Mice Expressing a Mammary Gland–Specific R270H Mutation in the *p53* Tumor Suppressor Gene Mimic Human Breast Cancer Development

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Abstract

The tumor suppressor gene *p53* has an apparent role in breast tumor development in humans, as ~30% of sporadic tumors acquire *p53* mutations and Li-Fraumeni syndrome patients carrying germ line *p53* mutations frequently develop breast tumors at early age. In the present study, conditional expression of a targeted mutation is used to analyze the role of the human R273H tumor-associated hotspot mutation in *p53* in mammary gland tumorigenesis. Heterozygous *p53*$^{R270H/+}$*WAP-Cre* mice (with mammary gland–specific expression of the *p53.R270H* mutation, equivalent to human R273H, at physiologic levels) develop mammary tumors at high frequency, indicating that the R270H mutation predisposes for mammary gland tumor development and acts in a dominant-negative manner in early stages of tumorigenesis. Spontaneous tumor development in these mice is further accelerated by 7,12-dimethylbenz(*a*)anthracene (DMBA) treatment at young age. The majority of spontaneous and DMBA-induced carcinomas and sarcomas from *p53*$^{R270H/+}$*WAPCre* mice is estrogen receptor α positive, and expression profiles of genes also implicated in human breast cancer appear similarly altered. As such, *p53*$^{R270H/+}$*WAPCre* mice provide a well-suited model system to study the role of *p53* in breast tumorigenesis and the responsiveness of mammary gland tumors to chemotherapeutics. (Cancer Res 2005; 65(18): 8166-73)

Introduction

Breast cancer is the most frequent tumor type among women worldwide with more than 1,000,000 new cases diagnosed every year (1). Human breast cancer is associated with different somatic alterations such as mutations in oncogenes and tumor suppressor genes, resulting in deregulation or loss of function of multiple, essential genes. The most frequently mutated gene in sporadic breast tumors is the tumor suppressor gene *p53* (2). The frequency of acquired *p53* mutations in primary breast carcinomas is ~30%, with the gene mutation often accompanied by loss of heterozygosity (LOH; ref. 3). