Mutations in Apc and p53 Synergize to Promote Mammary Neoplasia

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Abstract

Mutations of *Apc* and *p53* have both been implicated in human and murine mammary neoplasia. To investigate potential interactions between Apc and p53, we conditionally inactivated Apc in both the presence and the absence of functional p53. Apc deficiency on its own leads to the development of metaplasia but not neoplasia. We show here that these areas of metaplasia are characterized by elevated levels of both p53 and p21. In the additional absence of p53, there is rapid progression to neoplasia, with 44.4% of lymphoma-free mice developing a mammary tumor with earliest observed onset at pregnancy. To investigate the mechanism by which p53 deficiency accelerates neoplasia, we used the Rosa26R reporter strain as a marker of Cre-mediated recombination and show a role for p53 in the loss of Apc-deficient cells. This role seems limited to pregnancy and subsequent time points. We therefore show clear synergy between these two mutations in mammary gland neoplasia and present data to suggest that at least one mechanism for this acceleration is the p53-dependent loss of Apc-deficient cells. (Cancer Res 2005; 65(2): 410-6)

Introduction

Apc is expressed at high levels within the mammary epithelium. Loss of heterozygosity at 5q21 (the chromosomal location of human *APC*) was reported in sporadic tumors of the breast (1, 2), and recently, lost or reduced APC protein expression has been shown in human breast cancers (3, 4). Evidence for an association with human disease has also come from studies of the *APC* I1307K polymorphism, which has been shown to increase the risk of breast cancer in association with *BRCA* founder mutations (5). Perhaps most directly, Furuuchi et al. (6) reported the presence of somatic *APC* mutations in 18% of primary breast cancers, and Jin et al. (7) have shown the Apc promoter to be frequently hypermethylated in breast cancers. Within the mouse, there is clear evidence of a tumor suppressor role for Apc within the mammary epithelium, as mice heterozygous for the multiple intestinal neoplasia (Min) mutation in *Apc* develop mammary tumors (8). Furthermore, conditional inactivation of *Apc* in the mammary gland using Cre-loxP technology shows Apc deficiency to perturb development and lead to metaplasia, which in the additional absence of Tcf-1 resulted directly in acanthoma formation (9). It is therefore clear that mammary tumorigenesis in both mouse and human can be associated with mutation of Apc most likely as a consequence of increased β-catenin stability.

Elevated levels of β-catenin have been associated with poor prognosis in human adenocarcinomas of the breast (10). In the mouse, expression of an activated form of β-catenin driven by the mouse mammary tumor virus-long terminal repeat leads to both mammary gland hyperplasia and mammary adenocarcinoma (11). Similarly, expression of a transcriptionally active form of β-catenin lacking the NH_2-terminal 89 amino acids (ΔN89β-catenin) results in precocious development, differentiation, and neoplasia in both male and female murine mammary glands (12). Contrasting results have been obtained using a stabilized form of β-catenin produced after Cre-mediated excision of exon 3, which induces transdifferentiation into epidermis and squamous metaplasia of the mammary epithelium but fails to induce neoplasia (13). Together, these results indicate a key role for β-catenin in mammary gland physiology but show that expression of activated forms of β-catenin can produce different phenotypes probably as a consequence of different levels of expression (13).

The importance of p53 in breast epithelium is evident from its role in the mammary gland lactation cycle (14), the high frequency of mutations and altered expression of *p53* gene in breast tumors (15, 16), and the strong predisposition to mammary neoplasia associated with Li-Fraumeni syndrome. Recent data indicate that deregulated β-catenin not only drives processes that promote cancer but also leads to p53 activation (17). p53 also influences the Wnt signaling pathway, as it has been shown to down-regulate β-catenin (18). This raises the possibility that p53 deficiency may directly predispose to malignancy by deregulation of β-catenin.

Given the association between mutations in Apc and p53 with mammary neoplasia and the evidence for a feedback loop between p53 and β-catenin, we inactivated Apc specifically within the mammary gland on both wild-type and p53-deficient backgrounds. We show that in the absence of p53 there is rapid progression to neoplasia.

Materials and Methods

Mammary Gland Specific Inactivation of Apc. Mice homozygous for an *Apc* allele in which exon 14 are loxP flanked (termed Apc^{fl}) develop normally (19). Cre-mediated recombination of exon 14 leads to a frameshift mutation at codon 580. To examine the role of Apc in mammary gland tumorigenesis, we used a transgenic approach where Cre is under the control of the ovine β-lactoglobulin enhancer (*BLG-Cre*$^+$; ref. 20).

Genotyping of Mice. Detection of *Apc* and *p53* alleles was carried out by PCR as described previously (19, 21). The BLG-Cre transgene was detected as described previously (20). Outbred mice were used that were segregating for the Ola129/C3H and C57BL6J genomes. However, all mice were homozygous at the *Mom-1* locus for the *C3H* allele.

Mammary Gland Whole Mount. This procedure was carried out as described on the mammary gland Web site (http://mammary.nih.gov).

