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Tumorigenesis and Neoplastic Progression

Prolactin Promotes Mammary Pathogenesis Independently from Cyclin D1

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Epidemiological and experimental studies have revealed an important role for prolactin (PRL) in breast cancer. Cyclin D1 is a major downstream target of PRL in lobuloalveolar development during pregnancy and is amplified and/or overexpressed in many breast carcinomas. To examine the importance of cyclin D1 in PRL-induced pathogenesis, we generated transgenic mice (NRL-PRL) that overexpress PRL in mammary epithelial cells, with wild-type, heterozygous, or genetically ablated cyclin D1 in the FVB/N genetic background. Although loss of one cyclin D1 allele did not affect PRL-induced mammary lesions in nonparous females, the complete absence of cyclin D1 (*$D1^{-/-}$*) markedly decreased tumor incidence. Nevertheless, NRL-PRL/*$D1^{-/-}$ **females developed significantly more preneoplastic lesions (eg, epithelial hyperplasias and mammary intraepithelial neoplasias) than *$D1^{-/-}$ **females. Moreover, although lack of cyclin D1 reduced proliferation of morphologically normal mammary epithelium, transgenic PRL restored it to rates of wild-type females. PRL posttranscriptionally increased nuclear cyclin D3 protein in *$D1^{-/-}$ **luminal cells, indicating one compensatory mechanism. Consistently, pregnancy induced extensive lobuloalveolar growth in the absence of cyclin D1. However, transcripts for milk proteins were reduced, and pups failed to survive, suggesting that mammary differentiation was inadequate. Together, these results indicate that cyclin D1 is an important, but not essential, mediator of PRL-induced mammary proliferation and pathology in FVB/N mice and is critical for differentiation and lactation. *(Am J Pathol 2012, 181:294–302; http://dx.doi.org/10.1016/j.ajpath.2012.03.041)*

The hormone prolactin (PRL) is critical for mammary alveolar morphogenesis and differentiation.[1] Recent epidemiological studies have also implicated PRL in the risk of breast cancer, highlighting its importance in tumorigenesis. Elevated circulating PRL is associated with a higher risk of development of tumors that express estrogen receptor α (ERα^+) and with poorer patient outcomes, and PRL receptors (PRLR) are expressed at high levels in many cancers.[2,3] Moreover, particularly in women, the mammary gland is exposed to locally produced PRL, in addition to that from pituitary lactotrophs.[4–6] Murine transgenic experimental models with elevated mammary PRL have demonstrated the oncogenic potential of this hormone, and permit investigation of the mechanisms whereby PRL promotes breast cancer development and progression.[7]

Epithelial proliferation is a key feature of PRL-driven lobuloalveolar development during pregnancy,[1] and the cell cycle regulator, cyclin D1, has been reported to be a critical mediator of this process.[8] However, the role of cyclin D1 in PRL-induced pathogenesis has not been examined. The classic function of the D cyclins (D1, D2, and D3) is promotion of the G1 to S phase of the cell cycle, via regulation of their cyclin-dependent kinase partners, CDK4 and CDK6.[9,10] Activation of these kinases by D cyclins results in phosphorylation of retinoblastoma protein, leading to increased transcription of E2F-responsive genes, and subsequent mitosis. In addition, cyclin D1 has been shown to regulate multiple other processes relevant to oncogenesis, including other actions in cell cycle progression, adhesion and migration, responses to DNA damage, protein synthesis, metabolism, and differentiation, in many cases, independently of CDK4/6 or its kinase activity.[11–14] The expression of individual D cyclins is tissue

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specific, but redundancy permits compensation in many tissues.[10] Mammary lobuloalveolar proliferation has appeared to be an exception; genetic ablation of cyclin D1 ($D1^{-/-}$) in the C57BL/6 × 129SV genetic background prevented this event, culminating in lactation failure.[15,16] This phenotype is very similar to that observed in $Prlr^{-/-}$ females in the same mixed strain background.[1]

CCDN1 is amplified in a substantial subset of breast carcinomas, and cyclin D1 protein is overexpressed in many others (50% to 70%).[13,17,18] Many hormones and growth factors, including PRL and estrogen, activate its promoter.[19] In MCF-7 breast cancer cells, PRL increases transcription of cyclin D1,[20] which is required for the subsequent proliferative response.[21] PRL also induces nuclear accumulation of this cyclin in murine mammary epithelial cells.[22] The requirement for cyclin D1 in mammary tumorigenesis secondary to well-characterized oncogenes has been investigated in murine models in the C57BL/6 × 129SV genetic background. These experiments demonstrated that cyclin D1 was essential for some oncogenes, such as MMTV-driven *neu* (*erbB2*) and *v-Ha-ras*, but also that the cyclin D1 status of mice expressing *Myc* (alias c-*myc*) and *Wnt-1* driven by the same promoter did not affect tumor incidence or latency.[23,24]

Experimental models have demonstrated that augmented proliferation also is an important contribution of PRL to mammary tumorigenesis.[25–28] To investigate the requirement for cyclin D1 in PRL-induced pathology, we used a murine transgenic model of elevated mammary PRL, NRL-PRL. In this model, local PRL overexpression driven by the estrogen- and PRL-independent promoter, NRL, results in preneoplastic lesions, including epithelial hyperplasias and mammary intraepithelial neoplasias, similar to ductal carcinoma *in situ* in women, and eventually, invasive carcinomas, which resemble the clinical luminal subtype.[27,29] We generated NRL-PRL mice in the context of genetic ablation of *Ccdn1* in the FVB/N genetic background. We found that cyclin D1 was important, but not essential, for PRL-induced pathology. In nonparous females without cyclin D1, transgenic PRL was able to augment mammary epithelial proliferation, support alveolar development, and promote preneoplastic lesions and tumors, albeit at a lower level than with wild-type cyclin D1. PRL increased cyclin D3 expression posttranscriptionally, suggesting one compensatory mechanism. *Cyclin* $D1^{-/-}$ FVB/N females also displayed marked lobuloalveolar development during pregnancy, but expressed reduced levels of milk protein transcripts. Together, these observations indicate that cyclin D1 is not required for PRL-initiated mammogenic and tumorigenic signals in the FVB/N genetic background. Understanding the mediators of PRL actions in carcinogenesis will reveal potential sites for preventative and therapeutic interventions.

