Cyclin-dependent Kinase Inhibitor $p27^{Kip1}$ Is Required for Mouse Mammary Gland Morphogenesis and Function

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Abstract. We have studied the role of the cyclin-dependent kinase (Cdk) inhibitor $p27^{Kip1}$ in postnatal mammary gland morphogenesis. Based on its ability to negatively regulate cyclin/Cdk function, loss of p27 may result in unrestrained cellular proliferation. However, recent evidence about the stabilizing effect of p27 on cyclin D1–Cdk4 complexes suggests that p27 deficiency might recapitulate the hypoplastic mammary phenotype of cyclin D1–deficient animals. These hypotheses were investigated in postnatal p27-deficient ($p27^{-/-}$), hemizygous ($p27^{+/-}$), or wild-type ($p27^{+/+}$) mammary glands. Mammary glands from $p27^{+/-}$ mice displayed increased ductal branching and proliferation with delayed postlactational involution. In contrast, $p27^{-/-}$ mammary glands or wild-type mammary fat pads reconstituted with $p27^{-/-}$ epithelium produced the opposite phenotype: hypoplasia, low proliferation, decreased ductal branching, impaired lobuloalveolar differentiation, and inability to lactate. The association of cyclin D1 with Cdk4, the kinase activity of Cdk4 against pRb in vitro, the nuclear localization of cyclin D1, and the stability of cyclin D1 were all severely impaired in $p27^{-/-}$ mammary epithelial cells compared with $p27^{+/+}$ and $p27^{+/-}$ mammary epithelial cells. Therefore, p27 is required for mammary gland development in a dose-dependent fashion and positively regulates cyclin D–Cdk4 function in the mammary gland.

Key words: p27 • mammary gland • cyclin D1 • growth • apoptosis

Introduction

Initiation of cell division involves the integration of several growth signals, both stimulatory and inhibitory, that converge on the cell cycle machinery. The cell cycle is promoted by a family of cyclin-dependent kinases (Cdks),[1] and Cdk activity is negatively regulated by Cdk inhibitors (CKIs). Thus, CKIs lie at a critical point in this integrated network, serving as checkpoint controls for cell cycle progression (Sherr and Roberts, 1999). The CKIs can be subdivided into two categories, the Ink4 proteins, which are inhibitors of cyclin D–Cdk2/4 complexes, and the Cip/Kip proteins, which inhibit all cyclin–Cdk complexes. The Cip/Kip family includes $p27^{Kip1}$, $p21^{Cip1/Waf1}$, and $p57^{Kip2}$. $p27^{Kip1}$, originally identified as a Cdk inhibitory activity induced by antimitogenic signals (Polyak et al., 1994; Toyoshima and Hunter, 1994), is known to bind to cyclin D–Cdk4, cyclin E–Cdk2, and cyclin A–Cdk2 complexes (Soos et al., 1996).

Stimulation of cyclin D–cdk4 activity in early G_1 and cyclin E–cdk2 activity in mid-G_1 results in the sequential phosphorylation of their mutual target, the retinoblastoma gene product, pRb. Cdk-mediated hyperphosphorylation of pRb causes release of pRb-mediated inhibition of E2Fs and other factors that drive the cell cycle forward by transactivating genes required for S phase entry (Nevins, 1992; Kato et al., 1993; Johnson et al., 1993; Duronio and O'Farrell, 1995). Overexpression of p27 reversibly arrests cells in G_1, suggesting that p27 can limit the G_1 to S transition. Inhibition of G_1 progression by p27 requires the inhibition of Cdk activity, including cyclin D–Cdk4 and cyclin E–cdk2 complexes (Sherr, 1996). A direct physical association of p27 with cyclin–Cdk complexes can restrain Cdk

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[1]*Abbreviations used in this paper:* Cdk, cyclin-dependent kinase; CKI, Cdk inhibitor; dpc, days postcoitum; dpfw, days postforced wean; HH1, histone H1; IP, immunoprecipitation; PMEC, primary mammary epithelial cell; Stat, signal transducer and activator of transcription; TEB, terminal end bud; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP–biotin nick end labeling.

activity and maintain Rb in a hypophosphorylated state that sequesters E2F (Soos et al., 1996). By this mechanism, p27 inhibits progression into S phase (Weintraub et al., 1992, 1995). Consistent with these observations, cell culture studies have shown that expression of p27 is elevated in quiescent cells but declines as cells actively undergo DNA synthesis (Nourse et al., 1994).

Genetic evidence also suggests that p27 is a critical regulator of cellular proliferation in vivo. Mice with homozygous disruption of the p27 gene ($p27^{-/-}$) are 15–30% larger than their wild-type ($p27^{+/+}$) or hemizygous ($p27^{+/-}$) littermates due to hyperplasia in several organs, including spleen, thymus, pituitary, ovaries, and testes (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Tumors of the intermediate lobe of the pituitary were observed in $p27^{-/-}$mice by 6 mo of age, and both $p27^{+/+}$ and $p27^{-/-}$ mice were more susceptible to radiation- or chemically induced lung and colon tumors (Fero et al., 1998). Therefore, p27 acts as a tumor suppressor in many tissues, perhaps through its ability to inhibit cell cycle progression. Although homozygous gene inactivation has not yet been reported in any human tumors, hemizygous deletions have been detected (Pietenpol et al., 1995; Spirin et al., 1996; Takeuchi et al., 1998). Multiple human tumors exhibit abnormally low levels of p27 protein, including tumors of the breast (Clurman and Porter, 1998; Loda et al., 1997). Decreased p27 levels in breast tumors correlate with a poor patient prognosis, implying a regulatory role of p27 in cell cycle control in transformed mammary epithelium (Porter et al., 1997; Tan et al., 1997).

High cyclin D1 protein levels have been observed in a large percentage of breast tumors (Gillett et al., 1996; Fredersdorf et al., 1997; Robker and Richards, 1998; Medina, 1996). In addition, transgenic mice overexpressing cyclin D1 in the mammary glands develop ductal hyperplasias and tumors (Wang et al., 1994), suggesting that increased cyclin D1–Cdk4 activity may lead to uncontrolled growth of the mammary epithelium. Conversely, cyclin D1–deficient ($cyl^{-/-}$) mouse mammary glands display developmental abnormalities associated with a defect in cell cycle progression, including decreased proliferation, reduced lateral branching, and impaired differentiation (Fantl et al., 1995; Sicinski et al., 1995). Normally, the rudimentary ductal network of quiescent epithelial cells in the immature mouse mammary gland rapidly proliferates in response to increased production of pubertal hormones, such as estrogen, while undergoing extensive lateral epithelial branching. Pregnancy induces a second round of proliferation followed by differentiation to form the secretory epithelium responsible for milk production. The cessation of nursing induces involution, a process that eliminates the majority of the mammary epithelium through apoptosis within the time span of several days (for review see Medina, 1996). When stressed with the enormous proliferative pressure associated with pregnancy, $cyl^{-/-}$ mammary glands display delayed proliferation, decreased lateral branching, and incomplete differentiation; thus, $cyl^{-/-}$ mammary glands cannot support lactation (Fantl et al., 1994; Sicinski et al., 1995). These observations suggest that cyclin D1 is critical for mammary gland morphogenesis and function. Given the opposing nature of p27 to cyclin D1 activity, it might be expected that mammary glands from p27-deficient mice would display a hyperproliferative phenotype, in direct contrast with the decreased growth of the cyclin D1–deficient mammary gland.

Although several studies have shown that high levels of p27 inhibit both Cdk2 and Cdk4 activities, the activity of cyclin E–Cdk2 complexes appears to be more potently antagonized by p27 than any other Cdk-containing complex. Furthermore, there is increasing evidence to suggest that p27 may, in fact, promote the activity of Cdk4-containing complexes in primary mouse embryonic fibroblasts. Assembly, stability, and function of cyclin D1–Cdk4 complexes are greatly impaired in the absence of p27 in this cell culture system (Cheng et al., 1999). However, p27-deficient mouse fibroblasts did not exhibit overall cell cycle defects. This observation might be explained by the potential presence of other signaling pathways that allow for cell cycle progression in the absence of pRb phosphorylation, and these cells therefore may not be completely dependent on cyclin D1–cdk4 activity for cell cycle control. On the other hand, a cell type that displays growth defects in the absence of cyclin D1, such as the mammary epithelium, would provide an elegant model system in which to test this hypothesis. If p27 is required for the assembly and function of cyclin D1–cdk4 complexes, then loss of p27 in the mammary epithelium would result in a phenotype that mimics the hypoplastic phenotype of the cyclin D1–deficient mammary epithelium.

We investigated these hypotheses in $p27^{+/-}$and $p27^{-/-}$ mouse mammary glands at several stages of postnatal mammary gland morphogenesis. Transplantation of $p27^{-/-}$ epithelium into precleared mammary fat pads of wild-type mice allowed us to analyze the mammary glands at unique physiological stages, including pregnancy, lactation, and involution, thus circumventing the inability to observe $p27^{-/-}$ mammary gland differentiation due to the infertility of $p27^{-/-}$ females. We report that $p27^{+/-}$ mammary glands displayed increased proliferation and delayed involution. Intriguingly, $p27^{-/-}$ mammary glands displayed a decrease in epithelial proliferation with a marked delay in differentiation, similar to the phenotype of the $cyl^{-/-}$ glands. Cyclin D1–cdk4 assembly and activity, nuclear localization of cyclin D1, and the stability of cyclin D1 were all markedly reduced in $p27^{-/-}$ mammary glands. These results suggest that the absence of p27 results in a block in cell cycle progression in mammary epithelial cells, probably a result of the loss of cyclin D1–cdk4 activity.

Materials and Methods

Mouse Strains and Mammary Gland Transplantations

All mice were derived from $p27^{+/-}$ P1 on a mixed C57Bl/6-129/SvJ background strain, a gift from Dr. Andrew Koff (Memorial Sloan-Kettering Cancer Center, New York, NY) (Kiyokawa et al., 1996). All mice were housed in the Animal Care Facilities at Vanderbilt University School of Medicine in accordance with Association of Assessment and Accreditation of Laboratory Animal Care guidelines. Mice were genotyped by PCR analysis of genomic DNA using the following primers from the endogenous *p27* allele to generate a 206-bp product: primer 1, 5′-TCA AAC GTG AGA GTG TCT AAC GG-3′; and primer 2, 5′-AGG GGC TTA TGA TTC TGA AAG TCG-3′. The following primer from the neo cassette disrupting the *p27* gene (5′-ATA TTG CTG AAG AGC TTG GCG G-3′) was used to generate a 298-bp product with primer 1.