Tissue Sections and Immunohistochemistry. Sections (5 μm) were cut from paraffin-embedded tissue retrieved at dissection. Animals were injected with bromodeoxyuridine (70 mg/kg i.p. in sterile saline) 2 hours

Note: Supplementary data for this are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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before killing. Detection of bromodeoxyuridine incorporation was by rat antibody MCA2060 (1:100) from Serotec (Oxford, UK). Antigen retrieval was by 1 mol/L HCl, 10 minutes at 60°C. The antigen retrieval for c-*myc* and β-catenin was 30 to 50 minutes in a Tris-EDTA solution (pH 8) at 100°C. β-catenin was detected using a mouse monoclonal antibody supplied by Transduction Laboratories (San Diego, CA) used at 1:50 dilution and c-*myc* with a rabbit antibody (1:50 dilution) supplied by Upstate (Lake Placid, NY). P21 was detected by a Santa Cruz Biotechnology (Santa Cruz, CA) rabbit polyclonal antibody (M-19) diluted in 1:500, and antigen retrieval was 20 minutes in 10 mmol/L sodium citrate (pH 6.0). Cyclin D1 was detected with a mouse monoclonal antibody DCS-6 at a dilution 1:100 supplied by Novacastra (Newcastle upon Tyne, UK), and antigen retrieval was done using a high pH target retrieval solution from DAKO (Carpinteria, CA) at 99°C for 30 minutes. CD44 was detected by a rat antibody used at 1:50 supplied by BD PharMingen (San Diego, CA) using a 10 mmol/L sodium citrate (pH 6) buffer brought to the boil and then left to cool down for the antigen retrieval step. Finally, p53 was detected by a mouse monoclonal supplied by Labvision (Fremont, CA) (antibody p53 Ab-1) diluted at 1:50 and used with an EDTA buffer for antigen retrieval from Labvision at 99°C for 20 minutes.

LacZ Staining. The protocol was as described in Gallagher et al. (9) from Brisken et al. (22).

Results and Discussion

p53 and p21 Are Up-regulated in Metaplasia within *BLG-Cre$^+$Apc$^{fl/fl}$* Mice. We have shown previously that mammary glands from *BLG-Cre$^+$Apc$^{fl/fl}$* mice develop numerous small areas of metaplasia (9) and that β-catenin was dysregulated in these metaplasias (9). We confirm (Fig. 1*A* and *B*) that observation here and show the accumulation of β-catenin in the metaplastic areas to be predominantly nuclear. In accordance with this, we also saw up-regulation of several putative β-catenin/Wnt signaling pathway targets, including c-*myc* (Fig. 1*C*), cyclin D1, and CD44 (Supplementary Figs. A and B).

Mice overexpressing Wnt1, Wnt10B, or truncated β-catenin have all been shown to be predisposed to hyperplasia and malignant transformation in mammary epithelium (12, 23, 24). However, Apc deficiency alone very rarely progresses to neoplasia (9). In colorectal carcinogenesis, loss of APC occurs early, and conditional studies have shown loss of Apc to immediately confer a hyperproliferative state on intestinal epithelial cells (25). The precise relevance of these findings to the mammary epithelium remains to be fully explored. However, it is clear that many of the genetic alterations observed in colorectal cancer, such as loss of function of the tumor suppressor p53, are also key players in mammary gland neoplasia (26). P53 status is of particular relevance to Apc deficiency, with overexpression of β-catenin resulting in accumulation of p53 and the proposed existence of a feedback loop between β-catenin and p53 (17, 18). We therefore analyzed the expression of p53 and p21 and found up-regulation of p53 (Fig. 1*D*) and p21 (Fig. 1*E*) in a subset of cells in the metaplastic areas of squamous epithelium. This suggests that in mammary epithelial cells β-catenin dysregulation may be a signal for the p53 pathway and that increased p53 activity may provide a safeguard against oncogenic deregulation of β-catenin.

Mammary Tumorigenesis in *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$*. Given the proposed feedback loop between β-catenin and p53 and our finding that p21 and p53 are up-regulated in the areas of metaplasia, we intercrossed the floxed *Apc^{fl}* allele onto a p53$^{-/-}$ background and analyzed the phenotype in the mammary gland. *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$*, *BLG-Cre$^+$ Apc$^{fl/fl}$p53$^{+/+}$*, *BLG-Cre$^+$Apc$^{fl/fl}$ p53$^{+/-}$*, and *BLG-Cre$^-$Apc$^{fl/fl}$p53$^{-/-}$* mice were mated and allowed to give birth. The pups were removed at parturition and the mice were then aged until they become symptomatic of disease. Survival curves were then generated (Fig. 2).

