Tumorigenesis and Neoplastic Progression

Prolactin Potentiates Transforming Growth Factor α Induction of Mammary Neoplasia in Transgenic Mice

Lisa M. Arendt,^{*,†} Teresa A. Rose-Hellekant,[‡]
Eric P. Sandgren,[‡] and Linda A. Schuler[†]

From the Cellular and Molecular Biology Program,^{*} the
Department of Comparative Biosciences,[†] and the Department of
Pathobiological Sciences,[‡] University of Wisconsin-Madison,
Madison, Wisconsin

Prolactin influences mammary development and carcinogenesis through endocrine and autocrine/paracrine mechanisms. In virgin female mice, prolactin overexpression under control of a mammary selective nonhormonally responsive promoter, neu-related lipocalin, results in estrogen receptor α (ER α)-positive and ER α -negative adenocarcinomas. However, disease *in vivo* occurs in the context of dysregulation of multiple pathways. In this study, we investigated the ability of prolactin to modulate carcinogenesis when co-expressed with the potent oncogene transforming growth factor α (TGF α) in bitransgenic mice. Prolactin and TGF α cooperated to reduce dramatically the latency of mammary macrocyst development, the principal lesion type induced by TGF α . In combination, prolactin and TGF α also increased the incidence and reduced the latency of other preneoplastic lesions and increased cellular turnover in structurally normal alveoli and ducts compared with single transgenic females. Bitransgenic glands contained higher levels of phosphorylated ERK1/2 compared with single TGF α transgenic glands, suggesting that this kinase may be a point of signaling crosstalk. Furthermore, transgenic prolactin also reversed the decrease in ER α induced by neu-related lipocalin-TGF α . Our findings demonstrate that locally produced prolactin can strikingly potentiate the carcinogenic actions of another oncogene and modify ovarian hormone responsiveness, suggesting that prolactin signaling may be a potential therapeutic target. (Am J Pathol 2006, 168:1365–1374; DOI: 10.2353/ajpath.2006.050861)

The mammary gland undergoes dramatic changes in proliferation and apoptosis during puberty, pregnancy,

lactation, and involution. These changes are governed by multiple steroid and peptide hormones and by local and systemic growth factors. The study of hormonal interactions in the mammary gland has implicated many of these in carcinogenesis. Prolactin (PRL) increases proliferation, influences angiogenesis, and promotes cellular motility *in vitro*,¹ each of which is a critical element of neoplastic progression. Moreover, high levels of PRL receptor are expressed in a majority of human breast tumors.^{2–4} The role of PRL in human breast cancer, nevertheless, has been controversial. Correlations between circulating PRL and breast cancer development and progression are conflicting, and reduction of pituitary PRL with bromocriptine does not alter the disease course.¹ However, the association of elevated circulating PRL and postmenopausal breast cancer observed in a large prospective study within the Nurse's Health Study as well as growing appreciation of mammary autocrine/paracrine PRL activity in addition to its classic endocrine effects have reconciled many of the apparent conflicts.^{1,5,6} Recognition of the potential role of this endogenously produced PRL in breast disease has renewed interest in this understudied area and has pointed to the importance of experimental models that enable study of PRL in the complex *in vivo* context, taking into account both local and circulating PRL. Transgenic PRL overexpression under control of the metallothionein (MT) promoter results in mammary adenocarcinomas in virgin female mice,⁷ which, in contrast to humans, express negligible mammary PRL. We have shown that mammary PRL overexpression driven by a mammary selective nonhormonally responsive promoter, neu-related lipocalin (NRL), results in both estrogen receptor α (ER α)-positive and ER α -negative mammary adenocarcinomas and increased ER α expression in pre-

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Current address of T.A.R.-H.: Department of Physiology, School of Medicine, University of Minnesota-Duluth, Duluth, MN.

Address reprint requests to Linda A. Schuler, Department of Comparative Biosciences, University of Wisconsin, 2015 Linden Dr., Madison, WI 53706. E-mail: schulerl@svm.vetmed.wisc.edu.

neoplastic lesions in virgin females.⁸ This model provides a system to examine the roles of both local and circulating PRL in mammary oncogenesis in a defined transgenic system.

Although these studies demonstrate that PRL alone is sufficient to promote neoplasia and modulate estrogen receptor expression, in clinical disease, it would act in the context of dysregulation of multiple other pathways. Transforming growth factor α (TGF α) is a prime candidate molecule to evaluate in the context of local mammary PRL expression. The epidermal growth factor (EGF) family, including TGF α , plays important roles in breast cancer and like PRL has complex actions in normal mammary development.^{9–13} TGF α is overexpressed in 50 to 70% of human breast tumors.^{14,15} It binds to epidermal growth factor receptor (EGFR, ErbB1) and activates both EGFR homodimers and EGFR/ErbB2 heterodimers. Elevated TGF α , EGFR, and ErbB2 are associated with ER loss and antiestrogen resistance in breast tumors.^{15–17} TGF α is also a potent, well-characterized oncogene in transgenic mice.¹⁸ Transgenic mammary TGF α overexpression driven by several promoters, including whey acidic protein (WAP), MT, and mouse mammary tumor virus (MMTV), result in premature alveolar development, delayed involution after lactation, increased number of preneoplastic lesions, and eventually tumors.^{19–23}

Interactions between PRL receptor and EGFR pathways have been examined in several mammary cell lines. However, these studies have resulted in disparate conclusions: mutual inhibition, as observed in NMuMG and HC11 cells^{24–27}; positive interactions, as reported in SK-BR3 breast cancer cells²⁸; or variable outcomes in primary mammary epithelial cell cultures, depending on the concentration of EGF and physiological state of the cells.²⁹ These divergent results may reflect spurious differences in cell lines, limited sampling of pathways and end points, or alternatively, modulation of receptors and available downstream pathways by physiological context and/or accumulating neoplastic changes.

In view of the prevalence of TGF α overexpression in human breast cancer and the recently recognized ability of local mammary PRL to contribute to this disease, it is critical to establish the consequences of the interaction between these pathways in mammary cancer pathogenesis in the *in vivo* setting. Therefore, we investigated the effects of co-expression of mammary PRL and TGF α directed by the NRL promoter. We found that PRL and TGF α cooperate to dramatically reduce the latency of macrocyst development in virgin FVB/N females, the principal lesion elicited by transgenic TGF α in this strain. This was associated with increased phosphorylation of ERK1/2, a potential signal to mediators of dysregulated cell cycle progression. Furthermore, transgenic PRL countered the fall in ER α expression observed in NRL-TGF α glands. These findings demonstrate that PRL strikingly amplifies the neoplastic actions of another oncogene as well as potentially modifies ovarian hormonal responsiveness of the gland.

Materials and Methods

Reagents

The following antibodies were used for immunohistochemical and/or Western analyses: 5-bromo-2-deoxyuridine (BrdU) (MAS-250) from Accurate Scientific (Westbury, NY), ERK1/2 (9102) and phospho-ERK1/2 (Thr202/Tyr204; 9101) from Cell Signaling Technology (Beverly, MA), EGFR (SC-03) and ER α (SC-542) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and keratin 8 (RB-9095) from Labvision (Fremont, CA). Recombinant hPRL (Lot AFP795) was obtained through the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, and Dr. A. F. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center). TGF α was purchased from Peprotech (Rocky Hill, NJ).

Mice

NRL-PRL mice (line 1647-13, TgN(Nrl-Prl)23EPS; line 1655-8, TgN(Nrl-Prl)24EPS)⁸ and NRL-TGF α mice (line 1385-7, TgN(Nrl-TGF α)25EPS; T.A. Rose-Hellekant, unpublished data) were generated as described. The NRL promoter directs expression to mammary epithelial cells and is not altered by PRL or estrogen⁸ (T.A. Rose-Hellekant, unpublished data). All lines were maintained in the FVB/N strain background, and studies were conducted in virgin females. Tail biopsies were collected at weaning, and offspring were screened for the PRL transgene as described previously⁸ and for the TGF α transgene using the following polymerase chain reaction primers: forward, 5'-AAGGAAAGGTGTCTCAGGACAA-3', and reverse, 5'-CTGCTCCCTTCCCTGTCCTTC-3'. Female mice of the NRL-PRL 1655-8 but not the 1647-13 lineage have elevated circulating PRL.⁸ The studies described herein were performed in both NRL-PRL lines; for clarity, data only from line 1647-13 are included in the tables and Figures 3 to 7. Mice were housed and handled in accordance with the Guide for Care and Use of Laboratory Animals in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities. All procedures were approved by the University of Wisconsin-Madison Animal Care and Use Committee.

