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# A mammary-specific model demonstrates the role of the *p53* tumor suppressor gene in tumor development

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**Although alterations in the *p53* tumor suppressor gene are detected frequently in human breast cancers, mammary tumors are observed infrequently in $p53^{null}$ mice. This has led to the suggestion that absence of *p53* alone is not sufficient for induction of mammary tumors. However, early death of $p53^{null}$ mice from thymic lymphomas may obscure tumor phenotypes that would develop later. Therefore, $p53^{null}$ mammary epithelium was transplanted into cleared mammary fat pads of wild type *p53* BALB/c hosts to allow long-term analysis of mammary tumor phenotypes. Five treatments were compared for their effects on tumor incidence in hosts bearing transplants of $p53^{null}$ and $p53^{wt}$ mammary epithelium. The treatment groups were: (1) untreated; (2) continuous hormone stimulation with pituitary isografts; (3) multiple pregnancies; (4) DMBA alone; and (5) DMBA + pituitary isografts. The tumor incidences in $p53^{null}$ *vs* $p53^{wt}$ mammary transplants for each treatment group were 62% *vs* 0%, 100% *vs* 0%, 68% *vs* 0%, 60% *vs* 4% and 91% *vs* 14%, respectively. The mammary tumors that developed in the $p53^{null}$ mammary epithelium were all adenocarcinomas and were frequently aneuploid. These data demonstrate that the absence of p53 is sufficient to cause development of mammary tumors and that hormonal stimulation enhances the tumorigenicity of $p53^{null}$ mammary epithelium to a greater extent than DMBA exposure alone. This model provides an *in situ* approach to examine the molecular basis for the role of *p53* in the regulation of mammary tumorigenesis.** *Oncogene* (2000) **19,** 1052–1058.

**Keywords:** *p53*; knockout mice; DMBA; carcinogenesis; tumor incidence; hormonal stimulation

## Introduction

Altered expression and mutation of the *p53* tumor suppressor gene is observed frequently in human breast cancers (Elledge and Allred, 1994; Coles *et al*., 1992; Delmolino *et al*., 1993; Lehman *et al*., 1993), suggesting that abrogation of p53 function is a pivotal event in the natural history of breast tumors. However, it has been difficult to establish whether loss of p53 function is an early or late event in tumor development. Biochemical and functional analysis of p53 has demonstrated its participation in regulation of the cell cycle, apoptosis in response to DNA damage, senescence, DNA repair and centrosome stability. Disruption of any of these pathways could contribute to development of breast cancers and it remains unclear which of these biochemical functions of p53 is dominant in fulfilling its tumor suppressor activity in breast tissue.

Many mammary tumor models have been developed to study the process of tumorigenesis *in vivo*, but most have proven to be of limited utility in analysing the role of p53. In mammary tumors induced by chemical carcinogens (DMBA or NMU), the *ras* pathways are mutated preferentially and rarely result in altered expression or mutation of *p53* (McKenzie *et al*., 1997; Qing *et al*., 1997; Kito *et al*., 1996; Kumar *et al*., 1990; Jerry *et al*., 1994). Likewise, mammary tumors that arise from targeted overexpression of dominant-acting oncogenes do not appear to require disruption of the *p53* pathway (Ritland *et al*., 1997). Dominant-acting immortalizing viral genes have been used to disrupt p53 function in transgenic mice (Tzeng *et al*., 1993, 1998). Although p53 function appears to be impaired in mice expressing SV40 large T-antigen, the phenotype is complicated by the disruption of differentiation which is not observed in $p53^{null}$ mice, indicating that additional pathways are disrupted by T-antigen. Overexpression of *mdm2*, a cellular antagonist of p53, also disrupted differentiation of the mammary gland and resulted in late-appearing tumors, but acted through p53-independent pathways (Lundgren *et al*., 1997).

Together, these data would appear to challenge the significance of inactivation of p53 in the development of mammary tumors in rodents. Indeed, mammary tumors were observed infrequently in mice bearing homozygous deletions of the *p53* gene (Donehower *et al*., 1992; Jacks *et al*., 1994). DMBA-induced mammary tumors in $p53^{+/-}$ mice did not show loss of heterozygosity at the *p53* locus (Jerry *et al*., 1994). In contrast to the apparent lack of effect of altered p53 function alone, overexpression of *ras* or *wnt-1* oncogenes in p53-deficient mice led to more rapid development of mammary tumors which exhibited more aggressive features (Donehower *et al*., 1995; Hundley *et al*., 1997). Overexpression of a dominant-negative mutant of p53 alone failed to exhibit a phenotype, but rendered the mammary gland more

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sensitive to tumor induction by chemical carcinogens and oncogenes (Li *et al*., 1997, 1998). In each of these cases, inactivation of p53 function led to increased aneuploidy in the resulting tumors. Mammary tumors in p53-deficient mice bearing C3(1)-TAg transgene acquired more aggressive features and became metastatic (Maroulakou *et al*., 1997). Therefore, abrogation of p53 function can be a critical event in tumor formation, even in the presence of oncogenes. Absence of p53 may also contribute to the development of genetic instability and increased metastatic potential.

These models demonstrate the potential importance of *p53* status in mammary tumorigenesis, but it remains unclear whether absence of p53 alone increases susceptibility to mammary tumorigenesis. Therefore, the $p53^{null}$ allele was transferred to the BALB/c genetic background (Jerry *et al*., 1998) as the BALB/c genetic background had been shown to be relatively susceptible to mammary tumorigenesis (Medina, 1974). Transplantation of BALB/c-$p53^{null}$ mammary epithelium into wild type BALB/c hosts allowed observation of the effects of absence of *p53* in the mammary epithelium without complications associated with the effects of the $p53^{null}$ allele on other tissues. Using this model, we demonstrated that absence of *p53* renders the mammary epithelium exquisitely susceptible to tumor formation. Carcinogen treatment decreased significantly the latency period for tumor appearance, but did not alter the final tumor incidence in $p53^{null}$ mammary transplants. Surprisingly, continuous hormonal stimulation alone was shown to dramatically increase the incidence of mammary tumors in $p53^{null}$ transplants. The tumors were all adenocarcinomas and tended to be aneuploid. These data demonstrate that loss of p53 and hormonal stimulation synergize to create a potent tumorigenic environment. This animal model provides a valuable system with which to study p53-dependent mechanisms involved in mammary tumor development in the presence and absence of hormones.

## Results

The effect of $p53^{null}$ genotype on spontaneous mammary tumorigenesis and its interactions with carcinogenic stimuli were examined in this experiment. The effect of genotype was dramatic in the absence of carcinogen. No tumors developed in transplants of $p53^{wt}$ mammary epithelium in either the untreated control treatment group or those groups subjected to hormonal stimulation (Pituitary-stimulated or Multiparous groups; Figure 1a, open symbols). In contrast tumors, were frequent in the transplants of $p53^{null}$ mammary epithelium under all three treatment conditions (Figure 1a, closed symbols). The overall tumor incidence in $p53^{null}$ transplants was increased ($P<0.05$) from 61.5% (16/26) in the untreated recipients to 100% (25/25) in recipients bearing pituitary isografts (Table 1). Tumors arose more rapidly ($P<0.05$) in the mice bearing pituitary-isografts ($TE_{50}=37$ weeks) than in untreated mice ($TE_{50}=50$ weeks). Tumor incidence in the multiparous group was 68% (19/28) with the $TE_{50}$ reached at 46 weeks.

Treatment with DMBA and chronic hormonal exposure were chosen as carcinogenic stimuli because both have been well characterized with respect to mammary carcinogenesis in mice (Medina, 1982, 1996). The $p53^{null}$ genotype significantly increased tumor incidences ($P<0.05$) in both treatment groups in response to DMBA (Figure 1b; compare $p53^{null}$ shown by closed symbols and $p53^{wt}$ shown as open symbols). Treatment with DMBA alone resulted in a tumor incidence of 60% (15/25) in $p53^{null}$ outgrowths, but the tumor incidence in $p53^{wt}$ outgrowths was minimal (3.8%; 1/26). The combination of DMBA and pituitary-stimulation compared to DMBA alone resulted in a 90.5% (19/21) tumor incidence in $p53^{null}$ transplants compared to 14.3% (4/28) in $p53^{wt}$ transplants. As expected, the host glands of mice treated with DMBA and pituitary isografts yielded high tumorigenic incidence of 61% (17/28).

