Regulation of Cyclin D1 and p16INK4A Is Critical for Growth Arrest during Mammary Involution

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ABSTRACT

A coordinated growth arrest during mammary involution completes the dramatic changes in mammary cell proliferation seen during pregnancy and lactation. Signals regulating this arrest are poorly understood, despite their potential relevance to oncogenesis. Here we report that the arrest involves a unique pulse of p16INK4A expression in vivo, which accompanied decreased cyclin D1 expression and a shift to an active repressor E2F4 complex. We used INK4A/ARF−/− mice as well as cyclin D1 and p16INK4A transgenic strains to examine the physiological significance of these patterns. p16INK4A directly regulated the in vivo transition from E2F3 to E2F4 as the major E2F DNA binding activity, and its contribution to growth arrest was independent of cyclin D1. Transgenic cyclin D1 expression prevented normal terminal differentiation by abating the p16INK4A pulse, abolishing the shift from E2F3 to E2F4, derepressing E2F target genes, and expanding a stem cell population. The effects of cyclin D1 were reversed by restoring p16INK4A but were not seen in INK4A/ARF−/− mice. Our results indicate that cyclin D1 may contribute to tumorigenesis by altering cell differentiation and demonstrate a significant function for p16INK4A in development in vivo. These regulatory mechanisms used during mammary involution offer a potential explanation for the protective effect of pregnancy against breast cancer.

INTRODUCTION

Significant variations in breast cancer rates in different countries identify nongenetic factors as particularly important contributors to breast cancer (1). These variations are often attributed to simple cultural differences in the timing or numbers of pregnancies, but mechanisms by which these factors might alter breast cell proliferation remain unclear (2, 3). Rodent models identify a terminal differentiation event that alters the subsequent proliferative potential of the mammary gland by decreasing mammary stem cell numbers during involution (4, 5). We sought to identify molecular signals that might mediate this terminal differentiation event.

We focused on the regulation of cyclin D1 and its regulatory pathway because it is uniquely important in mammary development (6, 7). Moreover, cyclin D1 is located in a region on chromosome 11q13 that is commonly amplified and overexpressed in breast cancers (8, 9). The appearance of adenocarcinomas in MMTV-dIII-cyclin D1 transgenic mice demonstrates the oncogenic potential of cyclin D1 (10). Compared with other tissues (10–13), its potency in mammary tissues suggests that cyclin D1 may exhibit activities that uniquely contribute to mammary tumorigenesis.

Cyclin D1 works in concert with kinase partners (Cdk4/6) to phosphorylate pRb, which then regulates the E2F transcription complex. Release of active free E2F from inactive complexes with pRb may regulate some E2F-dependent transcriptional activation of genes controlling DNA synthesis (14). An alternative view holds that active repressor E2F/pRb complexes are the more significant regulators (15). Several growth-suppressing pathways converge on cyclin D1 and its kinase partners (16). Among the specific inhibitors, INK4A (p16) is especially interesting considering the frequency of p16INK4A mutations in human cancers (17) and its frequent inactivation in breast cancers (18, 19).

Although perturbations in cyclin D1/Cdk4, p16INK4A, or pRb are central to tumorigenesis (17), overexpressed G1 cyclins generally do not increase net cell proliferation, despite accelerating G1 to S progression (20). This paradox remains unresolved, especially in vivo. In contrast, the contribution of cell cycle regulators to alterations in terminal differentiation pathways has obvious implications for tumorigenesis (21–23). Here we report that regulated changes in cyclin D1 and p16INK4A independently contributed to aspects of growth arrest during normal mammary involution.

MATERIALS AND METHODS

Plasmids, Cell Lines, and DNA Constructs. Plasmids used included pGEM-cyclin D1 (24) and pBS-p16 provided by Drs. Jim Koh and Ed Harlow (25). Dr. Charles Sherr provided plasmids containing murine cDNAs for cyclin D1 (26), p16INK4A (27), and p19INK4A/ARF (28). pMMTV-p16 was constructed by replacing CMV enhancer and promoter sequences in pCMV-p16 (25) with the MMTV enhancer and promoter from plasmid pA9 (Ref. 29; Fig. 2). An injection fragment used to make transgenic mice was isolated from plasmid sequences by cutting at HindIII and NdeI sites. NIH3T3, murine erythroleukemia cells (MEL), and SAO52 cells were obtained from the American Type Culture Collection.