Mammary glands from virgin mice were collected in the diestrus phase of the estrous cycle as determined by cytological smear of vaginal cells. Before 6 wk of age, mice were not analyzed for phase of estrous. Mammary gland transplantations were performed as described (Edwards et al., 1996). In brief, the right and left no. 4 inguinal mammary glands of 21-d-old $p27^{+/+}$ recipient females were cleared of endogenous epithelium by surgically removing the mammary fat pad from the nipple to the lymph node. The no. 4 mammary glands of 6-wk-old $p27^{+/+}$or $p27^{-/-}$ female donors were harvested, and a 2-mm^3 segment was used for transplantation into the center of the remaining $p27^{+/+}$ recipient fat pad. Mammary glands were analyzed 6–12 wk after transplantation. Female mice were mated after 8 wk of age or 8 wk after transplantation. Mammary glands from pregnant mice were harvested at 16.5 days postcoitum (dpc). After 10 d of lactation, pups were withdrawn from $p27^{+/+}$ or $p27^{+/-}$ mothers, and glands were harvested at 1, 3, 5, and 21 days postforced wean [dpfw]). For reconstituted glands, pups were immediately withdrawn within 2–4 h of birth to analyze the "lactational" phenotype of the reconstituted mammary glands. (This was done to prevent precocious involution of the reconstituted mammary gland; during the transplant procedure, the primary epithelial duct was severed from the nipple. At lactation, the sealed primary duct becomes distended with milk, a condition which induces involution within 24 h.) As indicated, some mice of each genotype were given a 90-d release estrogen (0.1 mg)/progesterone (10 mg) pellets (Innovative Research of America), implanted subcutaneously between the scapulae. Mammary glands were harvested after 60 d of estrogen/progesterone exposure.

Western Blot Analyses

Mammary glands were harvested and homogenized immediately as described previously (Lenferink et al., 2000). Total protein (20 μg) was separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blot analyses were performed as described previously (Brantley et al., 2000) using the following antibodies: p27 (Transduction Laboratories); pRb and cyclin D1 (BD PharMingen); cyclin E, Cdk2, and Cdk4 (Santa Cruz Biotechnology, Inc.); α-lactalbumin and β-casein (Neomarkers); keratin-14 (Amersham Pharmacia Biotech); and signal transducer and activator of transcription (Stat)5a and phospho-Stat5 (Santa Cruz Biotechnology, Inc.).

Histological Analyses

Mammary glands were harvested and immediately fixed in 10% formalin (VWR Scientific). Hematoxylin-stained whole mount preparations of no. 4 mammary glands were prepared as described previously and photodocumented (Lenferink et al., 2000). Paraffin-embedded mammary glands were sectioned (5 μm), rehydrated, and stained with Mayer's hematoxylin and eosin B–phloxine (Sigma-Aldrich). For immunohistochemistry, sections were treated as described previously (Brantley et al., 2000) using rabbit polyclonal p27 antibody (Santa Cruz Biotechnology, Inc.) or cyclin D1 antibody (Upstate Biotechnology).

BrdU and TUNEL Analyses

A sterile solution of BrdU (10 mg/ml; Sigma-Aldrich) in PBS (pH 7.4) was administered to mice by intraperitoneal injection (0.1 mg/kg). Mammary glands were harvested after 3 h, paraffin-embedded, and sectioned. Immunohistochemical detection of BrdU incorporation was performed using a mouse monoclonal anti-BrdU antibody (Zymed Laboratories) according to the manufacturer's instructions. Detection of apoptosis in paraffin sections by terminal deoxynucleatidyl transferase–mediated dUTP–biotin nick end labeling (TUNEL) analysis was performed using terminal deoxynucleotidyl transferase (Intergen Co.) to label the ends of fragmented DNA with digoxigenin, which was immunohistochemically localized using a mouse monoclonal antidigoxigenin antibody (Intergen Co.) according to the manufacturer's instructions.

Isolation and Culture of Primary Mammary Epithelial Cells

10 inguinal mammary glands per genotype were harvested from 21-d-old siblings and digested at 37°C for 4 h in 100 U/ml hyaluronidase and 3 mg/ml collagenase A (Sigma-Aldrich) in PBS (pH 7.4). The resulting cell suspension was washed five times in PBS plus 10% FCS, plated on dishes coated with growth factor–reduced Matrigel (Becton Dickinson) in primary mammary epithelial cell (PMEC) medium (serum-free DME:F12 [50:50; GIBCO BRL], 5 ng/ml EGF [Clonetics], 5 ng/ml 17-β estradiol [Sigma-Aldrich], 5 ng/ml progesterone [Sigma-Aldrich], and 50 ng/ml insulin [Clonetics]), and cultured at 37°C, 5% CO_2. In some cases, monolayers were treated with cycloheximide (1 μg/ml; Sigma-Aldrich) for 0–90 min, washed with PBS, and lysed in 1% Nonidet P-40 (vol/vol). Extracts were collected as described above. Cyclin D1 protein was detected by Western blot analysis of equal amounts of protein (20 μg). For three-dimensional cultures, PMECs were plated within a thick layer of growth factor–reduced Matrigel. PMEC medium was layered on top of the polymerized Matrigel. Cultures were photodocumented using the Olympus DP-10 digital camera adapted to an Olympus CK-2 phase–contrast microscope.

FACS® Analysis

Proliferating unsynchronized PMECs were harvested by trypsinization, fixed in ice-cold methanol, and labeled with 50 μg/ml of propidium iodide (Sigma-Aldrich) as described previously (Lenferink et al., 2000). A total of 10,000 stained nuclei per sample was analyzed in a FACSCalibur™ flow cytometer (Becton Dickinson). DNA histograms were modeled using Modfit-LT Software (Verity).

Immunoprecipitation and Kinase Assays

Mammary gland extracts were prepared as described above. 500 μg of total protein was used for immunoprecipitation (IP) as described previously (Lenferink et al., 2000) using polyclonal antibodies against cyclin D1, Cdk2, and Cdk4 (Santa Cruz Biotechnology, Inc.). The precipitates were either used for in vitro kinase assays or resolved by SDS-PAGE followed by Western blot analysis. For kinase assays, IP products were washed in ice-cold PBS, then equilibrated to ice-cold kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 0.1 mM NaF, 0.1 mM Na_3VO_4, 1 mM ATP) for 20 min and resuspended in 20 μl of kinase buffer. Cdk2 IPs were assayed for kinase activity against 0.1 μg histone H1 (HH1; Roche Molecular Biochemicals), whereas Cdk4 IPs were assayed against 0.1 μg of a 46-kD fragment of pRb (Santa Cruz Biotechnology, Inc.). Kinase reactions were performed in a final volume of 30 μl in the presence of 5 μCi [γ-^{32}P]ATP (specific activity 3,000 Ci/mmol; Amersham Pharmacia Biotech) for 45 min at 30°C. The entire reaction volume was electrophoresed on an 8% polyacrylamide gel at 60 mA for 3 h at 4°C. Gels were dried at 80°C and exposed to autoradiographic film (16 h at −80°C).

Whole Organ Culture of Mammary Glands

Inguinal (no. 4) mammary glands were harvested from 5-wk-old mice under sterile conditions. Only the portion of the mammary gland between the nipple and the lymph node was used for culture. The mammary glands were cultured at the air–liquid interface on Costar 8-μ nitrocellulose filters in a chemically defined medium consisting of DME/F12 supplemented with 5 ng/ml 17-β estradiol, 5 ng/ml progesterone, 50 ng/ml recombinant human insulin, 5 μg/ml prolactin (Sigma-Aldrich), and 5 ng/ml EGF. Whole mammary glands were cultured in this manner for 0–10 d at which point whole mount hematoxylin staining was used to visualize the epithelium.

Results

p27 Expression Is Detected at All Stages of Postnatal Mammary Gland Morphogenesis

The steady state protein levels of p27 were measured at various stages of mammary gland development in wild-type C57Bl/6X129Sv/J female mice (Fig. 1 A). Similar levels of p27 protein expression were detected at all stages examined by Western blot analysis, including virgin, midpregnancy, lactation, and involution. Immunohistochemical detection of p27 in wild-type virgin mammary gland sections revealed intense staining for p27 within the epithelium with lower staining throughout the stroma (Fig. 1, B and E). Stromal staining was not due to background trapping of secondary antibody, since sections of wild-type mammary glands that were incubated in the presence of secondary antibody alone did not produce any stromal staining (data not shown). Staining for epithelial p27 protein was seen in the cytoplasm and nuclei of wild-type

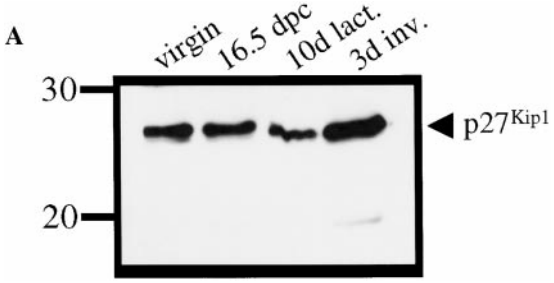
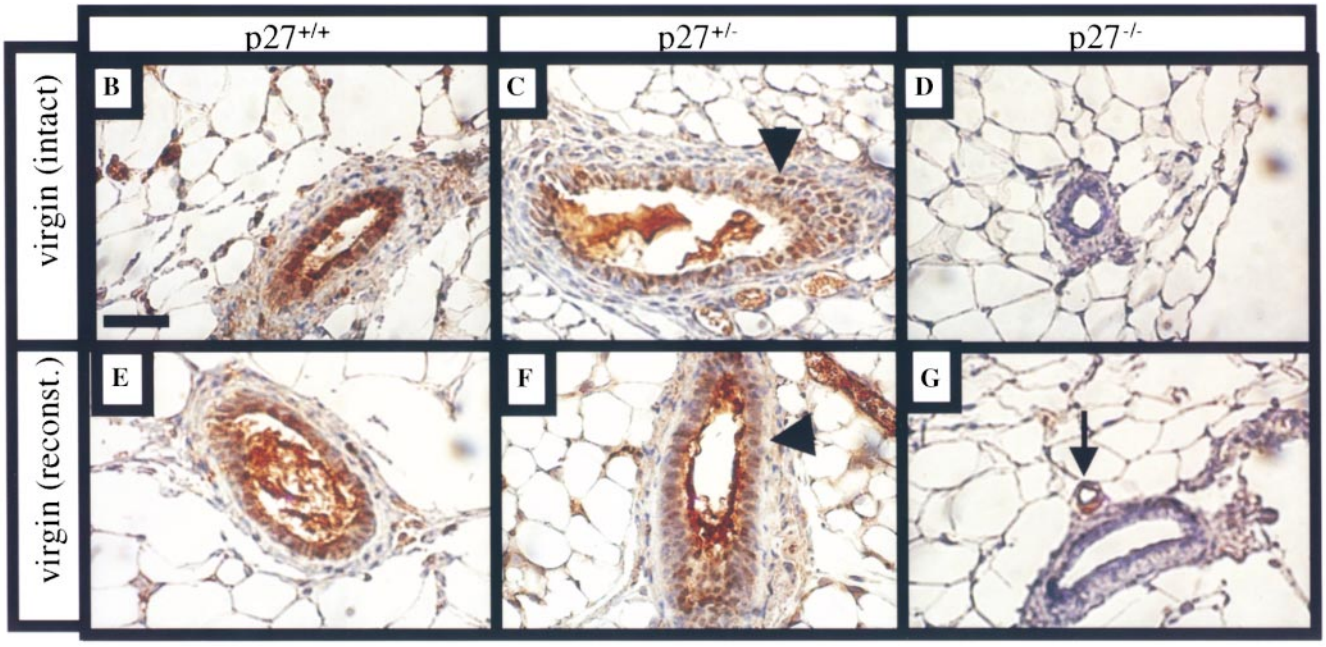


