Tumorigenesis and Neoplastic Progression

Prolactin–Growth Factor Crosstalk Reduces Mammary Estrogen Responsiveness Despite Elevated ER α Expression

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Most breast cancers that occur in women express estrogen receptor α (ER α). However, a large subset of these cancers either does not initially respond to anti-estrogen therapy or develops resistance to such treatment modalities. One postulated mechanism of this failure is signaling cross talk between hormones and local growth factors. To examine these complex interactions *in vivo*, we assessed the effects of estrogen on transforming growth factor α (TGF α)- and prolactin (PRL)-induced mammary tumorigenesis in transgenic mice. Both PRL and estrogen reduced the latency of TGF α -induced oncogenesis, resulting in tumors that were variably ER α -positive, but were progesterone receptor-negative. However, despite elevated ER α levels in NRL-PRL/TGF α glands, tumor latency was not reduced with increasing estrogen levels, nor increased after ovariectomy. Furthermore, PRL and TGF α in combination blocked the mitogenic effects of estrogen, dramatically reduced progesterone receptor levels, and diminished ER α down-regulation in response to circulating estrogen levels, in contrast to the other genotypes. Notably, however, ductal morphology remained responsive to estrogen, indicating that TGF α and PRL in combination can inhibit some, but not all, estrogenic signals. Both *in vitro* and *in vivo*, PRL and TGF α cooperatively enhanced Akt phosphorylation, which is associated with endocrine resistance in human disease. These findings provide insight into the interactions of PRL with growth factors during mammary oncogenesis and suggest combinatorial approaches that may result in improved therapeutic efficacy. (*Am J Pathol* 2009, 174:1065–1074; DOI: 10.2353/ajpath.2009.080719)

Strong epidemiological and experimental evidence has substantiated the importance of estrogen in the etiology of breast cancer. Increased exposure raises the risk of this disease,^{1–3} and conversely, ovariectomy can be used to prevent cancer in women with familial susceptibility,⁴ and treat premenopausal disease.⁵ The proliferative effects of estrogen appear to be mediated through estrogen receptor α (ER α), which is used both as a prognostic indicator and predictor of response to endocrine therapies. Although 50% to 70% of breast cancers express ER α , a significant subset do not respond to anti-estrogen therapy, and 40% of those that initially respond eventually relapse despite the continued presence of ER α .⁶ One postulated mechanism of this resistance is through enhanced cross talk among local growth factors. However, the contributions of potential participants are not well understood.

Prolactin (PRL) plays a critical role in mammary alveologenesis and lactation.⁷ Although its role in carcinogenesis has been controversial,^{8–10} recent large prospective studies have linked circulating PRL levels to pre- and postmenopausal breast cancer.¹¹ PRL itself is also synthesized within the mammary gland, permitting autocrine/paracrine action, and its production is higher in tumors than in normal tissue.¹² PRL increases the responsiveness of multiple tissues, including the mammary gland, to ovarian hormones,^{13,14} and elevates ER α levels in murine mammary epithelial cells.^{15,16} In breast cancer cells, overexpression of PRL increases ER α expression, as well as estrogen responsiveness,¹⁷ and PRL and estrogen cooperatively sustain ERK1/2 phosphorylation and enhance activating protein-1 (AP-1) activity,¹⁸ which

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is linked to cancer cell proliferation, survival, and invasion.^{19,20} Mice that overexpress PRL under control of the mammary selective, estrogen-insensitive promoter, NRL, develop both ER α positive and negative carcinomas of diverse histotypes.²¹ Together these observations suggest that PRL may be an important regulator of estrogen responsiveness in breast disease and that estrogens may be a key player in PRL-induced oncogenesis.

In contrast, epidermal growth factor (EGF) family members are generally inversely related to estrogen responsiveness. TGF α , a well-characterized mammary oncogene, binds the EGF receptor (EGFR) and activates EGFR homodimers or heterodimers with its preferred partner erbB2.^{22–24} Human studies have demonstrated an inverse correlation between EGFR and ER α in primary tumors,^{23,25} although the relationship between TGF α and ER α is less straightforward.^{26–28} Female mice that overexpress TGF α in mammary epithelial cells under control of the NRL promoter display reduced ductal ER α ,²⁹ although the tumors express variable levels of ER α .^{29,30} Transgenic PRL accelerates formation of these tumors in bitransgenic females, and enhances ER α expression in morphologically normal structures, compared with those in NRL-TGF α females.²⁹ However, bitransgenic tumors, like those in singly NRL-TGF α glands, display variable levels of ER α expression. These observations suggest that PRL and the EGFR ligand, TGF α , may exert opposite effects on estrogen-responsiveness and that this interplay may model clinically relevant responses to ovarian hormones in lesion development.

