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Delayed involution of the mammary epithelium in BALB/c-*p53*^{null} mice

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In mammals, weaning of neonates and subsequent milk stasis initiates removal of the secretory epithelium of the mammary gland by apoptosis. The *p53* tumor suppressor gene is induced rapidly following weaning of neonates, but its role in the process of involution has not been defined. Therefore, experiments were performed to identify the cell types in which the *p53* gene is expressed during involution and determine the consequences of its absence in BALB/c-*p53*^{null} mice. Both *p53* mRNA and protein were detected in the mammary epithelium within 48 h following weaning and resulted in an eightfold increase in levels of *p21*^{WAF1} mRNA. Induction of *p21*^{WAF1} mRNA was absent in BALB/c-*p53*^{null} mice, and therefore, was shown to be p53-dependent. The BALB/c-*p53*^{null} mice exhibited delayed involution of the mammary epithelium, as measured by 60% greater epithelial area compared to BALB/c-*p53*^{wt} mice through 5 days post-weaning. The delay was transient with no differences being apparent at 7 days post-weaning. Expression of the stromal protease stromelysin-1 was unaffected by the absence of p53 suggesting that stromal responses were intact. These data demonstrate that p53 participates in the first stage of involution initiated by the epithelium itself, but does not affect the second phase during which stromal proteases are induced.

Keywords: *p53*; mice; mammary involution; *p21*^{WAF1} apoptosis

Introduction

The role of the *p53* tumor suppressor gene in normal tissue development has been overshadowed by its critical role in human cancers (Almog and Rotter, 1997). However, differential expression of *p53* mRNA in embryos (Schmid *et al*., 1991; MacCallum *et al*., 1996) suggested that *p53* participates in development of a variety of tissues. Indeed, inappropriate expression of wild type *p53* transgenes in lens or kidney of mice led to not only increased apoptosis, but failure of differentiation as well (Godley *et al*., 1996; Nakamura *et al*., 1995). Conversely, absence of the *p53* gene resulted in impaired differentiation of neuronal cells *in vitro* (Eizenberg *et al*., 1996). Although embryonic development can occur in the absence of *p53*, embryonic mortality was increased in *p53*^{null} mice due to defects in neural tube closure (Armstrong *et al*., 1995; Sah *et al*., 1995). These data emphasize the importance of precise regulation of *p53* to ensure proper cellular differentiation and development.

Regulation of p53 levels is also important in tissues undergoing development postnatally. Early onset of thymic lymphomas is the major complication observed in *p53*^{null} mice. Impaired differentiation appears to be a significant factor as the lymphomas consist primarily of immature T-cells ($CD4^+/CD8^+$) (Donehower *et al*., 1995). In the prostate, apoptotic cell death following androgen ablation was reduced in *p53*^{null} mice resulting in delayed involution (Colombel *et al*., 1994).

The mammary gland, like the prostate, undergoes synchronous apoptotic cell death upon withdrawal of endocrine stimulation. The *p53* gene was implicated as a physiological regulator of mammary involution based on its rapid induction following weaning in mice (Strange *et al*., 1992). Inappropriate expression of *p53* within the mammary epithelium of transgenic mice caused the alveolar epithelium of the mammary gland to undergo apoptotic cell death demonstrating that the mammary epithelium remains sensitive to *p53*-induced apoptosis even in the presence of continuous hormonal stimulation (Li *et al*., 1994). Despite the correlative data, previous investigators did not detect a difference in involution in mice bearing the *p53*^{null} allele on mixed genetic backgrounds (Li *et al*., 1996). In an effort to reconcile these observations, histological procedures were employed to localize expression of *p53* mRNA and protein within involuting mammary tissues of BALB/c mice. The *p53*^{null} allele was transferred to BALB/c genetic background to determine the consequences of absence of *p53* on involution of the mammary epithelium on a uniform genetic background. The data demonstrate that *p53* is predominantly expressed by the mammary epithelium during early involution. Absence of *p53* resulted in delayed involution in BALB/c-*p53*^{null} mice.

Results

Expression of p53 *and* p21^{WAF1} *mRNAs in mammary tissue*

Removal of pups causes accumulation of milk products within the alveoli of the mammary gland and a decline in levels of lactogenic hormones in blood. These changes coincide with induction of *p53* mRNA within the mammary gland during the first 24–48 h following weaning (Figure 1). The induction was quantitated on separate blots and shown to be a >tenfold induction of *p53* transcripts compared to lactating tissue. The levels

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of *p53* mRNA remained elevated through 7 days post-weaning. The status of the $p21^{WAF1}$ gene, which is p53-responsive, was also determined as an indicator of whether these changes in levels of *p53* mRNA were sufficient to initiate a physiologic response. Expression of $p21^{WAF1}$ mRNA was very low during lactation, but was induced rapidly upon weaning of pups (Figures 1 and 2). The precise timing of initiation of *p53* and $p21^{WAF1}$ expression varied between 24–48 h post-weaning among individual animals despite standardized littersizes and forced-weaning during early lactation. Expression of $p21^{WAF1}$ declined to basal levels beyond day 5 post-weaning despite the continued expression of *p53* transcripts. The induction of $p21^{WAF1}$ mRNA during involution was absent in Balb/c-$p53^{null}$ mice (Figure 2, upper panel). The blot was rehybridized with a *gapdh* probe to compare loading among lanes (Figure 2, lower panel). This demonstrated that induction of $p21^{WAF1}$ in involuting mammary tissue is p53-dependent.