Mammary Gland Whole-Mount Analysis

Fourth inguinal mammary glands were pressed between two slides, fixed in 10% neutral buffered formalin overnight, and stored in 70% ethanol. Whole mounts were treated with 1:3 glacial acetic acid:ethanol for 1 hour, hydrated by graded ethanol, stained for 1 hour with hematoxylin, dehydrated with graded ethanol, and stored in glycerol until analysis. The whole mounts were analyzed for growth by measuring the percentage of the fat pad filled by ductal outgrowth from the nipple. Ductal growth was analyzed by dividing the area from the nipple to the leading edge of the ducts by the total area of the mammary gland.

Histological Examination of Mammary Tissue

Mammary glands were fixed in 10% neutral buffered formalin overnight, embedded in paraffin, and cut into 6- μ m sections. Morphological analysis was performed on hematoxylin and eosin-stained slides. Mice were injected with 200 mg/kg body weight BrdU (Sigma Chemical Co., St. Louis, MO) 1 hour before sacrifice to label cells undergoing DNA synthesis. Proliferation, apoptosis, and ER α expression were evaluated as described previously.⁸ BrdU, apoptotic, and ER α indices were evaluated separately for morphologically normal epithelial structures and lesions as described previously.⁸ Stromal density was assessed by counting fibroblast nuclei under $\times 400$ magnification. The counts from four fields of view were averaged for each mouse; 10 mice from each genotype were examined.

MCF-7 Cell Culture

PRL-deficient cells derived from the human mammary adenocarcinoma cell line MCF-7 were grown in RPMI-1640 containing 10% horse serum and 50 μ mol/L gangciclovir as reported previously.³⁰ For experiments, 10⁶ cells/60-mm plate were incubated in serum-free media for 24 hours before treatment with vehicle, 4 nmol/L PRL, and/or 30 ng/ml TGF α for times as stated in the legends. Cell lysates were harvested and analyzed by immunoblotting as described previously.³⁰

Western Analysis

Western analysis was performed as described previously.³⁰ In brief, 30 μ g protein of cellular lysate, or 60 μ g of mammary gland homogenate, was fractionated by standard Laemmli SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and then probed with appropriate antibodies (ERK1/2, 1:1000; phospho-ERK1/2, 1:5000; keratin 8, 1:1000; and EGFR, 1:400). Signals were visualized by enhanced chemiluminescence, followed by autoradiography. For some experiments, signals were quantified by densitometry (ImageQuant software, v.4.2a; Molecular Dynamics, Inc., Sunnyvale, CA).

Statistical Analyses

Latency data were subjected to Kaplan-Meier analysis and plotted as a function of the probability of a mouse remaining tumor free versus its age in months. Differences were detected using the Mantel-Haenszel test, which compares median survival among groups. Counted indices from immunohistochemistry sections were analyzed using Kruskal-Wallis test for nonparametric data, followed by the Mann-Whitney post test. Protein levels determined by immunoblotting were analyzed by one-way analysis of variance. Statistical analyses were performed using Prism v.3.02 (GraphPad Software, Inc., San Diego, CA). Differences were considered significant at $P < 0.05$.

Figure 1. Effect of transgene on tumor latency. Transgenic mice of all lines were monitored until tumors reaching 1.5 cm in diameter were detected (end stage). Nontransgenic littermates remained tumor free at ≥ 19 months of age. The latencies were compared by Kaplan-Meier analysis, and differences were detected using the Mantel-Haenszel test. NRL-PRL lines have a significantly longer tumor latency than NRL-TGF α females ($P < 0.002$) or NRL-TGF α /PRL bitransgenic lines ($P < 0.0001$). Both NRL-TGF α /PRL bitransgenic lines have a significantly shorter latency than TGF α females ($P < 0.0001$) and are not significantly different from each other.

Results

Bitransgenic Mice Develop Mammary Lesions Similar to NRL-TGF α Females

NRL-TGF α /PRL virgin females developed complex mammary macrocysts or neoplasms with 100% incidence

Table 1. Mammary Morphology at End Stage in Virgin NRL-TGF α /PRL Bitransgenic Mice

Lineage	Age (months)	Mammary abnormalities
PRL $\times$ TGF α	5.6 $\pm$ 2.1	Macrocysts (16/18) Complex/papillary (11/15) [†] Simple/papillary (4/15) [†] EH (10/18) Dilated ducts (9/18) Adenosis (10/18) MINs (1/18) [‡] Squamous changes (2/18) Squamous adenocarcinoma (2/18)
TGF α	11.2 $\pm$ 4.1	Macrocysts (35/36) Complex/papillary (14/27) [†] Simple/papillary (7/27) [†] Papillary/glandular (4/27) [†] Simple/glandular (1/27) [†] EH (16/36) Dilated ducts (1/36) Adenosis (5/36) Squamous changes (3/36) Papillary adenocarcinoma (1/36)
PRL [§]	16.7 $\pm$ 4.1	EH (9/11) Dilated ducts (11/11) MINs (11/11) [‡] Squamous changes (2/11) Adenocarcinoma (15/23) [¶]
Nontransgenic FVB/N	18.8 $\pm$ 5.3	No neoplasms present (58/61) Fibrosarcoma (1/61) Glandular adenocarcinoma (1/61) Adenosquamous carcinoma (1/61)

*Age at end stage is defined as tumor size reaching 1.5 cm diameter.