Animals and Histology. FVB inbred mice (Taconic Farms, Germantown, NY) were bred for analysis of gene expression. Unless otherwise noted, pregnancy samples were obtained on day 10 after vaginal plugs were identified. Lactation samples were obtained from mice 10 days after litters were born for most studies. Day two of lactation was used to evaluate the phenotype of MMTV-p16 mice losing litters. Involution was studied by removing pups from their mother on day 10 and obtaining samples on the indicated day after weaning (30). MMTV-cyclin D1 mice were described previously (10). Mammary glands were fixed in 4% paraformaldehyde in PBS for histological preparations. Whole mounts were prepared by dissecting the mammary fat pad from the pelt, fixing in 10% buffered formalin, staining with hematoxylin, dehydrating in graded series of alcohol, and clearing with xylene.

Immunostaining was performed using commercial antibodies (Santa Cruz Biotechnology) to mouse p16, human p16, and human cyclin D1 (HD11). For immunohistochemical staining, nonspecific mouse IgG1 was used as a negative control reagent. The detection reaction used the Vector Elite ABC kit (Vector, Burlingame, CA).

Eight lines of transgenic mice were developed by microinjection of the MMTV-p16 fragment using standard methods (10). For this study, we concentrated on mice of the TG.M16 and TG.M12 line, which demonstrated physiological and attenuated levels of INK4A expression. All animals received...
humane care following study guidelines established by the Massachusetts General Hospital Subcommittee on laboratory animal care.

**MMTV Infection and Analysis for Proviral Integrants.** Virgin female mice were infected with MMTV by i.p. injection of 10^7 EH-2 cells at 5–8 weeks of age. EH-2 is a rat XC cell line that produces a pathogenic hybrid MMTV consisting primarily of MMTV(C3H) sequences (31). To assay for infectious MMTV provirus in tumors, BgIII-digested genomic DNAs were hybridized with a 1.2-kb BamHI env probe from the parent plasmid, pUVH. We probed HindIII cut tumor DNA with the 1.2-kb BamHI env probe to detect 3’ flanking sequences and with a 2.4-kb XhoI-EcoRI gag-pol probe to detect 5’ flanking sequences. Finally, we probed BgIII cut tumor DNA with a 2.5-kb EcoRI V pol probe that hybridized with endogenous proviral sequences to control for DNA loading.

**Protein and RNA Expression Studies.** Dr. Rob Hurford kindly provided protein lysates from Rb+/+ and Rb−/− cells. Protein samples were obtained from mammary tissues by crushing mammary glands frozen in liquid nitrogen using a mortar and pestle (32). Mammary lysates were made by passing the resulting powders through a 20-gauge needle at 4°C in TNE buffer (50 mM Tris, 420 mM NaCl, 2 mM EDTA, 0.5% NP40, pH 8.0) and centrifugation to remove debris. Protein expression was determined using standard immunoblots containing 50 μg of total protein. Membranes were cut according to molecular weights of the proteins to be identified, and individual blots were successively incubated with the indicated following antibodies: (a) from Drs. Jim Koh and Ed Harlow: JC1 mouse monoclonal that specifically recognizes human p16INK4A, JC2 and JC8, which specifically recognize both mouse and human p16INK4A, (b) from Dr. Chuck Sherr: rabbit polyclonal antibodies against peptides for murine p16INK4A and p19ARF (33); and (c) a commercial mouse monoclonal antibody against actin (Boehringer). Secondary antibodies used were those included in an enhanced chemiluminescence detection kit (Amerham).

RNA blots were performed by standard techniques (10). RNase protection assays were performed using a kit from the Promega Corp. following the enclosed instructions.

**EMSA.** Tissue extracts for EMSAs were prepared from powdered mammary tissues by a modification of the Dignam method using extraction buffer [20 mM HEPES (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, and 25% glycerol]. Binding reactions included 10 μg of the indicated extracts. Gel shift activity was evaluated in standard EMSA binding buffer [20 mM HEPES (pH 7.4), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM MgCl2, and 5% glycerol] with salmon sperm DNA (0.1 μg/μl) as nonspecific competitor. Subsequently, complexes were resolved on 6 or 5% (for supershifts) nondenaturing polyacrylamide gels containing 1X TBE [0.09 M Tris-borate (pH 8.0), 0.002 M EDTA].

After labeling with [γ-32P]ATP using polynucleotide kinase, double-stranded oligonucleotides were purified by PAGE. The E2F consensus oligonucleotide contained the following sequence in the sense orientation: E2F

**RESULTS**

**Regulation of Cyclin D1 and INK4A (p16) in Mammary Glands Contributes to Regulation of E2F4 Activity during Mammary Involution.** The proliferative cycle of pregnancy includes several stages: (a) branching ductular proliferation during pregnancy; (b) lobuloalveolar proliferation and differentiated function during lactation; (c) growth arrest accompanied by a marked decrease in subsequent proliferative potential in the initial stages of involution (terminal differentiation); and (d) rapid tissue regression culminating in a return to normal cellularity 7 days after weaning (Fig. 1; Ref. 34). To identify patterns of regulation of cell cycle control genes during mammary involution, we compared expression of cyclin D1 and p16 on days 1, 2, 3, 5, and 7 after lactation with expression in mammary glands from virgin mice, pregnant mice on day 10, and lactating mice on day 10. Gene expression did not change throughout other time points in the estrous cycle of virgin mice, throughout pregnancy, or at any other time point during lactation.

Cyclin D1 remained at relatively constant levels in virgin mice and throughout pregnancy (Fig. 1, A and C). Cyclin D1 then decreased significantly to very low levels on days 1, 2, and 3 after weaning and returned to preproliferative levels on days 5 and 7 of involution. Following a pattern that was nearly the inverse of cyclin D1, p16INK4A protein appeared only on days 2, 3, and 5 after weaning and was absent from virgin mice, throughout pregnancy and throughout lactation (Fig. 1, A and D).

Although cyclin D1 is thought to regulate the G1-S transition by phosphorylating pRb, it remains unresolved whether free activating E2F or E2F-pocket protein repressor complexes ultimately mediate these effects in vivo. We therefore examined the functional status of E2F activity in vivo using EMSA coupled with supershift assays for the various E2Fs, DP1, and pocket protein family members. We focused on E2F activities as the critical readouts of the pRb pathway. Both cyclin-dependent kinase activity and pRb concentrations were too low in vivo for us to reliably detect changes. By comparing samples from actively growing tissues during lactation to histologically identical samples obtained 2 days later during mammary involution, patterns characteristic of growth arrest could be identified. The predominant E2F activity during lactation was supershifted by anti-E2F3 antibodies in wild-type mice (Fig. 1E, compare Lanes 1, 3, and 4). The predominant E2F activity during involution was supershifted by anti-E2F4 antibodies (Fig. 1E, compare Lanes 5, 7, and 8). In contrast, E2F activity was not supershifted by anti-E2F1, 2, or 5 antibodies in any stage of mammary development in any mice, and all complexes contained DP1 (not shown).

Given the association of the cyclin D1 and p16 regulation with changes in E2F activity that were consistent with the growth-inhibiting functions of E2F4, we sought to determine whether p16 contributes directly to the shift from E2F3 to E2F4 or to formation of pocket protein complexes. To answer this question, we obtained a knockout strain of mice that is nullizygous for the p16 locus (including both the p16INK4A transcript and the p19ARF transcript; Ref. 35). We first evaluated E2F activity in these INK4A/ARF−/− mice (Fig. 1F). We found no change in E2F EMSA activity during lactation in p16 nullizygous mice, consistent with its normal absence during that stage (not shown). However, E2F complex formation was lost in the INK4A/ARF−/− mice during involution (Fig. 1F, Lanes 1 and 3). The predominant binding activity was again E2F4 (Lanes 7 and 8), but the loss of p130-E2F2 interactions was apparent in supershift experiments (Lanes 9 and 10).

Growth arrest, terminal differentiation, and tissue regression occur together during normal mammary involution (34). To evaluate the role of the physiological pulse of p16INK4A in these processes, transgenic p16INK4A was targeted to mammary glands. Using MMTV enhancer and promoter sequences to regulate transcription, we targeted expression of a human p16INK4A cDNA (25) to mammary epithelium (Fig. 2A). We focused our studies on two transgenic lines among several founder lines that exhibited physiologically significant levels of p16. The higher expressing line was designated Tg.M16 and expressed p16 at levels similar to those found during normal mammary involution. A strain with lower expression levels was termed Tg.M12. Transgenic expression appeared in virgin mice immediately after puberty and was seen during all subsequent stages of mammary growth and development as expected of an MMTV-driven transgene.

The MMTV-p16 transgenic mice demonstrated a direct contribution of the pulse of p16 to the developmental switch to E2F4 and to
formation of E2F4-pocket protein complexes. We used Tg.MI2 mice for these biochemical studies because the Tg.MI6 mice lost their litters on day 2 of lactation, a time point preceding those used to study the normal mice. Premature expression of p16 induced E2F4 activity in the MMTV-p16 mice during lactation (Fig. 2B). Although addition of anti-E2F3 supershifted the E2F-complex in normal mice (Lane 1 versus Lane 5), no E2F3 supershift occurred in the MMTV-p16 mice (Lanes 3 versus Lane 6). In contrast, addition of anti-E2F4 supershifted the E2F-complex in MI2 mice (Lane 3 versus Lane 8), and no E2F4 supershift occurred in the wild-type mice during lactation (Lane 1 versus Lane 7). Increased E2F4-pocket protein complex formation supplemented this effect in the MMTV-p16 transgenic mice during involution (Fig. 2C). This was most evident in a comparison of the ratio of complexed-E2F to free E2F in the involuting MMTV-p16 mice (compare Lanes 3 and 7). This complexed E2F in the MMTV-p16 mice remained E2F4 (Lane 3 versus Lane 7), as seen previously in the wild-type mice (Lanes 1 and 7 here, and Lanes 1 and 7 in 1E). The pocket protein involved in the E2F4 complexes changed from the p130 observed in the wild-type mice to pRb (compare Lanes 1 and 9). Instead, antibodies to pRb supershifted the complexes in the MMTV-p16 transgenic mice (Lanes 3 and 12).

We used immunohistochemical techniques to demonstrate endogenous p16 protein expression in mammary epithelial cells lining lobuloalveolar structures during involution (Fig. 3B). Increased cellular growth rates, delayed growth arrest, or decreased tissue regression should increase epithelial cellularity at the end of mammary involution in INK4A/ARF−/− mice if p16 alone was sufficient to regulate those processes in mammary involution. We evaluated standard histological sections and found that p16 loss did not alter cellularity in mammary glands of INK4A/ARF−/− mice (Fig. 3, C and D). To evaluate the possibility that other INK4 proteins functioned in a redundant manner with p16, we stained histological sections using antibodies to p18INK4C and p19INK4D (Fig. 3, E and F). Both proteins were expressed in the same cells as p16INK4A. Finally, we did not observe expression of either p18INK4C (not shown) or p16 loci−β alternative reading frame (p19ARFβ) transcripts (Fig. 1D) in mammary tissue in any developmental stage.

