Ovarian hormones are not required for PRL-induced mammary tumorigenesis, but estrogen enhances neoplastic processes

Lisa M Arendt, Lindsay C Evans, Debra E Rugowski, Maria Jose Garcia-Barchino¹, Hallgeir Rui¹ and Linda A Schuler

Department of Comparative Biosciences, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA
¹Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Philadelphia 19107, USA

(Correspondence should be addressed to L A Schuler; Email: schulerl@svm.vetmed.wisc.edu)

(L M Arendt is now at Tufts University School of Medicine, Boston, Massachusetts 02111, USA)

(M J Garcia-Barchino is now at Genetics Department, Navarra University, Pamplona, Spain)

Abstract

Epidemiologic studies have demonstrated that increased prolactin (PRL) exposure raises the risk of invasive estrogen receptor α (ERα)-positive breast cancer in women. However, the mechanism(s) whereby this occurs and the interactions with estrogen itself in this disease remain poorly understood. In order to investigate the role of ovarian hormones in the disease process, we employed a transgenic model neu-related lipocalin (NRL)–PRL in which transgenic PRL is directed to mammary epithelial cells by the PRL- and estrogen-insensitive NRL promoter, mimicking the endogenous PRL expression within the breast observed in women. This high local exposure leads to mammary lesion development and eventually carcinomas. Ovariectomy (ovx), shortly after puberty, did not alter the incidence or latency of PRL-induced mammary carcinomas, consistent with the independence of PRL from circulating estrogens as a risk factor for invasive breast cancer in women. However, chronic estrogen administration to ovx NRL–PRL females decreased the latency of both ERα-positive and -negative tumors. We identified multiple mechanisms that may underlie this observation. Elevated estrogen exposure cooperated with PRL to increase epithelial proliferation and myoepithelial abnormalities, increasing the incidence of preneoplastic lesions. Critical components of the extracellular matrix secreted by the myoepithelium were reduced with age, and transgenic PRL raised transcripts for tenascin–C and maspin, both associated with tumor progression and poor prognosis in subclasses of clinical breast tumors. Mammary pERK1/2 and pAkt, but not phosphorylated Stat5, were markedly elevated by local PRL. Together, these findings indicate that PRL employs multiple mechanisms to promote mammary tumorigenesis.


Introduction

Although prolactin (PRL) is critical for physiologic development and differentiation of the mammary gland, its role in breast cancer remains poorly understood. Small early clinical studies and species differences in the sites of expression of this cytokine/hormone, which include the mammary gland itself in women (Clevenger et al. 2003, Zinger et al. 2003), obscured its role in the human disease. However, recent epidemiologic studies and experimental models have pointed to an important role for PRL in the development and progression of breast cancer (for reviews, Arendt & Schuler 2008, Tworoger & Hankinson 2008). Moreover, the expression of the PRL receptor (PRLR) in a majority of clinical tumors (for reviews, Ginsburg & Vonderhaar 1995, Clevenger et al. 2003, Tworoger & Hankinson 2008), and limited phenotype of the PRLR knockout mice apart from the mammary gland (Goffin et al. 2002) suggest that insight into the pathogenic activities of PRL may lead to preventative and therapeutic approaches with minimal side effects.

Epidemiologic studies have demonstrated a high correlation between circulating PRL and the risk of breast tumors that express estrogen receptor α (ERα also known as ESR1), which is independent of levels of circulating estrogen (for review, Tworoger & Hankinson 2008). When assay variability is taken into account, PRL exposure confers a risk only slightly weaker than that for estrogen itself (Tworoger & Hankinson 2006). PRL promotes estrogenic signals in a variety of experimental systems: it increases ER expression (Edery et al. 1985, Frasor & Gibori 2003, Gutzman et al. 2004a), and cooperatively activates the AP-1 transcriptional enhancer (Gutzman et al. 2005). These activities suggest potential interactions between estrogen and PRL in the pathogenesis of this disease.

In order to study the dynamic processes whereby PRL contributes to breast cancer, we have developed a novel transgenic mouse in which PRL is directed to mammary epithelial cells by a PRL- and estrogen-independent
promoter, neu-related lipocalin (NRL; Rose-Hellekant et al. 2003, Arendt & Schuler 2008). This local expression mimics that in normal mammary tissue (Clevenger et al. 2003, Zinger et al. 2003) and primary tumors in women (McHale et al. 2008). NRL–PRL nonparous females develop preneoplastic lesions, and eventually diverse, aggressive tumors after a long latency, similar to the human disease. Unlike most mouse models, many of these tumors express ERα, like the majority of clinical tumors in women (Rose-Hellekant et al. 2003). Both the ERα-positive (ERα+) and ERα-negative (ERα−) tumors that develop in these mice display transcript profiles similar to the luminal subtype of tumors observed in women (L M Arendt, personal communication). This model permits us to investigate the effects of hormonal milieu on the multiple cell types and structures that comprise this complex tissue over time. Here we have examined the interplay of aging and estrogen exposure on PRL-induced lesions. Our findings demonstrate that PRL can induce mammary tumors even in the postpubertal absence of ovarian hormones, associated with loss of integrity of the myoepithelial layer. However, estrogen can contribute to the disease process via multiple mechanisms, including augmenting proliferation of epithelial cells, and promoting abnormalities in the myoepithelium with age. These studies elucidate important hormonal interactions in breast cancer development and progression.