Comparison of the treatment effects on $p53^{null}$ transplants revealed a much greater tumorigenic response due to chronic hormonal stimulation than to DMBA. DMBA treatment alone failed to increase the overall tumor incidence in $p53^{null}$ transplants compared to untreated controls (Table 1; 60% *vs* 61.5%). The primary effect of DMBA was to significantly decrease ($P<0.05$) the tumor latency in $p53^{null}$ transplants (Table 1; $TE_{50}$ for nulliparous = 50 weeks *vs* $TE_{50}$ for DMBA = 35 weeks). Similarly, the additional effect of DMBA on tumor incidence in $p53^{null}$ transplants subjected to chronic hormonal stimulation by pituitary isografts decreased tumor latency ($P<0.05$) from 37 to 25 weeks (Table 1; $TE_{50}$ for Pituitary-stimulated *vs* DMBA-Pits). The overall tumor frequency in the DMBA treated group was slightly less than that of the Pituitary-stimulated group presumably because of a shorter observation period (48 weeks *vs* 60 weeks). In contrast, the effect of chronic hormone exposure due to the presence of pituitary isografts resulted in increases in tumor incidence of 38.5% (Table 1; Untreated *vs* Pituitary-stimulated) and 30.5% (Table 1; DMBA *vs* DMBA + Pituitary-stimulated). These data reveal a surprising interaction between the absence of *p53* and the biochemical effects of this hormone combination. Therefore, it appears that in the absence of the *p53* gene product, this hormone combination is a more potent tumorigenic stimulus than the carcinogen DMBA.

All glands without tumors were collected at the end of the experiment, stained with hematoxylin, and examined as whole mounts under the dissecting microscope. All transplants from $p53^{wt}$ mammary tissue presented normal duct or lobuloalveolar development depending on the absence or presence of pituitary isografts, respectively. A single exception was observed in one of the 25 non-tumor bearing glands from DMBA-treated mice in which a fine duct hyperplasia was present as a focal lesion. In contrast, six of 20 non-tumor bearing glands with $p53^{null}$ transplants exhibited fine duct hyperplasias and focal atypical duct hyperplasia, lesions that were observed in animals from both DMBA and untreated groups. Interestingly, no hyperplastic alveolar nodules, the common preneoplastic lesions occurring in MMTV-induced and spontaneous mammary tumors, were detected.

The histological appearance of tumors was uniform among the treatment groups. In general, the tumors were either moderate to poorly-differentiated adenocarcinomas (Figure 2). In some tumors, the nuclei were