At 200 days postpartum, none of the *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{+/+}$* (n = 16) developed overt symptoms of disease and were killed at this time point. This is in accordance with our previous observations (9) where we showed that mice allowed to pass through up to four complete lactations did not progress to neoplasia up to 1 year following the first lactation. Mammary tissue from *BLG-Cre$^+$Apc$^{fl/fl}$ p53$^{+/+}$* mice 200 days after involution had a similar appearance to mature virgin tissue, with few areas of metaplasia remaining and with occasional inflammatory cells surrounding the distorted ducts (Fig. 3*A* and *B*). This indicates that the areas of keratin and parakeratotic material observed at day 10 postpartum (Fig. 3*C* and *D*) are cleared during involution to restore a largely normal mammary gland with only few areas of metaplasia.

p53$^{-/-}$ mice develop lymphoma between 3 and 6 months of age. In accordance with this, 9 of 18 *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$* mice

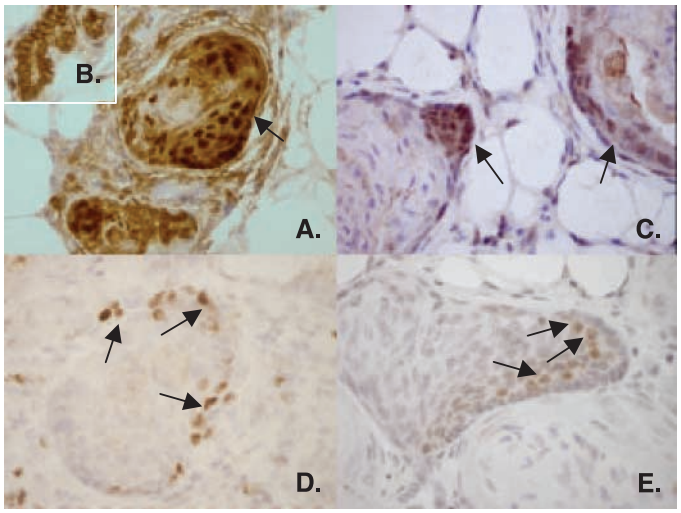

Figure 1. Immunohistochemical analysis of β-catenin, p53, p21,and c-*myc* in the metaplastic areas of *BLG-Cre$^+$ Apc$^{fl/fl}$* mice. *A-E,* sections of *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{+/+}$* 10 days postpartum; *arrows,* examples of positive staining. *A,* β-catenin staining in areas of metaplasia; β-catenin is up-regulated in the nuclei. *B,* β-catenin staining in normal epithelium with β-catenin in the adherens junctions. *C,* c-*myc* staining in areas of metaplasia. *D,* p53 staining in areas of metaplasia. *E,* p21 staining in areas of metaplasia. The same immunohistochemical analysis was done in metaplastic areas of *BLG-Cre$^+$Apc Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$* mice. An identical pattern of staining was observed compared with *BLG-Cre$^+$Apc$^{fl/fl}$ p53$^{+/+}$* mice, except no p53-positive cells were detected (data not shown).

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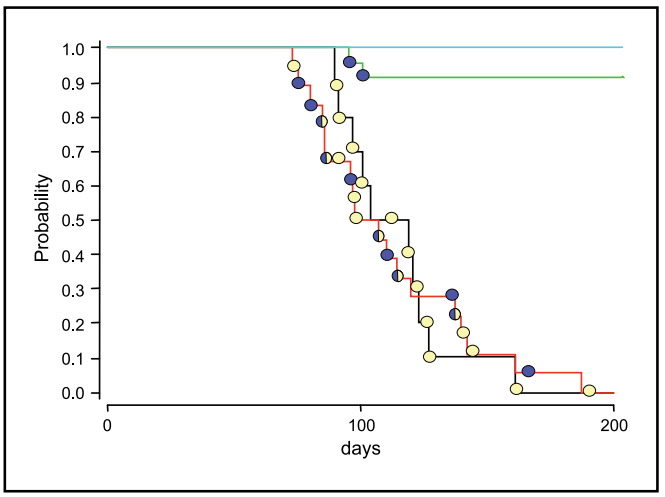

Figure 2. Kaplan-Meier survival plot of *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{+/+}$ (*blue line*, $n = 16$), *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ (*red line*, $n = 18$), *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{+/-}$ (*green line*, $n = 24$), and *BLG-Cre*$^{-}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ (*black line*, $n = 10$). *Blue circles,* mice with mammary lesions at necropsy; *yellow circles,* other illness, predominantly lymphoma; *segmented circles,* mice with both mammary lesions and other illness.