**Materials and Methods**

**Reagents**

5-Bromo-2-deoxyuridine (BrdU) was obtained from Sigma Chemical Co. and 17β-estradiol (E2) was purchased from Steraloids, Inc (Newport, RI, USA). The following antibodies were used for immunohistochemical analyses: pStat 5 (AX1) from Advantx BioReagents, LLP (El Paso, TX, USA), BrdU (MAS-250) from Accurate Scientific (Westbury, NY, USA), (ERα; SC-542) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), α-smooth muscle actin (α-SMA; A2547) from Sigma Chemical Co. ERK1/2 (9102) and phospho-ERK1/2 (Thr202/Tyr204, 9101) and Akt (9272) and pAkt S473 (9271) from Cell Signaling Technology (Beverly, MA, USA), and cytokeratin 8 (K8; RB-9095) was obtained from Lab Vision (Fremont, CA, USA).

**Genotyping mice**

FVB/N strain NRL–PRL mice (line 1647-13, TgN(Nrl–Prl)23EPS; line 1655-8, TgN(Nrl–Prl)24EPS) were generated and genotyped as described (Rose-Hellekant et al. 2003). Mice were housed and handled in accordance with the Guide for Care and Use of Laboratory Animals in AAALAC-accredited facilities, and all procedures were approved by the University of Wisconsin–Madison Animal Care and Use Committee.

**Ovariectomy and treatment with E2**

For some experiments, nonparous NRL–PRL and non-transgenic female mice were ovariectomized (ovx), subjected to sham surgery, or ovx and treated with E2 beginning at 12 weeks of age. For the latter, females received silastic capsules containing 20 μg E2, which were replaced every 6 weeks for the length of the experiment, maintaining circulating E2 levels at approximately those of estrus, as described (Medina et al. 2003). Animals were observed weekly for tumor development and considered to be end stage when tumor diameter reached 1·5 cm.

**Histological examination of mammary tissue**

Mammary tissue was fixed in 10% neutral buffered formalin for 18–24 h, embedded in paraffin, and cut into 6 μm sections. Mice were injected with 200 mg/kg body weight BrdU 1 h prior to killing to label proliferating cells. Cells containing BrdU and ERα were detected using immunohistochemistry (IHC) and quantitated as previously described (Rose-Hellekant et al. 2003). For detection of pStat 5, deparaffinized slides were rehydrated, boiled in citrate buffer, pH = 6·0, for antigen retrieval, blocked in 3% horse serum in PBS, and incubated with primary antibody diluted 1:750 overnight (Nevalainen et al. 2002). Signal was amplified using ImmPRESS anti-mouse Ig kit (MP-7402; Vector Laboratories, Burlingame, CA, USA). Histological structures were identified as described (Rose-Hellekant et al. 2003). One thousand cells in morphologically normal ducts, alveoli, and epithelial hyperplasias (EH) were analyzed from five females of each genotype. Immunohistochemical indices were statistically analyzed using the Kruskal–Wallis test followed by Mann–Whitney post test.

**Immunofluorescence**

Deparaffinized slides were rehydrated and exposed to 0·5% H2O2 in H2O to block endogenous peroxidase activity, boiled for 15 min in 0·1 M Tris buffer, pH 9·0, for antigen retrieval, then blocked in 1:100 rabbit serum in Tris-buffered saline–Tween20 (TBST). Slides were incubated with ERα antibody (1:500) for 1 h, rinsed, and incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:400) overnight (Molecular Probes, Eugene, OR, USA). Slides were rinsed, blocked, and incubated with BrdU (1:20), α-SMA (1:2500), or K8 (1:100) antibodies for 1 h, rinsed, and incubated with Texas Red conjugated goat anti-rabbit secondary antibody (1:400; Vector Laboratories) for BrdU and K8 or Alexa Fluor 568-conjugated goat anti-mouse secondary antibody (1:400; Molecular Probes) overnight.Slides were rinsed and mounted using VECTASHIELD mounting media with DAPI (Vector Laboratories). Images were aligned and quantitated using ImageJ v 1.36b (National Institutes of Health).
Western analyses

Western analyses were performed as previously described (Arendt et al. 2006). In brief, 30 μg of mammary gland homogenate from 12-week-old nonparous females was fractionated by standard Laemmli SDS-PAGE, transferred to polyvinylidene fluoride membranes, and then probed with antibodies as shown. Signals were visualized by enhanced chemiluminescence, followed by autoradiography.