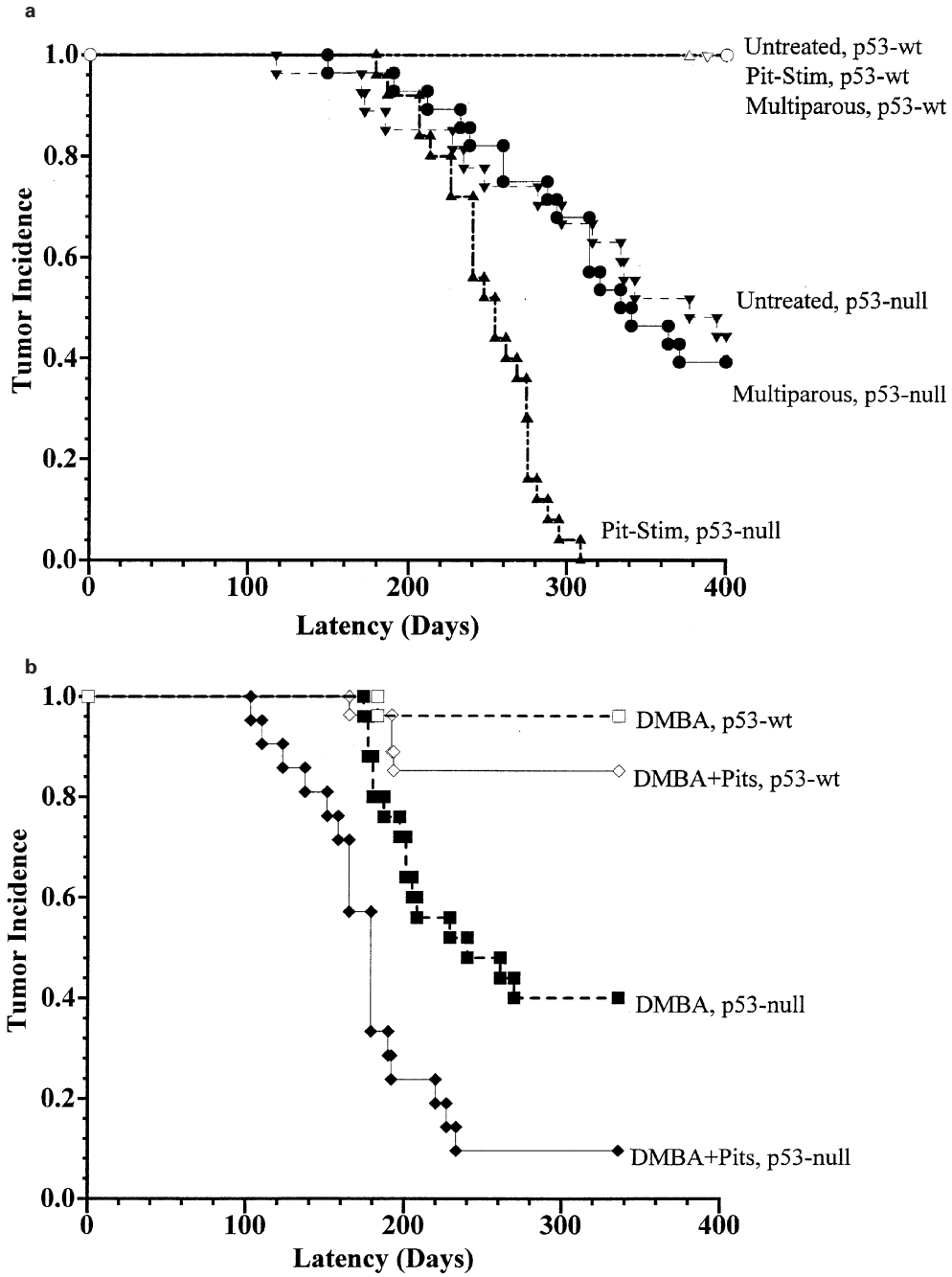


**Figure 1** Kaplan-Meier survival curves for mice bearing mammary transplants. (**a**) The tumorigenic effects of hormonal stimulation. Continuous hormonal stimulation by pituitary isografts induced a very high incidence of tumors in $p53^{null}$ transplants, whereas hormonal stimulation by multiple pregnancies caused no greater tumor incidence than was observed for $p53^{null}$ transplants in untreated hosts. This effect was evident only in $p53^{null}$ transplants. (**b**) The tumorigenic effects of carcinogen treatment. The effect of DMBA, both alone and in combination with hormonal stimulation, were exacerbated in glands bearing $p53^{null}$ transplants compared to those with $p53^{wt}$ transplants

extensively pleomorphic and the chromatin pattern was disorganized, indicating chromosomal abnormalities. For this reason, a subset of tumors was analysed for DNA content by flow cytometry. Rates of aneuploidy in the tumors from $p53^{null}$ mammary outgrowths were surprisingly high among all treatment groups, ranging from 29% to 100% (Table 2). Tumors that maintained normal ploidy had large S phase fractions (13–20%). The S phase fractions did not differ dramatically among tumors from different treatment groups. Although the DMBA + Pituitary isograft treatment yielded the lowest frequency of aneuploidy, further analysis revealed an inverse relationship between ploidy and latency in tumors (Figure 3). All tumors (6/6) appearing prior to 26 weeks post-transplantation were euploid. Of these early appearing tumors, five were derived from the DMBA + Pituitary-stimulated treatment group (which had the shortest overall latency) and one was derived from the untreated nulliparous group. Those tumors arising with a latency equal to or greater than 24 weeks post-transplantation were predominantly aneuploid (11/17) and were distributed

**Table 1** Effect of treatments on incidence of tumors in transplants of *p53*$^{null}$ and *p53*$^{wt}$ mammary epithelium

| *Treatment* | n[f] | *Tumors in transplants* | *%* | *$TE_{50}$*[g] *(weeks)* |
|---|---|---|---|---|
| *Untreated* [a] | | | | |
| *p53*$^{null}$ transplants | 26 | 16 | 61.5 | 50 |
| *p53*$^{wt}$ transplants | 27 | 0 | 0 | – |
| Host glands (*p53*$^{wt}$) | 27 | 0 | 0 | – |
| *Pituitary-stimulated* [b] | | | | |
| *p53*$^{null}$ transplants | 25 | 25 | 100[h] | 37[h] |
| *p53*$^{wt}$ transplants | 30 | 0 | 0 | – |
| Host glands (*p53*$^{wt}$) | 30 | 0 | 0 | – |
| *Multiparous* [c] | | | | |
| *p53*$^{null}$ transplants | 28 | 19 | 68 | 46 |
| *p53*$^{wt}$ transplants | 28 | 0 | 0 | – |
| Host glands (*p53*$^{wt}$) | 28 | 0 | 0 | – |
| *DMBA (4 mg)* [d] | | | | |
| *p53*$^{null}$ transplants | 25 | 15 | 60 | 35[h] |
| *p53*$^{wt}$ transplants | 26 | 1 | 3.8 | – |
| Host glands (*p53*$^{wt}$) | 28 | 2 | 7.1 | – |
| *DMBA (2 mg)+ Pituitary-stimulated* [e] | | | | |
| *p53*$^{null}$ transplants | 21 | 19 | 90.5[h] | 25[h] |
| *p53*$^{wt}$ transplants | 28 | 4 | 14.3 | – |
| Host glands (*p53*$^{wt}$) | 28 | 17 | 60.7 | 36 |

[a]Mammary epithelium from BALB/c-*p53*$^{null}$ and BALB/c-*p53*$^{wt}$ donors was transplanted into cleared mammary fat pads from BALB/c hosts at 3 weeks of age. [b]Pituitary isografts were implanted at 5 weeks of age. [c]Hosts were subjected to multiple cycles of pregnancy and involution. [d]DMBA was administered at four times at weekly intervals beginning at 8 weeks of age. [e]Pituitary isografts were implanted at 5 weeks of age. DMBA was administered two times at weekly intervals beginning at 8 weeks of age. [f]Number of glands bearing transplanted epithelium or, in the case of 'host glands' this is the number of mice each with eight glands/mouse. [g]$TE_{50}$ represents the time for 50% of the transplants to develop tumors. [h]$P<0.05$ compared to the untreated *p53*$^{null}$ transplants

among the treatment groups (two from DMBA + Pits; seven from DMBA alone; three from untreated nulliparous; five from Pituitary-stimulated). These data demonstrate that aneuploidy is a significant feature of mammary tumors arising from *p53*$^{null}$ transplants, but was inversely related to tumor latency.

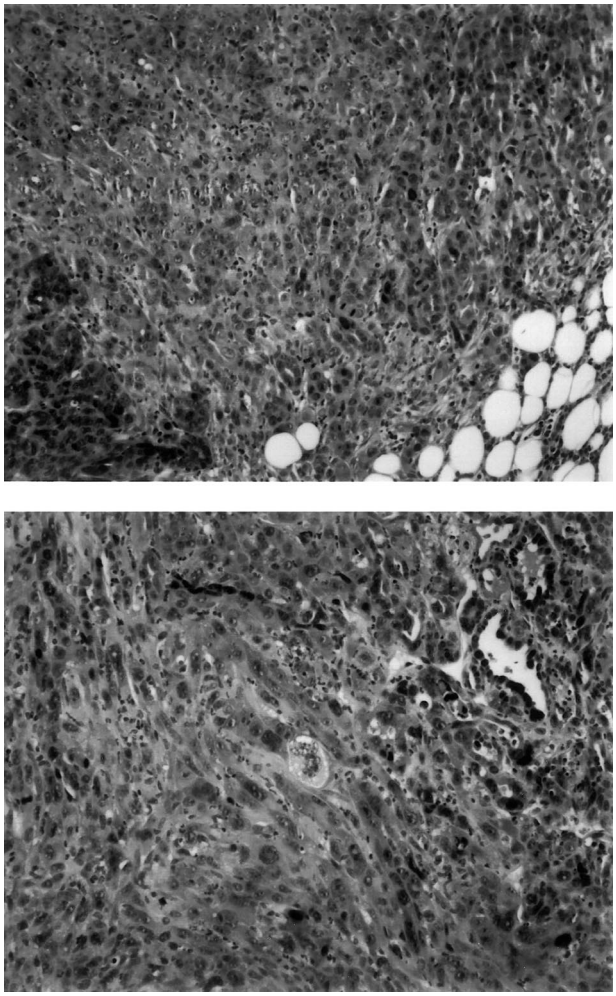

**Figure 2** Histological evaluation of mammary tumors. Tumors arising from the *p53*$^{null}$ transplants exhibited features typical of adenocarcinomas. The tumors were moderately to poorly differentiated as shown in this representative field (upper panel, 200× magnification). The remnants of the ductal architecture can still be distinguished in many regions. At higher magnification, the mitotic figures can be seen and hyperchromatic nuclei that are indicative of aneuploidy (lower panel, 400× magnification)

## Discussion

Rodent models of breast cancer would ideally recapitulate the histological and molecular pathogenesis of human disease. There are numerous rodent models that recapitulate the major features of human disease. For example, the majority of human breast cancers develop from the terminal ductal-lobular units (Wellings *et al*., 1975) and this histological origin is reflected in both rat and murine mammary tumors (Russo *et al*., 1990; Cardiff and Wellings, 1999). Carcinogen-induced mammary tumors in both rats and mice have identified critical periods of susceptibility during mammary gland development (Medina and Smith, 1999; Sivaraman *et al*., 1998) common to rodents as well as humans. Transplantable hyperplastic outgrowths provide a useful model of preneoplastic events and yielded much higher tumor frequencies than normal mammary glands following carcinogen treatment of hosts reflecting the enhanced risk associated with premalignant lesions (Medina, 1996; Medina *et al*., 1998;). Transgenic models have demonstrated the roles of specific pathways that, when altered, can induce mammary tumors (Amundadottir *et al*., 1995; Muller *et al*., 1998). Although these models have provided valuable insights into mechanisms of tumorigenesis, the resulting mammary tumors from many of the models lack alterations in p53 that are commonly observed in human breast cancers. Aneuploidy is also an infrequent feature in many of the models.

The frequent disruption of p53 function in human breast cancers suggests that this is a crucial event in the evolution of breast cancers. However, previous studies have not observed an increased susceptibility to mammary tumors in *p53*-deficient mice (Donehower *et al*., 1992; Jacks *et al*., 1994; Jerry *et al*., 1994). These experiments were limited by the use of mice with mixed genetic backgrounds (C57BL/6×129Sv) which are more resistant to mammary tumorigenesis than BALB/c mice (Medina and Smith, 1999, submitted). Second, the *p53*$^{null}$ mice succumb to thymic lymphoma at very early ages (less than 21 weeks) (Jacks *et al*., 1994) and preclude the detection of mammary tumors that may arise later. Indeed, mammary hyperplasias are observed frequently in *p53*$^{null}$ mice bearing the myc transgene (McCormack *et al*., 1998). Therefore, the *p53*$^{null}$ allele was backcrossed onto the BALB/c genetic

**Table 2** Relationships between treatments and aneuploidy in mammary tumors arising from $p53^{null}$ transplants

| | | *Aneuploid tumors* | | *Euploid tumors* | |
|---|---|---|---|---|---|
| *Treatment* | n | *Number aneuploid* [b] | *Aneuploid cells* [c] *% (Range)* | *Number euploid* [d] | *S phase fraction* [e] |
| Untreated | 4 | 3 (75%) | 36 (15–68) | 1 (25%) | 20% |
| Pituitary-stimulated | 5 | 5 (100%) | 56 (32–89) | 0 (0%) | – |
| DMBA (4 mg) | 7 | 3 (43%) | 25 (14–34) | 4 (57%) | 13.0% |
| DMBA (2 mg)+Pituitary-stimulated | 7 | 2 (29%) | 23 (18–28) | 5 (71%) | 13.5% |

[a]Treatment effects are described in Table 1. [b]The number of tumors (expressed as %) that had abnormal DNA content based on FACS analysis. [c]The mean percentage of cells that were aneuploid within a tumor sample that was aneuploid. [d]The number of tumors (expressed as %) that had normal DNA content based on FACS analysis. [e]The S phase fraction within euploid tumors provides an index of proliferation

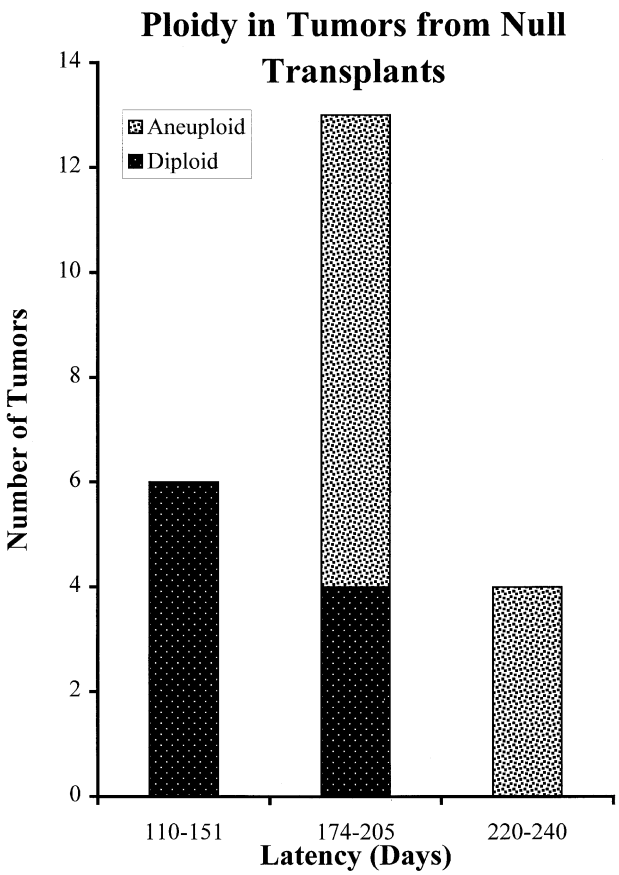


**Figure 3** Relationship between tumor latency and aneuploidy in mammary tumors from $p53^{null}$ transplants. Euploid tumors were associated with shorter latency, whereas aneuploid tumors were associated with longer latency periods. Aneuploid tumors were observed among all treatment groups