To examine the role of estrogen in the interactions between PRL and TGF α in tumorigenesis, we manipulated circulating estrogen in NRL-PRL/TGF α and NRL-TGF α females beginning shortly after puberty. In contrast to our prediction, tumor latency in NRL-TGF α females was accelerated with estrogen supplementation, and ovariectomy delayed tumors and decreased incidence. Moreover, in bitransgenic NRL-PRL/TGF α females, tumor latency was not altered by estrogen availability, despite elevated levels of ER α . PRL and TGF α cooperatively inhibited mitogenic responses to estrogen in morphologically normal ductal cells and altered steroid receptor patterning, leading to tumors that expressed variable levels of ER α , but were strikingly progesterone receptor (PR)-negative. Together, they also enhanced phosphorylation of Akt and ER α , pointing to one pathway that may mediate these events. These findings indicate that in combination, PRL and TGF α induce estrogen-insensitive disease, providing a model to examine control of ER α responsiveness in breast cancer.

Materials and Methods

Materials

5-bromo-2-deoxyuridine was obtained from Sigma Chemical Co. (St. Louis, MO), and 17 β -estradiol was purchased from Steraloids, Inc. (Newport, RI). The following antibodies were used for immunohistochemistry and Western blot analyses: 5-bromo-2-deoxyuridine (MAS-250) from Accurate

Scientific (Westbury, NY), ER α (SC-542) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), pS167-ER α (07–481) from Upstate-Cell Signaling Solutions (Lake Placid, NY), PR (A-0098) from DakoCytomation, Inc. (Carpinteria, CA), and Akt (9272) and pAkt S473 (9271) from Cell Signaling (Danvers, MA).

Genotyping and Maintaining Mice

NRL-PRL mice (line 1647-13, TgN[Nrl-Pr]23EPS) and NRL-TGF α mice (line 1385-7, TgN[Nrl-Tgfa]25EPS) were generated as described.^{21,29} All lines were maintained in the FVB/N strain background. Tail biopsies were collected at weaning and offspring were screened for the PRL²¹ and TGF α ²⁹ transgenes as described previously. Mice were housed and handled in accordance with the Guide for Care and Use of Laboratory Animals in facilities accredited by the Association for the Assessment and Accreditation for Laboratory Animal Care. All procedures were approved by the University of Wisconsin-Madison Animal Care and Use Committee.

Ovariectomy and Treatment with 17 β -Estradiol

Nonparous female mice were subjected to ovariectomy (ovx) or sham surgery, or ovariectomized and treated with 17 β -estradiol (E2) beginning at either 8 or 12 weeks of age for determining latency to tumor development. Females received silastic capsules (Dow Corning, Co., Midland, MI; 2 mm inner diameter $\times$ 3.2 mm outer diameter, 1.6 cm in length) containing 20 μ g E2, replaced every 6 weeks for the length of the experiment. This dose raises circulating E2 levels to about that observed at estrus.^{31,32} Animals were observed weekly for tumors, and considered to be end stage when tumor diameter reached 1.5 cm (defined as tumor latency). Due to the long latency of PRL-induced tumorigenesis, glands from NRL-PRL female controls were collected at 6 months of age for comparison with bitransgenic females.

Mammary Gland Whole Mount Analyses

At the time of tumor collection in NRL-TGF α and NRL-PRL/TGF α females or age-matched NRL-PRL and nontransgenic females, the contralateral chain of glands from four animals of each genotype was fixed in 10% neutral buffered formalin overnight and stored in 70% ethanol until processed. Whole mounts were stained with carmine alum, dehydrated with graded ethanol, cleared of fat using xylenes, and stored in glycerol until analysis.