Premature Expression of p16 INK4A in Mammary Epithelium Arrests Lobuloalveolar Development during Lactation. Although redundant expression of p18 and p19 proteins may preclude appearance of a mammary phenotype in the INK4A/ARF−/− mice, transgenic p16INK4A caused an obvious phenotype in the MI6 strain (Fig. 2, D–F, and Fig. 3, G–M). Sixty % of litters born to MI6 mothers died during their mothers’ first pregnancies (Fig. 2, D, MI6 column; P < 0.01 by χ2 analysis). The pattern of litter loss was consistent. Mothers initially fed their pups. All pups then died within the first 3 days of lactation, irrespective of the pups’ genotypes. Mammary glands of MI6 mothers losing their litters showed a marked loss of the ability to proliferate along lobuloalveolar lineages when compared with wild-type siblings (Fig. 3, compare I with H). Most of the ducts completely lacked alveoli and secretions (see arrows). These changes were apparent in thin ductular branches lacking alveoli and milk, which appeared within a background of ductular structures exhibiting more normal alveolar development. Immunostaining demonstrated the presence of transgenic p16 protein in the epithelial cells lining ductular and lobuloalveolar structures (Fig. 3G). Finally, whole-mount
Differences between wild-type and Tg.MI6 first litter survival were significant (P < 0.05 by \( H_1 \)) and premature expressing lower levels of p16 during lactation; MP6, through lactation was compared between first and subsequent litters in the following genotypes: WT, MI6, MI2, MI4, and wild-type virgin (WT V) mammary glands. The MI6 lactating sample is from day 2 of lactation when MI6 mice typically lost their litters. The MI2 lactation sample is from day 10, and the MI2 involution sample is from day 2. Pregnancy samples from day 10 in both transgenic strains were directly compared on the right side of this panel (Comparison, MI6 P versus MI2 P). p16 protein levels expressed during lactation in the MI2 strain were very similar to physiological p16 levels during involution. B, an EMSA compared E2F activity in MMTV-p16 (MI2) mammary glands during lactation (Gene I) with lactating wild-type (Gene W). Specificity was demonstrated by cold oligonucleotide competitions (Lanes 2 and 4). Lanes 1 and 3 demonstrated no change in E2F/pocket protein complex activity in the p16 overexpressing mice. Supershifts were performed using the indicated antibodies (Anti: E2F3, E2F4, p130, and pRb). In this case, p16 expression increased E2F4 activity during lactation, as indicated by comparing the wild-type E2F3 supershifts (compare wild-type Lanes 1, 5, and 7 with the E2F4 supershifts in the MI2 mice (compare MI2 Lanes 3, 6, and 8). An antibody to p130 (Anti: p130) supershifted the complexed activity equally in the wild-type (Lane 9) and MI2 mice (Lane 10). No supershifts were seen using antibodies to pRb (Anti: pRb, Lanes 11 and 12). C, an EMSA compared E2F activity in MMTV-p16 (MI2, Gene I) mammary glands to wild-type mice (Gene W) on day two of involution. Specificity was demonstrated by cold oligonucleotide competitions (Lanes 2 and 4). Lanes 1 and 3 demonstrated increased E2F/pocket protein complex activity in the p16-expressing mice identified by the increased intensity of the complexed band (Complex). Supershifts were performed using the indicated antibodies (Anti: E2F3, E2F4, p130, and pRb). As before, the activity during wild-type involution was predominantly free E2F4 (compare wild-type Lanes 1, 5, and 7). In contrast, the activity in the MI2 mice was predominantly complexed E2F4 (compare Lanes 3, 6, and 8). Moreover, in this case the antibody to p130 (Anti: p130) did not supershift the complexed activity in the MI2 mice (Lane 10), but supershifts were instead detected using an antibody to pRb (Anti: pRb, Lane 12). D, litters born to MMTV-p16 transgenic mothers died in the first days of lactation. The percentage of litters surviving through lactation was compared between first and subsequent litters in the following genotypes: WT, wild-type, MI6, Tg.MI6 prematurely expressing p16 during lactation; MI2, Tg.MI2 prematurely expressing lower levels of p16 during lactation; MP6, Tg.MP6 overexpressing cyclin D1; and MP6XMI6, doubly transgenic Tg.MP6/Tg.MI6 expressing both cyclin D1 and p16. Differences between wild-type and Tg.MI6 first litter survival were significant (P < 0.01 by \( \chi^2 \)). The differences between the first and second litter survival rates of Tg.MI2 were significant (P < 0.05 by \( \chi^2 \)). E, increased inhibitory effects of p16INK4A were seen after passage through mammary involution in a second p16INK4A transgenic line. We show numbers of offspring surviving on each of the indicated days after birth, comparing the outcome of first and second litters born to MI2 mothers. F, the mean weight of mice in surviving litters at weaning on day 21 born to Tg.MI2 mothers during their second pregnancy (open columns) was significantly decreased compared with litters born during their first pregnancy (black columns).
increased during involution in the transgenics. This prolongation of increased cyclin D1 expression during involution was also associated with loss of p16INK4A protein expression throughout involution, although neither p18 nor p19 levels were affected (Fig. 4A). This decrease in cyclin D1 expression during involution was not as marked in INK4A/ARF−/− null mice as in wild-type mice, although increased p18INK4C in the p16 null mice supported the view that p18 function may be redundant with p16 during mammary involution (Fig. 4A, additional bottom panels).