background for nine generations (Jerry *et al*., 1998) in order to assess mammary tumorigenesis. These mice were sufficiently congenic to allow transplantation of the $p53^{null}$ mammary epithelium into wild type BALB/c hosts. This approach allowed extended observation of the effects of the $p53^{null}$ allele in mammary glands without complications associated with effects of the $p53^{null}$ allele on other tissues.

The transplantation of BALB/c-$p53^{null}$ mammary epithelium provided clear and striking support for the notion that loss of *p53* function is a major determinant of susceptibility to mammary tumorigenesis. This was evident in the absence of tumors in $p53^{wt}$ mammary transplants in nulliparous hosts, whereas tumors were detected in 61.5% of the $p53^{null}$ transplants in these same hosts. Surprisingly, continuous hormonal stimulation with pituitary isografts resulted in tumors in 100% of $p53^{null}$ transplants, which is in stark contrast to the total absence of mammary tumors in $p53^{wt}$ transplants. DMBA treatment did not affect the final incidence of mammary tumors in $p53^{null}$ epithelia, but did result in a decreased tumor latency (Table 1).

The relatively small effect of DMBA treatment on mammary tumor appearance, as compared to hormonal stimulation, was unexpected. Treatment with DMBA is known to cause adduct formation in genomic DNA which, if improperly repaired, would result in mutations (Ross and Nesnow, 1999). In contrast, pituitary isografts provide a mitogenic stimulus similar to that encountered during mid-pregnancy (Christov *et al*., 1993), and therefore, is thought to act to expand a population of cells bearing initiating mutations. Given the role of p53 in recognizing and ensuring DNA repair, the effect attributable to DMBA was surprisingly small. The dramatic effect of chronic hormonal stimulation on $p53^{null}$ mammary epithelia suggests that, although DNA repair is impaired by the absence of *p53*, the major effect of *p53* in mammary epithelial cells must involve other cellular events. Examples of alternate mechanisms leading to tumors in $p53^{null}$ epithelia may include failure to ensure proper segregation of chromosomes or regulation of transcriptional activity of genes involved in redox cycling or angiogenesis.

The effects of hormone stimulation were assessed by two different procedures and yielded different results. Hormone stimulation by pituitary isografts provides continuous exposure to levels of prolactin, estrogen and progesterone similar to those attained in mid-pregnancy. This treatment results in continuous lobuloalveolar differentiation of the mammary gland and caused a very high incidence of mammary tumors in the $p53^{null}$ epithelial transplants. In contrast, hormonal stimulation by multiple parities provides sequential rounds of lobuloalveolar differentiation, lactation and involution with consequent fluxes in hormone levels. The latter procedure increased tumor incidence and decreased latency in $p53^{null}$ mammary transplants, but only modestly. The two procedures do not provide equivalent hormonal and developmental stimuli to the mammary gland, and thus, are not necessarily comparable. Continuous hormone stimulation by pituitary isografts identified a significant synergism between hormones and p53 status on tumor development. The results also raise the question of which of the three hormones elevated by pituitary stimulation is critical for tumorigenesis and what cellular and molecular events in these mammary glands are normally regulated by wild type p53 protein.

Disruption of *p53* function has been associated with increased genetic instability in mammalian cells (Bouffler *et al*., 1995; Fukasawa *et al*., 1996, 1997; Donehower *et al*., 1995). With this result in mind, the incidence and degree of aneuploidy in mammary tumors was analysed by flow cytometry. Only *p53*$^{null}$ mammary tumors were analysed because too few tumors were obtained from *p53*$^{wt}$ transplants to provide meaningful results. Overall, 57% (13/23) of the mammary tumors from *p53*$^{null}$ epithelial transplants exhibited aneuploidy. The euploid tumors were largely restricted to the DMBA treated groups where nine of the ten euploid tumors were observed. The relationship between latency and ploidy (Figure 3) suggests that tumors developing after a longer latency period are more likely to be aneuploid and that genetic instability is not an obligate feature of mammary pathogenesis associated with loss of p53 function. A similar conclusion was reached in analysis of mammary tumors arising in *wnt-1*/*p53*$^{null}$ bitransgenic mice (Donehower *et al*., 1995). The results reported herein, are the first that we are aware of that evaluate p53-dependent aneuploidy in mammary tumors as a function of tumor latency. The results suggest that the absence of the *p53* gene is affecting multiple cellular events and that the mechanisms generating aneuploidy are affecting cells over a long time period and are not necessary for tumor formation.