Materials and Methods

Reagents

5-Bromo-2-deoxyridine (BrdU) was purchased from Sigma-Aldrich (St. Louis, MO), and 17β-estradiol was obtained from Steraloids (Newport, RI). Antibodies were purchased from the following sources: BrdU (MAS-250) from Accurate Scientific (Westbury, NY), estrogen receptor α (ERα; SC-542), cyclin D2 (SC-53637), cyclin D3 (SC-182), and Grb2 (SC-255) from Santa Cruz Biotechnology (Santa Cruz, CA); cyclin D1 (CP 236 B) from Biocare Medical (Concord, CA), and rat anti-cytokeratin 8 (Troma1) from Developmental Studies Hybridoma Bank, University of Iowa. Secondary antibodies, anti-rat and anti-rabbit, were obtained from BioGenex (San Ramon, CA) and signals detected with 3,3′ diaminobenzidine from Vector Laboratories (Burlingame, CA).

Mice

Cyclin D1 heterozygous mice that had been backcrossed into the FVB/NJ strain (FVB.129S2(B6)-*Ccnd1*^{tm1Wbg}/J) were purchased from Jackson Laboratories (Bar Harbor, ME). NRL-PRL mice [line 1647-13, TgN (Nrl-Prl)23EPS; line 1655, TgN(Nrl-Prl)24EPS] were generated and maintained in the FVB/N strain as described.[27] Offspring were genotyped for the PRL transgene and cyclin D1 using the primers shown in Table 1. All mice were housed and handled in accordance with the Guide for Care and Use of Laboratory Animals in Association for the Assessment and Accreditation for Laboratory Animal Care–accredited facilities. All procedures were approved by the University of Wisconsin–Madison Institutional Animal Care and Use Committee.

17β-Estradiol Treatment

For some experiments, Silastic pellets containing 20 μg of 17β-estradiol were implanted subcutaneously in intact female mice starting at 8 weeks of age, and replaced every 6 weeks until the animal was euthanized at 1 year of age. This dose has been shown to supplement circu-

Table 1. PCR Primers

Target	Primer
Genetic manipulations	
Prolactin transgene	F, 5′-CCTCCTCATTCTCTGCTCTTC-3′
	R, 5′-CCAATCACCCTTGCTCTAAACCC-3′
Cyclin D1 ablation	5′-TAGCAGAGAGCTACAGACTTCG-3′
	5′-CTCCGTCTTGAGCATGGCTC-3′
	5′-CTAGTGAGACGTGCTACTTC-3′
Real-time PCR	
Cyclin D1	F, 5′-CGCCCTCCGTATCTTACTTCAA-3′
	R, 5′-CTCACAGACCTCCAGCATCCA-3′
Cyclin D2	F, 5′-GCTCTGTGCGCTACCGACTT-3′
	R, 5′-CCACGCTTCCAGTTGCAAT-3′
Cyclin D3	F, 5′-CGACTTCCTGGCCTTGATTC-3′
	R, 5′-CAAAGGTGTAATCTGTAGCACAGA-3′
β-Casein	F, 5′-GCAGAAACTTCAGAAGGTGAATCTC-3′
	R, 3′-TGACTGGATGCTGGAGTGAACT-3′
γ-Casein	F, 5′-GGTCAACCTAAACCAGCAGAAAA-3′
	R, 5′-TGTGCAACATTGGGAAAAGG-3′
Whey acidic protein	F, 5′-CGCTCAGAACCTAGAGGAACAAG-3′
	R, 5′-TGATACACTCTGTGCCCTCAATG-3′
Cytokeratin 8	F, 5′-TGAACAACAAGTTCGCCTCCTT-3′
	R, 5′-TCCACTTGGTCTCCAGCATCT-3′
18S	F, 5′-CGCCGCTAGAGGTGAATTTCT-3′
	R, 5′-CGAACCTCCGACTTTCGTTCT-3′

lating 17β-estradiol levels to approximately those at estrus.[30] Uteri were weighed at the time of collection to confirm the positive effect of the pellets. Uterine weight did not differ in untreated mice and was significantly increased in response to 17β-estradiol in all genotypes (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).

Examination of Mammary Tissue

Histological assessments were performed on hematoxylin and eosin–stained sections. Sections of some genotypes and treatment groups were stained for BrdU, ERα, cyclin D1, and/or cyclin D3 (primary antibodies: BrdU, 1:40; ERα, 1:1000; cyclin D1, 1:200; cyclin D3, 1:200), and apoptosis was determined by morphological criteria as described.[27] The proportion of epithelial cells undergoing proliferation, apoptosis, and those expressing ERα and cyclin D3 was quantified in three to five mice of each genotype by counting 2000 cells in at least 10 different fields. One thousand cells from at least five distinct microscopic fields were counted in tumors to determine the proportion of cells expressing detectable cyclin D1. For gross evaluation of epithelial structures, mammary whole mounts were prepared as described.[27]

Immunoblot Analysis

Western blot analyses of mammary homogenates were performed as previously described[28] Signals were quantified by densitometry (ImageQuant software, v.4.2a; Molecular Dynamics, Sunnyvale, CA).