Histological Examination of Mammary Tissue

Mammary tissue was fixed and examined as previously described.^{21,29} Mice were injected with 200 mg/kg body weight 5-bromo-2-deoxyuridine 1 hour before sacrifice to label cells undergoing DNA synthesis (see supplemental Figure S1A at <http://ajp.amjpathol.org>). Proliferating, apo-

ptotic, ER α - and PR- positive cells were detected and quantified as described previously.²¹

Immunofluorescence

Deparaffinized slides were rehydrated and exposed to 0.5% H₂O₂ in H₂O to block endogenous peroxidase activity, boiled for 15 minutes in 0.1 M Tris, pH 9.0, for antigen retrieval, then blocked in 5% bovine serum albumin in Tris-buffered saline with Tween. Slides were incubated with PR antibody (1:250) for 1 hour, rinsed, and incubated with Alexa Fluor 488-conjugated goat-anti-rabbit secondary antibody (1:400) overnight (Molecular Probes, Eugene, OR). Slides were rinsed, blocked, and incubated with ER antibody (1:500) for 1 hour, rinsed, and incubated with Alexa Fluor 568-conjugated goat-anti-rabbit secondary antibody (1:400) overnight. Slides were rinsed and mounted using VECTASHIELD mounting media with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Images were aligned using ImageJ v. 1.36b (National Institutes of Health). The number of cells co-expressing ER α and PR were counted, and expressed as a proportion of total ER α -expressing cells.

MCF-7 Cell Culture

MCF-7 cells were grown in RPMI-1640 containing 10% horse serum and 50 μ mol/L gangciclovir as reported previously.³³ For experiments, 10⁶ cells/100-mm plates were incubated in serum-free media for 24 hours before treatment with vehicle, 4 nmol/L PRL, and/or 5.5 nmol/L TGF α for 15 or 120 minutes. For some experiments, cells were pretreated with DMSO or 20 μ mol/L MG132 for 1 hour before PRL/TGF α treatment. Western analyses were performed as described previously.²⁹

Statistical Analyses

Counted indices from immunohistochemistry sections were analyzed using the Kruskal-Wallis test for nonparametric data, followed by the Mann-Whitney post test. Statistical analyses were performed as described using Prism v. 4.03 (GraphPad Software, Inc., San Diego, CA).

Results

PRL Overrides the Sensitivity of TGF α -induced Tumorigenesis to Estrogen

To examine how estrogen modulates TGF α and PRL-induced oncogenesis, nonparous females that overexpressed TGF α either singly or in combination with PRL under control of the estrogen-insensitive NRL promoter³⁰ were treated with postpubertal ovariectomy (ovx), sham surgery, or ovx with continuous supplementation of 17 β -estradiol (E2). Bitransgenic females developed locally invasive macrocystic tumors, characteristic of the TGF α -induced tumor morphology in this strain. In NRL-PRL/TGF α females, tumor incidence and latency were not

Figure 1. Prolactin altered sensitivity of TGF α -induced tumorigenesis to estrogen. Bitransgenic NRL-PRL/TGF α and single transgenic NRL-TGF α females underwent ovx, sham surgery, or ovx with 17 β -estradiol (ovx-E2) supplementation after puberty as described in the Materials and Methods. Ovariectomized females had significantly decreased uterine weights (30 ± 20 mg; mean $\pm$ SD) as compared with sham treated females (80 ± 20 mg), while those from E2-treated ovariectomized females were significantly increased (150 ± 40 mg). Latency was defined as the time required for the tumors to reach 1.5 cm in diameter (end stage). **A:** Bitransgenic NRL-PRL/TGF α females in all treatment groups developed tumors with 100% incidence, without differing in latency. **B:** Ovariectomized NRL-TGF α females developed tumors with reduced incidence (42%) and increased latency; E2-treated ovariectomized NRL-TGF α females developed tumors with 100% incidence and decreased latency. (Differences among treatments for this genotype, $P < 0.001$). **A,B:** Latencies were compared by Kaplan-Meier analysis, and differences were detected using the Mantel-Haenszel test. **C-F:** Percentage of ER α (+) (**C,D**) and PR (+) (**E,F**) cells present in tumors. Each symbol represents a single tumor. ER α expression was variable regardless of treatment, with no significant differences between genotypes or treatments. No epithelial cells in either NRL-PRL/TGF α or NRL-TGF α tumors expressed detectable PR, in contrast to normal ductal structures (see supplemental Figure S1C at <http://ajp.amjpathol.org>).