Localization of p53 *and* p21 *mRNAs by* in situ *hybridization*

In situ hybridization was employed to directly assess the contributions of the mammary epithelium and stroma (lymph node, adipocytes and fibroblasts) to the overall level of *p53* mRNA observed by Northern blot analysis.

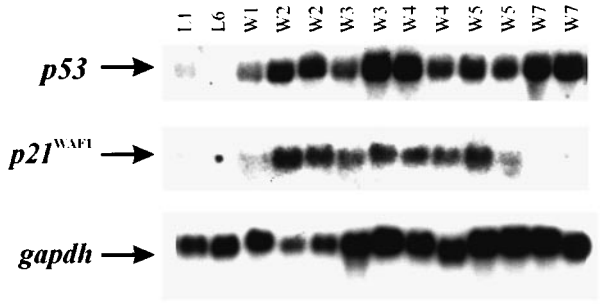

Figure 1 Patterns of *p53* mRNA expression in normal murine mammary glands. Total mammary gland RNA was isolated from BALB/c females during lactation and post-weaning involution. Ten μg of total mammary RNA from mice at days 1 or 6 of lactation (L1, L6) and days 1–7 post-weaning (W1, W2, W3, W4, W5, W7) were analysed by Northern blot. After transfer to nylon membranes, the RNAs were hybridized to ^{32}P-dCTP-labeled probes for *p53*, $p21^{WAF1}$ or *gapdh*

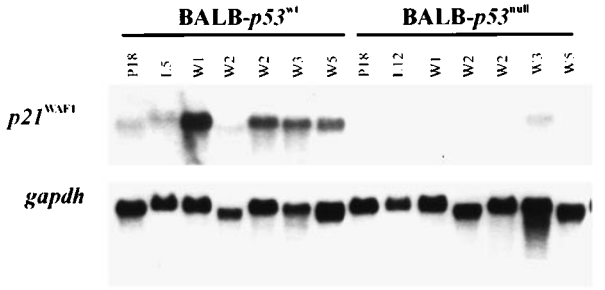

Figure 2 Expression of $p21^{WAF1}$ in BALB/c-$p53^{wt}$ and BALB/c-$p53^{null}$ mice during involution. Total mammary gland RNAs (10 μg/lane) were from mice at 18 days of pregnancy (P18), days 5 or 12 of lactation (L5, L12) and days 1–5 post-weaning (W1, W2, W3, W5). The blot was hybridized sequentially with ^{32}P-dCTP-labeled probes for $p21^{WAF1}$ and *gapdh*

Expression of *p53* mRNA in the mammary epithelium of lactating females was generally below the limit of detection with digoxigenin-labeled cRNA probes. Therefore, these sections were counterstained with fast red to visualize glandular structures (Figure 3a and b). Although infrequent, intense hybridization of the antisense cRNA was observed within isolated alveoli of lactating mammary tissue (Figure 3a). Hybridization of the antisense cRNA probe to cytoplasmic mRNA was detected in some, but not all cells within an alveolus indicating that induction of *p53* transcripts was regulated by local factors. Serial sections containing these cells hybridized with sense cRNA probes did not show any detectable signal confirming the specificity of detection (Figure 3b). In contrast to lactating tissue, *p53* mRNA was detected in the majority of the alveolar epithelial cells within 24 h after removal of pups (Figure 3c); and therefore, the counterstain was omitted in these sections. Sense probes did not hybridize (Figure 3d). Adjacent intramammary lymph nodes were also nearly devoid of hybridization providing an internal negative control. Accumulation of immunoreactive p53 protein was evident in alveolar cells by 2 days post-weaning in BALB/c-$p53^{wt}$ mice (Figure 4a). Some cells with detectable levels of p53 in the nucleus appeared to be undergoing apoptosis. The absence of immunoreactivity in tissues from BALB/c-$p53^{null}$ mice demonstrated the specificity of the staining (Figure 4b). Although both *p53* mRNA and p53 protein were induced in parallel during early weaning, p53 protein was detected in fewer cells.

Expression of $p21^{WAF1}$ mRNA was also localized by *in situ* hybridization. Antisense-cRNA probes labeled with ^{33}P-UTP were used to allow quantitation of the level of transcripts. Expression of $p21^{WAF1}$ was limited to the mammary epithelium. There was an eightfold induction of $p21^{WAF1}$ mRNA levels within the first 24 h after removal of pups (Figure 5) which was statistically significant ($P<0.001$). These data confirmed that expression of both *p53* and $p21^{WAF1}$ mRNAs were specifically induced within the mammary epithelium within 48 h following milk stasis in Balb/c-$p53^{wt}$ mice.