Real-Time PCR

RNA was isolated from mammary lysates using RNeasy Mini Kit (Qiagen, Valencia, CA), and levels of transcripts determined by quantitative real-time PCR analysis as described previously.[31] The primers used are shown in Table 1.

Statistics

Statistical analyses were performed as described using Prism version 4.03 (GraphPad Software, San Diego, CA).

Results

Absence of Cyclin D1 Reduces but Does Not Prevent PRL-Induced Lesions and PRL-Augmented Proliferation

In light of the association between PRL and cyclin D1 in mammary epithelial proliferation revealed by several experimental approaches, we examined cyclin D1 expression in PRL-induced mammary carcinomas that developed in the context of wild-type cyclin D1. As shown in Figure 1A, the proportion of cells expressing cyclin D1 correlated moderately positively with the rate of proliferation (Spearman $r = 0.5647$; $P < 0.0004$), consistent with an important role for this cell cycle regulator in diverse, established PRL-induced primary tumors.

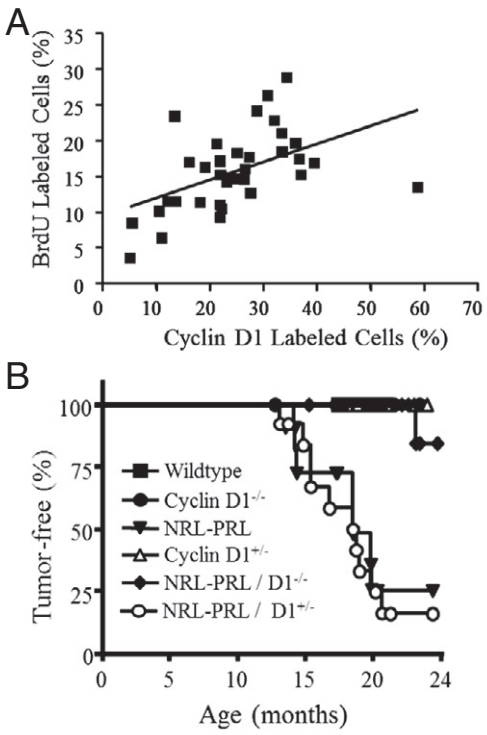

Figure 1. A: Cyclin D1 expression correlates with the rate of proliferation in PRL-induced tumors. A panel of diverse primary mammary tumors that developed in NRL-PRL females in the presence of wild-type cyclin D1 was examined by IHC for cyclin D1 and BrdU incorporation as described in *Materials and Methods* ($n = 35$; panel as described[29]). Each symbol represents a single tumor. The correlation coefficient was determined using Spearman's nonparametric test ($r = 0.57$; $P < 0.0004$). **B:** The absence of cyclin D1 reduces the incidence and increases the latency of tumors in NRL-PRL females. Females of all genotypes were monitored until tumors reached 1.5 cm in diameter, the mice developed significant health problems, or they reached 24 months of age (end stage). Absence of one cyclin D1 allele did not alter the incidence or latency of PRL-induced tumors ($P = 0.83$). However, loss of both alleles significantly reduced tumor incidence ($P = 0.001$), and tended to increase tumor latency ($P = 0.0528$, one-tailed Student's *t*-test). Latencies were compared using the Kaplan-Meier test, and differences between groups were detected using the Mantel-Haenszel test.

To evaluate the requirement for cyclin D1 in PRL-promoted tumor development, we generated NRL-PRL mice with wild-type, heterozygous, or genetically ablated cyclin D1 in the FVB/N genetic background. Nonparous females of all genotypes were evaluated at 24 months of age, when tumors reached 1.5 cm in diameter, or mice developed significant health problems (end stage). As shown in Figure 1B and Table 2, NRL-PRL females with wild-type cyclin D1 developed palpable mammary tumors with a high incidence, consistent with previous studies.[27] Heterozygosity for cyclin D1 did not alter the incidence or latency of PRL-induced tumors. However, ablation of cyclin D1 reduced the incidence to 12.5%, and the latency tended to be longer than those with wild-type cyclin D1 levels ($P = 0.0528$, one-tailed Student's *t*-test). Interestingly, although tumors that arose in NRL-PRL females with both or only a single cyclin D1 allele displayed varying histotypes (Table 2; Figure 2, A–C), tumors that developed in NRL-PRL/*D1*$^{-/-}$ females were adenosquamous carcinomas.

Despite the paucity of carcinomas, transgenic PRL induced many preneoplastic lesions in the absence of

Table 2. Effect of Cyclin D1 Status on Mammary Carcinogenesis in Nonparous Females

Genotype	Tumor incidence at end stage*	Tumor latency mean ± SD (months)	Tumor histotypes (%)
Wild-type (FVB/N)	0/10 (0%)	N/A	N/A
NRL-PRL	8/1 8/11 (72.7%)†	18.0 ± 2.9‡	Adenocarcinomas: Glandular (25) Papillary (25) Adenosquamous (12.5) Carcinosarcomas (37.5)
Cyclin D1$^{+/-}$	0/7 (0%)	N/A	N/A
NRL-PRL/*D1*$^{+/-}$	9/11 (81.8%)	17.6 ± 2.3	Adenocarcinomas: Glandular (22.2) Papillary (55.6) Adenosquamous (22.2)
Cyclin D1$^{-/-}$	0/12 (0%)	N/A	N/A
NRL-PRL/*D1*$^{-/-}$	2/1 2/16 (12.5%)†	22.1 ± 2.4‡	Adenocarcinomas: Adenosquamous (100)

*End stage is defined as a tumor reaching 1.5-cm diameter in size or 2 years of age.
†Incidences differ significantly ($P < 0.001$, chi-square test).
‡Latencies trend toward a significant difference ($P = 0.0528$, one-tailed Student's *t*-test).

cyclin D1 that were readily apparent on histological examination (Figure 2, D–F, Table 3). NRL-PRL/*D1*$^{-/-}$ females displayed significantly more epithelial hyperplasias that were larger and more widespread than in *cyclin D1*$^{-/-}$ females (Table 3). Furthermore, although mammary intraepithelial neoplasias were scarce in *D1*$^{-/-}$ mammary glands, they were readily apparent in NRL-PRL/*D1*$^{-/-}$ females. These data indicate that the lack of D1 does not abrogate PRL-initiated pathogenesis, but rather suggest that it slows lesion progression. However, the limited healthy lifespan of these mice precludes analysis of longer-term effects on tumor development.

To better understand the underlying mechanism, the rates of proliferation and apoptosis of morphologically normal mammary epithelium were determined. BrdU-labeled and ERα-labeled epithelial cells were observed in ducts of NRL-PRL/*D1*$^{-/-}$ females (Figure 2, G and H, respectively). Transgenic PRL increased both proliferation and apoptosis in the presence of wild-type cyclin D1 compared to nontransgenic females (Figure 3, A and B), as previously reported.[27,28] As expected, rates of proliferation were very low in *cyclin D1*$^{-/-}$ glands. However, PRL in the context of cyclin D1 ablation was able to augment proliferation to levels of nontransgenic glands (Figure 3A). The absence of cyclin D1 significantly increased apoptosis, which strikingly was reduced by transgenic PRL to wild-type levels (Figure 3B). This opposite net effect of transgenic PRL on apoptosis, depending on cyclin D1 status, suggests crosstalk between these factors in otherwise distinct pathways. Similar patterns were observed in PRL-induced hyperplasias (Figure 3, A and B), although as expected,[27] levels of prolifer-

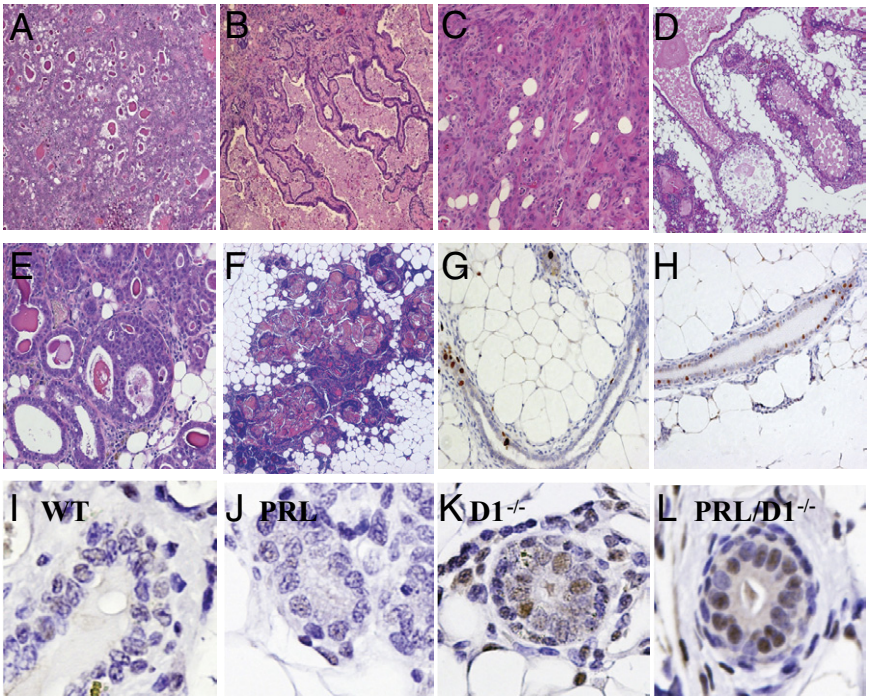

Figure 2. A–H: Diverse mammary carcinomas and lesions develop in NRL-PRL and NRL-PRL/*D1*$^{-/-}$ females. **A:** Glandular adenocarcinoma with eosinophilic secretions from an NRL-PRL female. **B:** Papillary adenocarcinoma from an NRL-PRL female. **C:** Adenosquamous carcinoma from an NRL-PRL/*D1*$^{-/-}$ female. **D:** Irregular (degenerative) ductal epithelium and dilated ducts in NRL-PRL/*D1*$^{-/-}$ female. **E:** Mammary intraepithelial neoplasia (MIN) within an epithelial hyperplasia in NRL-PRL/*D1*$^{-/-}$ female. **F:** Epithelial hyperplasia in an NRL-PRL/*D1*$^{-/-}$ female. **G:** BrdU-labeled epithelial cells in a duct of an NRL-PRL/*D1*$^{-/-}$ female. **H:** ERα-labeled epithelial cells in a duct of an NRL-PRL/*D1*$^{-/-}$ female. **I–L:** Immunohistochemical localization of cyclin D3 expression in mammary glands of end-stage females. **I:** Wild-type (WT); **J:** NRL-PRL; **K:** *Cyclin D1*$^{-/-}$; **L:** NRL-PRL/*D1*$^{-/-}$. Original magnification: ×200 (**A**, **C**, **D**, **E**, **F**, and **I–L**); ×100 (**B**); ×400 (**G** and **H**).