[†]Subset of tissues examined in each category.

[‡]MIN, mammary intraepithelial neoplasia.

[§]From Ref. 8.

[¶]More than one tumor was present in several individuals.

^{||}From Ref. 31.

Figure 2. Macrocysts, preneoplastic lesions, and ER α expression in mammary glands of virgin bitransgenic female mice. Macrocysts from NRL-PRL (1655-8) $\times$ TGF α female (**A**) and NRL-PRL (1647-13) $\times$ TGF α female (**B**) with areas of simple and papillary cystic epithelial lining. **C:** Focus of adenosis in mammary gland of NRL-PRL (1647-13) $\times$ TGF α female. **D:** Macrocyst from NRL-PRL (1647-13) $\times$ TGF α female with simple to dense glandular epithelium. **E:** Higher magnification of **D** showing cellular detail. Epithelial lining varies from simple to multilayered and in many areas forms solid foci. There is moderate variability in nuclear size and shape and occasional mitotic figures (**arrow**) and apoptotic bodies (**arrowheads**). **F:** Macrocyst with papillary lining with high ER α expression in TGF α female. **G:** Macrocyst with papillary lining that is ER α negative in NRL-PRL (1655-8) $\times$ TGF α female. **H:** BrdU-labeled epithelial cells in duct from NRL-PRL (1647-13) $\times$ TGF α female. **I:** Apoptotic bodies (**arrowheads**) in duct from NRL-PRL (1647-13) $\times$ TGF α female. **J:** Phospho-ERK 1/2 staining in duct from NRL-PRL (1647-13) $\times$ TGF α female. Original magnification in **A**, **B**, and **C**, $\times 25$; in **D**, $\times 100$; and in **E**, **F**, **G**, **H**, **I**, and **J**, $\times 400$.

(Figure 1; Table 1). Although these end-stage lesions were similar to those that developed in the mammary glands of NRL-TGF α virgin females, the macrocysts formed with greatly decreased latency in the bitransgenic mice (Figure 1; Table 1). The latency to development of macrocysts was not significantly different between the bitransgenic lines, despite the presence of elevated circulating PRL in only one of the NRL-PRL lines, consistent with the importance of locally produced PRL.

The macrocysts in the bitransgenic glands were highly pleomorphic structures, morphologically similar to mammary lesions found in female mice overexpressing TGF α under control of the NRL promoter (T.A. Rose-Hellekant, M.D. Schroeder, J.L. Brockman, M.N. Gould, L.A. Schuler, and E.P. Sandgren, unpublished data), as well as the WAP³¹ and MT promoters²¹ in the FVB/N train

background. Microscopically, the multiloculated macrocysts consisted of a single layer of simple or papillary epithelium surrounding proteinaceous fluid (Figure 2, A and B). The cells were uniform in size with an increased nuclear-to-cytoplasmic ratio, one to two nucleoli in each nucleus, and highly vacuolated cytoplasm, suggesting high secretory activity. Focal areas within the cysts of NRL-PRL/TGF α bitransgenic mice displayed a more complex histotype, with combinations of both papillary and simple epithelium present around the same lumen and foci with multiple cell layers in the cyst lining and occasionally solid centers (Figure 2, D and E). This complex histotype was found more often in bitransgenic females than NRL-TGF α females (Table 1). For clarity, only the analysis of mice derived from NRL-PRL line 1647-13 (normal circulating PRL) is shown; glands derived from

Figure 3. Stromal density, ER α expression, proliferation, and apoptosis rates in macrocyst lesions in NRL-TGF α and bitransgenic NRL-TGF α /PRL females. **A:** Stromal density surrounding macrocysts (HPF, high powered field; magnification, $\times 400$). **B:** Percentage of ER α + cells present in mammary macrocysts. Each symbol represents a single macrocyst. **C and D:** Proliferation (**C**) and apoptosis (**D**) indices in macrocysts. BrdU labeling, apoptotic indices, and stromal density were determined as described in Materials and Methods and expressed as means $\pm$ SD. Letters represent significant differences using Kruskal-Wallis test with Mann-Whitney post test ($P < 0.05$).