In vivo *involution in BALB/c-*p53^{null} *mice*

The histological appearance of tissues from BALB/c-$p53^{wt}$ and BALB/c-$p53^{null}$ mice were indistinguishable during lactation. However, differences were detected at 2 and 5 days post-weaning. Extensive loss of lobuloalveolar structures was evident at 5 days after forced weaning in tissues from BALB/c-$p53^{wt}$ mice (Figure 6a), whereas enlarged ducts filled with residual secretory products were still present in BALB/c-$p53^{null}$ mice (Figure 6b). Sections shown in Figure 6a and b were stained with pan-keratin antibodies to unequivocally identify the epithelium and counterstained with hematoxylin. The epithelial surface area was quantitated by image analysis of keratin-positive area. (Counterstain was omitted on sections used for image analysis.) The area of mammary epithelium was 60% greater in glands from BALB/c-$p53^{null}$ mice at days 2 and 5 post-weaning compared to the BALB/c-$p53^{wt}$ controls (Figure 6c). These estimates of epithelial area compare well with previous reports based on morphometric analysis of the involuting mammary gland (Walker *et al*., 1989). Although this method may

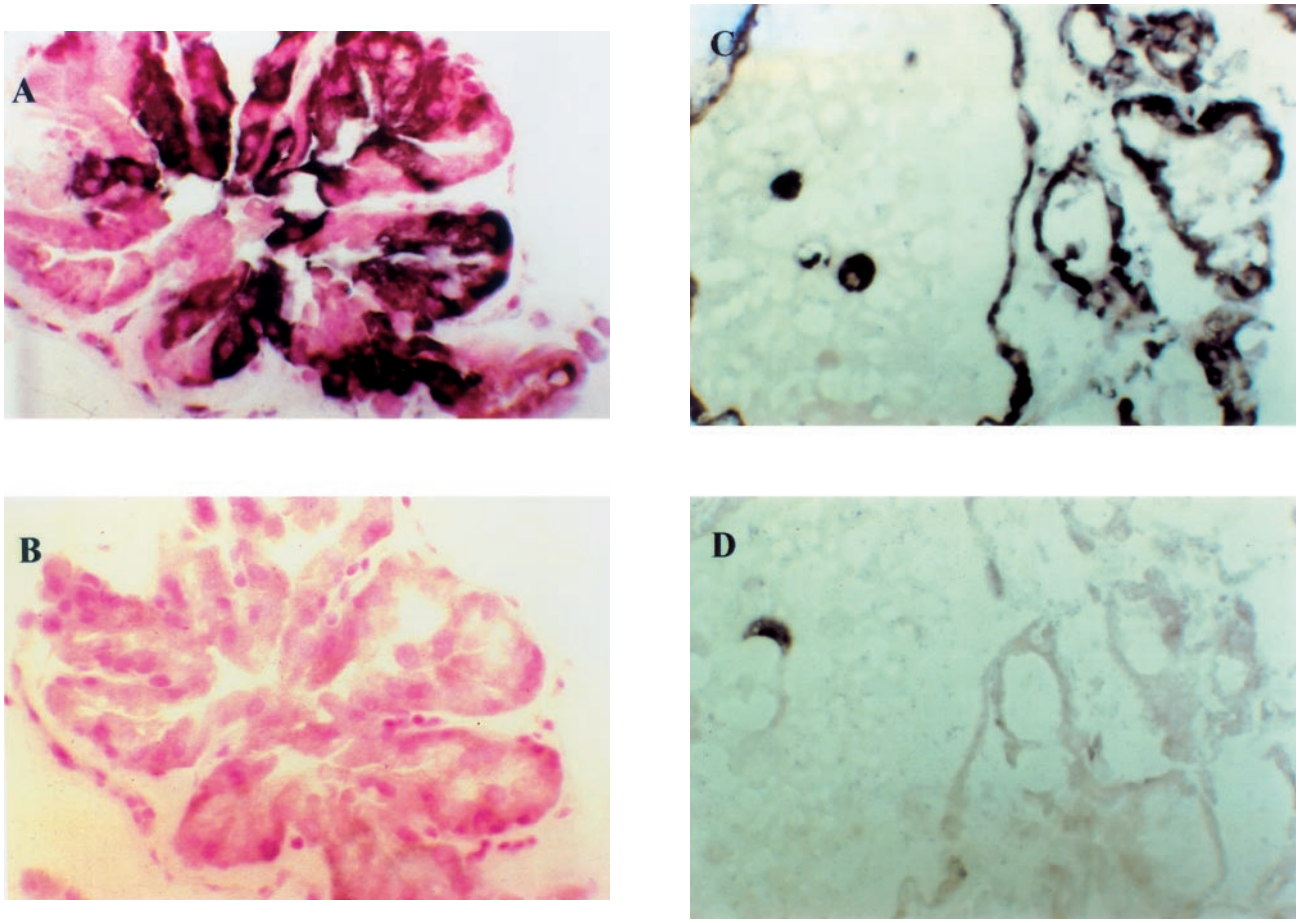

Figure 3 Localization of *p53* mRNA by *in situ* hybridization of frozen sections (6 μm thick) with digoxigenin-labeled cRNA probes. (**a**) Antisense cRNA hybridization was limited to focal areas in lactating mammary tissue indicated by dark purple staining. Light red cytoplasm and nuclei are from Fast Red counterstain. (**b**) The sense cRNA probe was hybridized to an adjacent section and counterstained with Fast Red. Note the absence of hybridization in the cells detected by the antisense probe. (**c**) Intense hybridization of antisense cRNA was observed 24 h after removal of pups. Counterstain was omitted resulting in a purple color. The hybridization was cytoplasmic demonstrating that the reactivity is specific for cytoplasmic mRNA. (**d**) Minimal reactivity was observed for sense probes at W1. Magnifications were 400× for panels (**a**) and (**b**) and 200× for panels (**c**) and (**d**)