altered by ovx or E2-supplementation, regardless of whether treatment was initiated at 8 (Figure 1A, see supplementary Table S1 at <http://ajp.amjpathol.org>) or 12 weeks of age (data not shown). In contrast, ovx at 12 weeks of age significantly decreased the incidence of tumor development and increased latency in NRL-TGF α females (Figure 1B).³⁰ E2-treated NRL-TGF α females developed these tumors with an accelerated latency, reducing it to that of bitransgenic females. No nontransgenic or NRL-PRL females developed palpable tumors over this time period, as expected.²¹ Steroid hormone receptor levels in the tumors did not vary with respect to genotype or treatment. Tumors expressed variable levels of ER α (Figure 1, C–D; see supplemental Figure S1B at <http://ajp.amjpathol.org>), and were strikingly PR negative (Figure 1, E–F; see supplemental Figure S1C at <http://ajp.amjpathol.org>). Further, tumors in both bitransgenic and

Figure 2. Mammary glands from bitransgenic females demonstrated estrogen responsiveness. Glands from ovariectomized females of all genotypes (**A,D,G,J**) demonstrated thin ducts with little alveolar development, consistent with loss of ovarian progesterone. Glands from sham-treated females (**B,E,H,K**) displayed variable alveolar budding. Glands from E2-treated ovariectomized females of all genotypes (**C,F,I,L**) demonstrated dilated ducts. E2-treated glands from ovariectomized NRL-PRL/TGF α and NRL-PRL females (**C,D**) also displayed some alveolar budding. Focal epithelial hyperplasias were evident in many animals of these genotypes, regardless of ovarian hormonal status (marked with **arrows**). Glands from PRL and NonTG control females were collected at 6 months of age or after tumor development in bitransgenic and TGF α females and prepared as described in Materials and Methods. (Representative of four animals of each genotype).

NRL-TGF α females demonstrated similar proliferation rates, regardless of treatment (see supplemental Figure S2 at <http://ajp.amjpathol.org>), suggesting that once they have formed, estrogen is not a major mitogenic factor.

Ductal Morphology in Glands of Bitransgenic Females Remains Responsive to Ovarian Hormones

Although the latency to tumor development in the bitransgenic females was insensitive to E2, we investigated other potential mammary responses to E2 availability in whole mounts. To elucidate the role of PRL in any changes, we compared end stage bitransgenic glands to those from 6-month-old (age-matched) NRL-PRL, NRL-TGF α and nontransgenic females. In response to ovx,

glands from females of all genotypes were filled primarily with thin ducts and showed no alveolar development (Figure 2A,D,G,J), although occasionally bitransgenic females demonstrated localized dilated ducts (Table 1). Glands from ovariectomized bitransgenic and NRL-PRL females developed small focal hyperplasias (arrows, Figure 2, A and G), which were not present in glands from either ovariectomized nontransgenic or NRL-TGF α females. Glands from sham-treated females from all genotypes demonstrated variable levels of alveolar development (Figure 2, B,E,H,K), with hyperplasias in glands from NRL-TGF α and bitransgenic females, but not 6-month-old NRL-PRL or nontransgenic females. Females from all genotypes responded to E2 treatment with ductal dilation (Figure 2, C,F,I,L; Table 1). Glands from E2-treated bitransgenic and 6-month-old NRL-PRL fe-

Table 1. Mammary Abnormalities at End Stage* in NRL-PRL/TGF α and NRL-TGF α Mice

	PRLxTGF α			TGF α		
	ovx	sham	E2	ovx	sham	E2
Epithelial hyperplasias	4/7	9/12	4/10	1/12	5/13	6/10 [†]
Dilated ducts	2/7	9/12	10/10	0/12	4/13	10/10 [†]
MIN [‡]	0/7	1/12	0/10	0/12	3/13	1/10
Squamous changes	0/7	1/12	1/10	1/12	6/13	3/10
Adenosis	5/7 [§]	12/12 [§]	7/10	0/12	4/13	8/10 [†]

*End stage is defined as tumor size reaching 1.5 cm diameter or 1 year of age. [†]E2-increased lesions in NRL-TGF α glands ($P < 0.04$, Chi-square test). [‡]MIN, mammary intraepithelial neoplasia. Treatment did not statistically alter the incidence of any lesion in NRL-TGF α mice. [§]Increased incidence in NRL-PRL/TGF α glands compared with similarly treated NRL-TGF α glands ($P < 0.04$, Chi-square test).

males demonstrated some alveolar budding, and bitransgenic females also developed epithelial hyperplasias (Table 1). E2 enhanced the development of multiple abnormalities including dilated ducts, epithelial hyperplasias, and most frequently, adenosis lesions compared with either ovariectomized or sham-treated NRL-TGF α females but did not alter the incidence in bitransgenic females (Table 1).