Figure 1. p27 is expressed in the mammary epithelium at all stages of postnatal morphogenesis. (A) Western blot analysis detecting p27 protein in $p27^{+/+}$ mammary glands harvested at the indicated stages of mammary gland development: pregnancy at 16.5 dpc; lactation at postnatal day 10; involution at 3 dpfw. Molecular weights (in kD) are indicated at left. (B–G) Immunohistochemical analysis of p27 expression in no. 4 intact virgin mammary glands harvested from 6-wk-old females or wild-type mammary fat pads reconstituted with tissue of the indicated genotype at 5 wk after transplantation. Arrowhead indicates nuclear localization of p27. Arrow in G indicates host vessel stained positive for p27. Panels shown are representative of results obtained for $n = 3$ per genotype, including reconstitutions.



mammary glands, but was primarily localized to the nuclei of both intact $p27^{+/-}$ glands (Fig. 1 C) and wild-type fat pads reconstituted with $p27^{+/-}$ epithelium (Fig. 1 F). Although intermediate p27 staining was detected in $p27^{+/-}$ glands compared with wild-type glands, detection of p27 expression was absent in intact $p27^{-/-}$ mammary glands (Fig. 1 D) and in the epithelium of wild-type mammary fat pads transplanted with $p27^{-/-}$ cells (Fig. 1 G). Blood vessels from wild-type fat pads transplanted with $p27^{-/-}$ tissue stained positive for p27 (Fig. 1 G, arrow), since the vasculature was derived from the wild-type host (for review see Young, 2000). These results confirm the loss of *p27* gene expression in $p27^{-/-}$ mice and demonstrate epithelial expression of p27 at various stages of postnatal mammary gland morphogenesis.

Loss of One p27 Allele Causes Increased Mammary Epithelial Growth, Whereas Loss of Both p27 Alleles Results in Decreased Growth

To determine the consequences of a reduction or loss in p27 expression on mammary gland morphogenesis, whole mount preparations and histological sections of virgin $p27^{+/+}$, $p27^{+/-}$, and $p27^{-/-}$ mammary glands were examined (Fig. 2). At 21 d of age, mammary glands from $p27^{+/-}$ mice demonstrated a consistent increase in the size of terminal end buds (TEBs) compared with age-matched wild-type controls (Fig. 2 A). TEBs are thought to represent the most highly proliferative region of the progressing mammary epithelium (for review see Medina, 1996). Furthermore, $p27^{+/-}$ mammary epithelium consistently penetrated a greater area of the mammary fat pad (compare the relative location of the TEBs to the centrally located lymph node [see asterisk] of the no. 4 mammary gland), suggesting an increased rate of ductal growth (Fig. 2 A). Mammary epithelium in $p27^{-/-}$ mice displayed the opposite phenotype, characterized by a decrease in the size and number of TEBs and a reduced penetrance through the fat pad (Fig. 2 A). These differences persisted through 35 (Fig. 2, B and C) and 70 d (Fig. 2, D and G). At 35 d, TEBs were present in wild-type glands and penetrated to the distal edge of the lymph node. The strikingly prominent TEBs of the $p27^{+/-}$ mammary glands progressed well past the lymph node, whereas the leading edge of the $p27^{-/-}$ mammary epithelium lacked obvious TEBs. It was also apparent that the primary ducts of the $p27^{-/-}$ mammary glands had a substantial decrease in secondary branches compared with wild-type or $p27^{+/-}$ mammary glands.

At 70 d, TEBs regressed from wild-type glands, but remained apparent in age-matched $p27^{+/-}$ mice at identical phases of estrous (Fig. 2, D and E). Mammary glands from $p27^{-/-}$ mice did not display TEBs. The decreased epithelial stromal ratio in the $p27^{-/-}$ mammary glands was apparent in histologic sections, and the diameter of the ductal lumena appeared to be decreased in size at this stage

when the mammary glands were fully matured (Fig. 2, F and G). The number of TEBs per no. 4 mammary gland at 35 d (Fig. 2 B) and the number of epithelial structures visible per 100× field in sections taken from mammary glands at 70 d (Fig. 2 F) were counted for all three genotypes and are shown in Fig. 2 I.

Although $p27^{-/-}$ mice are known to have altered ovarian function, it is thought that basal levels of the ovarian hormones that regulate virgin mammary gland morphogenesis are present in relatively equal concentrations in wild-type versus p27-deficient mice (Tong et al., 1998). However, ovarian production of nidatory estrogen required for embryonic implantation is reduced in p27-deficient animals. Based on this, we wanted to determine if increased concentrations of ovarian hormones could rescue the hypoplastic phenotype of p27-deficient mammary glands. Slow release (90-d) estrogen/progesterone pellets were implanted under the dorsal skin of 180-d-old virgin mice. After 60 d, the mammary glands were harvested for analysis. Mammary epithelium from all three genotypes displayed lobular bud formation (Fig. 2 H). The epithelium of $p27^{+/-}$ mice was more abundant than that seen in wild-type glands and displayed areas which may represent focal hyperplasias (Fig. 2 H, arrowhead). Mammary epithelium in $p27^{-/-}$ glands still exhibited reduced secondary branching compared with controls. These results demonstrate that in the absence of p27, mammary epithelium has fewer TEBs during early development and persistently reduced secondary branching. In contrast, $p27^{+/-}$ mammary glands display an increased rate of ductal development with increased numbers of TEBs.

Altered Cell Growth of the Mammary Epithelium Due to Loss of p27 Is an Epithelial Cell Autonomous Phenotype

To determine whether the $p27^{-/-}$ phenotype was intrinsic to the mammary epithelium and not secondary to stromal or hormonal influences, $p27^{-/-}$ mammary tissue was transplanted into wild-type mammary fat pads cleared of endogenous breast epithelium. This technique has been used to reconstitute mammary fat pads with donor mammary epithelium, which will permeate the fat pad and differentiate in a manner consistent with the native gland. p27 protein expression was detected in mammary glands reconstituted with $p27^{+/+}$ or $p27^{+/-}$ epithelium (see Fig. 1, E and F) but not in mammary glands reconstituted with $p27^{-/-}$ cells (Fig. 1 G). After 6 wk, mammary glands reconstituted with $p27^{+/+}$, and $p27^{+/-}$ epithelium exhibited extensive ductal branching with prominent TEBs (Fig. 3, A and B, arrows). In contrast, $p27^{-/-}$ epithelium displayed decreased lateral branching. There was a marked reduction of TEBs in the mammary glands reconstituted with $p27^{-/-}$ tissue, similar to what is seen in intact mammary glands from $p27^{-/-}$ animals (see Fig. 2, C, E, and G). These characteristics persisted for >12 wk after transplantation (data not shown). These results suggest that the hypoplastic phenotype of $p27^{-/-}$ mammary glands is independent from hormonal or stromal factors.

PMECs harvested from $p27^{+/+}$, $p27^{+/-}$, and $p27^{-/-}$ mice were cultured in a three-dimensional extracellular matrix in the presence of mitogenic hormones to further investigate the epithelial autonomy of the $p27^{-/-}$ and $p27^{+/-}$ phenotypes (Fig. 3, C and D). Tubulogenesis in $p27^{+/+}$ and $p27^{+/-}$ PMECs occurred within 5 d of culture (21.4 ± 7.3 and 32.1 ± 5.3 branches per 400× field in $p27^{+/+}$ and $p27^{+/-}$ PMECs, respectively). $p27^{-/-}$ PMECs failed to induce tubular structures within this time frame (0 branches per 400× field). After 15 d in culture, $p27^{-/-}$ PMECs began to organize into rudimentary tubules (data not shown), suggesting that $p27^{-/-}$ mammary epithelial cells are capable of growth and tubulogenesis but in a delayed fashion compared with cells containing p27. In contrast, complex branch formations occurred at a more rapid rate in $p27^{+/-}$ PMECs compared with wild-type cells.

Loss of One p27 Allele Causes Increased Proliferation of the Mammary Epithelium; Loss of Both p27 Alleles Causes Decreased Proliferation

We next measured mammary epithelial proliferation by labeling virgin 5-wk-old mice with BrdU and assessing BrdU incorporation into chromosomal DNA (Fig. 4, A–C). When TEBs were examined, a 2.6-fold increase in BrdU incorporation into $p27^{+/-}$ compared with $p27^{+/+}$ mammary epithelial cells was observed (Fig. 4, A and E; $P < 0.001$, $n = 6$). Although TEBs are rare in $p27^{-/-}$ mammary glands, the structures which most closely resembled TEBs were analyzed for BrdU incorporation and displayed a 2.6-fold decrease compared with wild-type TEBs (Fig. 4, A and E; $P < 0.006$, $n = 6$). BrdU incorporation into ductal epithelium was also examined (Fig. 4 B). As expected, the overall rate of BrdU labeling was decreased in ductal epithelium compared with that in TEBs for each genotype analyzed. However, quantification of BrdU-labeled cells demonstrated that $p27^{+/-}$ ducts contained 1.8-fold more BrdU-positive nuclei than wild-type ducts, whereas $p27^{-/-}$ glands displayed a 2-fold decrease in BrdU incorporation compared with wild-type ducts (3.1 ± 0.36%, 5.58 ± 0.49% [$P < 0.04$], and 1.38 ± 0.12% [$P < 0.02$] for $p27^{+/+}$, $p27^{+/-}$, and $p27^{-/-}$, respectively). The altered epithelial proliferation correlated with increased epithelial content in $p27^{+/-}$ glands and decreased epithelial content in $p27^{-/-}$ glands (in 10 randomly chosen 400× fields, $p27^{+/+}$ equals 7,832 cells, $p27^{+/-}$ equals 10,490 epithelial cells, and $p27^{-/-}$ equals 5,502 epithelial cells). Proliferation in wild-type mouse fat pads that had been transplanted with $p27^{+/+}$, $p27^{+/-}$, or $p27^{-/-}$ mammary epithelium was examined at 5 wk after transplantation (Fig. 4 C). Again, BrdU incorporation in $p27^{+/-}$ mammary epithelium (31.2 ± 4.6%) was elevated compared with wild-type mammary epithelium (17.1 ± 4.4%), whereas BrdU labeling was decreased in $p27^{-/-}$ epithelium (6.7 ± 3.0%). Labeling of purified PMECs in culture demonstrated that a monolayer of $p27^{+/-}$ PMECs had a higher rate of BrdU incorporation compared with wild-type PMECs (Fig. 4, D and E), whereas $p27^{-/-}$ PMECs had a decreased rate of BrdU labeling. Flow cytometric analysis of PMEC nuclei stained with propidium iodide demonstrated a larger G_1 with a smaller S phase fraction in $p27^{-/-}$ cells compared with wild-type cells (Fig. 4 F, $P < 0.045$, S phase comparison, Student's t test). In contrast, $p27^{+/-}$ cells displayed an increase in the percentage of cells in S phase ($P < 0.02$, Student's t test) with a lower proportion of cells in G_1 compared with wild-type cells, consistent with an accelerated G_1 to S transition. These results suggest that p27 function is critical for the growth of the mam-