Table 3. PRL Increases Mammary Abnormalities in *Cyclin D1*$^{-/-}$ Glands

	Cyclin D1$^{-/-}$	NRL-PRL/*D1*$^{-/-}$	NRL-PRL/*D1*$^{+/+}$
Epithelial hyperplasias*	4/12†	11/16	11/11
	Focal: 2/12	Focal: 2/16	Focal: 0/11
	Multifocal: 2/12	Multifocal: 2/16	Multifocal: 0/11
	Diffuse: 0/12	Diffuse: 7/16	Diffuse 11/11
Mammary intraepithelial neoplasias	1/12†	11/16	10/11

Mammary glands were histologically examined at end stage.
Analysis performed by one-tailed chi-square test.
*Topographical distribution of hyperplasias as defined.[32]
†Indicates reduced frequency compared to NRL-PRL/*D1*$^{-/-}$ ($P < 0.05$).

ation were higher in lesions, compared to morphologically normal structures. Together, these results indicate that cyclin D1 is not required for PRL-induced responses, but both proliferation and neoplastic processes are facilitated by its presence.

The ability of PRL to increase epithelial growth in the absence of cyclin D1 was evident in mammary whole mounts and histological sections from these end-stage females. As expected from previous reports,[15,16] wild-type and *cyclin D1*$^{-/-}$ glands displayed normal ductal development and branching, as well as some alveolar budding, but developed alveoli were rare (Figure 3C). By contrast, glands of NRL-PRL females with wild-type cyclin D1 exhibited many lobuloalveoli and hyperplasias, as previously reported.[27] Consistent with the ability of PRL to augment proliferation in the absence of cyclin D1, transgenic PRL also induced limited lobuloalveolar development in NRL-PRL/*D1*$^{-/-}$ animals.

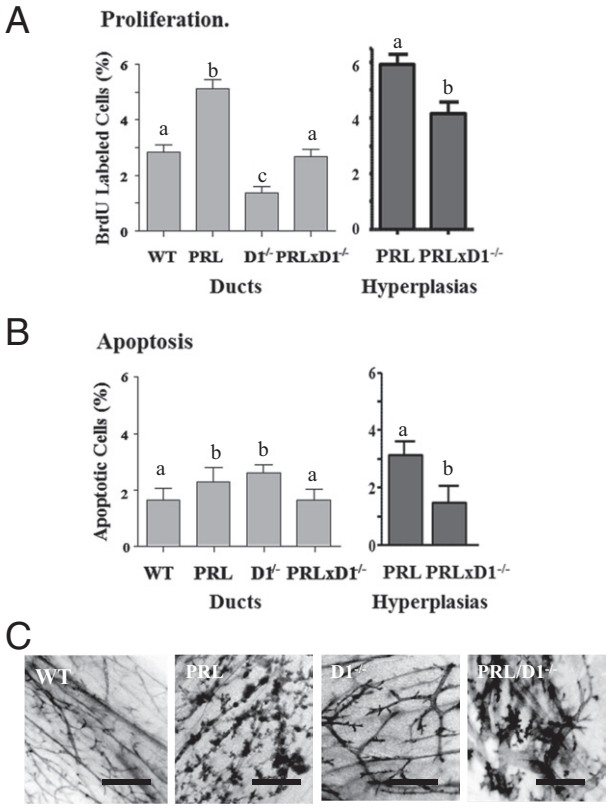

Figure 3. **A** and **B:** Transgenic PRL induces proliferation of morphologically normal ductal and hyperplastic epithelia in end-stage glands in the absence of *cyclin D1*$^{-/-}$ (**A**), and differently affects apoptosis depending on cyclin D1 status (**B**). Rates of proliferation and apoptosis were determined as described in *Materials and Methods*. Data are expressed as the mean ± SEM ($n = 3$–5). Analyses were performed by the Kruskal-Wallis test with the Mann–Whitney posttest ($P < 0.05$). Different letters (a, b, c) indicate statistically significant differences among genotypes. **C:** NRL-PRL/*D1*$^{-/-}$ females exhibit significant lobuloalveolar development, albeit reduced compared to NRL-PRL females with wild-type cyclin D1. Glands from all genotypes, including *cyclin D1*$^{-/-}$, displayed normal ductal development. Representative whole mounts at the end stage of each genotype as indicated. Scale bar = 2 mm. WT, wild type.

PRL Further Increases Cyclin D3 Expression in the Absence of Cyclin D1

Although cyclin D2 and cyclin D3 can compensate for the loss of cyclin D1 in many tissues,[33] the lack of lobuloalveoli in pregnant C57BL/6 × 129SV *cyclin D1*$^{-/-}$ mice[15,16] suggested that the mammary gland may be an exception. In light of the observed PRL-induced mammary pathology in the absence of cyclin D1 in our study, we examined the effect of elevated local PRL on expression of the other D cyclins. As shown in Figure 4, A–C, mammary transcripts for both cyclin D2 and D3 were increased in the absence of cyclin D1 as previously reported.[15,16] However, transgenic PRL did not further increase these mRNAs. Analysis of protein expression, however, revealed a more complex picture. PRL tended to raise total mammary cyclin D2 and D3 protein levels in *D1*$^{-/-}$ glands, but not in those expressing wild-type levels of cyclin D1 (Figure 4, D–F). Immunohistochemistry verified the low levels of cyclin D3 in mammary glands with wild-type cyclin D1 (Figures 2, I and J, and 4G), although it was readily detectable in uteri of these individuals (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). In glands of *D1*$^{-/-}$ females, cyclin D3 expression was clearly evident in some epithelial as well as stromal cells (Figure 2K). Transgenic PRL strikingly elevated cyclin D3 protein levels in *D1*$^{-/-}$ glands, most notably in a subset of the cells lining the lumens of the epithelial structures, indicating posttranscriptional action (Figures 2L and 4G).

