tikk et al., 2014b; Tworoger et al., 2013; Tworoger and Hankinson, 2008). The PRL-induced increase in MEC progenitor/stem subpopulations aligns with a current hypothesis that these cells may be cells of origin for tumors. These long-lived cells would be able to accumulate mutations and generate large numbers of daughter cells, compatible with the time frame for tumorigenesis (Lim et al., 2009; Molyneux et al., 2010). Luminal progenitors, in particular, are receiving attention as cells of origin for some breast cancers, supported by studies of mouse models and emerging data from patient samples (reviewed in Sreekumar et al., 2015; Visvader and Stingl, 2014). Our data revealed that PRL inhibited transcriptional programs critical for maturation of these cells, resulting in expansion of this susceptible MEC subpopulation. Furthermore, the PRL-induced expression of the PR-A isoform and co-activators for ERα suggest mechanisms underlying the cooperation of PRL with ovarian steroids and hormone replacement therapies to increase breast cancer risk in postmenopausal women (Tikk et al., 2014b). Overexpression of PR-A, but not PR-B, in mouse models leads to hyperplasia (Shyamala et al., 1998) compatible with the tumorigenesis observed in NRL-PRL females. Moreover, the estrogen-induced PR-A expression in ductal epithelia of nonparous females (Aupperlee et al., 2005) supports the association between PRL and ERα^+ ductal carcinomas identified in epidemiologic studies (Tikk et al., 2014b; Tworoger et al., 2013; Tworoger and Hankinson, 2008). A growing body of literature documents higher expression of PR-A in more aggressive breast cancers and resistance to anti-estrogen therapies (reviewed in Diep et al., 2015; Grimm et al., 2016). Together, our findings illuminate the role of PRL and crosstalk with ovarian steroids in the regulation of the mammary epithelial hierarchy, and raise new hypotheses concerning its role in the risk for breast cancer.

EXPERIMENTAL PROCEDURES

Mice

NRL-PRL mice (line 1647-13, TgN(Nrl-Prl)23EPS), which secrete transgenic rat PRL from mammary epithelia, were maintained in the FVB/N strain background. Transgenic (NRL-PRL) and WT FVB/N mice were housed and handled in accordance with the Guide for Care and Use of Laboratory Animals in AAALAC-accredited facilities. All procedures were approved by the University of Wisconsin-Madison Animal Care and Use Committee.

Manipulation of Ovarian Steroid Hormone Activity

For some experiments, activity of ovarian steroids was manipulated. After pubertal ductal elongation was complete (8–10 weeks of age), females were ovariectomized, and left as controls (Ovx) or treated with the estrogen receptor antagonist, ICI 182,780 (ICI; 167 mg/kg/week subcutaneously), supplemental 17β-estradiol (E; silastic capsules containing 20 μg of 17β-estradiol [Arendt et al., 2009]), or supplemental 17β-estradiol together with progesterone (E + P; 20 μg of 17β-estradiol and 20 mg of progesterone). Glands were harvested after 4 weeks of treatment for analysis.

Histologic and Immunocytochemical/Fluorescent Analyses

Cranial mammary glands were fixed, embedded in paraffin, and sectioned as described in Supplemental Experimental Procedures. Morphology was assessed on H&E-stained slides. Sections were immunostained for ERα, PR, and cytokeratins as described.

Analysis of Isolated MECs

MECs were examined by flow cytometry, fluorescence-activated cell sorting, transplantation analyses, colony-forming assays, or transcript analyses (Figure S5, see Supplemental Experimental Procedures). For flow cytometry, cells were labeled with the following antibodies: CD24-PE, CD49f-FITC, CD31-Pacific Blue, CD45-Pacific Blue, and in some cases CD61-APC. CD45 and CD31 were used to deplete endothelial cells and lymphocytes, termed Lin$^+$ cells. The gating strategies are shown in Figure S6.

To assess CFCs, an assay for progenitor activity, we plated freshly isolated MECs onto 2.5 × 10^5 irradiated NIH-3T3 feeder cells (see Supplemental Experimental Procedures). To assess stem cell activity, we quantified mammary repopulating units (MRUs) by limiting dilution transplantation (see Supplemental Experimental Procedures). MECs from nontransgenic FVB/N and NRL-PRL donors were transplanted into contralateral glands to control for potential differences in recipients, and epithelial outgrowths were assessed after 6 weeks. The frequency of MRUs was calculated

by the method of maximum likelihood (http://bioinf.wehi.edu.au/software/limdil) (Hu and Smyth, 2009).

RNA was purified from isolated MECs using RNeasy kits (Qiagen, Valencia, CA), or from MECs sorted directly into TRIzol LS (Life Technologies, #10296-028), and real-time qRT-PCR reactions were carried out as described in Supplemental Experimental Procedures. Primers used are shown in Table S1.

Statistical Analyses

Statistical analyses were performed using Prism v.5 (GraphPad Software, San Diego, CA). Differences were considered significant at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.08.011.

AUTHOR CONTRIBUTIONS

K.A.O. contributed to the conception, design, execution, and analysis of all experiments and writing of the manuscript. M.P.S. contributed to the design, surgery, and analysis of experiments. S.S. contributed to surgery and histological analysis. C.E.B. contributed to surgery, flow cytometry, and histological analysis. L.A.S. contributed to the conception, design, and interpretation of experiments, manuscript writing, and funding.

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