underestimate the actual difference because the large distended lumens of alveoli in tissues from BALB/c-$p53^{null}$ mice tended to dilute the area occupied by epithelium per field, the effect of *p53* genotype was statistically significant ($P<0.001$). The histological appearance of the BALB/c-$p53^{null}$ tissues at 5 days post-weaning approximated that of 2 days post-weaning in BALB/c-$p53^{wt}$ controls. Comparison of the area of epithelium for mammary glands from BALB/c-$p53^{wt}$ at 2 days post-weaning and BALB/c-$p53^{null}$ at 5 days post-weaning (Figure 6c) emphasized this similarity that was noted histologically.

Despite the differences in remodeling during early involution, milk protein gene expression was extinguished with normal kinetics in the BALB/c-$p53^{null}$ tissues (Figure 7). The majority of *α-lactalbumin* mRNA was absent from tissues of both genotypes within 48 h post-weaning. The results for expression of *β-casein* mRNA were identical (data not shown). *Stromelysin-1* expression was also induced normally at 5 days post-weaning indicating that stromal responses were intact in involuting mammary glands of BALB/c-$p53^{null}$ mice. Therefore, the major difference observed in BALB/c-$p53^{null}$ mice was a delay in the initial collapse of the alveoli during involution.

Discussion

Rapid induction of *p53* mRNA during involution on the mammary gland was observed in association with apoptotic cell death providing a plausible mechanism by which involution may be regulated (Strange *et al*., 1992). Although levels of *p53* mRNA remain elevated for at least 7 days post-weaning, functional responses were transient. Induction of $p21^{WAF1}$ mRNA was detected by Northern blot only during 1–5 days post-weaning (Figures 1 and 2). The reason for absence of functional responses to *p53* beyond 5 days post-weaning is not clear, but may be attributed to the presence of functional antagonists of p53 (e.g. *mdm2, mdmX*) which are expressed in involuting mammary tissues (DJJ, unpublished observations). This may also represent a shift from primarily alveolar cells during early weaning to primarily ductal cells at later stages. Ductal cells, but not alveolar cells, have been shown to express elevated levels of Bcl-2 (Pullan *et al*., 1996) which may allow ductal cells to tolerate elevated levels of p53. The transient induction of $p21^{WAF1}$ mRNA proved to be a very sensitive indicator of p53 function as only basal levels of $p21^{WAF1}$ mRNA were observed in mammary tissue from BALB/c-$p53^{null}$ mice (Figure 2).

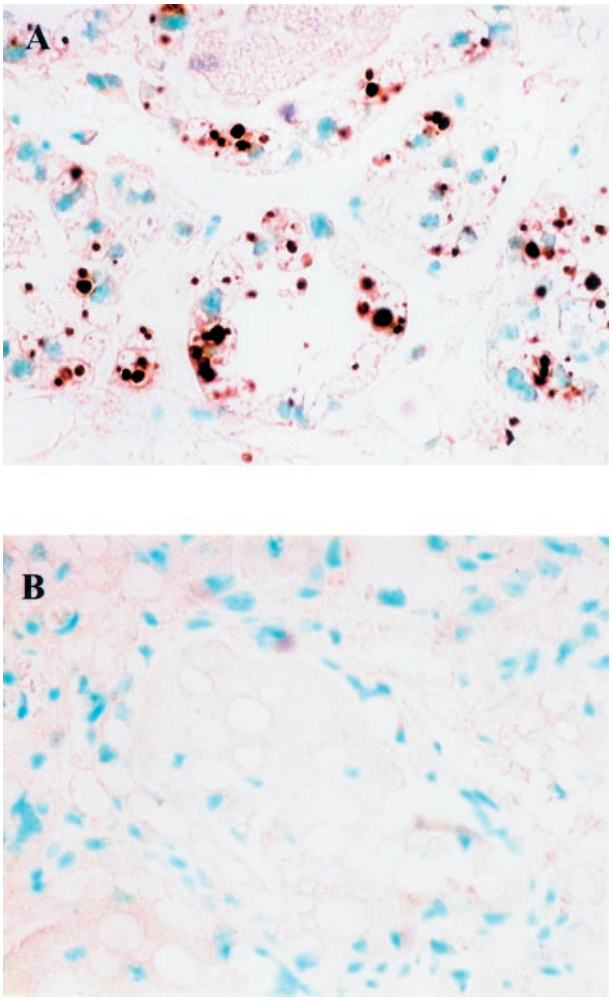

Figure 4 Dectection of p53 protein by immunohistochemical staining in involuting mammary tissue. (**a**) Nuclear p53 staining (brown-staining) was detected in epithelial cells throughout the sections on day 2 post-weaning. Morphological appearance of cells often indicated that cells were undergoing apoptosis. Staining was also present in endothelial and some stromal cells. (**b**) Immunoreactivity was absent in tissue from BALB/c-$p53^{null}$ mice including cells within the lumen with apoptotic nuclei. Sections were counter-stained with methyl green to visualize morphology in both panels (**a**) and (**b**): Magnification was 400×