We next evaluated the role of cyclin D1 in regulation of E2F activity. We first demonstrated a 10-fold increase in free E2F activity that was identified as E2F3 by supershift experiments during lactation (Fig. 4B, compare Lanes 3 and 1 in the lactation panel, and Lanes 5 and 6). Cyclin D1 had even stronger effects during mammary involution at the time that mammary cells should stop dividing. We first found that E2F activity was predominantly in a complexed form during involution in the cyclin D1 transgenic mice and that cyclin D1 therefore abolished the appearance of a free E2F form (Fig. 4B, involution panels, compare Lanes 3 and 1). The complexed activity was predominantly E2F3 activity in the cyclin D1 mice (Fig. 4B, Lanes 5–8), and the complexes contained p130 (Lane 10). Thus, cyclin D1 overexpression induced additional E2F3 during both lacta-
tion and involution. This E2F3 remained in the free form in lactating cyclin D1 mice.

Taken together, our data provide important in vivo evidence that cyclin D1 acts to derepress an active repressor form of E2F (Fig. 4C). The E2F proteins regulate a variety of well-characterized genes needed for progression through S-phase. The response of these E2F target genes to transgenic cyclin D1 expression should differentiate between its potential function as an activator of E2F targets that promote G1-S progression during lactation or as a derepressor of E2F target genes during involution. We therefore evaluated the response of a standard set of E2F target genes (36) to perturbations of cyclin D1 and p16 expression during lactation and involution (Fig. 4D). Prolongation of cyclin D1 expression into involution caused a striking and consistent pattern of alterations in E2F target gene regulation that was not seen in either the INK4A/ARF−/− or MMTV-p16 mice. None of the three genetic alterations had marked effects on E2F target gene activity during lactation (Fig. 4D, first four lanes of each panel), although cyclin D1 overexpression slightly increased c-myc levels. In contrast, the expression level of each E2F target gene examined was contrast, the expression level of each E2F target gene examined was not seen in either the INK4A/ARF−/− or MMTV-p16 mice. None of the three genetic alterations had marked effects on E2F target gene activity during lactation (Fig. 4D, first four lanes of each panel), although cyclin D1 overexpression slightly increased c-myc levels. In contrast, the expression level of each E2F target gene examined was markedly increased during involution in the MMTV-cyclin D1 transgenics. Furthermore, neither loss nor gain of p16 affected any of the same set of target genes at that time. We also found no change in casein levels during lactation, demonstrating no change in differentiated function in any of the strains.

The absence of E2F target gene regulation in mice that have either lost or gained p16 during involution contrasts with the effects of cyclin D1 and suggests that cyclin D1 and p16 regulate somewhat different functions during involution. We confirmed this possibility by mating MMTV-cyclin D1 mice to MMTV-p16 mice, where we found that overexpression of cyclin D1 did not repair the lactational phenotype in the MI6 strain (Fig. 2D).