Proliferation of Morphologically Normal Ducts of Bitransgenic Females is Unresponsive to Estrogen

Since PRL, TGF α , and E2 are all mammary mitogens, we examined how these factors interacted in proliferation of morphologically normal ductal epithelial cells of adult, age-matched 6-month-old animals. Transgenic PRL and TGF α individually significantly increased the basal rate of proliferation of ductal epithelium in all treatments compared with nontransgenic glands ($P < 0.05$). The ducts of both single transgenic females, like those in nontransgenic animals, were very responsive to circulating steroids (Figure 3, B–D). Chronic E2 administration to ovariectomized females increased proliferation over twofold compared with ovx alone in each of these genotypes. In the bitransgenic females, proliferation was higher than in sham-treated nontransgenic animals, even after removal of ovarian steroids (Figure 3A, $P < 0.05$). However, PRL and TGF α cooperatively rendered the epithelial cells refractory to the mitogenic (Figure 3A) and anti-apoptotic (see supplemental Figure S3 at <http://ajp.amjpathol.org>) effects of E2, in contrast to the ductal appearance in the whole mounts (Figure 2, A–C). Moreover, the rate of ductal proliferation in the bitransgenic ovx-E2-treated females was significantly less than that of single transgenic females receiving the same treatment ($P < 0.0005$).

PRL and TGF α Cooperatively Decrease Ductal PR Expression While Enhancing ER α Expression

As another measure of steroid hormone responsiveness, we investigated ER α and PR expression within the ducts, and the effect of circulating E2 on this pattern. To quantify ER α and PR expression, we separately detected each using immunohistochemistry, and quantified the proportion of labeled cells. Together, transgenic PRL and TGF α significantly increased the number of epithelial cells ex-

pressing ER α in all treatment groups compared with females from all other genotypes (Figure 4A, $P < 0.02$). However, bitransgenic females were the only genotype that did not exhibit the close correlation between ER α and PR expression previously observed.³⁴ In this genotype, the proportion of cells expressing PR was about threefold less than that expected from the level of ER α expression (Figure 4A).

The proportion of cells expressing ER α varies inversely with circulating levels of E2 in women, as well as other species.³⁵ As predicted from these reports, ovx increased the proportion of epithelial cells expressing ER α in females of all genotypes, compared with sham-treated animals (compare Figures 4, A and B), and E2 treatment of ovariectomized females reduced ER α in all genotypes ($P < 0.0001$; Figure 4B). However, ER α expression in the bitransgenic glands was less responsive to estrogen administration. In contrast to the 60% decline in the number of cells containing detectable ER α in the nontransgenic and single transgenic glands, ER α expression was reduced only about 30% in the NRL-PRL/TGF α glands, and remained higher than sham-treated animals of the other

Figure 3. Estrogen exposure did not enhance proliferation in NRL-PRL/TGF α ductal epithelial cells (A), in contrast to single transgenic and nontransgenic mice (B,C,D). Proliferation rates were determined in age-matched females of all genotypes at 6 months of age using 5-bromo-2-deoxyuridine labeling, quantified as described in the Materials and Methods, and expressed as mean $\pm$ SD. Different lowercase letters denote statistical differences among the treatment groups within a genotype as determined by the Kruskal-Wallis test followed by Mann-Whitney post test ($P < 0.05$). Ductal proliferation was higher in ovariectomized NRL-PRL/TGF α females than intact single transgenic females ($P < 0.05$), and higher in ovx-E2-treated NRL-PRL and NRL-TGF α females than similarly treated bitransgenic females ($P < 0.0005$).

Figure 4. Morphologically normal ducts of NRL-PRL/TGFα females exhibited reduced PR expression and attenuated ERα down-regulation. **A:** PR was low compared with ERα expression in NRL-PRL/TGFα females, but not other genotypes. ERα and PR expression in ducts of age-matched 6-month-old nonparous females was determined as described in the Materials and Methods and expressed as mean ± SD. The asterisk denotes the significantly elevated ERα expression in the bitransgenic females, compared with the other genotypes ($P < 0.02$). **B:** Down-regulation of ERα expression following chronic estrogen administration to ovariectomized females was decreased in NRL-PRL/TGFα females. Asterisks denote the significantly reduced proportion of cells expressing ERα following E2 treatment within each genotype ($P < 0.0001$). Differences were determined by the Kruskal-Wallis test followed by Mann-Whitney post test.

genotypes, suggesting reduced sensitivity to ligand-induced down-regulation (Figure 4B).