Quantitative real-time PCR

cDNA was synthesized from 1 μg RNA using random hexamers (Amersham Biosciences) and MMLV reverse transcriptase (Promega) as described (Gutzman et al. 2007). PCR was performed in a 25 μl reaction volume of 1 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 3 μl cDNA sample, and 300 nM of each primer (120 nM for 18S primers), using the primers shown in Supplementary Table 1, available in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/cgi/full/content/JOE-09-0221/DC1. Reactions were cycled for 15 min at 95 °C for 15 s/60 °C for 1 min on the Applied Biosystems 7300 real-time PCR system. Results were calculated using the comparative Ct method and normalized to 18S RNA, and analyzed for statistical significance using the nonparametric Mann–Whitney test.

Statistical analyses

Statistical analyses were performed using Prism v.4.03 (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered significant at P<0.05.

Results

Estrogen treatment enhances PRL-induced tumorigenesis

Studies in breast cancer cells in vitro have demonstrated cooperative interactions between PRL and estrogen at many levels. To elucidate the contribution of circulating estrogen to PRL-induced mammary tumorigenesis in vivo, we altered the ovarian hormonal environment of nonparous 12-week-old NRL–PRL females and nontransgenic littermates by either ovx, sham surgery, or ovx followed by continuous administration of E2 to about normal estrus levels. Sham-treated females developed mammary carcinomas of diverse histotypes with a latency similar to our previous study (Fig. 1A, Supplementary Table 2, available in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/cgi/full/content/JOE-09-0221/DC1, Rose-Hellekant et al. 2003). Ovx did not alter tumor incidence or latency. However, E2 significantly shortened the latency to tumor development, without altering incidence (Supplementary Table 2, Fig. 1A, P=0.02). Ovx females that were continuously treated with E2 for this prolonged period exhibited generally poorer health than age-matched sham or ovx females, particularly urinary tract abnormalities (Pearse et al. 2009). This resulted in earlier mortality independent of mammary tumors than the other treatments, which may have obscured an increase in incidence with estrogen administration. Ovarian hormonal status did not favor the development of a specific tumor histotype (Supplementary Table 2). Levels of ERα expression were highly variable among tumors in all treatment groups and also were not correlated with specific carcinoma histotypes (Fig. 1B). Tumor latency for ERα+ and ERα− tumors did not differ, regardless of treatment group; E2 treatment significantly decreased latency of both tumor subclasses (P<0.05; one-way ANOVA, Tukey–Kramer post test). These results suggest that although postpubertal ovarian hormones are not necessary for PRL–induced tumor development, PRL and E2 can cooperatively enhance mammary oncogenesis. Furthermore, this interaction does not appear to be mediated by ERα within epithelial cells of established tumors, although this does not preclude a role for this site of crosstalk at an earlier stage in oncogenesis.
Table 1 Histologic mammary abnormalities in aged<sup>a</sup> neu-related lipocalin–prolactin (NRL–PRL) and nontransgenic FVB/N females

<table>
<thead>
<tr>
<th></th>
<th>NRL–PRL (ovx)</th>
<th>sham</th>
<th>ovx-E&lt;sub&gt;2&lt;/sub&gt;</th>
<th>FVB/N (ovx)</th>
<th>sham</th>
<th>ovx-E&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial hyperplasias</td>
<td>7/33</td>
<td></td>
<td>13/25&lt;sup&gt;*&lt;/sup&gt;</td>
<td>10/30</td>
<td></td>
<td>2/9</td>
</tr>
<tr>
<td>MIN</td>
<td>9/33&lt;sup&gt;*&lt;/sup&gt;</td>
<td>12/25&lt;sup&gt;†&lt;/sup&gt;</td>
<td>21/30&lt;sup&gt;*,&lt;/sup&gt;</td>
<td>0/9</td>
<td>0/9</td>
<td>0/8</td>
</tr>
<tr>
<td>Squamous changes</td>
<td>3/33&lt;sup&gt;*&lt;/sup&gt;</td>
<td>9/25</td>
<td>9/30</td>
<td>2/9</td>
<td>5/9</td>
<td>1/8</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>10/33&lt;sup&gt;†&lt;/sup&gt;</td>
<td>10/25&lt;sup&gt;†&lt;/sup&gt;</td>
<td>11/30&lt;sup&gt;†&lt;/sup&gt;</td>
<td>1/9</td>
<td>0/9</td>
<td>1/8</td>
</tr>
</tbody>
</table>

MIN, mammary intraepithelial neoplasia.  *Significantly different than in other treatment groups of the same genotype using the χ² test, P<0.05;  †Significantly higher in NRL–PRL females than in nontransgenic littermates in the same treatment groups using the χ² test, P<0.05.

<sup>a</sup>NRL–PRL females were 19-3 ± 1.1 months old, when the tumor reached 1.5 cm in diameter; nontransgenic FVB/N females were examined at 24 months of age in the absence of tumor development.