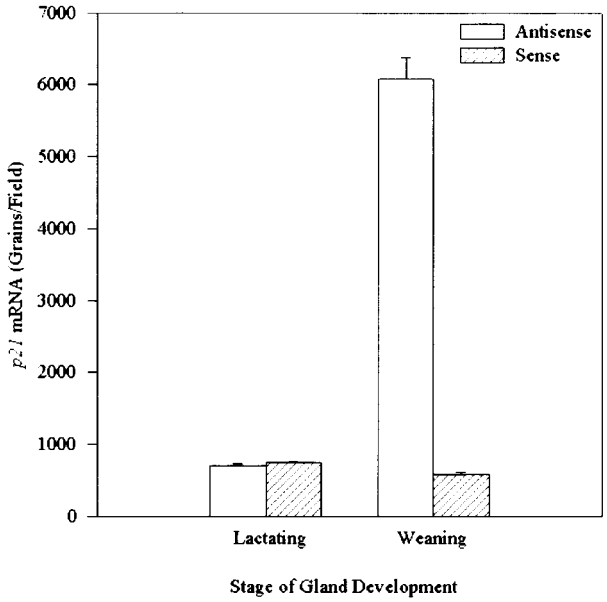

Figure 5 Quantitation of $p21^{WAF1}$ mRNA by *in situ* hybridization with ^{33}P-labeled cRNA probes. Hybridization of the antisense probes was localized to the secretory epithelium in day 1 post-weaning mammary tissue. The level of induction was quantitated by counting grains of emulsion per field to obtain an average of 10 fields per tissue for each of three mice at 21 days of lactation (L21) and four mice at 1 day post-weaning (W1): Eightfold induction of $p21^{WAF1}$ mRNA was detected by the antisense probe at W1 ($P<.05$), whereas there was no difference between hybridization of sense and antisense probes at L21

Therefore, the major effects of p53 would be expected to be evident during the period of 1–5 days post-weaning.

The mammary gland is composed of epithelial cells as well as multiple cell types which make up the stroma. Therefore, *p53* mRNA and protein were localized by histological procedures to ensure that epithelial cells were the source of the differences in expression. As expected, both *p53* mRNA and protein were most prevalent within the epithelium during early involution. However, the appearance of p53 protein was much more heterogeneous than was the expression of *p53* mRNA. Likewise, induction of $p21^{WAF1}$ mRNA tended to be more heterogeneous resulting in asynchronous expression among alveolar cells. The overall responses were a >tenfold increase in *p53* mRNA and a concomitant eightfold induction of $p21^{WAF1}$ mRNA. Thus, levels of p53 protein were sufficient to elicit a biological response.

The anticipated consequence of p53 induction would be apoptotic cell death based on previous reports (Li *et al*., 1994). Indeed, cells with detectable p53 protein often appeared apoptotic. Qualitative differences in apoptosis were observed between BALB/c-$p53^{wt}$ and BALB/c-$p53^{null}$ mice when analysed by the TUNEL method (data not shown, DJJ). However, this was a relatively insensitive measure due to asynchronous induction and rapid removal of apoptotic cells (Quarrie *et al*., 1995). Maximal levels of apoptosis in involuting mammary tissue are only 4–5% (Li *et al*., 1994), yet >80% of the epithelial area is lost during involution (Munford, 1963; Walker *et al*., 1989). Therefore, epithelial area was chosen as a more robust means to quantitate responses. It allows analysis of differences in cell death as well as changes due to remodeling.

The absence of *p53* resulted in delayed dismantling of the alveoli during early involution of the mammary gland. The effect on morphology was most striking because integrity of the alveoli was maintained for up to 5 days post-weaning. This period coincides with the period during which p53 would be expected to be active based on the induction of $p21^{WAF1}$. The delay in involution was transient with no difference detected at 7 days post-weaning (Figure 6c). These data are in agreement with that of Feng *et al*. (1995), but appear to contradict reports by Li *et al*. (1996). The apparent discrepancy is most likely attributable to the use of mice bearing the $p53^{null}$ allele on C57BL/6×129/Sv genetic backgrounds, the transient effect of *p53* and differences in stage of lactation when involution was initiated (Li *et al*., 1996, 1997). Genetic heterogeneity would be expected to increase variability which could obscure the transient effect of *p53*. Preliminary observations (DJJ) revealed that involution progresses

more rapidly in C57BL/6 × 129/Sv mice making selection of time points more critical. It was for these reasons that the *p53*^{null} allele was backcrossed onto the BALB/c genetic background where involution is more