developed lymphoma with an onset between 73 and 187 days postpartum. Of those mice developing lymphoma, nine showed no indication of mammary neoplasia, with histology comparable with that observed in *BLG-Cre*$^{+}$*APC*$^{fl/fl}$*p53*$^{+/+}$ mice at similar time points. The remaining nine *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ mice developed mammary lesions with an onset between 75 and 161 days of age (Fig. 2). Histologic analysis revealed various categories of mammary disease. Four animals showed a phenotype comparable with that observed in *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{+/+}$ mice at day 10 postpartum but with more marked distortion of the normal structure with increased distension of glandular spaces by keratin and parakeratotic material (Fig. 3*E* and *F*). There was no evidence of tumors in these mice. A second group of animals showed localized changes, including strong epithelial proliferation, fibroblastic and inflammatory responses (Supplementary Figs. C and D), distorted acinar architecture with abundant paucicellular keratin material, and focal calcification (Supplementary Fig. E). However, there was no evidence of tumor formation in these mice. The final category comprised four animals (44.4% of the animals not affected by lymphoma), which showed clear tumor formation (Fig. 4*A*), with an onset between 80 and 161 days. In one animal, three independent tumors were observed (Fig. 4*C* and *D*). The tumors were marked by elevated levels of both mitosis and apoptosis compared with normal tissue and by inflammatory infiltrate (Supplementary Fig. F). In most tumors, abundant keratin formation was noted suggestive of keratoacanthoma (Supplementary Fig. G). The single tumor lacking keratin was composed of epithelial cells with a papillary architecture with minimal squamous differentiation (Supplementary Figs. H and I). In two tumors, a mass of laminated keratin with a thick rim of epithelium was observed (Supplementary Fig. J). No evidence of invasion or metastasis was observed in any of the tumors, and they were therefore probably benign.

BLG-Cre^{+}*Apc*$^{fl/fl}$*p53*$^{+/-}$ mice developed an intermediate phenotype, with 12.5% mice developing a mammary tumor between 95 and 260 days ($n = 24$; Fig. 2). Two mice developed well-circumscribed mammary tumors characterized by a rim of epithelium around a mass of laminated keratin. These were comparable with tumors seen in the *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ mice (Fig. 4*D*).

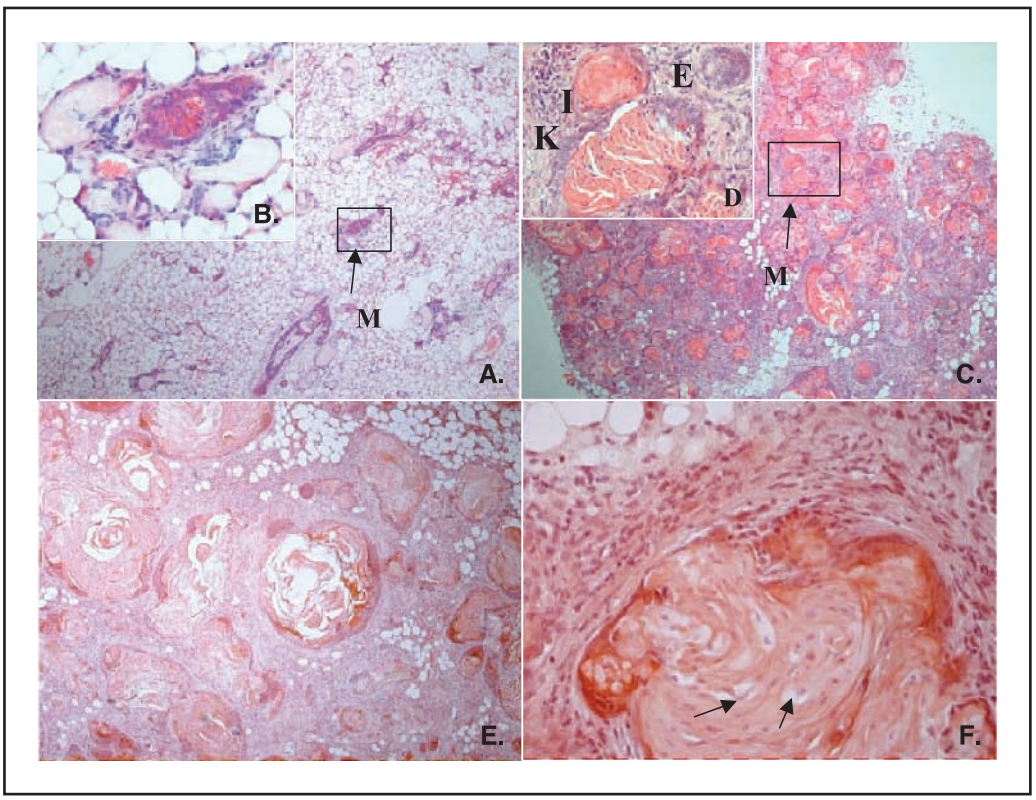

Figure 3. H&E-stained sections of mammary glands. *A* and *B,* mammary glands of *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$ animals contain metaplasias at day 10 postpartum that are almost completely cleared after 200 days. *A,* glands from *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{+/+}$ mice at 200 days postpartum (magnification, ×40). *Inset B,* ×400 higher magnification of the *boxed area*. Mammary tissue has the general appearance of a mature virgin with ductal structures and few areas of metaplasia (*M*) remaining. *C,* glands from *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{+/+}$ mice at 10 day postpartum (magnification, ×40). *Inset D,* ×400 higher magnification of the *boxed area*, showing a hyperproliferative epidermal cyst containing ghost cells and keratinized structures (*K*), accompanied by an acute stromal inflammatory reaction (*I*), and epithelial structures (*E*). *E* and *F,* histologic characteristics of *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ mice at day 10 postpartum, showing heterogeneously differentiation. Few normal alveolar structures are present, with massive squamous metaplasias containing ghost cells (*E,* magnification, ×40; *F,* magnification, ×200).