Estrogen and PRL augment the development of preneoplastic lesions

To determine the interactions of these factors in lesion development, we histologically examined glands of nontransgenic and NRL–PRL females subjected to these prolonged manipulations of estrogen levels. Both NRL–PRL and nontransgenic females developed multiple abnormalities with age, with substantial variability among individuals (Table 1). Interestingly, EH were found in more intact females of both genotypes, suggesting a role for other ovarian factors, such as progesterone. We have previously shown that the EH that develop in NRL–PRL females express higher levels of ER<sub>α</sub> than those in nontransgenic females (Rose-Hellekant et al. 2003, Arendt et al. 2006). PRL-induced EH exposed to sustained E<sub>2</sub> in the current study exhibited a higher rate of proliferation, as assessed by the proportion of cells exhibiting BrdU incorporation (ovx: 6.9 ± 0.3%; ovx+E<sub>2</sub>: 9.9 ± 0.6%; mean ± S.E.M., P<0.0001 by Student’s t-test). In addition, mammary intraepithelial neoplasia (MIN), which resemble ductal carcinoma in situ (DCIS) in women, commonly developed in NRL–PRL females with all treatments but not nontransgenic females. The incidence of these lesions was significantly enhanced by E<sub>2</sub> (P=0.033; Table 1).

These data indicate that postpubertal ovarian hormones are not required for the development of PRL–induced mammary lesions, similar to effects on tumorigenesis. Consistent with previous reports in this species (Shimkin 1948, Nandi et al. 1995), estrogen alone did not induce lesions in nontransgenic females. However, E<sub>2</sub> can enhance the development and/or proliferation of PRL–induced preneoplastic lesions, which may contribute to the reduction in tumor latency observed.

Epithelial proliferation increases with age and E<sub>2</sub> exposure

Estrogen is believed to regulate proliferation via paracrine factors, since ER<sub>α</sub>-expressing cells in women or mouse models rarely contain evidence of this activity (Clarke et al. 1997, Shyamala et al. 2002). However, as women age, the proportion of cells that express ER<sub>α</sub> increases and colocalization of ER<sub>α</sub> with proliferation markers occurs more frequently (Shoker et al. 1999). In order to examine these events in the aging mouse and effects of long-term alterations in PRL and E<sub>2</sub> exposure on this process, we quantified mammary proliferation using BrdU incorporation, and ER<sub>α</sub> expression by IHC in nontransgenic and NRL–PRL nonparous females at 6 and 24 months of age. As shown in Fig. 2A, proliferation of ductal epithelium was significantly higher in older nontransgenic females, compared with those at 6 months of age. Manipulation of available estrogen by either ovx or ovx followed by continuous administration of E<sub>2</sub> demonstrated a strong dependence on available estrogen. Consistent with our earlier reports (Arendt & Schuler 2008), transgenic PRL increased ductal proliferation (Fig. 2A and B). The rate in NRL–PRL glands was significantly greater than that in nontransgenic glands at both ages and treatments, except for the elderly sham-treated animals. The reasons for this are unclear; these data may reflect inhibitory factors produced by the intact aged ovary.

Aging alters ER<sub>α</sub> expression

Although proliferation was enhanced, ER<sub>α</sub> levels were not significantly altered with aging in sham–treated nontransgenic (Fig. 2C) or NRL–PRL females (Fig. 2D). Exposure to ligand downregulates ER<sub>α</sub> (Alarid 2006); consistently, E<sub>2</sub>-treated ovx females of both genotypes exhibited lower numbers of ER<sub>α</sub>-expressing cells than ovx females at both 6 months and 2 years of age. However, the extent of this reduction was substantially greater in the younger females: E<sub>2</sub>-treatment reduced ER<sub>α</sub> by about 50% at 6 months in both genotypes but <25% in aged females. This dysregulation of proliferation and ER<sub>α</sub> expression with age resembles reports in women (Clarke et al. 1997), and suggests that ER<sub>α</sub>-expressing cells may also proliferate more frequently in aged mice. To directly examine this question, we examined colocalization of BrdU with ER<sub>α</sub> expression using immunofluorescence. However, any effect was obscured by high variability among individuals (data not shown).

Prolonged E<sub>2</sub> administration alters the pattern of ER<sub>α</sub> expression in elderly mice and reduces the integrity of the myoepithelial layer

In light of the reduced downregulation of ER<sub>α</sub> in the elderly ovx mice receiving E<sub>2</sub>, we investigated the location of this receptor. In the normal mammary epithelium, ER<sub>α</sub> and
progesterone receptor (PR) are expressed together within luminal epithelial cells (for review, Anderson et al. 2000, Shyamala et al. 2002). In glands of elderly ovx and sham-treated females of both genotypes, as well as 6-month-old females regardless of genotype or treatment, ERz labeling was detected in a subset of ductal cells (Fig. 3A). In these glands, ERz-labeled nuclei (green) were centrally located in cells expressing the luminal marker, cytokeratin 8 (K8; red, Fig. 3C). PR was similarly located (not shown). The ducts in these females were surrounded by a continuous layer of myoepithelial cells, detected with the marker, (α-SMA; Fig. 3E). However, long-term E2 administration to ovx females shifted ERα (Fig. 3B) expression to a more basal pattern by 2 years of age. Glands of NRL–PRL females exhibited a similar pattern (data not shown), indicating that this was independent of PRL status. Despite this altered position, ERα-staining cells also expressed K8, indicating that these cells were of the luminal lineage (Fig. 3D). However, in these aged E2-treated females, the α-SMA staining was no longer continuous around the ducts, suggesting abnormalities in the myoepithelial layer (Fig. 3F).