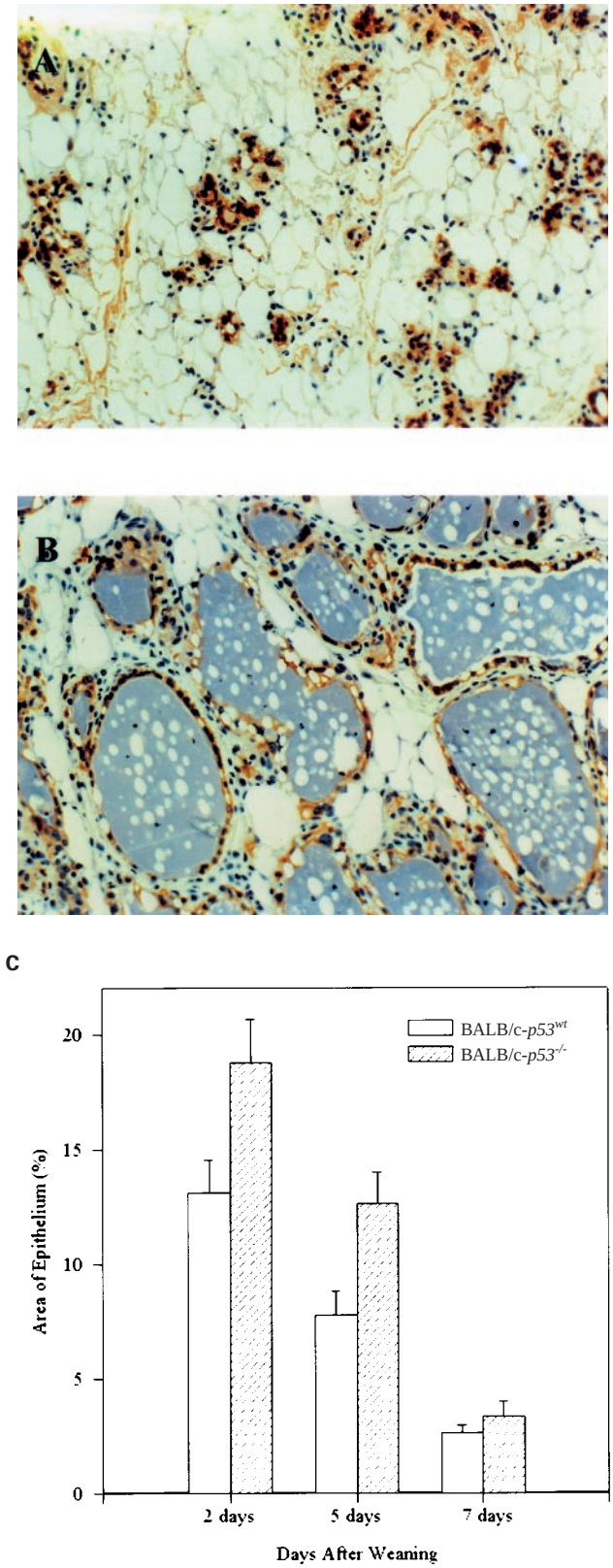

Figure 6 The effect of absence of *p53* on morphological changes during involution. Epithelial cells were unequivocally identified immunohistochemical detection of keratins (brown staining). The sections were counter-stained with hematoxylin to visualize nuclei. Extensive disruption of alveoli and introgression of the stroma was observed in BALB/c-*p53*^{wt} tissues (**a**); whereas integrity of alveoli was largely maintained in BALB/cp-*p53*^{null} tissues at 5 days post-weaning. Magnification for panels **a** and **b** was 200×. (**c**) Image analysis was performed on keratin-stained sections to quantitate the amount of epithelium present at 2, 5 and 7 days post-weaning for each of the genotypes. Delayed involution was evident at 2 and 5 days post-weaning, but not at 7 days. Counterstain was omitted on sections used for image analysis

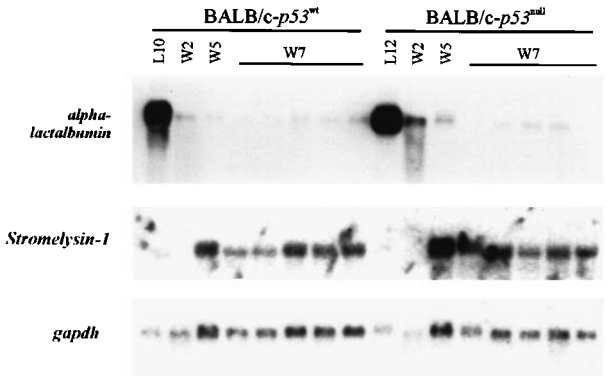

Figure 7 Expression of markers of differentiation and involution in tissues from BALB/c-$p53^{wt}$ and BALB/c-$p53^{null}$ mice. Upper panel: Expression of *α-lactalbumin* mRNA was extinguished with similar kinetics in both genotypes despite differences in alveolar structures. Middle panel: Induction of *stromelysin-1* mRNA was normal in both genotypes as well. Lower panel: Expression of *gapdh* mRNA was used as an internal hybridization control

protracted and has been extensively characterized (Strange *et al*., 1992; Atwood *et al*., 1995; Lund *et al*., 1996). Our data also differ in that mice were force-weaned at peak lactation as is typical for studies of involution. Mice in later lactation were utilized in the previous report (Li *et al*., 1996); a time when involution is taking place due to the decreased demands of neonates as they begin to eat solid food.