NRL-PRL line 1655-8 were similar. The stroma surrounding these lesions in bitransgenic mice had significantly increased density (Figure 3A), indicated by increased numbers of fibroblast nuclei surrounding the macrocyst lining, compared with stroma adjacent to NRL-TGF α macrocysts. Two bitransgenic females developed squamous adenocarcinomas (classified based on recommendations of the Annapolis Pathology Panel³²). The majority of mammary tumors and macrocysts developed in the cranial glands, although preneoplastic lesions were found in all mammary glands examined. Unlike NRL-TGF α females, virgin NRL-PRL females did not develop these macrocysts but instead developed adenocarcinomas of varying histotype at a longer latency, as described previously.⁸

Macrocysts Display Variable ER α Expression

PRL has been shown to regulate responsiveness of multiple reproductive tissues to ovarian hormones *in vivo*,³³ and endogenously synthesized PRL increases ER α *in vitro*.³⁴ In contrast, elevated TGF α , EGFR, and ErbB2 are generally associated with ER α loss in breast tumors. Therefore, we were interested in examining the net outcome of PRL and TGF α interaction on ER α expression. Within the cells lining the macrocysts, ER α expression varied widely among individual cysts in both bitransgenic and single transgenic NRL-TGF α females (Figures 2, F and G, and 3B). Furthermore, despite the decreased latency to macrocyst development in bitransgenic females, the proliferation rates in the macrocysts of these females did not differ from NRL-TGF α females (Figure 3C). Apoptosis levels in macrocysts of bitransgenics were significantly greater than NRL-TGF α females ($P < 0.05$; Figure 3D), which may reflect the influence of PRL on apoptosis. Overall, these results suggest that once the macrocysts have developed, the transgenes do not strongly modulate proliferation or ER α expression.

PRL and TGF α Cooperatively Induce Other Preneoplastic Lesions

Although the most common preneoplastic lesion in the bitransgenic animals was the macrocyst, other lesions were present in all mammary glands examined. Lesions were present with an increased incidence in end-stage glands of bitransgenic females compared with end-stage glands from NRL-TGF α females, even though the latter were twice as old (5.6 versus 11.2 months; Table 1). Epithelial hyperplasias (EHs), common in the older single transgenic females, were present in a majority of the bitransgenic glands. In addition, many of these glands also exhibited dilated ducts, associated with expression of PRL alone, and adenosis lesions (Figure 2C), found at a lower frequency with monotransgenic TGF α expression (Table 1). The decreased latency of these abnormalities demonstrates strong cooperation between PRL and TGF α in development of these lesions.

PRL Increases Proliferation and Apoptosis in Morphologically Normal Structures of Bitransgenic Females

To determine the cellular events underlying the interaction between the transgenes, we examined the rates of proliferation and apoptosis in normal appearing mammary structures. Glands of 6-month-old NRL-TGF α females exhibited increased rates of proliferation in both alveoli and ducts (Figure 4, A and B) and decreased rates of apoptosis in ducts (Figure 4, C and D) compared with nontransgenic controls, similar to WAP-TGF α animals.³⁵ Bitransgenic females demonstrated significantly higher proliferation in both alveoli (Figure 4A) and ducts (Figure 4B) compared with the NRL-TGF α glands, although not different from NRL-PRL animals.⁸ Bitransgenic animals also exhibited significantly more alveolar apoptosis than NRL-TGF α mice (Figure 4C), comparable with NRL-PRL lines. Similarly,

Figure 4. Proliferation rates, apoptosis indices, and ERα expression in morphologically normal alveoli and ducts. **A** and **B**: BrdU labeling in alveoli (**A**) and ducts (**B**). **C** and **D**: Apoptosis indices in alveoli (**C**) and ducts (**D**). **E** and **F**: ERα expression in alveoli (**E**) and ducts (**F**). Analysis was performed on mammary glands of 6-month-old virgin nontransgenic (NonTG), NRL-TGFα (TGFα), NRL-PRL 1647-13 (PRL), and bitransgenic NRL-TGFα/PRL 1647-13 (PRL x TGFα) females. Labeling was determined as described in Materials and Methods, and data are expressed as means ± SD. Different lowercase letters denote statistical differences among structures of the different lines determined by the Kruskal-Wallis test followed by Mann-Whitney post test ($P < 0.05$). NonTG and NRL-PRL proliferation and apoptosis rates were described previously.⁸

EHs in bitransgenic females showed significantly increased proliferation and apoptosis compared with hyperplasias in NRL-TGFα females (Figure 5, A and B). NRL-PRL females had very few EHs at this age.

Figure 5. Proliferation (**A**), apoptosis (**B**), and ERα (**C**) expression in epithelial hyperplasias in 6-month-old NRL-TGFα and NRL-TGFα/PRL (line 1647-13) bitransgenic virgin females. NRL-PRL and nontransgenic (NonTG) females did not develop epithelial hyperplasias by this age. Labeling was determined as described in Materials and Methods, and data are expressed as means ± SD. Different lowercase letters denote statistical differences among structures of the different lines determined by the Kruskal-Wallis test followed by Mann-Whitney post test ($P < 0.05$).

TGFα Lowers ERα Expression in Morphologically Normal Structures

Transgenic mammary PRL elevates ERα expression in mammary structures.⁸ In contrast, glands of NRL-TGFα females displayed significantly reduced ERα expression in alveoli and ducts compared with nontransgenic controls at 6 months of age (Figure 4, E and F). Interestingly, age-matched bitransgenic females had more ERα, particularly in ducts, than both NRL-PRL and nontransgenic animals (Figure 4, E and F). Furthermore, ERα expression in EH was more than three times higher in bitransgenic glands than in NRL-TGFα females (Figure 5C). These data indicate that PRL can modulate the effect of TGFα on ERα expression in morphologically normal structures and early lesions.

PRL and TGFα Induce Developmental Abnormalities

To assess interactions of these transgenes during mammary development, we evaluated mammary whole mounts at 13 weeks of age and at end stage (Figure 6). In normal female mice, ducts in the mammary gland grow from the nipple past a central lymph node by 4 weeks of age, reach the border of the mammary fat pad with some remaining terminal end buds by 6 weeks, and have filled the fat pad with a ductal network by 12 weeks of age.^{35,36} Glands of NRL-TGFα females (Figure 6, C and D) demonstrated ductal development similar to nontransgenic females (Figures 6, G and H) but also displayed widespread alveolar development, as reported for MMTV-TGFα females.¹⁹ In contrast, NRL-PRL females demonstrated retarded ductal growth into the mammary fat pad compared with nontransgenic glands (Figure 6E; Table 2). These glands also exhibited dilated ducts and hyperplasias, which were more prominent by end stage (Figure 6F), consistent with abnormalities observed histologically (Table 1).

Whole mounts from bitransgenic females demonstrated characteristics of each individual transgene (Figures 6, A and B). Similar to glands of NRL-PRL females,

Figure 6. Representative whole mounts from inguinal glands of the different genotypes at 13 weeks of age and end stage. **A** and **B**: Bitransgenic NRL-PRL (line 1647-13) $\times$ TGF α . **C** and **D**: TGF α . **E** and **F**: NRL-PRL (line 1647-13). **G** and **H**: nontransgenic mice (NonTG). The first column represents inguinal mammary whole mounts from 13-week-old females (**A**, **C**, **E**, and **G**). The second column represents inguinal mammary whole mounts collected at end stage, after the mice developed an adenocarcinoma or macrocyst reaching 1.5 cm in diameter (**B**, **D**, **F**, and **H**). Mammary whole mounts from NonTG female cage mates were also collected at this time. Mammary glands were collected and prepared as described in Materials and Methods. The large dark oval in each mammary gland is a lymph node.

bitransgenic glands displayed decreased ($P = 0.07$) ductal penetration into the mammary gland (Table 2) and increased epithelial development and hyperplasias, apparent by 13 weeks of age. These hyperplasias appeared to be enlarged compared with NRL-TGF α females (compare Figure 6, A and B versus C and D).

Mammary Glands from Bitransgenic Females Contain Elevated ERK1/2 Activity

Although both transgenic PRL and TGF α are produced by mammary epithelial cells, these ligands are secreted and their respective receptors are present on stromal and

Table 2. Extent of Inguinal Mammary Fat Pad Filled by Ducts*

	<i>n</i>	13 weeks	<i>n</i>	End stage
PRL $\times$ TGF α	3	64 $\pm$ 18 [†]	4	78 $\pm$ 14
TGF α	3	100 $\pm$ 0	4	100 $\pm$ 0
PRL	3	62 $\pm$ 5 [‡]	3	96 $\pm$ 7
Non-TG	3	100 $\pm$ 0	3	100 $\pm$ 0

*Expressed as percentage of mammary gland filled, mean $\pm$ SD.

[†]Different from Non-TG controls at $P = 0.07$.

[‡]Significantly different from Non-TG controls ($P = 0.01$).

A. P-ERK 1/2

B. P-ERK 1/2

C. EGFR

Figure 7. PRL raises levels of phosphorylated ERK1/2 above TGF α alone. **A**: Mammary glands from 3-month-old NRL-TGF α and bitransgenic NRL-PRL (line 1647-13) $\times$ TGF α virgin females were disrupted with a polytron, and lysates were examined for phosphorylated ERK1/2, total ERK1/2, and keratin 8 by Western analysis. **B**: Signals in **A** were quantitated densitometrically and expressed as phosphorylated ERK1/2/total ERK1/2/keratin 8 signal ($n = 3$, means $\pm$ SD). Different lowercase letters denote statistically significant differences after one-way analysis of variance ($P < 0.02$). **C**: EGFR levels in the mono- and bitransgenic glands examined by Western analysis as in **A**, corrected for keratin 8 expression. ($n = 3$, means $\pm$ SD).

epithelial cells.^{37–39} In addition, both ligands can initiate multiple signaling cascades in their targets. Therefore many potential mechanism(s) may mediate the interaction between these transgenes to create the mammary phenotype described here. ERK1/2 is an important kinase in the proliferative response to many growth factors, including PRL and TGF α .^{40–42} To examine the net effect on this kinase cascade in glands destined to develop macrocysts, but before morphological abnormalities, we compared levels of phosphorylated ERK1/2 in glands of 3-month-old NRL-TGF α and NRL-TGF α /PRL mice. As shown in Figure 7, A and B, relative levels of activated ERK1/2 were higher in bitransgenic than single TGF α transgenic glands ($P < 0.02$), demonstrating positive crosstalk to this end point. In contrast, levels of EGFR were similar at this time (Figure 7C); levels of ErbB2 were very low in both genotypes (not shown). To determine whether crosstalk to ERK1/2 could occur within breast cancer cells, we acutely stimulated MCF-7-derived cells with PRL and/or TGF α . Each ligand alone transiently increased phosphorylated ERK1/2 as expected (Figure 8A). Although no interaction between them was observed after 15 or 45 minutes of stimulation, these factors together significantly prolonged phosphorylation of these kinases, evident after 120 minutes (Figure 8B, $P < 0.01$). The importance of signal duration, including phosphorylation of mitogen-activated protein kinases, is increasingly recognized for multiple activities, including proliferation and apoptosis.^{40,43}

Discussion

In this study, we have shown that mammary transgenic PRL dramatically synergizes with TGF α to promote de-

A. Time course of P-ERK1/2

B. P-ERK1 (120 min)

Figure 8. PRL and TGF α together prolong ERK1/2 phosphorylation. **A:** Western analysis of levels of phosphorylated ERK1/2 in PRL-deficient 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 30 ng/ml TGF α for the times shown. Cellular lysates (30 μ g protein) were analyzed for phosphorylated ERK1/2 and total ERK1/2 by Western analysis. **B:** Fold change in phosphorylated ERK1/2 levels after 120 minutes incubation with ligand compared with vehicle-treated controls from three independent experiments (means $\pm$ SD). Different lowercase letters denote statistically significant differences after one-way analysis of variance ($P < 0.05$).

velopment of locally aggressive, highly secretory mammary lesions with greatly decreased latency compared with single transgenic animals. Furthermore, transgenic PRL reduced the latency of other mammary abnormalities and countered the fall in ER α expression induced by transgenic TGF α . Importantly, the two transgenic PRL lines exhibited similar cooperation with TGF α , demonstrating that mammary PRL with or without elevated circulating PRL is sufficient for this synergy. These findings show that local PRL can powerfully potentiate the carcinogenic actions of another oncogene and modify apparent ovarian hormonal responsiveness. Together, they suggest that PRL may play an important role in mammary tumorigenesis and may be an effective therapeutic target.