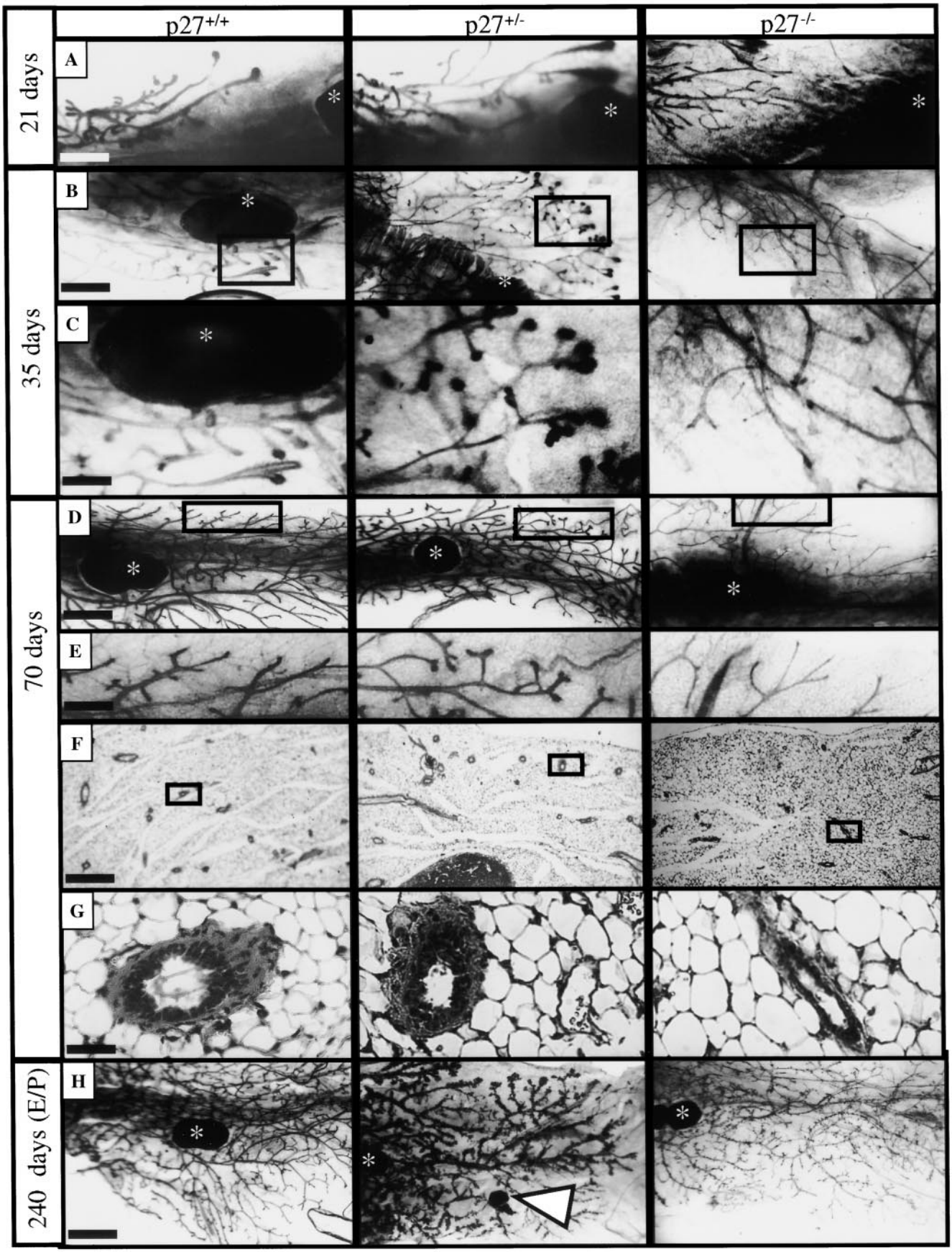


Figure 2 (continues on facing page)

mary gland. Whereas loss of one p27 allele results in markedly increased epithelial cell proliferation, loss of both p27 alleles reduces breast epithelial proliferation.

Total Loss of p27 Affects the Formation and Function of Cyclin D1–Cdk4 Complexes in Mammary Epithelial Cells

To elucidate the mechanism by which proliferation is increased in $p27^{+/-}$ and decreased in $p27^{-/-}$ mammary glands, the abundance of cyclin D1, cyclin E, Cdk2, and Cdk4 proteins was examined. Western blot analyses of virgin mammary extracts revealed that levels of Cdk2, Cdk4, and cyclin E were not affected significantly by the relative amount of p27 protein, whereas cyclin D1 levels were significantly lower in $p27^{-/-}$ glands (Fig. 5 A). Phosphorylated pRb was detected in lysates from all three genotypes, as shown by the slowly migrating immunoreactive species. However, hyperphosphorylation of ppRb was greater in $p27^{+/+}$and $p27^{+/-}$ lysates compared with $p27^{-/-}$ lysates. Cyclin D1 from $p27^{+/+}$ and $p27^{+/-}$ mammary glands coprecipitated similar levels of p27 and Cdk4, even though there was significantly less total p27 available in $p27^{+/-}$ glands compared with $p27^{+/+}$ glands (Fig. 5 B). In contrast, experiments performed with Cdk2 antibodies showed a relative decrease in the amount of Cdk2-associated p27 in $p27^{+/-}$ mammary extracts but increased levels of Cdk2-associated cyclin E. As expected, cyclin D1 antibodies were unable to precipitate p27 in $p27^{-/-}$ glands. However, most strikingly coprecipitation of Cdk4 with cyclin D1 was eliminated in the absence of p27, even though both proteins were present in the $p27^{-/-}$ extracts. Antibodies directed against Cdk2 were able to coprecipitate cyclin E in the absence of p27. These data suggest that the gene dosage of *p27* influences the association between cyclin D1 and Cdk4 but not of cyclin E with Cdk2.

The kinase activities of immunoprecipitated Cdk4 complexes were measured in $p27^{+/+}$, $p27^{+/-}$, and $p27^{-/-}$ mammary gland extracts using a 46-kD COOH-terminal fragment of Rb as a substrate (Fig. 5 C). Cdk4 immunoprecipitates from $p27^{+/-}$ extracts were able to phosphorylate p46-Rb at a level similar to that observed with $p27^{+/+}$ extracts. Cdk4 activity against p46-Rb was barely detectable in lysates from $p27^{-/-}$ glands. Phosphorylation of HH1 by Cdk2 immunoprecipitates was comparable in extracts from each genotype tested. These results suggest that the loss of p27 does not affect the kinase activity of Cdk2, whereas Cdk4 relies on the presence of p27 in mammary epithelium for its interaction with cyclin D1 and maximal kinase activity against Rb.

To examine cyclin D1 stability in the absence of p27, PMECs were treated with cycloheximide, and their content of cyclin D1 protein was followed over time (Fig. 5 D). The level of cyclin D1 in $p27^{+/+}$ and $p27^{-/-}$ PMECs decreased as a function of exposure to cycloheximide. However, cyclin D1 levels decreased at a faster rate in $p27^{-/-}$ PMECs ($t_{1/2} < 15$ min) compared with wild-type PMECs ($t_{1/2} \sim 30$–45 min). This is consistent with previous cell culture studies in which cyclin D1 displayed a reduced stability in the absence of p27 (Cheng et al., 1999).

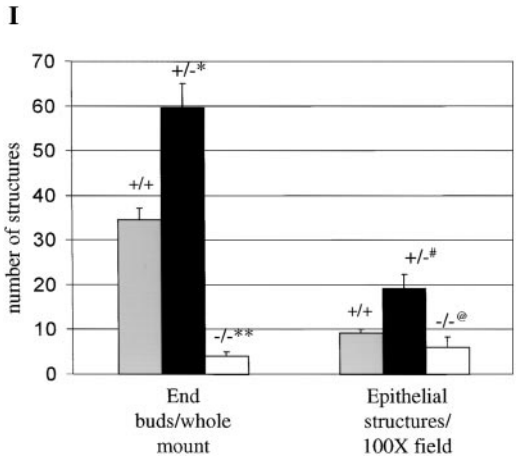


Figure 2. Altered ductal growth and branching in $p27^{+/-}$ and $p27^{-/-}$ mammary glands. (A–E) Whole mounts of virgin no. 4 mammary glands at 21, 35, and 70 d. Asterisk denotes lymph nodes. All samples were photographed at the same magnification. Boxed areas (B and D) are shown enlarged directly below each panel (C and E) to show detail. Arrows indicate TEBs. (F–G) Hematoxylin and eosin–stained sections of mammary glands from virgin mice of each genotype at 70 d are shown. (H) Whole mount staining of virgin mammary glands from 240-d-old mice treated for 60 d with slow release estrogen–progesterone pellets. Arrowhead denotes area of focal hyperplasia. Panels shown are representative of results obtained for $n = 3$ per genotype. (I) Quantification of the number of TEBs per mammary gland at 35 d of age ($n = 10$ per genotype, see Fig. 2 B) and the number of epithelial structures per 100× field of hematoxylin and eosin–stained section of mammary gland from mice at 70 d of age ($n = 10$ fields per each of 6 glands per genotype, see Fig. 2 F). Numbers are presented as the average plus or minus the SEM; $^{*}P < 0.003$, $^{**}P < 0.0001$, $^{\#}P < 0.03$, $^{@}P < 0.01$, Student's *t* test. Bars: (A, B, D, and H) 400 μm; (C, E, and F) 100 μm; (G) 25 μm.

Nuclear Localization of Cyclin D1 Is Impaired in $p27^{-/-}$ Mammary Glands

The subcellular localization of cyclin D1 in the mammary epithelium was examined by immunohistochemistry (Fig. 6). Cyclin D1 staining was observed in glands from each genotype, but it was markedly reduced in $p27^{-/-}$ mammary glands, consistent with its rapid turnover (see Fig. 5 D). Cyclin D1 staining was observed in both the cytoplasmic compartment and nuclei of virgin $p27^{+/+}$, with the majority of nuclei being completely devoid of cyclin D1 (Fig. 6). Cyclin D1 was evident in 17.8% of $p27^{+/-}$ nuclei, a 1.8-fold increase in the frequency of cyclin D1 nuclear localization over wild-type mammary epithelial cells ($P < 0.045$). In contrast, $p27^{-/-}$ epithelium displayed almost no nuclear cyclin D1 (0.0625%; $P < 0.001$). These results suggest that in the absence of p27, nuclear localization of cyclin D1 is severely impaired.