Cyclin D1$^{-/-}$ *Glands Exhibit Elevated ERα Expression, and Respond to 17β-Estradiol Supplementation and the Hormonal Milieu of Pregnancy with Lobuloalveolar Development, but Lactation Failure*

To examine the effects of the absence of cyclin D1 on other indicators of hormonal responsiveness in the FVB/N

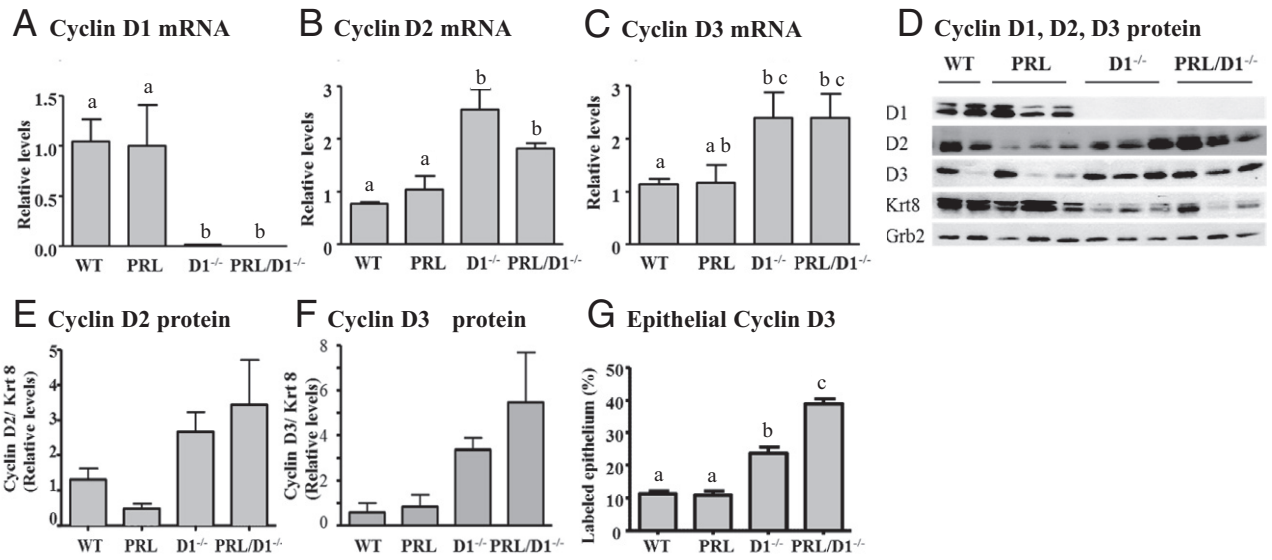

Figure 4. Transgenic PRL does not further increase cyclin D2 and cyclin D3 mRNA in *cyclin D1*$^{-/-}$ glands, but tends to increase cyclin D3 protein. mRNA levels of cyclin D1 (**A**), cyclin D2 (**B**), and cyclin D3 (**C**) were quantitated by RT-PCR as described in *Materials and Methods*. **D:** Western blot analyses of mammary lysates from individual end-stage mice for cyclin D1 (D1), cyclin D2 (D2), cyclin D3 (D3), cytokeratin 8 (Krt8), and Grb2, as shown. Quantitation of cyclin D2 (**E**) and cyclin D3 (**F**) relative to keratin 8 levels from the Western blot analyses (**D**). **G:** Proportion of epithelial cells that are positive for cyclin D3 protein by immunohistochemistry (as described in *Materials and Methods*). **E–G:** Data are expressed as the mean ± SEM ($n = 3$). Different letters (a, b, c) indicate statistically significant differences among genotypes, detected using the Kruskal-Wallis test, followed by the Mann–Whitney posttest ($P < 0.05$). WT, wild type.

genetic background, we examined ERα expression in glands of age-matched 1-year-old nonparous females. The absence of cyclin D1 elevated the proportion of cells in normal mammary structures that expressed detectable ERα to levels comparable to those induced by transgenic PRL in wild-type glands (Figure 5A), but this was not further elevated by PRL. Increased numbers of ERα^+ cells in *D1*$^{-/-}$ glands may reflect the low proliferative activity (Figure 3A); segregation of proliferation and ERα expression is observed in normal mammary tissue.[34] Mammary epithelial structures in all genotypes responded readily to elevated systemic estrogen. Supplementation with 17β-estradiol beginning after ductal elongation had occurred (8 weeks) elicited alveolar budding when examined at 1 year of age regardless of the presence of cyclin D1 (Figure 5, B and C). This treatment also increased uterine weight, although to a lesser extent in *D1*$^{-/-}$ females, compared to those with wild-type cyclin D1 (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).

Likewise, pregnancy induced extensive lobuloalveolar development in nulliparous females of all genotypes. Mammary glands of *cyclin D1*$^{-/-}$ females were morphologically similar to those with wild-type D1 at 24 hours postpartum (Figure 6A). However, although apparently healthy pups were born in comparably sized litters to mothers of all genotypes, survival of pups born to *cyclin D1*$^{-/-}$ mothers regardless of transgenic PRL status was dramatically reduced (Table 4). Although secretions were apparent in alveolar lumens of *D1*$^{-/-}$ mothers, transcripts for milk proteins were significantly less than those of mothers with wild-type D1, regardless of transgenic PRL status (Figure 6, B–D). These data indicate that mammary glands of FVB/N females are able to robustly proliferate in response to physiological combinations of mammogenic hormones during pregnancy in the absence of cyclin D1. However, cyclin D1 itself is necessary for functional lactation, and compensatory mechanisms are not adequate.