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A third animal developed a tumor composed of epithelial cells with a papillary architecture with minimal squamous differentiation and no keratin production, which resembled the phenotype of a single tumor in the *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ cohort (Fig. 4*C*).

Comparison of the survival curves of the *BLG-Cre*$^{-}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ (n = 10) and *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ showed no statistical differences presumably as a reflection of the high penetrance of lymphoma in all p53-deficient genotypes. However, a marked difference was observed in mammary neoplasia, with none of the *BLG-Cre*$^{-}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ mice developing mammary lesions even after several rounds of pregnancy. Furthermore, no mammary lesions were observed in any of the 16 *Apc*$^{fl/+}$*p53*$^{-/-}$ mice (*BLG-Cre*$^{+}$ or *Cre*$^{-}$). These observations are consistent with two previous studies of conditional inactivation of p53 using either a K14-Cre (27) or a MMTV-Cre transgene (28), which respectively showed either no tumor predisposition or delayed onset between 11.5 and 24 months of age.

The Role of p53 in Suppressing Apc-Mediated Mammary Neoplasia. We have shown that p53 deficiency clearly accelerates Apc-mediated neoplasia in the mammary gland. There seem to be several likely mechanisms. One relates to the role of p53 in down-regulation of β-catenin, as it is probable that levels of β-catenin are important in mediating tumorigenesis. Another possible mechanism is that p53 may function to remove *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$ recombined cells. We hypothesize that in the absence of functional p53 many of the *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$ recombined cells fail to be removed by a p53-dependent pathway and remain to be potential founders of neoplasia. To address the first hypothesis, we analyzed the levels of β-catenin by immunohistochemistry in *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{+/+}$ and *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ mice. To test the second hypothesis, we used the *ROSA26R* reporter allele (29) to score the retention of recombined cells using the surrogate LacZ marker.

β-Catenin Immunohistochemistry in the Absence of p53. In the *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{+/+}$ mice, β-catenin is strongly up-regulated in the nuclei of the metaplastic squamous epithelium. As described above (Fig. 1), this metaplastic change triggers accumulation of both p53 and p21 in a subset of cells. One would predict that, in the absence of the p53-negative feedback loop, the levels of β-catenin would be further raised. Figure 5 shows immunohistochemical analysis of β-catenin of *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{+/+}$ glands at days 10 and 200 postpartum (Fig. 5*A* and *B*) and of *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ glands at days 10 and 20 postpartum (Fig. 5*C* and *D*; later analysis was precluded by lymphoma onset). At each time point examined, no p53-dependent difference in the expression level or pattern was noted. Our immunohistochemical analysis therefore offers no support for increased deregulation of β-catenin in the absence of p53. However, given the limitations of this approach in accurately assessing protein levels on a per cell basis, we cannot formally exclude this mechanism.

p53-Mediated Loss of Apc-Deficient Cells. Previously, we studied the level of recombination in the mammary gland directed by the BLG-Cre promoter using the *Rosa26R* reporter allele (9), which showed Cre-mediated recombination reaching nearly 100% of epithelial cells by day 10 of lactation. To address the hypothesis that p53 may mediate loss of *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$ recombined epithelial cells, we generated *BLG-Cre*$^{+\mathrm{Apc}^{fl/fl}}$*p53*$^{+/+}$*Rosa26R*$^{+}$, *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$*Rosa26R*$^{+}$, and *BLG-Cre*$^{+}$*Apc*$^{fl/+}$*p53*$^{+/+}$*Rosa26R*$^{+}$ mice. Mammary glands were then analyzed for β-galactosidase activity at various time points.

As shown previously (9), the BLG-Cre^{+} transgene is active in the virgin mammary gland. Whole mounts of 12 weeks virgins mammary glands from control *BLG-Cre*$^{+}$*Apc*$^{fl/+}$*p53*$^{+/+}$*Rosa26R*$^{+}$ mice showed widespread recombination of the epithelial cells (Fig. 6*A*). By contrast, *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{+/+}$*Rosa26R*$^{+}$ (Fig. 6*B*) and *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$*Rosa26R*$^{+}$ (Fig. 6*C*) virgin whole mounts showed almost no evidence of β-galactosidase activity. At parturition, whole mounts show high efficiency recombination for the control *BLG-Cre*$^{+}$*Apc*$^{fl/+}$*p53*$^{+/-}$*Rosa26R*$^{+}$ (Fig. 6*D*). By contrast, whole mounts made at parturition in *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{+/+}$*Rosa26R*$^{+}$ (Fig. 6*E*) and *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$*Rosa26R*$^{+}$ (Fig. 6*F*) both showed only focal LacZ staining. Taken together, these results suggest strong selection against cells bearing a recombined *Apc*$^{fl/fl}$ allele during virgin mammary development and pregnancy. These data are also consistent with the previously reported retarded development of the virgin mammary gland in *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$ mice (9). This process seems to be independent of p53 activity as both *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{+/+}$*Rosa26R*$^{+}$ and