Complete Absence of p27 Impairs Growth and Differentiation of the Mammary Epithelium during Pregnancy and Lactation

Comparison of mammary glands from pregnant (16.5 dpc) $p27^{+/+}$ and $p27^{+/-}$ mice revealed no morphological differences (Fig. 7, A and B). Lobuloalveolar development appeared to occur normally in $p27^{+/-}$ glands (Fig. 7, E and F). Because $p27^{-/-}$ females are infertile, the effect of p27 deficiency on the mammary epithelium during pregnancy

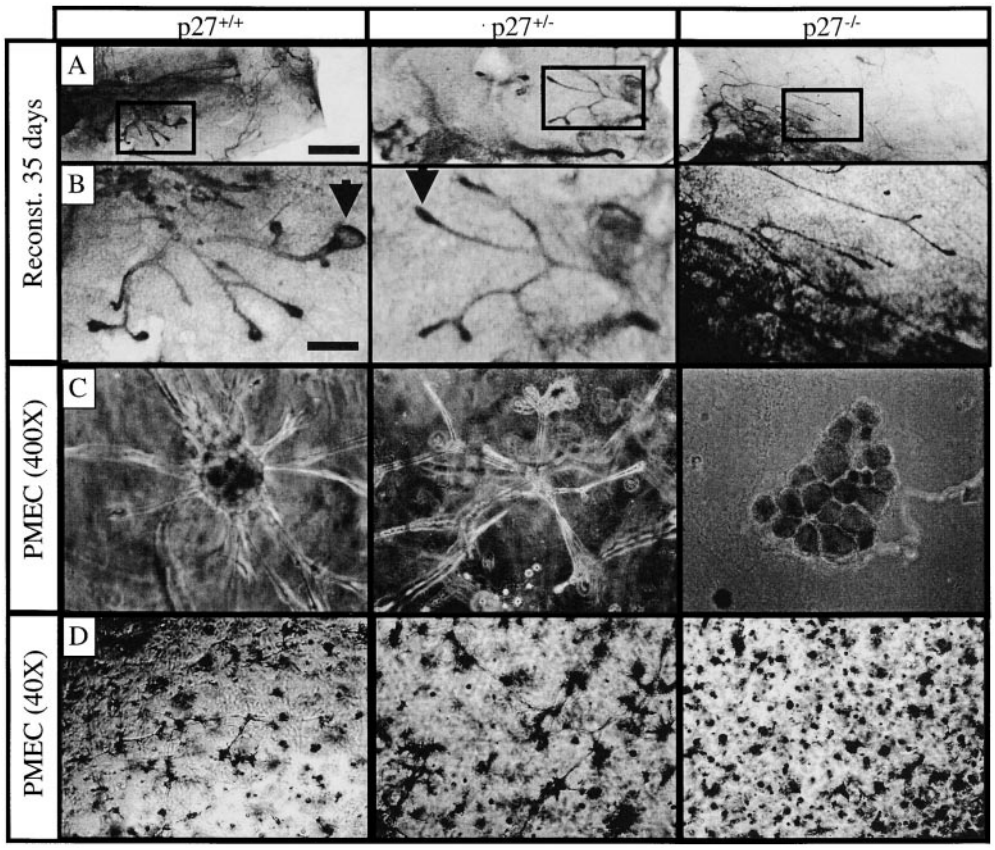


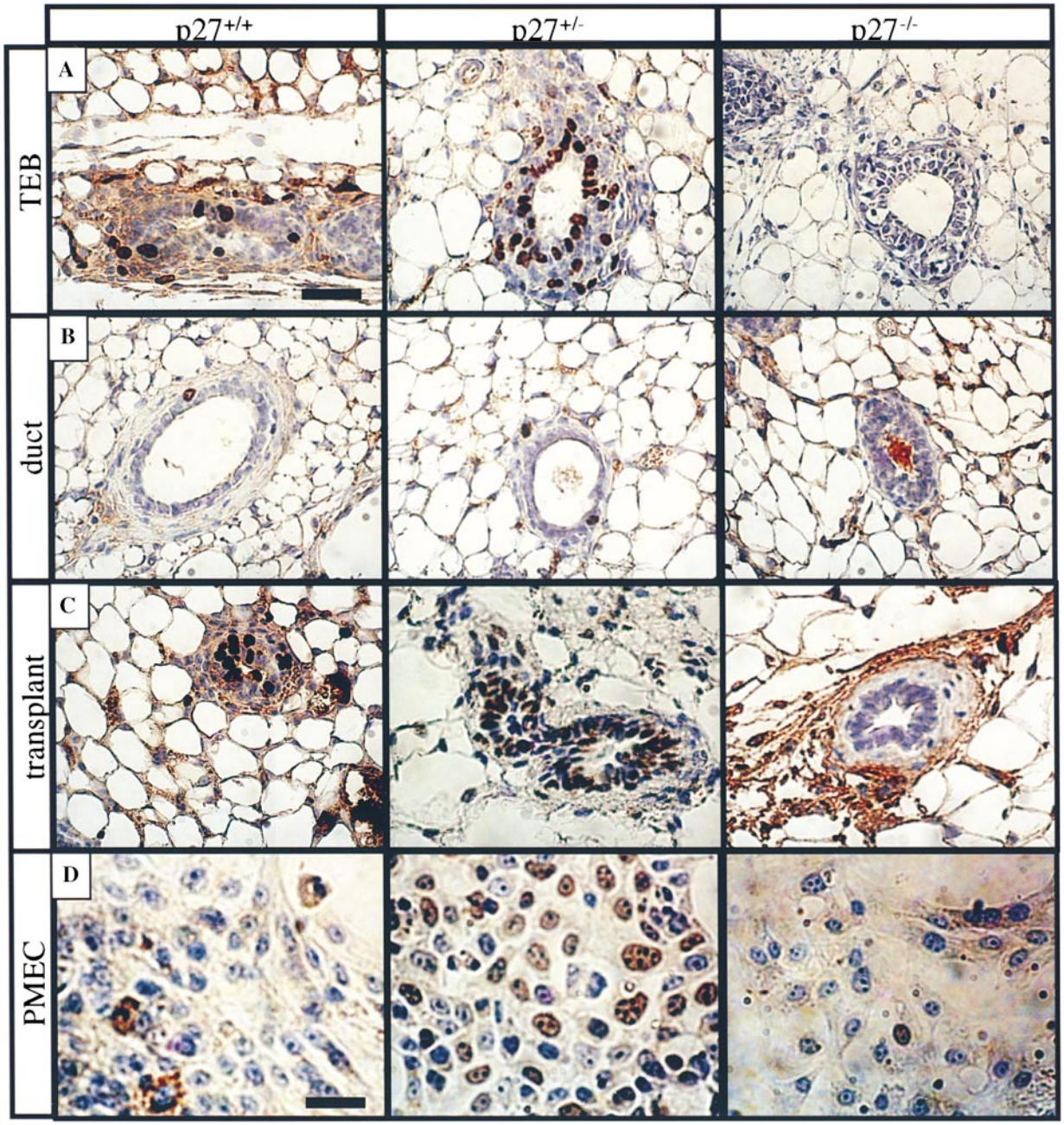
Figure 3. The morphological effects of p27 haploinsufficiency and of p27 deficiency are epithelial autonomous. (A and B) Whole mounts of wild-type mammary glands reconstituted with $p27^{+/+}$, $p27^{+/-}$, or $p27^{-/-}$ tissue 5 wk after transplantation. Boxed areas are enlarged to show detail. Arrows indicate TEBs. (C and D) Primary mammary epithelial cells from virgin $p27^{+/+}$, $p27^{+/-}$, or $p27^{-/-}$ females (n = 3 mice per genotype) cultured in Matrigel. Representative photomicrographs shown were captured at 400× magnification (C) or at 100× (D) after 5 d; n > 20 colonies/genotype. Bars: (A) 400 μm; (B) 100 μm.

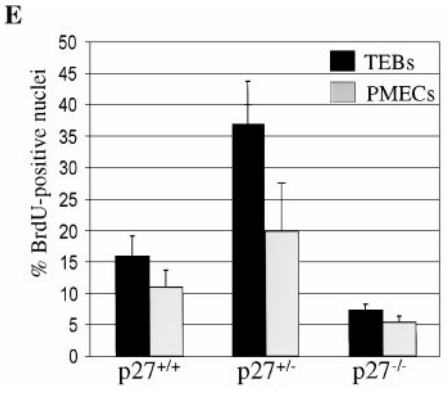
was investigated in wild-type mammary glands reconstituted with either $p27^{-/-}$ or wild-type tissue (Fig. 7, C and D). Contralateral control mammary glands reconstituted with wild-type epithelium appeared histologically indistinguishable from intact wild-type glands at 16.5 dpc (Fig. 7, C and G compared with A and E). However, severely impaired growth of the p27-deficient epithelium was apparent (Fig. 7, D and H), whereas the native mammary gland from the same animal displayed the morphological changes associated with pregnancy (data not shown). This suggests that p27 is required in the mammary epithelium for proper morphogenesis during pregnancy.

No morphological differences were observed between $p27^{+/+}$ and $p27^{+/-}$ lactating mammary glands (Fig. 8, A, B, E, and F). Indeed, $p27^{+/-}$ lactating alveoli were filled with lipid-containing secretions, and $p27^{+/-}$ mothers were able to successfully nurse litters from sequential pregnancies, suggesting that $p27^{+/-}$ mammary glands are able to differentiate (Fig. 8, B and F). Examination of mammary glands reconstituted with $p27^{+/+}$ epithelium showed histological evidence of lactation, including distended alveoli filled with lipid-containing secretions and a marked increase in the ratio of epithelial to stromal cells (Fig. 8, C and G). In contrast, mammary glands reconstituted with $p27^{-/-}$ tissue (n = 12) showed minimal increase in epithelial tissue with an abundance of visible stroma throughout the gland (Fig. 8, D and H). Although $p27^{-/-}$ ducts became distended with eosinophilic secretions, distention of the alveoli was never observed, suggesting an inability to form lobuloalveolar structures and achieve functional differentiation.

To visualize the development and differentiation of the $p27^{-/-}$ epithelium in the context of its native mesenchyme, whole organ culture of mammary glands was used (Fig. 8, I and J). Glands were cultured for 1–10 d in a chemically defined medium containing estrogen, progesterone, prolactin, EGF, and insulin, a combination of factors that have been used to mimic pregnancy and induce differentiation of whole mammary glands in culture. Glands harvested from 4-wk-old wild-type or p27-deficient mice and cultured for 1 d both displayed the characteristic phenotypes of their respective genotypes (Fig. 8 I). Note the thin ducts of the $p27^{-/-}$ glands and the apparent lack of TEBs. After 10 d in culture, wild-type mammary glands responded to the lactogenic hormones by producing abundant lobuloalveolar clusters with an increase in the total epithelial content of the cultured gland (Fig. 8 J). In contrast, $p27^{-/-}$ glands did not demonstrate lobuloalveolar differentiation, nor did they display the increase in epithelia observed in the wild-type organ cultures. These results confirm the in vivo data obtained with mammary gland transplants and extend this observation to include the development of $p27^{-/-}$ epithelium in the context of its native mesenchyme.