To examine the effects of PRL and TGFα on colocalization of ERα and PR, we examined expression patterns using immunofluorescence. In sham-treated nontransgenic females, PR colocalized with ERα in >90% of ERα+ cells (Figure 5, J–L). This is similar to humans, where 96% of mammary epithelial cells that express ERα also express PR.³⁶ This association was disrupted in the transgenic females, most notably the bitransgenic NRL-PRL/TGFα animals (Figure 5, A–I). In ducts of intact sham-treated bitransgenic females, ERα colocalized with PR in only 45% of ERα cells (Figure 5, A–C). These data indicate that PRL and TGFα cross talk alters expression patterns of ERα and PR within morphologically normal structures.

PRL and TGFα Cooperatively Enhance Akt Activity

PRL and TGFα each initiate multiple signaling cascades, and the outcome of their cross talk may be dictated by the phenotype of the responding cell. We have previously shown that transgenic PRL and TGFα in combination increase mammary levels of pERK1/2 above TGFα alone.²⁹ Another possible mediator of PRL and TGFα cross talk is Akt. This kinase has been associated with endocrine resistance in breast cancer,^{37,38} suggesting that this pathway also may contribute to the estrogen-

insensitive tumorigenesis in NRL-PRL/TGFα females. To determine the net effect on this kinase cascade *in vivo*, we examined glands from NRL-TGFα and bitransgenic adult females before the appearance of lesions. Although individuals varied considerably in the level of activation of this pathway, glands from 3-month-old bitransgenic females demonstrated significantly higher Ser473 pAkt levels, compared with glands from age-matched NRL-TGFα females ($P < 0.05$, Figure 6, A,B). These results suggest that cooperative signaling through Akt may contribute to tumorigenesis in NRL-PRL/TGFα glands.

To examine the underlying mechanism, we turned to cultured breast cancer cells. Length of signal duration has been shown to increase effectiveness to multiple actions, including proliferation and apoptosis.³⁹ *In vitro*, co-treatment with PRL and TGFα²⁹ or family member EGF,⁴⁰ prolongs ERK1/2 activation compared with either treatment alone. Therefore, we examined the effect of these factors in combination on activation of Akt in MCF-7 derived cells *in vitro*. As shown in Figure 6C, treatment with PRL or TGFα alone transiently increased Ser473 pAkt. No additional response to these factors in combination was evident at 15 minutes. After 2 hours, Akt activation was attenuated, compared with that at 15 minutes ($41.8\% \pm 23.4$, mean ± SD; Figure 6, C and D). However, PRL and TGFα together strikingly prolonged Ser473 pAkt levels ($P < 0.01$). This was associated with increased phosphorylation of ERα on Ser167 (Figure 6, E and F), which was most evident in the presence of MG132, blocking proteasomal degradation of ERα. Phosphorylation of ERα at this site, which is downstream of Akt in these cells, increases DNA binding and transcriptional activity, and is associated with ligand-independent activation.^{41,42}

Discussion

Despite the critical role that ERα plays in prognosis and therapeutic options in breast cancer, the control of ERα expression and responsiveness is not well understood. Here we used unique transgenic models to assess the interaction of TGFα and PRL with E2. Our studies demonstrate that although both PRL and E2 enhanced TGFα-mediated tumorigenesis, TGFα and PRL in combination reduced selective E2 responses. Despite the elevated ERα in the bitransgenic glands, neither ovariectomy nor increased serum E2 altered tumor latency or turnover of cells within normal structures. Further, PR was dramatically reduced, and ERα down-regulation in response to circulating E2 was attenuated. However, E2 continued to modulate ductal morphology, indicating that not all estrogenic actions were affected. These findings demonstrate important interactions between PRL and this EGFR ligand in breast pathogenesis.