Myoepithelial cells surround the ducts and alveoli, forming a structural barrier between the luminal epithelial cells and surrounding stroma. They secrete components of the basement membrane, which contribute to signals maintaining epithelial integrity and hormonal responsiveness, including ERα expression (Gudjonsson et al. 2002, Haslam & Woodward 2003, Novaro et al. 2003, Naylor et al. 2005). In order to determine whether the apparent abnormalities in the myoepithelial layer of E2-treated aged females were associated with altered levels of key components of the extracellular matrix (ECM), we compared levels of transcripts for some of the molecules implicated in these activities using quantitative real-time (qRT)-PCR. Laminin 1α mRNA, which is expressed exclusively by myoepithelial cells (Kenny & Bissell 2003), was significantly decreased in glands of E2-treated 2-year-old nontransgenic females, compared with those of 6-month-old females with the same treatment, consistent with the morphologic changes (Fig. 3G). Similarly, collagen type IV α1a mRNA was strikingly reduced in 4/5 of the glands of E2-treated nontransgenic females with age (Fig. 3H).

PRL alone accelerates loss of the myoepithelial layer associated with increases in transcripts of genes implicated in tumor invasion.

In order to determine whether PRL can modulate the integrity of the myoepithelial layer, we compared the effect of age and E2 availability in nontransgenic females and
NRL–PRL mice. Nontransgenic mice at 6 months of age with all treatments demonstrated continuous α-SMA staining (Fig. 4A–C), which was only slightly reduced in the 24-month-old ovx and sham-treated females (Fig. 4D and E). However, as noted above, α-SMA staining in E2-treated ovx aged FVB/N females demonstrated generalized discontinuities (Figs 3F and 4F). In contrast, although glands of 6-month-old ovx NRL–PRL mice displayed normal α-SMA distribution (Fig. 4G), glands of both sham- and E2-treated NRL–PRL females were already abnormal at this age (Fig. 4H and I). In the older NRL–PRL females, α-SMA staining became markedly abnormal with all treatments (Fig. 4J–L). The reduction in the myoepithelial α-SMA-expressing cells relative to K8-expressing luminal cells was quantitatively confirmed by western analyses (Fig. 5A).

Myoepithelial cells also secrete many factors including protease and angiogenic inhibitors, which inhibit invasion and vascularization, contributing to their natural tumor suppressor activity (for reviews, Barsky & Karlin 2005, Faraldo et al. 2005, Gudjonsson et al. 2005, Polyak & Hu 2005). Because of the pronounced PRL-induced disruption of this layer, we compared levels of transcripts for some of the implicated mediators using qRT-PCR in aged sham-treated NRL–PRL and nontransgenic females. Tenascin-C is a component of the ECM, which interacts with integrins. Its expression is increased upon invasion through the basement membrane, and therefore it is used as a stromal marker for epithelial malignancies of various organs, including the breast (for reviews, Jones 2001, Orend & Chiquet-Ehrismann 2006). As shown in Fig. 5B, transcripts for this glycoprotein were elevated in aged NRL–PRL glands. Maspin is a serine protease inhibitor secreted by myoepithelial cells. Maspin is considered to be a component of myoepithelial tumor suppressor activity because it can inhibit invasion and angiogenesis and promote apoptosis (Barsky & Karlin 2005, Bailey et al. 2006). Surprisingly, maspin transcripts were also elevated in the NRL–PRL glands compared with nontransgenic glands (Fig. 5C). In contrast, levels of fibronectin mRNA, another ECM component synthesized by the stroma rather than myoepithelial cells, were not altered by PRL (Fig. 5D).

PRL increases phosphorylation of multiple signaling mediators

In order to better understand the signaling pathways whereby local PRL may contribute to these pathogenic processes, we examined mammary glands of 12-week-old nonparous females, prior to the development of detectable lesions. Stat5 is a critical mediator of PRL in alveologenesis, and mammary overexpression of Stat5 in mouse models reduces apoptosis at involution, and eventually results in tumors. Furthermore, high levels of tyrosine phosphorylated Stat5 (pStat5) are found in a subset of well-differentiated clinical tumors (Wagner & Rui 2008). To determine whether transgenic local PRL elevates pStat5, we examined glands of 12-week old nonparous intact females, prior to the...
development of any visible pathology. Surprisingly, nontransgenic and NRL–PRL females exhibited similar levels of pStat5 in mammary lysates by immunoblotting (data not shown). Even at 6 months of age, nuclear pStat5 was detectable in similar proportions of mammary epithelial cells (MECs) in nontransgenic and NRL–PRL glands by IHC (Fig. 6A). However, when glands of aged (18–24 month old) NRL–PRL females containing plentiful preneoplastic lesions were examined, levels of pStat5 were significantly higher in EH than morphologically normal structures (Fig. 6B), suggesting a role for this pathway in the development of some lesions.