To further determine the extent to which $p27^{-/-}$ epithelium was able to differentiate on a molecular level, the expression of milk proteins was examined. A low level of β-casein was detected in lactating glands reconstituted with $p27^{-/-}$ epithelium at 16.5 dpc, whereas it was strongly expressed in lactating wild-type glands and glands reconstituted with wild-type mammary tissue (Fig. 8 K, left). The expression of α-lactalbumin was not detected in glands reconstituted with $p27^{-/-}$ epithelium. This decreased expres-





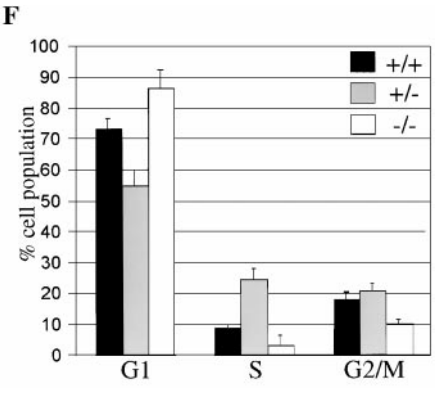


Figure 4. Proliferation is increased in $p27^{+/-}$ but decreased in $p27^{-/-}$ mammary epithelium. (A and B) Immunohistochemical detection of BrdU incorporation in mammary glands of 5-wk-old virgin mice (n = 6 per genotype). TEBs and ducts are shown. Genotypes are indicated. (C) BrdU incorporation into nuclei of wild-type mammary glands transplanted with $p27^{+/+}$, $p27^{+/-}$, or $p27^{-/-}$ mammary tissue. (D) BrdU incorporation into purified PMECs harvested from virgin mice of the indicated genotypes (n = 3 per genotype). (E) Quantification of BrdU incorporation in TEBs and in PMECs. The average number of BrdU-positive epithelial cells per single randomly chosen 400× field was determined. The proliferative index was calculated as follows: ([number of BrdU-labeled cells]/[total number of cells]) × 100. Each value represents the average of 10 fields from each of 6 individually analyzed mammary glands per genotype. (F) Flow cytometric analysis of propidium iodide–stained PMEC nuclei from virgin $p27^{+/+}$, $p27^{+/-}$, or $p27^{-/-}$ mice. Results are shown as the mean SEM of six mice per genotype. Bars, 25 μm.

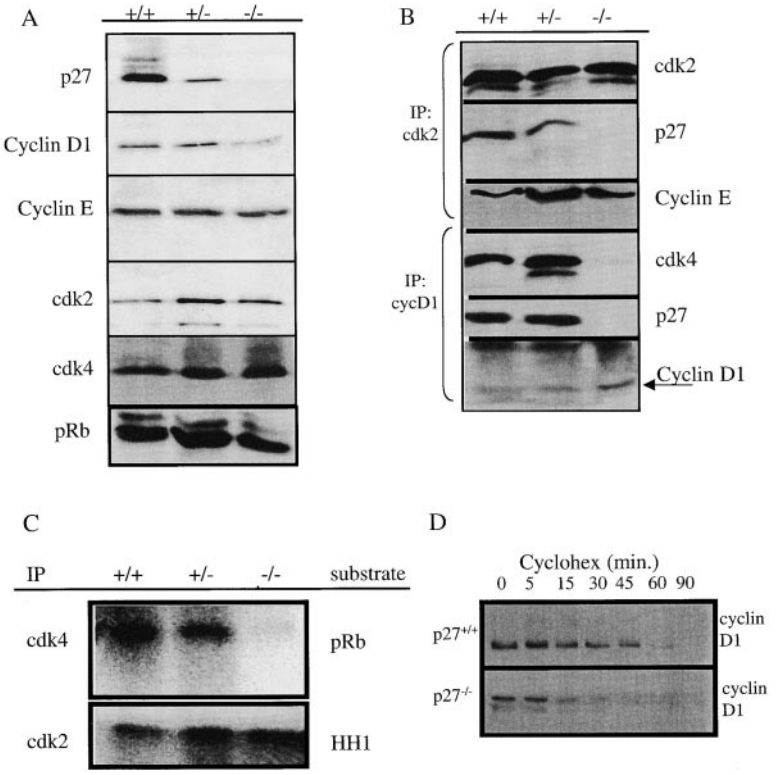
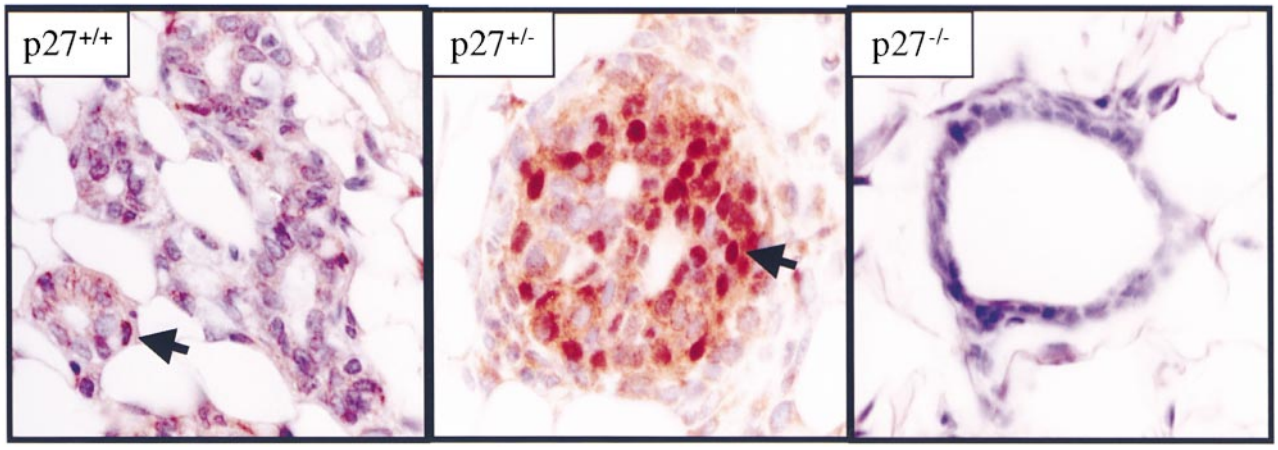


Figure 5. Loss of cyclin D1–Cdk4 activity and decreased cyclin D1 stability in $p27^{-/-}$ mammary glands. (A) Western blot analysis of whole mammary gland extracts from virgin mice of each genotype was used to examine the content of p27, cyclin D1, cyclin E, Cdk2, Cdk4, and Rb. (B) IP using Cdk2 or cyclin D1 antibodies was performed on mammary gland extracts from mice of each genotype. Immunoprecipitates were subjected to Western blot using antibodies against p27, cyclin E, cyclin D1, Cdk2, or Cdk4 as indicated. (C) Kinase activity of Cdk4 and Cdk2 precipitates pRb (46 kD) or HH1 as indicated in Materials and Methods. (D) $p27^{+/+}$ or $p27^{-/-}$ PMECs were cultured for 0–90 min in the presence of cycloheximide (5 μg/ml). Whole cell extracts were analyzed for the presence of cyclin D1 by Western blot analysis.

sion of lactational proteins was not due to a decrease in $p27^{-/-}$ epithelial content relative to wild-type glands, since data were normalized to the content of keratin-14, a marker of epithelial cells. The expression and phosphorylation status of Stat5a were also examined, since this transcription factor is known to be required to initiate the transcription of several milk protein genes (Henninghausen et al., 1997). Although the levels of Stat5a were equal regardless of the genotype examined, it was observed that phosphorylation of Stat5a was decreased at 16.5 dpc in wild-type mammary fat pads reconstituted with $p27^{-/-}$ epithelium but not in fat pads reconstituted with wild-type epithelium (Fig. 8 K, right). These results further support that $p27^{-/-}$ mammary epithelial cells are

A



B

	% cyclin D1-positive nuclei	Total cells counted	Total # cyclin D1-positive nuclei
p27+/+	9.99%	2010	200
p27+/-	17.8%	650	116
p27-/-	0.0625%	1050	1

Figure 6. Impaired nuclear localization of cyclin D1 in the absence of p27. (A) Immunohistochemical detection of cyclin D1 protein in 6-wk-old virgin $p27^{+/+}$, $p27^{+/-}$, or $p27^{-/-}$ glands. (B) Quantification of the total number of cyclin D1–positive nuclei.

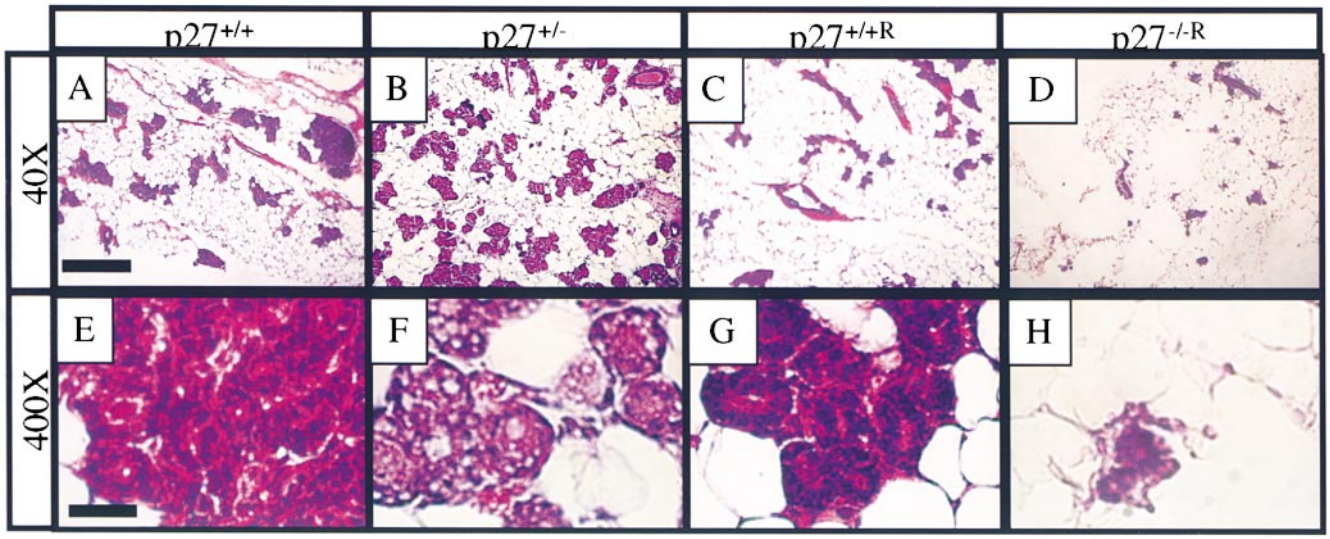


Figure 7. Mammary glands reconstituted with $p27^{-/-}$ epithelium display impaired morphogenesis during pregnancy. (A–D) Hematoxylin and eosin staining of mammary glands from mice of the indicated genotypes at d 16.5 of pregnancy. Analysis of glands reconstituted with wild-type ($p27^{+/+R}$) and $p27^{-/-}$ ($p27^{+/+R}$) tissue was performed. Panels are shown at lower magnification to show the extent of epithelial development and at higher magnification (E–H) to show the detail of lobuloalveolar development. Results are representative of 12 independent analyses. Bars: (A–D) 100 μm; (E–H) 25 μm.

unable to differentiate in response to pregnancy and lactation.