Increased cellular growth rates, delayed growth arrest, or decreased tissue regression should increase epithelial cellularity at the end of mammary involution, if cyclin D1 is primarily regulating those processes in mammary development. Surprisingly, at the end of a single pregnancy cycle, we found no difference in epithelial cellularity between wild-type and MMTV-cyclin D1 mice when involution was completed (Fig. 5, A and B). In these photomicrographs, mammary tissue appears as islands of epithelial cells in a background of stromal tissue. The numbers of epithelial islands and the number of cells in individual islands were equivalent in cyclin D1-expressing and normal tissues.

The histological appearance of MMTV-cyclin D1 mammary glands could not be distinguished from wild-type mice for several months.
after completion of a single pregnancy. Evidence for subsequent focal nodular growth of lobuloalveolar epithelium first appeared 4 months later when focal hyperplastic nodules were demonstrated in whole-mount preparations of the MMTV-cyclin D1 transgenic mice (Fig. 5, compare F with E). These renewed hyperplastic changes (Fig. 5, D versus C) occurred in mice that had completed a single pregnancy and were not pregnant at the time of harvest. These changes have not been observed in older nulliparous MMTV-cyclin D1 mice (not shown). In contrast to the initial diffuse hyperplastic changes in pregnant cyclin D1 transgenic mice (10), these delayed lesions were focal, stable, and exhibited progressive acquisition of atypical cellular characteristics (Fig. 5J). We also examined whole-mount preparations of MMTV-cyclin D1/MMTV-p16 transgenic mice and INK4A/ARF−/− mice (Fig. 5, G and H) and found that loss of p16 alone was not sufficient to account for the changes seen in the MMTV-cyclin D1 mice. Finally, these initial hyperplastic and dysplastic changes were followed by adenocarcinomas 6–8 months later in the cyclin D1 transgenic mice (Fig. 5J), which expressed the human cyclin D1 at high levels (Fig. 5, K and L).

To determine whether cyclin D1 expanded a population of potential stem cells, we analyzed clonality of tumors arising in the MMTV-cyclin D1 transgenic mice. Although retroviral infections are usually used to identify collaborating oncogenes in transgenic experiments, they also provide a measure of clonality in tumors. We initially sought to identify oncogenes collaborating with cyclin D1 in mammary tumorigenesis by infecting wild-type mice and MMTV-cyclin D1 transgenic mice with a cloned strain of MMTV. Importantly, MMTV infection of the cyclin D1 transgenics did not accelerate tumorigenesis when compared with the infected wild-type mice (Fig. 6A). Tumor onset was the same as we observed previously in uninfected MMTV-cyclin D1 transgenics, 505 ± 113 days compared with 534 ± 35 days (10). Despite finding no evidence of collaboration between cyclin D1 and MMTV insertions, we did find that the tumors in the MMTV-cyclin D1 transgenic were derived from multiple independent infection events (Fig. 6B). Thus, tumor masses appeared at the same rate in the MMTV-infected wild-type and MMTV-infected cyclin D1 mice but arose from an expanded population of infectable target cells in the MMTV-cyclin D1 transgenic mice.

DISCUSSION

Although perturbations in cyclin D1/Cdk4, p16, or pRb are central to tumorigenesis (17), their cell cycle functions are not essential for completion of most normal cell division cycles (6, 7, 35, 37). This paradox is emphasized by the general inability of G1 cyclins to increase net proliferation in cells despite accelerating G1 to S progression (20). In contrast, the potential contributions of cell cycle regulators to terminal differentiation have obvious implications for tumorigenesis (21–23). Despite the potential importance of their role in terminal differentiation, surprisingly little is known about this function in vivo.