NRL-TGFα and NRL-PRL/TGFα bitransgenic females, regardless of levels of circulating estrogen, developed histotypically similar tumors that were variably ERα+ and strikingly PR-. E2 supplementation and transgenic PRL similarly reduced the latency of these tumors in nonparous females, suggesting that both E2 and PRL

Figure 5. Prolactin and TGF α altered patterns of ER α and PR expression. ER α (red) and PR (green) were visualized in 4,6-diamidino-2-phenylindole-stained nuclei (blue); nuclei expressing both ER α and PR appear yellow. Bitransgenic females (**A–C**) demonstrated low levels of PR relative to ER α expression, compared with the other genotypes. (NRL-TGF α , **D–F**; NRL-PRL, **G–I**; nontransgenic females, **J–L**). Original magnification for all images = $\times 600$.

signals enhance proliferation of a population targeted by TGF α for tumor development. Since PRLR and ER α are expressed in both mammary epithelial cells as well as stroma,^{43,44} these cooperative interactions may be mediated by either paracrine or autocrine signaling cross talk. The etiology of ER α + / PR– tumors in humans is not understood. Although ER α + / PR– tumors may evolve from ER α + / PR+ tumors, the cellular origin of these tumor subsets may be different. Clinically, ER α + / PR– tumors have higher levels of EGFR and HER2/erbB2 compared with ER α + / PR+ tumors, and PR status can predict benefit from tamoxifen therapy independently of ER α expression.⁴⁵ Although PR expression has been evaluated clinically as a marker for ER α activity, the absence of PR expression in the tumors may instead reflect strong cross talk among ER α and growth factor-initiated signals that can down-regulate PR.⁴⁶

This dysregulation of steroid receptor expression was evident in the end stage lesions of the single transgenic NRL-TGF α glands. In the presence of both transgenic

TGF α and PRL, the dissociation between ER α and PR expression was already pronounced even in morphologically normal ducts of intact females, which demonstrated enhanced levels of ER α , but reduced PR expression. This interaction of the two growth factors agrees with observations from cultured murine primary epithelial cells, in which induction of PR by progesterone and PRL was inhibited when EGF was present.¹⁶ However, in MCF-7 cells, growth factor signaling has been shown to have divergent effects on PR expression, which may in part be due to differences in cell culture conditions.^{47–49} Interestingly, the ER α rich / PR poor pattern of steroid receptor expression in bitransgenic glands resembles that found in the normal mammary gland during lactation. At that time, circulating hormones, which include PRL, dramatically increase the number of epithelial cells expressing ER α ,⁵⁰ and PR expression is inversely proportional to the secretory activity of the gland.⁵¹ In this hormonal environment, the gland is refractory to the changes in circulating estrogen.^{52,53}

Figure 6. PRL and TGF α together enhanced Akt phosphorylation. **A:** Mammary glands from 3-month-old NRL-TGF α and bitransgenic NRL-PRL/TGF α nonparous females were disrupted with a polytron, and lysates from individual animals were examined for pAkt and total Akt by Western analyses. **B:** Signals from glands were quantitated densitometrically and expressed as pAkt/total Akt ($n = 3$, mean $\pm$ SD). The **asterisk** denotes a statistically significant difference between these genotypes by one-tailed Student's t -test ($P < 0.05$). **C:** Western analyses of pAkt in MCF-7 cells after stimulation with PRL and/or TGF α . Cells were serum starved for 24 hours before incubation with vehicle, 4 nmol/L PRL, and/or 5.5 nmol/L TGF α for 15 or 120 minutes. **D:** Fold change pAkt after 120 minutes incubation with ligand compared with vehicle-treated controls from three independent experiments, calculated as % of co-treatment (mean $\pm$ SD). Different lowercase letters denote statistically significant differences among treatments by one-way analysis of variance with Bonferroni post test ($P < 0.01$). **E:** Western analyses of pS167 ER α in MCF-7 cells. Cells serum-starved as in (C) were pretreated with DMSO or 20 μ mol/L MG132 for 1 hour before vehicle, 4 nmol/L PRL, and/or 5.5 nmol/L TGF α for 120 minutes. **F:** Fold change in pER α after 120 minutes incubation with ligand compared with vehicle-treated controls from three independent experiments, calculated as % of co-treatment (mean $\pm$ SD). Different lowercase letters denote statistically significant differences between treatments by one-way analysis of variance with Bonferroni post test ($P < 0.05$).