In summary, transplantation of *p53*$^{null}$ mammary epithelium offers a versatile model system in which to study the effects of the *p53* pathway on tumorigenesis. The results demonstrate that absence of *p53* renders the mammary epithelium highly susceptible to tumor development. The dramatic effects of hormonal stimulation on tumor incidence in *p53*$^{null}$ mammary transplants suggest that *p53* plays a pivotal role in regulating hormone-induced molecular processes.

## Materials and methods

### *Animals*

The BALB/c-*p53*$^{null}$ mice were derived by backcrossing the *p53*$^{null}$ allele (Jacks *et al*., 1994) onto the BALB/c genetic background as described previously (Jerry *et al*., 1998). Tissues for transplantation were obtained from mice at the 9th backcross generation. Procedures for transplantation of mammary epithelium into cleared mammary fat pads has been described previously (Medina, 1973, 1996). In these experiments, 21–24-day-old BALB/c females were used as transplant recipients. The endogenous mammary epithelium was surgically removed from the 4th inguinal glands to provide a cleared mammary fat pad. Single ducts were dissected from mammary glands of 8–10-week-old BALB/c-*p53*$^{null}$ and wild type BALB/c females and transplanted into the cleared mammary fat pads of recipients. Both *p*53$^{null}$ and *p*53$^{wt}$ mammary ducts were transplanted into contralateral glands of each recipient to ensure an identical host environment. Twenty-eight to thirty mice bearing transplants were prepared for each treatment group. One group of mice bearing transplants were not subjected to further manipulations to provide the untreated control group. The pituitary-stimulated group was prepared by implanting pituitary isografts under the kidney capsule at 5 weeks of age. The Multiparous treatment group was subjected to 3–5 cycles of pregnancy, lactation (10 days) and involution (10 days). The DMBA alone treatment group received 1 mg of 7,12-dimethylbenz($\alpha$)anthracene in oil by gavage four times at weekly intervals beginning at 8 weeks of age. The DMBA + pituitary isograft group received pituitary transplants at 5 weeks followed by 1 mg DMBA by gavage two times at weekly intervals beginning at 8 weeks of age. The two groups receiving DMBA were monitored weekly for tumors for up to 48 weeks. The remaining treatment groups were monitored for up to 60 weeks. In the DMBA treated groups, two mice each were lost due to treatment. A decreased number of successful takes was observed for the *p53*$^{null}$ transplants (125/141 = 88%) compared to the *p53*$^{wt}$ transplants (139/142 = 98%).

### *Histological procedures*

Mammary tumors were removed surgically and fragments were fixed in 10% neutral buffered formalin, then embedded in paraffin. Five micron sections were stained with hematoxylin and eosin for histopathological examination. Tissues that did not form tumors during the observation period were removed after 48 weeks (DMBA and DMBA + pituitary isograft treatments) or 60 weeks (untreated, pituitary-stimulation, and multiparous treatments). These tissues were fixed, defatted, then stained with hematoxylin (Medina, 1973) to allow examination of the entire gland for the presence of outgrowths and focal tumors. Wholemounts in which epithelium was absent represented unsuccessful transplants and were removed from the analyses.

### *Analysis of aneuploidy*

Flow cytometry was performed as described previously (Li *et al*., 1998). Cell suspensions were obtained by pepsin digestion of four to five paraffin sections (50 $\mu$m each). The suspension was filtered, then stained with propidium iodide. The sample was analysed on a FACScan flow cytometer and 20 000 events were collected from each sample. DNA histograms were produced using ModFit LT software (Verity Software House, Topsham, ME, USA).

### *Statistical analysis*

The tumor-free survival data were analysed using PROPHET 5.0 (BBN Systems and Technologies). Differences among survival functions were analysed using the Mantel-Cox test. The differences in tumor incidences were determined by the chi square procedure (Peto, 1974). Differences were considered statistically significant if $P < 0.05$.

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