The shortened latency to a similar dominant lesion in the bitransgenic and TGF α monotransgenic gland, albeit with a more complex histotype and surrounding reactive stroma, indicates that PRL potentiates TGF α signals at some mammary targets. Our data demonstrate that crosstalk to ERK1/2 is one mechanism that may mediate this interaction and that this can occur within epithelial cells. These kinases have been shown to be important for neoplasia in several transgenic models, including MMTV-ErbB2/TGF α ,⁴¹ in part by their signals to cell cycle progression.^{40,44} However, PRL and TGF α share other signaling cascades observed in various model systems, many of which have been implicated in mammary carcinogenesis, including other mitogen-activated protein kinases, phosphatidylinositol 3-kinase-AKT, and STATs.^{1,9,45,46} These pathways, as well as proximal

cross-activation of the other's receptor and modulation of distal feedback loops, present multiple opportunities for interactions within common target cells. In addition, PRL and TGF α may also target distinct cell subpopulations, permitting collaboration in lesion development via secondary secreted factors. Interestingly, some morphological abnormalities, such as ductal dilation, were common in NRL-PRL mammary glands but not those expressing transgenic TGF α and were observed in many but not all bitransgenic glands. This suggests that PRL exerts some actions that are only modestly modulated by TGF α and may reflect a different receptor distribution and/or use of distinct signaling pathways.

The positive interaction between PRL and TGF α in proliferation of morphologically normal structures and hyperplasias contrasts with the opposing effect of these oncogenes on apoptosis. Whereas TGF α alone decreased apoptosis, PRL in combination with TGF α restored apoptosis to that of nontransgenic controls. This PRL-induced increase in cell turnover is further evidence of distinct activities of these two factors. The apparent inability of PRL to promote survival *in vivo* is consistent with the similar level of apoptosis in transplanted PRL^{+/+} and PRL^{-/-} epithelium⁴⁷ but differs from effects observed *in vitro*,^{1,48} underscoring the importance of *in vivo* studies.

In addition to these observations in adult glands, cooperation between transgenic TGF α and PRL was also evident in pubertal ductal morphogenesis. In the normal mouse, paracrine stromal/epithelial signals elicit ductal elongation at adolescence, orchestrated by estrogen, growth hormone, and local growth factors, including EGF family members.^{12,49} Deficits in EGFR, ErbB2, and amphiregulin all result in profound defects in ductal growth and defective terminal end buds. Interestingly, transgenic overexpression of TGF α alone also slightly retards ductal penetration.²¹ TGF α levels are tightly controlled in the peri-pubertal murine gland⁵⁰; overexpression may disrupt appropriate signaling patterns at critical times, perhaps by competing with amphiregulin for the EGFR. The latter is considered to be the critical EGFR ligand at puberty^{51,52} and binds EGFR with a lower affinity than TGF α .⁵³ In the current study, transgenic PRL also delayed ductal morphogenesis, perhaps reflecting PRL suppression of estrogen-induced ductal proliferation, as reported in studies of ovariectomized mice by Hovey et al.³⁸ The accentuated defective ductal elongation observed in the current study indicates that PRL crosstalk with TGF α further confounds the precise temporal and spatial signals that regulate this event.

Our data also demonstrate the robust ability of PRL to elevate ER α levels *in vivo*. Estrogen, EGFR, and TGF α have complex interactions in the normal mammary gland as well as breast tumors. Although estrogen increases the secretion of TGF α and TGF α can at least partially mediate estradiol-stimulated growth of mammary epithelial cells,^{54–57} estrogen and another EGFR ligand, EGF, oppose one another's signals via decreasing expression of their respective receptors *in vitro*.⁵⁸ EGFR and ER α also are inversely related in human breast tumors.^{15–17} In our model, transgenic TGF α reduced ER α expression in

morphologically normal structures, consistent with these clinical findings. However, PRL overexpression in combination with TGF α was able to restore ER α levels in these structures and dramatically elevated expression in EH, overriding this aspect of the transgenic TGF α phenotype. Recent studies have demonstrated that estrogen hastens progression from premalignant EH to ER α -negative tumors.^{59,60} Our data suggest that PRL may be an important modulator of ER α expression *in vivo*. Interestingly, ER α expression varied widely among individual macrocysts at end stage in both NRL-TGF α and bitransgenic females in our model. This may reflect loss of the normal regulation of ER α by some subset of lesions, similar to that occurring in human disease. Studies in progress will determine the functional significance of the PRL-induced increase of these receptors in lesion development and tumor progression.

Despite the established role of PRL in mammary gland development and lactation, attention has only been recently focused on its actions in breast cancer and the contribution of locally synthesized PRL to oncogenic processes in this gland. Our data demonstrate that prolonged exposure to increased mammary PRL cannot only lead to neoplasia but also potentiation of processes driven by other mammary oncogenes such as TGF α , decreasing the latency of lesion development and increasing the apparent estrogenic responsiveness of the early lesions. Understanding the actions of this hormone in tumorigenesis and interactions with characterized players in breast cancer will direct efforts toward improved diagnostic and treatment modalities for human disease.

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