PRL also activates other downstream mediators that have been implicated in breast cancer, and displays positive crosstalk with growth factors to these signaling cascades (Arendt et al. 2006, 2009, Carver & Schuler 2008). Approximately half of primary breast tumors in women express more activated ERK1/2 than surrounding tissue, although the relationship to clinical outcome is not clear (Santen et al. 2002, Milde-Langosch et al. 2005). Activation of the PI3K–Akt pathway also has been associated with breast cancer and anti-estrogen resistance (for reviews, Dillon et al. 2007, Liu et al. 2007). As shown in Fig. 6C and D, glands from 12-week-old NRL–PRL mice exhibited enhanced levels of both pERK1/2 and pAkt, compared with nontransgenic littermates. Both pERK1/2 and pAkt were detected by IHC in both epithelial cells and stroma, although pERK1/2 tended to be stronger in stroma (data not shown). These data suggest that PRL signals to these non-Jak2/Stat5 pathways also may contribute to early changes leading to tumor development.

**Discussion**

While increased PRL exposure raises the risk of invasive ERα+ breast cancer in women, the mechanism(s) whereby this occurs, and interactions with estrogen itself in this disease remain poorly understood. Using our mouse model of augmented mammary PRL exposure, we demonstrated that post-pubertal ovarian hormones are not necessary for PRL-induced lesion and tumor development, consistent with the independence of PRL as a risk factor for invasive breast cancer independent of circulating estrogens in women (Tworoger &

![Figure 4](https://example.com/image4.png)

Figure 4 PRL and 17β-estradiol diminish the myoepithelial layer surrounding morphologically normal structures. At 6 months of age, ovx (A), sham- (B) and ovx/E2-treated (C) nontransgenic mice demonstrated normal α-SMA staining around ducts. At 2 years of age, ovx/E2-treated (F) nontransgenic females exhibited decreased α-SMA staining, compared with those subjected to ovx alone (D) or sham surgery (E). Abnormalities in α-SMA staining were present in sham- (H) and ovx/E2- (I) treated NRL–PRL females at 6 months of age, compared with ovx NRL–PRL females (G) of the same age. Pronounced abnormalities were present in all treatment groups at 2 years of age (J–L) in NRL–PRL females, with the greatest discontinuities in the E2-treated group (L). Morphologically normal structures were stained for α-SMA (red) and counterstained with DAPI (blue) as described in Materials and Methods. Representative micrographs. Original magnification of all images: 600×.

![Figure 5](https://example.com/image5.png)

Figure 5 Transgenic PRL reduces the relative level of α-SMA: keratin 8 protein expression, and raises mammary tenascin C and maspin transcripts, without altering fibronectin 1. Protein homogenates and RNA from whole mammary glands of sham-treated 2-year-old nontransgenic (FVB) and NRL–PRL nonparous females were examined for levels of α-SMA and keratin 8 protein by western analyses (A), and myoepithelial products by qRT-PCR (B–D), as described in the Materials and Methods. (A) Data are expressed as the ratio between α-SMA and keratin 8 staining (mean ± S.D., N = 3). (B–D) Data are expressed as mean ± S.E.M., N = 5. Asterisk indicate a significant difference between genotypes by Student’s t-test (*P < 0.05; ***P < 0.0001).
Hankinson 2006). However, elevated estrogen exposure cooperated with PRL to increase epithelial proliferation and myoepithelial abnormalities, increasing the incidence of preneoplastic lesions, and decreasing the latency of both ER\(\alpha^+\) and ER\(\alpha^-\) mammary tumors. Critical components of the ECM secreted by the myoepithelium were reduced with age, and transgenic PRL raised transcripts for tenascin-C and maspin, both associated with tumor progression and poor prognosis in subclasses of clinical breast tumors. These studies suggest that myoepithelial cells may be important and understudied effectors of hormonal contributions to breast cancer, which may become increasingly important with age.

The potent oncogenic activity of PRL at the mammary gland is underscored by the ability of local overexposure to promote tumors even in the absence of post-pubertal ovarian hormones. However, chronic estrogen exposure significantly reduced the latency of PRL-induced tumors. Lifetime estrogen exposure is one of the strongest risk factors for development of invasive breast cancer in women (The Endogenous Hormones and Breast Cancer Collaborative Group 2002, Writing Group for the Women’s Health Initiative Investigators 2002, Eliassen et al. 2006). Although E\(2\) alone could not elicit preneoplastic hyperplasias or MINs, as previously observed in this species (Shimkin 1948, Nandi et al. 1995), our studies revealed multiple mechanisms by which E\(2\) can accelerate PRL-induced lesion development. ER\(\alpha\) and PRLR are expressed in both mammary epithelial and stromal cells (Bera et al. 1994, Hovey et al. 2001, Mueller et al. 2002); their coexpression in many epithelial cells (Grimm et al. 2002) suggests that intracellular crosstalk may contribute to this outcome (Gutzman et al. 2005, Silva & Shupnik 2007). The ability of E\(2\) to reduce the latency of both PRL-induced ER\(\alpha^+\) and ER\(\alpha^-\) tumors indicates that direct action on established tumor cells themselves is not required. However, the E\(2\)-induced increases in lesions and proliferation of normal and hyperplastic structures suggest that intracellular crosstalk may be important early but may be reduced with progression and loss of ER\(\alpha\) expression in some tumors. The significance of the E\(2\)-induced restriction of ER\(\alpha\) expression to a distinct subpopulation of luminal cells with age is not known. Green and colleagues noted a similar location of ER\(\alpha\) in MINs induced by transgenic C3(1) SV40Tag (Yoshidome et al. 2000). Prolonged chronic exposure to estrogen may promote expansion and/or stability of a distinct epithelial subpopulation. Some evidence suggests that a subpopulation of basally located cells may have progenitor cell characteristics (for reviews, Smith et al. 1984, Smith & Chepko 2001), although any relationship to ER\(\alpha\)