Loss of One p27 Allele Decreases Apoptosis and Delays Involution of the Mammary Gland

Involution of $p27^{+/-}$ and $p27^{+/+}$ mammary epithelium was examined at 1, 3, 5, and 21 dpfw (Fig. 9, A–H). By 3 dpfw, delayed involution was apparent in $p27^{+/-}$ glands with an excess of epithelium and a reduced level of visible adipocytes compared with $p27^{+/+}$ glands (Fig. 9, B compared with F). At 21 dpfw, the wild-type mammary gland resembled the relatively quiescent ductal network of a virgin gland, whereas abundant epithelium remained in $p27^{+/-}$ mammary glands, including persistent lobuloalveolar structures and ducts distended with lipid-containing secretions (Fig. 9, D compared with H). These changes persisted as long as 40 dpfw (data not shown).

TUNEL analysis was performed to detect cells undergoing apoptosis during involution (Fig. 9, I–L). Approximately 64% of the total number of $p27^{+/+}$ epithelial nuclei stained TUNEL positive at 3 dpfw (Fig. 9, I and M), consistent with previous reports (Strange et al., 1992). However, only 29.9% of $p27^{+/-}$ nuclei were TUNEL positive at this stage (Fig. 9, K and M; $P < 0.007$). The peak of apoptosis in wild-type involuting mammary glands occurred at 3 dpfw. In contrast, apoptosis in $p27^{+/-}$ mammary glands did not peak until 5 dpfw (43.6%), after which the rate of apoptosis declined. These results suggest that $p27^{+/-}$ glands undergo reduced and delayed apoptosis, resulting in a persistence of breast epithelium after cessation of lactation. Involution in $p27^{-/-}$ mammary epithelium is not reported herein, since differentiation and growth did not occur in a manner that would make results interpretable.

Discussion

We report the first in vivo evidence that the absence of p27 within the mammary gland impairs cell cycle progression, whereas loss of only a single p27 allele has an opposite effect, an acceleration of G_1 to S progression. In this manner, stoichiometric levels of p27, ranging from no p27 (by homozygous-targeted disruption of the *p27* gene) to abnormally high levels of p27 (by gene overexpression or increased protein stability) may determine the growth potential of the mammary epithelium. We demonstrate that haploid gene inactivation of *p27* causes a decrease in p27 levels within the mammary epithelium, correlating with increased proliferation, increased lateral branching, elevated lobuloalveolar development, and delayed involution. These observations are consistent with previous studies demonstrating that antisense-mediated inactivation of p27 results in increased cell proliferation (Coates et al., 1996) and the low levels of p27 protein reported in several human tumors (Clurman and Porter, 1998), further supporting its role as a tumor suppressor.

However, intriguingly $p27^{-/-}$ mammary glands showed decreased proliferation. This defect appears to be intrinsic to the epithelium, since $p27^{-/-}$ epithelium transplanted into wild-type females also exhibited decreased proliferation. This confirms that the hormonal environment of $p27^{-/-}$ females is not the ultimate determinant of this hypoproliferative phenotype. Epithelial–stromal interactions are known to influence mammary gland morphogenesis. Since a small amount of $p27^{-/-}$ stromal tissue is transplanted along with the $p27^{-/-}$ epithelium, one concern was that the $p27^{-/-}$ stroma may have caused the retarded proliferation of the mammary epithelium. This possibility was addressed by purifying primary mammary epithelial cells from $p27^{-/-}$ mice (Fig. 3). Under these ex vivo conditions, $p27^{-/-}$ cells recapitulated the decreased branching and

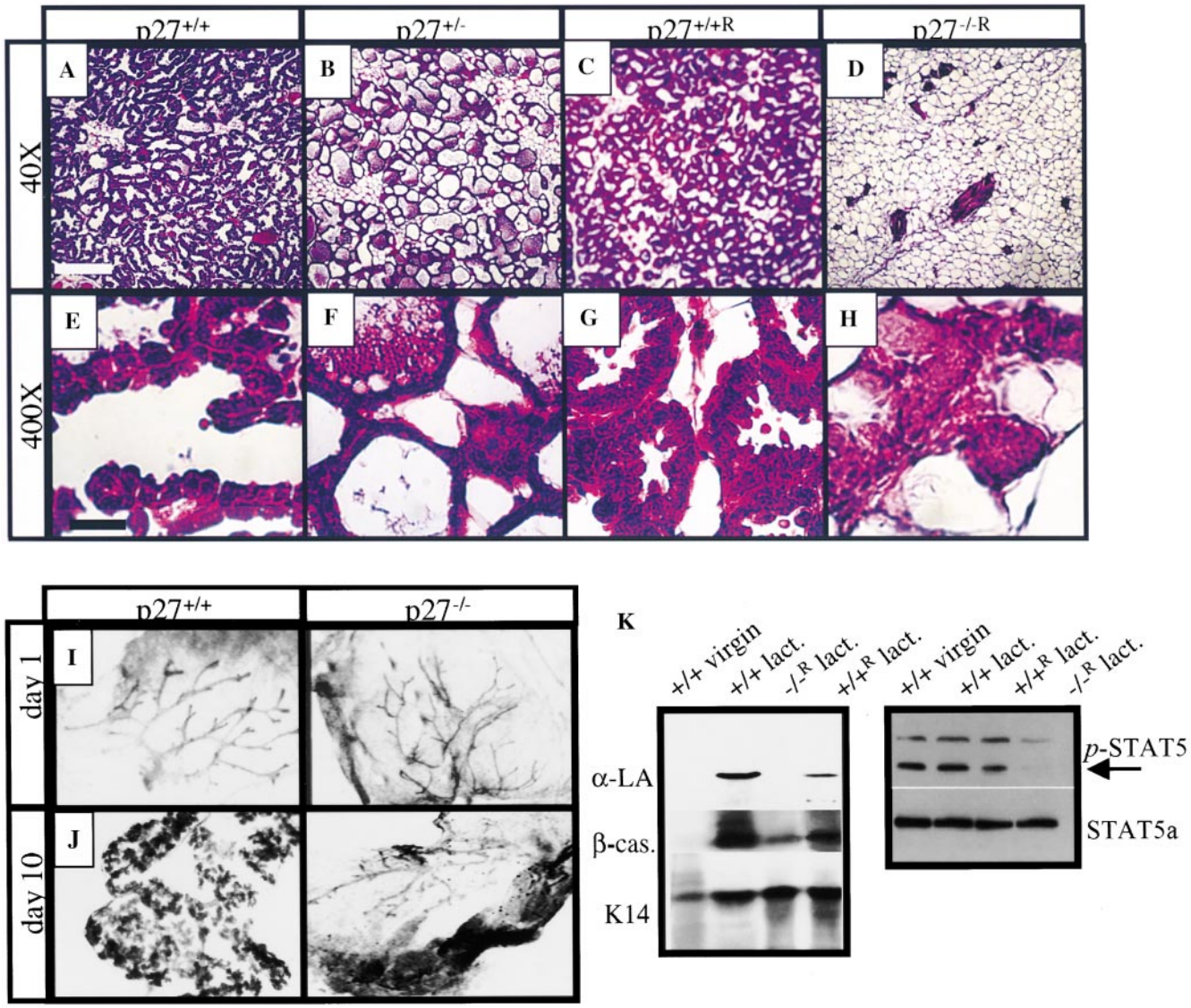


Figure 8. Mammary glands reconstituted with $p27^{-/-}$ epithelium display impaired function during lactation. (A–D) Hematoxylin and eosin staining of glands taken postpartum at 10 d (for intact glands) or 2 h (for reconstituted glands). Upper row shows low magnification (A–D); bottom row shows high magnification (E–H). (I and J) Whole mammary glands from $p27^{+/+}$ and $p27^{-/-}$ mice were cultured for 10 d in the presence of mammogenic hormones (described in Materials and Methods) to determine the ability of $p27^{-/-}$ glands to differentiate within the context of $p27^{-/-}$ stroma. Whole mounts are shown at 1 and 10 d of culture. Representative panels are shown; $n = 10$ per genotype. (K) Western blot analyses were used to examine the content of β-casein (β-cas), α-lactalbumin (α-LA), keratin-14 (K14), Stat5a, and phospho-Stat5 proteins in virgin $p27^{+/+}$ and lactating $p27^{+/+}$ glands or lactating wild-type glands reconstituted with $p27^{-/-}$ or $p27^{+/+}$ epithelium at 2 h postpartum. Results are representative of three independent analyses. Bars, 25 μm.

proliferation observed in vivo. Our results confirm that p27 is required in the mammary epithelium for proliferation and morphogenesis to proceed at a normal rate.