The signaling events leading to reduced PR expression in glands from NRL-PRL/TGF α females are likely to be complex. Both *in vivo* and *in vitro*, PRL and TGF α cooperatively induce prolonged activation of ERK1/2, as we have shown previously,²⁹ and Akt as shown herein. Both kinases have been implicated in phosphorylation of coactivators, such as AIB1/SRC-3,⁵⁴ and ER α itself, which has been shown to alter transcriptional activity.^{42,55} Mice overexpressing AIB1 under control of the MMTV promoter demonstrate elevated glandular ER α expression with reduced PR expression and increased levels of phosphorylated Akt,⁵⁶ similar to glands from NRL-PRL/TGF α females. Both PRL and TGF α also individually activate AP-1 through phosphorylation of ERK 1/2,^{57,58} and this cross talk may alter transcription through AP-1 sites in the PR promoter, which can either enhance or reduce PR expression depending on the complement of available transcriptional activators or suppressors.^{59,60} In addition to critically impacting PR transcription, modulation of ERK1/2 activity may also increase PR degradation via phosphorylation on serine residues leading to proteasomal destruction.⁶¹

Proliferation of normal structures and development of tumors in NRL-TGF α mice was markedly responsive to E2

availability, although proliferation within tumors themselves did not differ with ovx or supplemental E2, suggesting acquired loss of estrogen sensitivity. Surprisingly, co-expression of mammary PRL greatly reduced E2-sensitivity in bitransgenic females, despite the elevated ER α expression in morphologically normal and preneoplastic structures. In contrast to the other genotypes, long term manipulation of E2 availability did not alter latency to tumor development or epithelial turnover, and steady state levels of ER α itself suggested reduced sensitivity to E2-induced ER α down-regulation. The apparent insensitivity of bitransgenic glands to these endpoints does not appear to be due to maximized epithelial proliferation, since proliferation in NRL-TGF α animals when supplemented with E2 was greater than that in NRL-PRL/TGF α females. Consistently, ovx also failed to reduce proliferation in the bitransgenic glands. Rather, these data suggest that TGF α and PRL together may inhibit some, but not all, estrogenic signals. Interestingly, in contrast to proliferation, ductal morphology of bitransgenic females did reflect the ovarian hormone environment, like all of the other genotypes. Together, our observations suggest differential effects of PRL and TGF α on discrete E2-initiated pathways leading to cellular turnover and differentiation toward secretion.

Possible mediators include cross talk at E2-activated AP-1 sites,^{62,63} interactions with the transcription factor Stat 5, and/or ligand-independent activation of ER α . Of interest in this regard, recent studies of mice with a "knocked-in" mutant ER α revealed that uterine epithelial proliferation was driven by nonclassical tethered estrogen driven signals, rather than signals mediated by a classical ERE. However, fluid accumulation and hyperemia required the classical mechanism.⁶⁴ Similarly, postnatal proliferation of mammary epithelial cells was decreased by induced expression of the AP-1 inhibitor, Tam67.⁶⁵ Further, PRL and TGF α both activate Stat 5 isoforms,⁶⁶ which can negatively interfere with some E2-induced signals.⁶⁷ Alternatively, TGF α and PRL together may activate ER α in the absence of ligand directly or through modulation of coregulatory proteins by established pathways (Akt, MAPKs), consistent with our data herein, promoting a subset of ER α -driven actions and precluding further responses to ligand.⁴⁶ PRL and TGF α cross talk to these distinct E2-driven pathways is under investigation.

The dramatic reduction in E2 sensitivity to some endpoints appears to be dependent on the combination of PRL and TGF α . Like the NRL-TGF α mice, age-matched NRL-PRL females exhibited E2-responsive ductal structures and rates of proliferation. However, ovariectomized NRL-PRL females demonstrated focal hyperplasias that were not present in ovariectomized NRL-TGF α females, suggesting that PRL-induced pathology is independent from ovarian steroids. PRL alone appears to target a different tumor precursor population than that affected by TGF α and these factors together. Single transgenic NRL-PRL females develop primarily adenocarcinomas, as well as a different complement of preneoplastic lesions over a more protracted time frame.²¹ Experiments to

examine the role of E2 in PRL-mediated tumorigenesis are underway.

Our findings show that PRL and TGF α induce variably ER α + PR– tumors, which are insensitive to E2. These characteristics are similar to human disease, in which women diagnosed with ER α + /PR– breast tumors have a worse prognosis and the disease is often refractory to treatment with tamoxifen.⁶⁸ This class of tumors is more likely to be aneuploid, proliferate faster, and is more frequent in older patients than ER α + /PR+ tumors.⁴⁵ Given that women diagnosed with ER α + /PR– disease have a worse long-term prognosis, it is critical to find alternate strategies to treat this particular illness. Combinatorial approaches based on targeting PRLR or EGFR/erbB2, as well as ER α , may result in improved therapeutics, enhancing survival in women afflicted with this class of tumors.

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