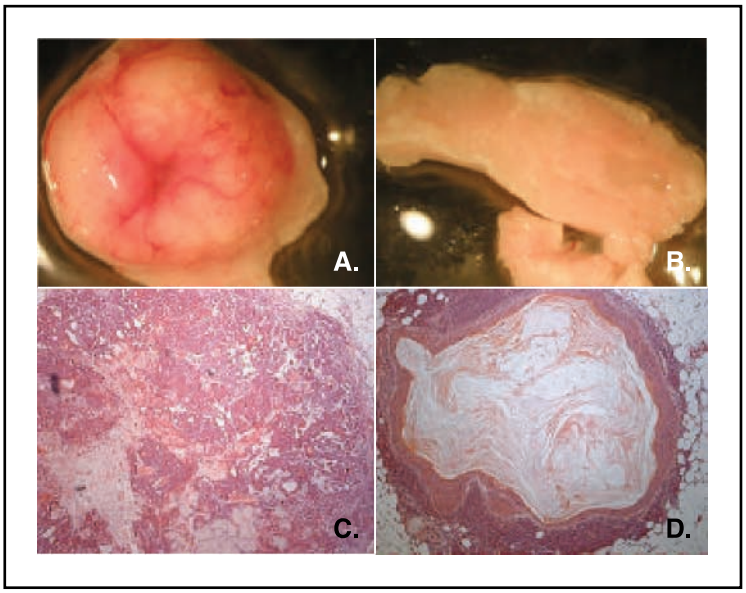

Figure 4. Histologic characterization of tumors arising in *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ mice. *A,* macroscopic appearance of a tumor developing at parturition. *B,* macroscopic appearance of a comparable normal mammary gland. *C* and *D,* histologic characteristics of mammary tumors developing in a *BLG-Cre*$^{+}$*Apc*$^{fl/fl}$*p53*$^{-/-}$ mouse 74 days postpartum. Three independent tumors were detected in a single animal. *C,* one tumor showed papillary architecture with minimal squamous differentiation (magnification, ×40). *D,* two other tumors were composed of a mass of laminated keratin with a rim of epithelium with varying thickness (magnification, ×40).

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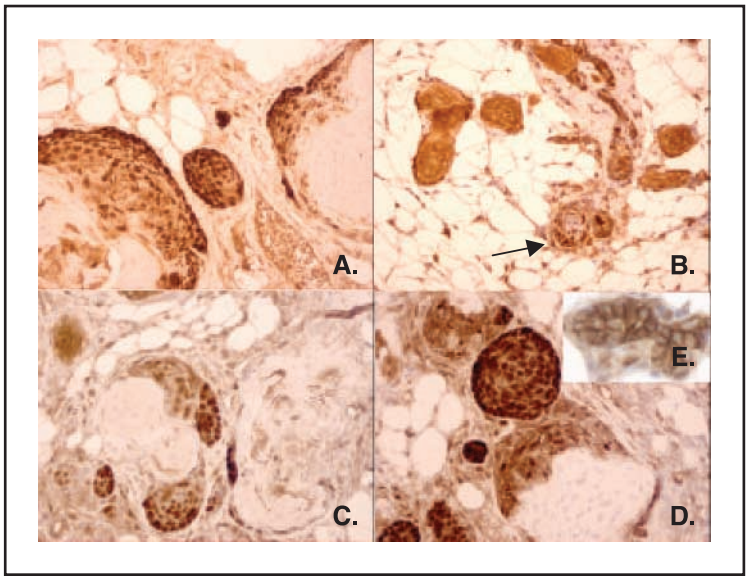

Figure 5. Immunohistochemical analysis of β-catenin expression in the metaplastic areas (×200). *A, BLG-Cre$^+$Apc$^{fl/fl}$p53$^{+/+}$* mice at day 10 postpartum. β-catenin is detected in the nuclei of epithelial cells within the areas of metaplasia and in the adherens junction of normal epithelium. *B, BLG-Cre$^+$Apc$^{fl/fl}$p53$^{+/+}$* mice 200 days postpartum. Few areas of metaplasia remains (*arrows*). Intensity of the staining per epithelial cells is comparable with that observed in *A*. *C, BLG-Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$* mice 10 days postpartum. *D* and *E, BLG-Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$* mice 20 days postpartum. β-catenin is detected in the nuclei of epithelial cells of the areas of metaplasia (*D*) and in the adherens junction of normal epithelium (*inset E*). β-catenin levels per epithelial cell of *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$* seem similar to that seen in *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{+/+}$* glands.

BLG-Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$Rosa26R$^+$ show similar patterns of recombination (Fig. 6*B*, *C*, *E*, and *F*).