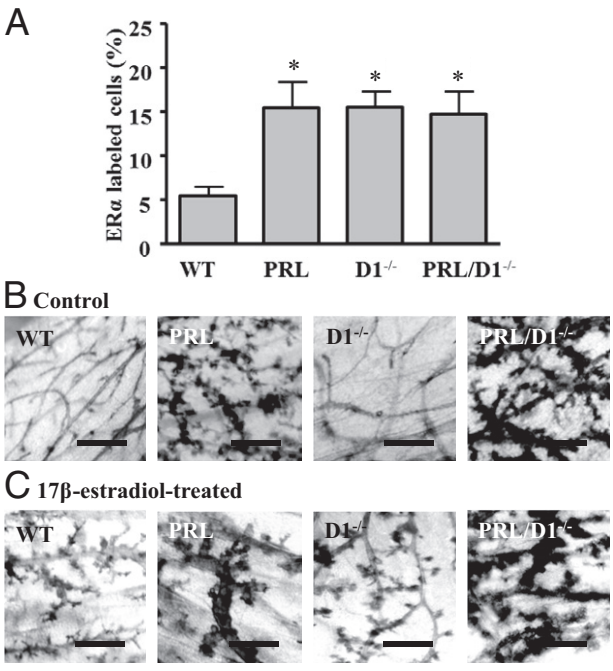

Figure 5. A: ERα is elevated in *cyclin D1*$^{-/-}$ glands of 1-year-old nonparous females. Morphologically normal epithelial cells expressing detectable ERα by IHC were quantified as described in *Materials and Methods* (mean ± SEM, $n = 5$). *$P < 0.05$ compared to WT. **B** and **C:** Nonparous females of all genotypes respond to long-term 17β-estradiol treatment with enhanced lobuloalveolar development. Intact nonparous females of all genotypes were untreated (**B**), or supplemented with 17β-estradiol beginning at 8 weeks of age (**C**), and glands were collected at 1 year of age (see *Materials and Methods*). Representative whole-mounted glands from each genotype as indicated. Scale bar = 1 mm. WT, wild type.

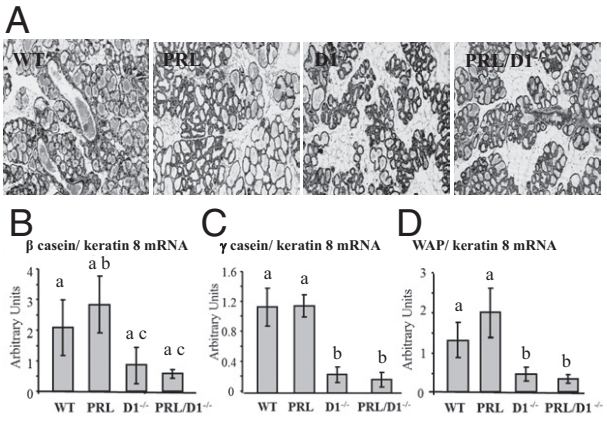

Figure 6. Cyclin D1 status does not affect mammary development during pregnancy, but is essential for optimal milk protein synthesis. Nulliparous females of the different genotypes were bred to wild-type FVB/N males, and pup health and mammary morphology were assessed 24 hours after parturition. **A:** Mammary glands at day 1 postpartum of each genotype as indicated. Original magnification, ×100. **B–D:** Mammary glands of *cyclin D1*$^{-/-}$ mothers contain lower levels of transcripts for milk proteins β casein (**B**), γ casein (**C**), and WAP (**D**) compared to cytokeratin 8, regardless of PRL transgene status. Levels of specific RNAs in mammary lysates from mothers of different genotypes were determined by real-time PCR as described in *Materials and Methods* (mean ± SD, n = 3 to 5). Different letters (a, b, c) indicate statistically significant differences among genotypes by analysis of variance followed by the Tukey Multiple Comparison test ($P < 0.05$).

Discussion

Cyclin D1 is a physiological mediator of lobuloalveolar proliferation induced by PRL and cooperating hormones during pregnancy.[35] Using a transgenic model of elevated local PRL exposure in combination with germline deletion of *Ccnd1* in the FVB/N genetic background, we demonstrated herein that cyclin D1 is also required for maximal PRL-promoted mammary tumorigenesis. However, even in the absence of cyclin D1, transgenic PRL increased mammary epithelial proliferation, induced early lesions, and promoted carcinomas, albeit with a reduced incidence and longer latency. PRL posttranscriptionally increased epithelial cyclin D3 expression, revealing one underlying compensatory mechanism. Mammary epithelium of cyclin D1–deficient females also proliferated in response to other hormones, including supplemental estrogen and the complex endocrine milieu of pregnancy. Nonetheless, despite extensive lobuloalveolar development during gestation, pups failed to survive due to apparent lactational failure. Together, our data indicate that compensatory mechanisms, including cyclin D3, are able to partially replace cyclin D1 in PRL-induced proliferation, but cannot compensate for cyclin D1 in PRL-induced differentiation. These studies point to distinct actions of cyclin D1 in hormonal regulation of mammary function, as well as genetic differences among mouse strains.

Circulating PRL is associated primarily with ERα^+ breast cancer,[3] modeled by experimental PRL-induced carcinomas.[29] The significant correlation between proliferation and cyclin D1 expression in the tumors that develop in NRL-PRL females shown here is consistent with the observed high cyclin D1 expression in ERα^+ clinical tumors.[11,13] PRL increases expression of cyclin D1 via transcription,[20] as well as nuclear accumulation and stabilization of the protein.[22] Although mammary transcripts for both cyclin D2 and D3 were elevated in *D1*$^{-/-}$ FVB/N females in the current study, similar to reports in other genetic backgrounds,[15,16] PRL did not further increase levels of these mRNAs. The apparent lack of PRL-stimulated transcription of these other D cyclins in the mammary gland *in vivo* suggests that regulatory signals to these genes are cell specific; Stat5, a prominent mediator of PRL in the mammary gland,[36] mediates activation of both the cyclin D2 and D3 promoters in response to other cytokines in other cell types.[37,38] However, our studies showed that PRL increased cyclin D3 protein in luminal epithelial cells of NRL-PRL/*D1*$^{-/-}$ females. Nuclear accumulation of cyclin D3, like cyclin D1, is regulated by glycogen synthase kinase-3β–dependent phosphorylation, subsequent nuclear export and proteasomal degradation,[39,40] a pathway inhibited by PRL in mammary epithelium.[22] This mechanism is likely to contribute to the ability of PRL to induce the mammary epithelial proliferation and pathology in the absence of cyclin D1 observed in our studies. In light of the incomplete compensation observed in our study, it is interesting to note that cyclin D3 was less effective than cyclin D1 in stimulating mitosis of hepatocytes.[41]