We found a novel pulse of p16 expression during normal mammary involution that has not been observed previously in other tissues. The contribution of p16 to normal growth arrest in a mature tissue is particularly interesting because a physiological role for this specific antagonist of cyclin D signaling has not yet been identified (38). This physiological p16 pulse directly regulated E2F4/pocket protein complex formation, and it directly contributed to growth arrest. However, growth arrest progressed normally in the INK4A/ARF−/− strain, indicating that this pulse is not required for growth arrest. Although...
to promote additional E2F4-pocket protein assembly with pRb. Interestingly, the most significant effect of all of these regulatory changes was simultaneous derepression of all E2F target genes only during involution in the cyclin D1 transgenics.

Why are two interdependent signaling molecules regulated during mammary involution? The phenotype of the two MMTV-p16 transgenic lines clearly implicates p16 as a regulator of growth arrest. However, the phenotype of the MMTV-cyclin D1 mice identifies a far more complex issue, suggesting that cyclin D1 actually regulates the proliferative potential of the mammary gland after passage through pregnancy and involution. Consistent with every other test using cyclin D overexpression, increased cyclin D1 did not promote additional cell divisions in the cyclin D1 mice. Instead, it derepressed a critical set of E2F target genes during a transition to a terminally differentiated state.

At least four models are consistent with our findings: (a) the delayed development of focal nodular hyperplasias suggests that these hyperplastic nodules might expand from an expanded population of stem cells that failed to undergo normal terminal differentiation; (b) alternatively, the focal proliferations might represent stochastic escape from global repression of the G1-S regulatory machinery; (c) the loss of normal growth arrest during involution in the cyclin D1 mice may abrogate a “checkpoint” elimination of mammary epithelial cells that have accumulated genetic mutations during the growth cycle of pregnancy; and (d) target cells for cyclin D1 in transformation might be expanded during pregnancy and lactation, independent of cyclin D1 effects during these periods. In this scenario, the critical “execution” period for the effects of cyclin D1 may not be until after involution when the cells would normally be quiescent.

The MMTV result is particularly provocative. In the first two models, we would expect to find expanded targets for MMTV infections, because both mechanisms increase the dividing cell populations that are needed to support retroviral integration. However, our infected cyclin D1 transgenics did not exhibit accelerated tumorigenesis, despite the presence of new exogenous proviruses. Although this might result from an inefficient infection of the mice, the presence of multiple new exogenous proviruses in the majority of tumors argues against it. Furthermore, MMTV infection accelerates tumorigenesis in several other transgenic mouse models (44–46). More likely, this result reflects the intimate involvement of cyclin D1 in the pathways of most or all of the targets for MMTV insertion mutations including both Wnts and Fgfs (47, 48).

Delayed menarche, pregnancy at a young age, multiple pregnancies, multiparity, breast feeding, and early menopause all decrease the incidence of breast cancer in humans (1, 49). Many mechanisms for this protective effect have been proposed (50). Mechanistic studies in rodents identified enhanced terminal differentiation as one key result of pregnancy (4, 51). The pattern of litter loss in the M12 strain of MMTV-p16 mice provides a potential confirmation of this effect. Loss of litters in the second and subsequent pregnancies confirms that the proliferative capacity of the mammary glands decreases after passage through pregnancy.

We identified normal and critical functions for regulated changes of both cyclin D1 and p16 levels during mammary involution. Although cyclin D1 is known to regulate G1-S progression, its functions in differentiation are poorly understood. Although the significance of p16INK4A loss in tumorigenesis has been repeatedly shown in numerous studies, little is known about its normal functions. Importantly, the ability to regulate growth arrest in the involuting mammary gland is one p16INK4A function that is clearly independent of p16INK4AARF (28). Future studies of both cyclin D1 and p16INK4A regulation and function during mammary involution may clarify the nature of the
protective effect of pregnancy, which has otherwise been approachable only as an epidemiological risk factor in human breast cancers.

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We sadly report that our friend, colleague, and co-author, Dr. Zeljko Nikolic, died of a recurrent Ewing’s sarcoma on August 27, 2001 while we were revising the manuscript. He was training in the Massachusetts General Hospital Pediatric Residency program at the time of his death. His loss is doubly tragic because it occurred 12 years after his initially successful treat- ment and because his contributions to pediatric oncology would have been profound.

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