We next scored the pattern of recombination at day 10 postpartum. At this point, the mammary glands of control *BLG-Cre$^+$Apc$^{fl/+}$p53$^{+/-}$Rosa26R$^+$* mice (Fig. 6*G*) have largely involuted, and all the remaining epithelial cells show recombination at the *Rosa26R* allele. Analysis of both *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{+/+}$Rosa26R$^+$* (Fig. 6*H*) and *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$Rosa26R$^+$* mice (Fig. 6*I*) showed low levels of recombination compared with the wild-type control, but notably the levels of recombination were higher compared with parturition. This suggests an expansion of the few areas of recombination observed at parturition, a phenomenon more marked in the absence of p53 (Fig. 6*I*). At day 27 postpartum, there was a clear difference between *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{+/+}$Rosa26R$^+$* and *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$Rosa26R$^+$* mice, with few recombined cells retained in the presence of p53 (Fig. 6*K*), but significant areas retained in the absence of p53 (Fig. 6*L*). These data therefore support the p53-dependent loss of recombined cells at later stages postpartum. Notably, mammary tumors arising within the *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$Rosa26R$^+$* background report 100% Rosa26R$^+$ recombination as predicted (Fig. 6*M*). The most attractive explanation for the loss of cells is through the engagement of p53-dependent apoptosis. However, we detected no elevation in the levels of apoptosis at days 10 and 23 postpartum by either terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling or caspase-3 positivity (data not shown). This suggests either that the hypothesized increase in apoptosis was below the threshold of detection at these time points or that cell loss was occurring through other undetected mechanisms, such as low-level induction of necrosis.

An alternative explanation for these observed differences is that, in the absence of p53, the areas of metaplasia undergo rapid expansion. Indeed, as described above, there is an increase in the number of *BLG-Cre$^+$Apc$^{fl/fl}$* recombined cells between parturition and 10 day postpartum, and this was more marked in the absence of p53. However, we observed no difference in the numbers of cells cycling in the metaplastic areas by bromodeoxyuridine incorporation between genotypes at the time points analyzed. Thus, in the metaplastic areas in *BLG-Cre$^+$APC$^{fl/fl}$p53$^{+/+}$* mice, 5 ± 1.5% and 6.2 ± 2.8% cells were cycling at birth and at day 10 postpartum respectively, compared with values of 5.4 ± 3.4% and 3 ± 1.3% cells in comparable areas in the *BLG-Cre$^+$Apc$^{fl/fl}$p53$^{-/-}$* mice.

These results show that p53 has little or no role in the selection against *BLG-Cre$^+$Apc$^{fl/fl}$* recombined cells in the virgin mammary gland. The role of p53 during pregnancy is somewhat less clear, as data from the Rosa26R$^+$ cross did not support p53-dependent loss of recombined *BLG-Cre$^+$Apc$^{fl/fl}$* cells at this stage, yet two of the mice in our cohort developed tumors at parturition, clearly indicating a tumor suppressive role for p53. This role may reflect either subtle differences in cell loss not detected by the Rosa26R reporter assay or alternative mechanisms of p53-dependent tumor suppression. Beyond parturition, we present evidence that there is strong p53-mediated selection against *BLG-Cre$^+$Apc$^{fl/fl}$* recombined cells and that the duct trees subsequently become repopulated with *BLG-Cre$^+$Apc$^{fl/fl}$* unrecombined cells. In the absence of p53 activity, significant numbers of *BLG-Cre$^+$Apc$^{fl/fl}$* recombined cells persist in the mammary gland, and this may represent either the driving mechanism or a contributing mechanism to neoplastic development.

P53 function therefore seems critical during pregnancy and beyond but not to be relevant to the loss of *BLG-Cre$^+$Apc$^{fl/fl}$* recombined cells in the virgin gland. There is considerable evidence for such a differential role of p53 within the literature. First, treatment with placental hormones has been shown to result in nuclear accumulation of p53 protein in the mammary epithelium, transcriptional activation of target genes, and apoptosis in response to ionizing radiation (30). This shows that p53 function is subject to hormonal regulation and may explain why pregnancy exerts a protective effect against breast cancer in humans (31, 32). Second, p53 deficiency has been shown to abrogate the protective effect of estrogen and progesterone hormone stimulus against carcinogen-induced mammary tumorigenesis (33). Furthermore, the exposure to pregnancy levels of estrogen and progesterone has been shown to induce nuclear sequestration of p53 and to block proliferation on carcinogen challenge (34). Finally, p53 mRNA levels are increased at midpregnancy (35), an increase dependent on