Because proliferation occurs in $p27^{-/-}$ glands, albeit delayed, it is likely that there are other mechanisms in place that incompletely circumvent the loss of p27 with the subsequent loss of cyclin D1–Cdk4 activity. Many signals are responsible for regulating proliferation within the mammary epithelium, including estrogen, growth hormone, prolactin, progesterone, adrenal steroids, and various growth factors (Medina, 1996). These pathways may be able to partially compensate for the loss of p27 and the loss of cyclin D1–Cdk4 activity. Interestingly, previous reports suggest a possible role for cyclin D1 in directly binding ER and potentiating ER-mediated transactivation of ER-responsive genes (Zwijsen et al., 1997). The ability of cyclin D1 to interact with ER is independent of its interaction with Cdks and therefore may not require the presence of p27. Other studies have shown that cyclin E–cdk2 complexes have additional functions, downstream of the Rb pathway, that drive the cell cycle into S phase (Lukas et al., 1997). It is possible that this or another signaling pathway may allow a low level of proliferation to occur, independent of the level of Rb phosphorylation. Indeed, the level of Rb phosphorylation is reduced in $p27^{-/-}$ mammary glands, consistent with the delay in G_1 progression, but Rb phosphorylation is not completely eliminated by the loss of p27 (Fig. 5). It is possible that low levels of Rb

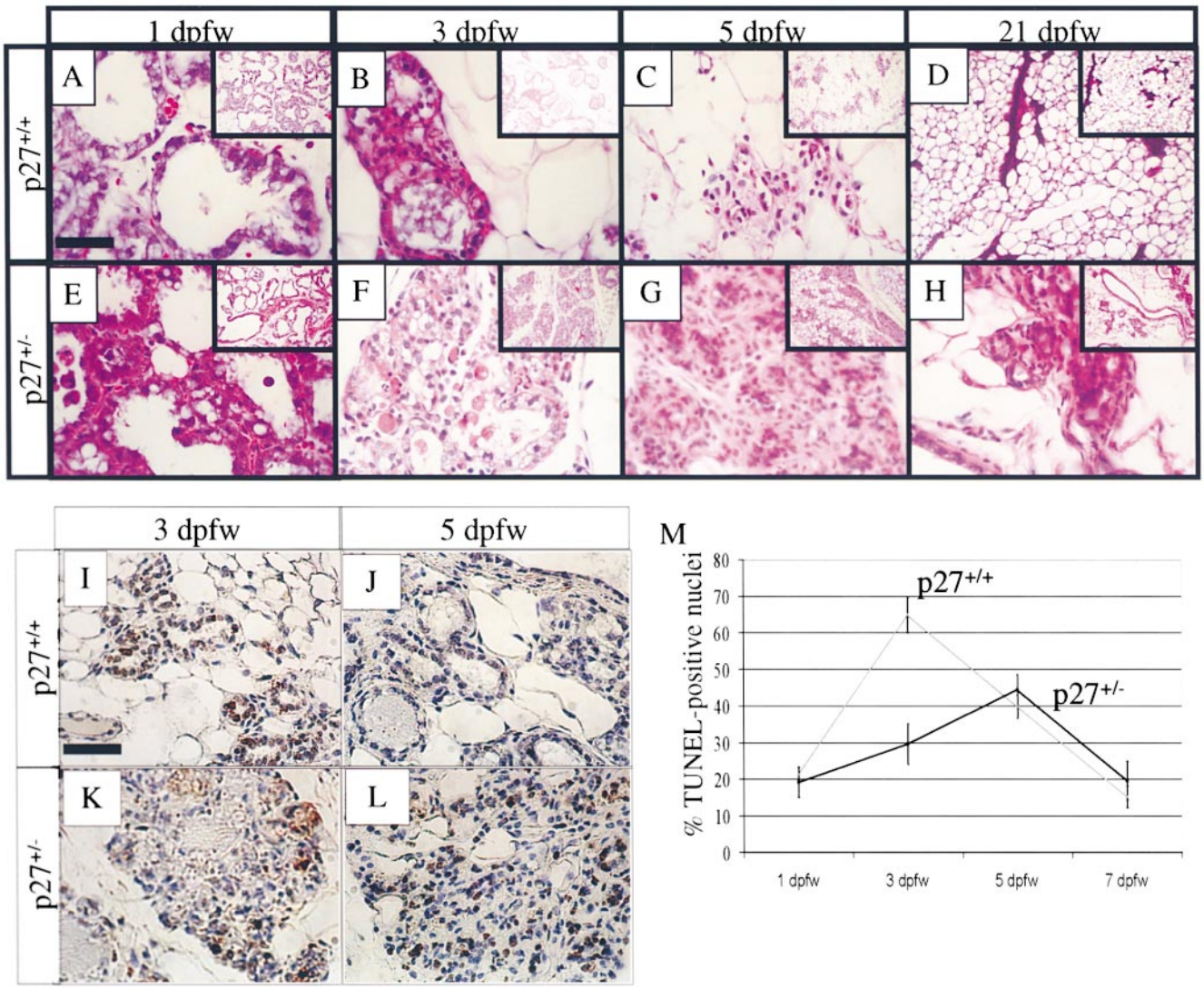


Figure 9. Postlactational involution is delayed in $p27^{+/-}$ mammary glands. (A–H) Hematoxylin and eosin staining of mammary gland sections taken from mice at 1, 3, 5, and 21 dpfw. Inset displays a low magnification of each of the indicated mammary glands shown. (I–L) The level of apoptosis was measured in involuting mammary glands using TUNEL analysis. (M) Quantification of TUNEL-positive nuclei per total number of nuclei in 10 randomly chosen 400× fields per each of six mice per genotype. Quantification of TUNEL-positive nuclei was performed as described in Materials and Methods at 1, 3, 5, and 7 dpfw. Bars, 25 μm.

phosphorylation by cyclin E–Cdk2 are sufficient to initiate a reduced rate of G_1 progression. Alternatively, other cyclin D isoforms (such as cyclin D2 and cyclin D3) may be able to interact with Cdks to initiate Rb phosphorylation in $p27^{-/-}$ mammary glands. Many cell types express combinations of D-type cyclins, such as D1–D2 or D2–D3 (Tam et al., 1994). However, we did not detect cyclin D2 in virgin glands from all three mouse genotypes (data not shown). This is consistent with previous studies demonstrating that cyclin D1 is the predominant D-type cyclin within the mammary gland. During periods of increased proliferation such as pregnancy, *cyclin D2* and *cyclin D3* mRNAs were not detected by in situ hybridization in the mouse mammary gland, whereas levels of *cyclin D1* mRNA were dramatically elevated (Sicinski and Wienberg, 1997). This observation highlights the dependence of the mammary epithelium on cyclin D1 to undergo rapid proliferation and suggests that Rb phosphorylation by cyclin D2– or D3–containing complexes in the mammary gland is unlikely. However, these possibilities would require further investigation.

The effects of p27 deficiency became most pronounced during pregnancy. Cyclin D1–deficient mammary glands also suffer from proliferative defects that are most pronounced during pregnancy (Sicinski and Weinberg, 1997). Like cyclin D1 deficiency, loss of p27 results in impaired lobuloalveolar development and production of lactational proteins, suggesting that $p27^{-/-}$ mammary glands would be unable to support lactation. Strikingly similar defects were observed in mice that are deficient for the genes encoding the prolactin receptor or STAT5a (Henninghausen et al., 1997; Ormandy et al., 1997). It is interesting to speculate that the prolactin receptor, which operates through the JAK/STAT signaling pathway, may ultimately increase expression of cyclin D1 to initiate rapid proliferation and differentiation of the mammary epithelium during preg-

nancy. If cyclin D1, prolactin receptor, and Stat5a are involved in this linear pathway that controls growth and differentiation, then dependence of cyclin D1 on p27 would explain the similarity of the $p27^{-/-}$ mammary gland to those that are devoid of either cyclin D1, prolactin receptor, or STAT5a.

Although we present evidence that the mammary glands of p27-deficient mice are severely hypoplastic, many other organs of p27-deficient mice are hyperplastic, containing an increased number of cells. These include liver, retina, gonads, and pituitary (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). This observation suggests that different organs respond to the loss of p27 in different manners. This speculation is supported by a recent report of $p27^{+/-}$ and $p27^{-/-}$ mice exhibiting increased susceptibility to chemical- or radiation-induced tumor formation in lungs, stomach, and colon, whereas mammary tumors were not reported in $p27^{-/-}$ mice (Fero et al., 1998). The observations presented herein that p27 deficiency in the mammary epithelium results in the same phenotype as the cyclin D1–deficient mammary epithelium is in direct contrast to a recent report in which p27 deficiency can actually correct the retinal hypoplasia of cyclin D1–deficient animals (Tong and Pollard, 2001). The correction of the $cyl^{-/-}$ retinal phenotype by loss of p27 suggests that p27 may function antagonistically to cyclin D1–cdk activity within the retina. These data taken with our observations presented herein demonstrate the tissue specificity of p27 function and confirm that p27 can indeed perform dual roles. Mammary hypoplasia due to p27 deficiency may be related to the unique dependence of the mammary epithelium on cyclin D1 to initiate rapid proliferation, such as during puberty and pregnancy. Since p27 is required for cyclin D1–Cdk4 complex formation and function, the mammary gland may also have a specific requirement for p27, whereas other epithelia may be able to compensate for the loss of p27 and/or the loss of cyclin D1–Cdk4 function.

The inability of $p27^{-/-}$ mammary epithelial cells to form lobuloalveolar structures and lactate (Figs. 8 and 9) suggests that p27 plays a direct role in differentiation of the mammary gland. There is precedent evidence regarding the role of p27 in differentiation; for example, the complete loss of p27 prevents terminal differentiation of the ovarian corpus luteum, oligodendrocytes, and skin keratinocytes (Robker and Richards, 1998; Tong et al., 1998). On the other hand, this impaired differentiation may be secondary to the proliferative defect due to loss of p27. For example, a critical level of mammary epithelial cells may be required to allow terminal differentiation to proceed, or perhaps any given mammary cell must divide a minimum number of times before the onset of differentiation. These speculative hypotheses require further investigation.

The data presented herein support the notion that p27 levels regulate proliferation within the mammary epithelium to the extent that a reduction of p27 produces the opposite effect on proliferation compared with the complete loss of p27. This apparently paradoxical observation is perhaps expected given the recently identified role of p27 in cyclin D1–Cdk4 stabilization and function (Cheng et al., 1999). These mechanisms by which p27 regulates proliferation, differentiation, and function of the mammary epithelium are proposed as follows. p27 protein encoded by two functional *p27* alleles ($p27^{+/+}$) allows for stabilization of cyclin D1–Cdk4 complexes with abundant p27 remaining for association with and inhibition of cyclin E–Cdk2 complexes for the normal control of G_1 progression. Heterozygous inactivation of a single *p27* allele ($p27^{+/-}$) reduces the level of p27. Upon mitogenic signaling, cyclin D1–Cdk4 complexes sequester p27, resulting in a relatively greater reduction of free p27 in $p27^{+/-}$ than in $p27^{+/+}$ cells. Under these conditions, cyclin E–Cdk2 complexes bind in stoichiometric excess with the reduced levels of free p27, a situation in which Cdk2 can phosphorylate p27 and target it for ubiquitin-mediated degradation (Pagano et al., 1995; Sheaff et al., 1997). In this manner, a reduction of p27 (as it occurs in $p27^{+/-}$ cells) is permissive for cyclin E–Cdk2 function, Rb phosphorylation, and an accelerated G_1 to S transition. The delayed postlactational involution noticed in $p27^{+/-}$ glands (and its potential implications for epithelial transformation) remain unexplained. However, the excessive proliferation may indirectly abrogate this process if in fact exit from the cell cycle is required for the epithelium to undergo postlactational apoptosis. In the absence of p27 ($p27^{-/-}$), stable association of cyclin D1 and Cdk4 does not occur, resulting in reduced Rb phosphorylation and cell cycle arrest in early G_1. Whether this delayed proliferation indirectly accounts for the impaired lactational differentiation of these glands will require further research. Nonetheless, a direct requirement of both p27 alleles for the formation of TEBs and lactation cannot be ruled out at this time. The data presented herein support a model for the role of p27 in both activation and inhibition of cell cycle progression and demonstrate a critical role for p27 in regulation of mammary epithelial proliferation, morphogenesis, and function.

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