Although apoptosis appeared to be decreased in the involuting glands from BALB/c-$p53^{null}$ mice, apoptosis was nonetheless present (data not shown, DJJ). Therefore, it is possible that *p53* is important for apoptosis of only a subpopulation of cells that are responsible for maintaining alveolar architecture. The myoepithelial cells, which form a barrier between the stroma and the luminal epithelial cells, may be the critical population that dies in response to *p53* resulting in rapid collapse of the alveoli. Extended survival of this subpopulation of cells may allow the lobular morphology to be maintained despite apparent apoptotic cell death of the more prevalent luminal epithelium. Therefore, it will be important to discriminate the effects of p53 on these populations of cells in future analyses.

These data support and refine the biphasic model of involution proposed by Lund *et al*. (1996). In this model, the initial phase of involution is characterized by collapse of secretory alveolar structures, then apoptosis of the secretory luminal epithelial cells and myoepithelium. This is followed by a secondary phase in which stromal proteases degrade the basement membrane and adipose tissue replaces the epithelial cells. In BALB/c-$p53^{null}$ mice, it appears that the initial phase, in which the alveoli are dismantled, is largely abrogated. Despite the presence of intact alveoli, signals for induction of stromal proteases persist as expression of *stromelysin-1* was induced normally at 5 days post-weaning in BALB/c-$p53^{null}$ mice (Figure 7). Erosion of the basement membrane by stromal proteases would induce apoptosis in mammary epithelial cells by a p53-independent pathway (Witty *et al*., 1995; Sympson *et al*., 1994; Boudreau *et al*., 1995, 1996) and recovery of involution would be anticipated by 7 days post-weaning. Therefore, abrogation of the initial phase of involution in BALB/c-$p53^{null}$ mice is supplanted by compensatory loss of luminal epithelial cells induced by the surrounding stromal cells. It has been suggested that incomplete involution of the mammary epithelium during the post-weaning period may render breast tissue susceptible to tumor development (Henson and Tarone, 1994), and therefore, the completeness of the compensatory mechanism may be crucial in determining susceptibility to subsequent tumor development.

Materials and methods

Mice and tissues

Mice bearing the *p53*-null allele on a mixed genetic background (C57BL/6 × 129/Sv) (Jacks *et al*., 1994) were obtained from the laboratory of Tyler Jacks (MIT, Boston, MA, USA) and were maintained at the University of Massachusetts. The genotypes of individuals were determined by multiplex PCR as described by Jacks (Jacks *et al*., 1994). The $p53^{null}$ allele from these mice was back-crossed for at least five generations onto the BALB/cMed strain (referred to as BALB-$p53^{null}$ mice). Females were mated to obtain mammary tissue from pregnant, lactating and involuting glands. Involuting glands were obtained from females that were force-weaned after 5–10 days lactation. Littersizes were standardized at 4–6 pups for these studies.

Northern blot hybridization

Total RNA was extracted from mammary glands by acid guanidine-thiocyanate-phenol-chloroform procedures (RNazolB or Ultraspec; Biotecx, Houston, TX, USA). Typically 10 μg of total RNA/lane were separated on 1.2% denaturing agarose gels in MOPS-formaldehyde buffer. RNA was transferred to nylon membranes (Zetabind; CUNO, Meriden, CT) by capillary action and cross-linked to the membrane with UV light. Membranes were washed in 2 × SSC at room temperature, then 0.1 × SSC, 0.5% SDS for 30 min at 65°C to remove residual Coomassie blue dye from the loading buffer. Membranes were prehybridized at 45°C for at least 4 h in buffer containing 50% formamide as described by Derman *et al*. (1981). Membranes were hybridized with ^{32}P-labeled cDNA probes in the 50% formamide buffer overnight at 45°C, then washed sequentially in 2 × SSC/0.2% SDS at room temperature and 0.1 × SSC/0.2% SDS for 1 h at 55–60°C. The membranes were exposed to BioMax X-ray film (Kodak) for up to 3 days at −70°C, then developed. In some cases, the signal was quantitated using a phosphorimager (Molecular Dynamics).

Preparation of probes

The mouse *p53* cDNA was excised from the pLR10 expression plasmid provided by G Lozano (MD Anderson Cancer Center, Houston, TX, USA). The coding regions of cDNAs for mouse $p21^{WAF1}$ (El-Deiry *et al*., 1993), *α-lactalbumin* (Clone p450; Lothar Hennighausen), stromelysin-1 (Zena Werb), and rat *gapdh*(Fort *et al*., 1985) were isolated from plasmids and used as probes. The complete coding region of *β-casein* mRNA was amplified by RT–PCR, then cloned into pBluescript. Probes for Northern blotting were purified by gel electrophoresis, then labeled with ^{32}P-dCTP using random primed labeling method (Feinberg and Vogelstein, 1983).

Single-stranded cRNA probes for detection of *p53* by *in situ* hybridization were labeled with UTP-digoxigenin

according to the manufacturer's specifications (Boehringer-Mannheim, Indianapolis, IN, USA). Probes for detection of $p21^{WAF1}$ mRNA by *in situ* hybridization were prepared in a similar fashion, except 1.5 μg of linearized template was labeled with 120 μCi of ^{33}P-UTP (NEN/Dupont).