![Figure 6](image-url)

Figure 6 NRL–PRL glands exhibit elevated pStat5, pERK1/2, and pAkt. (A and B) Phosphorylated Stat5 (pStat5) was examined in morphologically normal structures of age-matched 6-month-old nontransgenic and NRL–PRL intact nonparous females (A), and normal structures and epithelial hyperplasias (EH) in glands of 2-year-old NRL–PRL females. (B) pStat5-labeled cells were detected and quantitated as described in Materials and Methods and expressed as mean ± S.D. Lower case letters denote significant differences using ANOVA followed by Student–Newman–Keuls multiple comparison test (\(P < 0.05\)). (C) Glands of NRL–PRL females at 12 weeks of age contain elevated pERK1/2 and pAkt, compared with nontransgenic littermates. Each lane represents mammary homogenate from a single 12-week-old NRL–PRL female or nontransgenic littermate (FVB), examined by immunoblotting as shown. (D) The ratio of phosphorylated: total kinase for each animal shown in (C) was calculated as described in the Materials and Methods. An asterisk indicates a significant difference between genotypes by a one-tailed Student’s t-test (\(*P < 0.05\)).
expression is controversial (Asselin–Labat et al. 2006, Booth & Smith 2006, Sleeman et al. 2007). The growing repertoire of tools to characterize these discrete cell subpopulations will enable further characterization of the ERa+ cells identified here, and implications for actions of other neoplastic factors, such as PRL. E2 also enhanced the development of age-dependent abnormalities in the myoepithelial layer, as discussed further below. Although myoepithelial cells do not express ERa, they do express ERβ (Barsky & Karlin 2005), suggesting possible direct and/or indirect effects on these cells. Finally, E2 may promote mammary lesions via targets outside the mammary gland. Emerging evidence from several laboratories has demonstrated that elevated systemic E2 can mobilize bone marrow-derived stromal and endothelial progenitors to enhance the growth, stromal infiltration, and vascularization of transplanted mammary tumors, including those that do not express ER. (Gupta et al. 2007, Suriano et al. 2008).

The myoepithelial layer is an important determinant of the mammary microenvironment, secreting components of the ECM that modulate responsiveness to many endocrine/paracrine factors, including hormones. The importance of these cells in maintaining epithelial organization and preventing invasion and migration has earned them recognition as natural tumor suppressors (Adriance et al. 2005, Barsky & Karlin 2005, Polya & Hu 2005). We found that the structural integrity of this layer diminished with age, associated with lower expression of laminin-1α and collagen IV. These key components of the ECM not only regulate hormonal responsiveness of the breast (Haslam & Woodward 2003, Novaro et al. 2003, Naylor et al. 2005) but also decline with tumorigenesis (Nakano et al. 1999, Maatta et al. 2001, Gudjonsson et al. 2002). Local PRL dramatically hastened the loss of this layer. PRL elicited these changes even in ovx females, although estrogen could enhance the loss. Whey acid protein (WAP)–PRL Balb/c females, in which transgenic PRL expression is elevated during pregnancy, also develop myoepithelial abnormalities (Manhes et al. 2006). The ability of PRL to induce this pathologic feature in markedly different hormonal milieus in distinct mouse strains confirms the potency of this action.

In the current study, PRL-induced elevation of the myoepithelial transcripts, tenasin C and maspin, was readily apparent even in highly heterogeneous whole mammary glands. Tenasin-C is strongly expressed in the stroma in areas of wound healing, inflammation and neoplasia (for reviews, Jones 2001, Orend & Chiquet-Ehrismann 2006). Its activation of integrin-mediated signals influences growth factor receptor expression and activation, and the cytoskeleton. It can also augment vascularization, associated with increased expression of proteases. These activities are consistent with its association with increased metastases in mouse models (Calvo et al. 2008) and clinical tumors (Minn et al. 2005). The PRL-induced increase in its expression in our studies indicates functional breeches in the barrier imposed by the myoepithelium and basement membrane, and suggests that this may contribute to lesion progression. Our finding that maspin transcripts also were elevated in NRL–PRL glands is in seeming conflict with the portrait of this gene as a tumor suppressor (Barsky & Karlin 2005, Bailey et al. 2006). However, recent reports indicate that elevated maspin mRNA may be a negative prognostic indicator for patients with node negative breast cancer (Bieche et al. 2003, Tsoli et al. 2007), suggesting a more complicated biological role for this protein. Interestingly, other transcripts elevated in myoepithelial cells of DCIS lesions in women (Polayk & Hu 2005), including insulin-like growth factor–2 (IGF–2), SOCS–3 and the AP–1 proteins, JunD and c-Fos, have been reported to be elevated by PRL at mammary targets (Tam et al. 2001, Brisen et al. 2002, Hovey et al. 2003, Gutzman et al. 2004b). Together, our data suggest that effects on myoepithelial cells may contribute to the oncogenic actions of PRL. The exacerbation of these myoepithelial abnormalities with age and estrogen exposure supports further investigation of the effect of post-menopausal hormonal therapies on this mammary subpopulation.