The role of cyclin D3 in breast cancer is less studied than that of cyclin D1. Cyclin D3 is elevated in some clinical breast cancers, frequently in conjunction with cyclin D1.[42–44] Experimental overexpression of cyclin D3 results in squamous cell carcinomas in mice,[45] demonstrating that this D cyclin also can contribute to mammary oncogenesis. In addition, cyclin D3 may confer distinct phenotypic features to mammary cancers. Like those tumors that developed in MMTV-cyclin D3 mice, both of the carcinomas that developed in NRL-PRL/*D1*$^{-/-}$ females exhibited a squamous histotype. This phenotype contrasts with the adenocarcinomas that develop in MMTV-cyclin D1 females,[46] suggesting distinct actions of different D cyclins on either a subpopulation of epithelial cells or cellular functions. Indeed, limited studies have demonstrated that cyclin D family members determine different substrates of activated CDK4/6,[47] and associate with distinct transcriptional regulators in breast cancer cells *in vitro* (see below).

In addition to effects on D cyclins, PRL alters expression of the cell cycle regulators p21Cip/WAF1 and cyclin

Table 4. Pups of *cyclin D1*$^{-/-}$ Mothers Exhibit Poor Survival

Genotype	Litter size at birth Mean ± s.d.	Pup survival (%) Mean ± s.d.
Wild type (FVB/N) n = 5	7.4 ± 1.5	84.0 ± 12.1
NRL-PRL n = 4	9.0 ± 1.1	86.9 ± 12.5
Cyclin D1$^{-/-}$ n = 7	7.0 ± 1.4	21.1 ± 17.7*
NRL-PRL/D1$^{-/-}$ n = 9	7.0 ± 1.4	35.7 ± 43.0

Nulliparous females of all genotypes were bred to nontransgenic males. Litter size was observed on PND 0 and survival on PND 1, when tissues were collected for analysis.

Analysis performed by Student's *t*-test.

*Indicates reduced frequency compared to wild-type and NRL-PRL mice.

B1 in breast cancer cells.[21] These targets of PRL, complemented by direct and indirect actions of other hormones in the complex environment of pregnancy, are likely to contribute to the extensive lobuloalveolar development in *D1*$^{-/-}$ periparturient females observed here.

Compensation for the loss of cyclin D1 in lobuloalveolar growth was not evident in the first descriptions of *cyclin D1*$^{-/-}$ mice, which were carried out in the C57BL/6 × 129SV genetic background.[15,16] However, in addition to our study, Wagner and colleagues[43] also recently reported reduced mammary cyclin D1 dependence in the FVB/N strain, and Haslam and colleagues[48] reported extensive alveologenesis during pregnancy in BALB/c *D1*$^{-/-}$ females. Together, these studies suggest that the original strain background, which is known to display reduced hormonal responsiveness,[48,49] may be unusual in this aspect. These reports underscore the importance of genetic background, which needs to be taken into account because mouse models are used to dissect complex biological processes, such as oncogenesis. This is particularly critical in light of the role of cyclins in hormonal actions in breast cancer,[13] and the potential role of cyclin D1 in tumor progenitor populations.[23,24,50] Strain differences also present the opportunity to identify the genetic loci that dictate the distinct phenotypes; these studies also may elucidate factors underlying susceptibility and resistance to breast cancer in women.

In contrast to the ability of compensatory mechanisms to support proliferation and support marked lobuloalveolar development in the absence of cyclin D1 in both FVB/N as well BALB/c females, pups failed to survive, indicating that cyclin D1 itself is essential for lactational competence. The reduced transcripts for milk proteins in *D1*$^{-/-}$ mammary glands shown here indicate that this may result from incomplete differentiation rather than inadequate alveolar capacity. Using a "knocked-in" cyclin D1 mutant that is unable to activate CDK4/6, Landis and colleagues[51] demonstrated that lobuloalveolar development is independent of kinase activity. Cyclin D family members have been shown to exert kinase-independent actions on transcription via multiple mechanisms in a variety of systems.[11,13,14] An elegant study examining cyclin D1–associated proteins in mouse embryos determined that about one third of the identified proteins were transcription factors.[52] Our data suggest that these actions may be less readily compensated in the mammary gland. In support of this, Mullany and colleagues[41] found that substantial subsets of transcripts were distinct in hepatocytes overexpressing individual D cyclins. Indeed, cyclin D1 is a strikingly stronger activator of the transcriptional activity of ERα than either D2 or D3.[53,54] Additional study will be required to dissect the specific targets of individual D cyclins in the mammary gland and genetic modulation of these responses.

In light of the accumulating evidence implicating PRL in the development and progression of ERα^+ tumors[3] and increased expression of cyclin D1 in early lesions and carcinomas, especially luminal tumors,[11,13,17] it is important to understand the relationship between these factors in breast cancer. Our studies here demonstrate the importance of cyclin D1 in PRL-induced mammary proliferation and pathogenesis, but also reveal other mediators likely to include cyclin D3 in the murine FVB/N genetic background. Understanding the web of signals that generate the array of phenotypes and variation in therapeutic responsiveness of the luminal subtype of breast cancer will illuminate strategies to prevent and treat this disease.

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