In situ *hybridization*

Preliminary work showed that frozen sections (6 μm thick) produced the most sensitive detection. Slides were allowed to come to room temperature, then equilibrated in 2 × SSC for 2 min. The tissues were treated with acetic anhydride solution (125 μl acetic anhydride in 50 ml of 0.1 M TEA-HCl, 0.9% NaCl buffer) for 10 min, rinsed in 2 × SSC, then dehydrated through graded ethanols. Lipids were extracted in chloroform for 5 min, then rehydrated to 70% ethanol. Hybridization solution (10% dextran sulfate, w/v; 0.5 mg/ml tRNA; 4 mg/ml salmon sperm DNA; 0.5 mg/ml heparin; 1 × Denhardt's; 4 × SSC; 50% formamide) was prepared in advance, and stored frozen. Just prior to use, 1 μl of 5 M DTT and the appropriate cRNA probe were added.

Expression of *p53* was determined *in situ* by addition of the digoxigenin-labeled cRNA to a concentration of approximately 10 ng/ml of hybridization buffer and allowed to hybridize at 50°C in a humidified chamber for 14 h. The coverslips were removed and tissues were washed in 4 × SSC. Nonspecifically hybridized probe was removed by treatment with RNase (Boehringer Mannheim; 20 μg/ml RNase in 0.5 M NaCl; 1 mM EDTA; 10 mM Tris-HCl, pH 8.0) for 30 min at 37°C. The tissues were washed sequentially in 2 × SSC, 1 × SSC and 0.5 × SSC containing 10 mM DTT for 15 min at room temperature. After a final stringent wash in 0.1 × SSC; 10 mM DTT at 55°C for 30 min, the tissues were equilibrated in 2 × SSC. The tissues were incubated in 2% BSA; 2 × SSC; 0.05% Triton X-100 for 1.5 h at room temperature prior to addition of anti-digoxigenin antibody-alkaline phosphatase conjugate (Boehringer-Mannheim) diluted 1 : 250 in Antibody Buffer (50 mM Tris-HCl, pH 7.5; 0.1 M NaCl; 02% Triton X-100). The sections were incubated with the antibodies for 14 h at 4°C. Excess antibody was removed by washing in Alkaline Phosphatase Buffer (0.25 M Tris-HCl, pH 9.5; 0.1 M NaCl; 0.05 M $MgCl_2$). Color substrate was then diluted in the Alkaline Phosphatase Buffer (22.5 μl NBT; 17.5 μl X-phosphate; 1.2 g levamisole in 5 ml buffer) and added to the slides. After 3 h at room temperature, the reactions were terminated. Tissues were mounted with Aqua-Polymount (Polysciences). In cases where there was minimal reactivity, tissues were counterstained with Fast Red prior to cover-slipping to visualize the tissue architecture.

Hybridization of the $p21^{WAF1}$ cRNA probes differed in that the buffer contained 2 × SSC (10% dextran sulfate, w/v; 0.5 mg/ml tRNA; 4 mg/ml salmon sperm DNA; 0.5 mg/ml heparin; 1 × Denhardt's; 2 × SSC; 50% formamide) and were incubated at 55°C. After hybridization for 14 h, the tissues were washed sequentially in 1 × SSC at room temperature, 50% formamide in 2 × SSC at 52°C, followed by RNase digestion (100 μg/ml in 0.5 M NaCl; 1 mM EDTA; 10 mM Tris-HCl, pH 8.0) for 30 min at 37°C. The tissues were washed again in 50% formamide in 2 × SSC at 52°C, then dehydrated in graded alcohols diluted in 0.1 × SSC. The slides were dipped in emulsion, exposed for 2 weeks at 4°C, developed, and then counterstained in toluidene blue. Levels of expression were quantitated by counting grains in five fields on each of two replicate slides using software provided by Bioquant (R and M Biometrics, Nashville, TN, USA) using procedures described by Petersen *et al*. (1996).

Immunohistochemistry

Paraffin-embedded sections (5 μm thick) were deparaffinized in xylenes followed by rehydration in graded alcohols to phosphate-buffered saline (PBS). The tissues were subjected to either antigen retrieval (MacCallum *et al*., 1996) or digestion with 0.1% trypsin to expose antigens for detection of p53 protein and keratins, respectively. Nonspecific binding was blocked in 5% Maleate Buffer, Magic Blocker™ (Boehringer Mannheim) with 0.75% Triton X-100 followed by Avidin Blocker (Vector Laboratories). The tissues were incubated 14 h at 4°C with primary antibodies specific for p53 protein (CM5 diluted 1 : 200, Novacastra, UK) or cytokeratins (obtained from Biogenex, diluted 1 : 50 or Dako, diluted 1 : 500) in 2% Maleate Buffer, Magic Blocker without Triton X-100. The complexes were detected by the ABC method using Vectastain Elite reagents (Vector Laboratories) and diaminobenzidene color substrate as suggested by the manufacturer. Sections used for detection of p53 protein were counterstained with methyl green dehydrated through alcohols and xylenes, then mounted with Permount. Sections used for detection of keratins were treated similary except that a hematoxylin counterstain was used. Image analysis was performed on tissues that were not counterstained. The area of epithelium was quantitated in at least three fields per section using NIH Image software. The procedures were detailed by Petersen *et al*. (1996). Two-way ANOVA was performed with genotype and stage of development as independent factors (JMP-IN, SAS Institute, Cary, NC, USA).

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