Our examination of activated mediators revealed that prolonged exposure to elevated PRL enhances a complex matrix of signaling pathways. Surprisingly, morphologically normal structures in NRL–PRL glands did not display elevated pStat5, the well-characterized mediator of most PRL-induced physiologic mammary growth and differentiation (Wagner & Rui 2008). This difference from the response to acute administration (Nevalainen et al. 2002) may result from the temporally constant exposure in this transgenic model, or a shift in the spectrum of pathways activated by autocrine PRL. The modest rise in pStat5 in HE in elderly NRL–PRL females suggests that this pathway may play a role in the proliferation of these preneoplastic lesions (Wagner & Rui 2008). However, our observation that post-pubertal ovarian hormones are not required for PRL-induced lesions, in combination with the recent report that ovarian hormones, but not PRL, are necessary for mammary Stat5 expression (Santos et al. 2008), suggests that Stat5 is not the primary mediator of PRL-promoted oncogenesis. In contrast, transgenic PRL markedly elevated pERK1/2 and pAkt in glands of relatively young females (12-weeks-old). These kinases are major downstream effectors of PRL in some breast cancer cell lines in vitro (Gutzman et al. 2007); in the intact mammary gland, the elevated phosphorylation that we observed may indicate direct activation via the PRLR, or indirect stimulation via secondary paracrine factors. Of note, elevated tenasin–C facilitates ERK1/2 phosphorylation, which can itself stimulate tenasin–C expression (Orend & Chiquet-Ehrismann 2006). Although myoepithelial PRLR has not been reported, identification of target cells that may express low levels of this receptor has been hampered by the lack of antibodies which recognize the murine PRLR. Both ERK1/2 and Akt are nodes of potent crosstalk between PRL and other oncogenic growth factors, such as TGFα/EGF (Arendt et al. 2006, 2009, Huang et al. 2006) and IGF–1 (Carver & Schuler 2008) in breast cancer cells in vitro.
These kinases are implicated in many processes in mammary tumorigenesis and progression, including proliferation, survival, the epithelial to mesenchymal transition and altered relationship with the ECM, and invasion (Vivanco & Sawyers 2002, Sebolt-Leopold & Herrera 2004, Turley et al. 2008), as well as treatment resistance (Svensson et al. 2005, Liu et al. 2007).

Transgenic PRL promotes development of murine mammary tumors that resemble the luminal-type tumors in women (Arendt et al. ms in prep). Its ability to potently stimulate proliferation of luminal epithelial cells (Rose-Hellekant et al. 2003, Arendt et al. 2006, Oakes et al. 2007) suggests one component of its oncogenic activity. Our data indicate that effects on nonluminal components, including the myoepithelial cell layer, may also play roles in disease processes. In clinical oncogenesis, PRL is likely to cooperate with other neoplastic factors, including estrogen, as shown herein, as well as growth factors (Arendt et al. 2006). Interestingly, PRL in combination with TGFβ reduces responsiveness to E2 in mouse models in vivo (Arendt et al. 2009), pointing to misunderstood complexities among these factors. Understanding the actions of PRL and the interplay with other factors implicated in mammary tumorigenesis will lead to improved preventative and therapeutic strategies for this pervasive and devastating disease.

Declaration of interest

The authors have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This publication was made possible by Grant Number K01-RR021858 (L M A) from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), R01-CA78312 (L. A S) and R01-CA101841 and R01-CA118740 (H R). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NCRR or NIH.

Acknowledgements

The authors thank Tara Graffewller-Huseth for assistance with sample collection and analysis, and Drs Kathleen O’Leary and Caroline Alexander for helpful discussions.

References


Booth BW & Smith GH 2006 Estrogen receptor-alpha and progesterone receptor are expressed in label-retaining mammary epithelial cells that divide asymmetrically and retain their template DNA strands. *Breast Cancer Research* 8 R49.


Estrogen enhances PRL-induced neoplasia · L. M Arendt and others


Nakano S, Iyama K, Ogawa M, Yoshio K, Sado Y, Oohashi T & Ninomiya Y 1999 Differential tissue expression and localization of type IV collagen alpha1(IV), alpha2(IV), alpha5(IV), and alpha6(IV) chains and their mRNA in normal breast and in benign and malignant breast tumors. Laboratory Investigation 79 281–292.


Received in final form 20 July 2009
Accepted 24 July 2009
Made available online as an Accepted Preprint 27 July 2009