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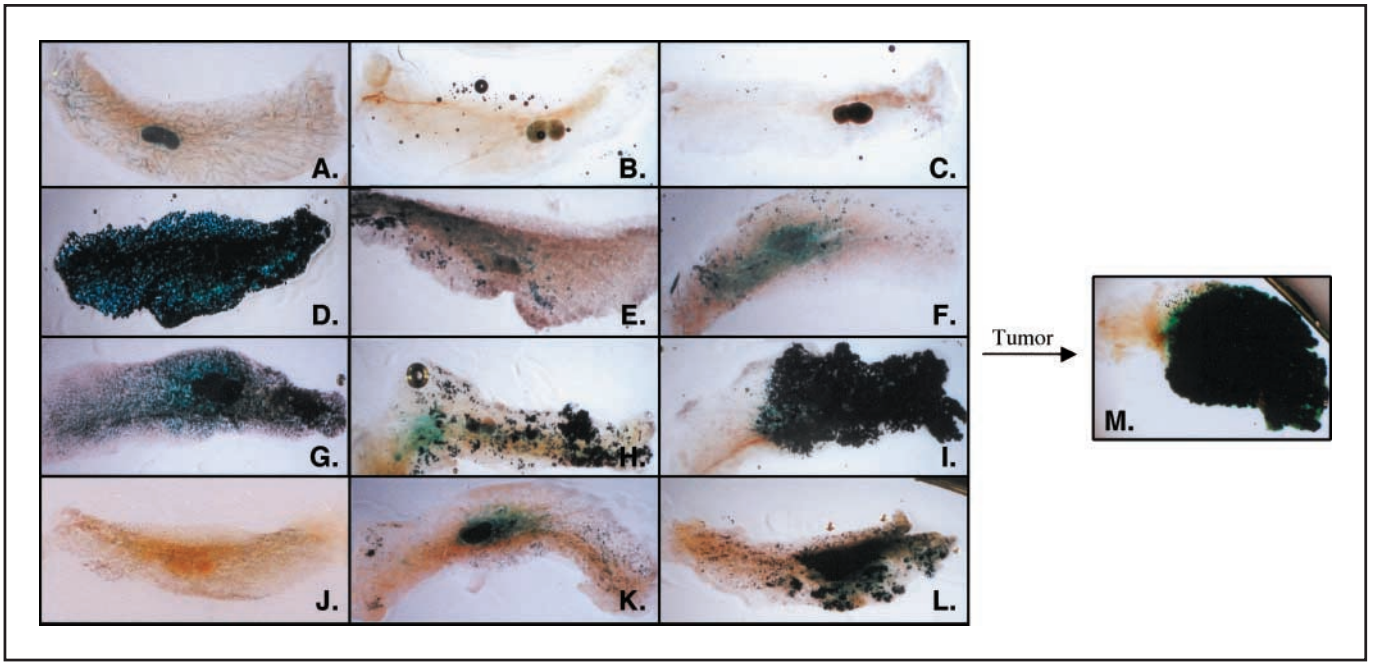

Figure 6. Pattern of BLG-Cre-mediated recombination using the flox-STOP Rosa26 reporter strain where LacZ positivity indicates Cre-mediated recombination. *A-C,* 12-week-old virgin glands; *D-F,* parturition glands; *G-I,* glands 10 days postpartum; *J,* glands 200 days postpartum; *K-L,* glands 27 days postpartum; *M,* mammary tumor detected 19 days postpartum. *A, BLG-Cre^{+}Apc$^{fl/+}$p53$^{+/+}$Rosa26R^{+}*; *D*, *G*, and *J, BLG-Cre^{+}Apc$^{fl/+}$p53$^{+/-}$Rosa26R^{+}*; *B*, *E*, *H*, and *K, BLG-Cre^{+}Apc$^{fl/fl}$p53$^{+/+}$Rosa26R^{+}*; *C*, *F*, *I*, *L*, and *M, BLG-Cre^{+}Apc$^{fl/fl}$p53$^{-/-}$Rosa26R^{+}*; *A*, *D*, *G*, and *J,* control glands illustrating the pattern of recombination in the presence of Apc. *B* and *C,* near absence of LacZ staining in *BLG-Cre^{+}Apc$^{fl/fl}$* virgin glands, consistent with loss of *Cre^{+}Apc$^{fl/fl}$* recombined cells. *D,* high levels of LacZ staining indicating high levels of recombination in control gland at parturition. *E* and *F,* focal LacZ staining indicating low-frequency retention of *BLG-Cre^{+}Apc$^{fl/fl}$* recombined cells at parturition. Level of retention is similar in the presence (*E*) or absence (*F*) of p53. *H*, *I*, *K*, and *L,* focal LacZ staining indicating variable retention of *BLG-Cre^{+}Apc$^{fl/fl}$* recombined cells in days 10 and 27 postpartum mammary glands. Highest proportion of recombined cells is observed in the absence of p53 (*I* and *L* are p53 deficient compared with *H* and *K*). The tumor (*M*) comprehensively stained for LacZ, which indicates that the tumor is fully derived from *BLG-Cre^{+}Apc$^{fl/fl}$* recombined cells. *K,* the blue staining of the lymph node is an artifact of the staining process.

binding of the transcription factor NF1-C2 to the mouse p53 promoter.

Taken together, our data indicate that mutations in p53 and Apc synergize in promoting mammary tumorigenesis in the mouse. Remarkably, the protective effect of p53 seems constrained to lactation and beyond, which is consistent with data suggesting that p53 is inactive in the quiescent gland. Concerning the mechanism by which p53 promotes Apc-mediated tumorigenesis, we provide no support for the notion of further deregulated β-catenin levels in the absence of p53, although we cannot exclude such a role. We do however show that p53 is involved in the loss of Apc-deficient epithelial cells and that this provides a ready mechanism for p53-accelerated neoplasia in the absence of Apc.

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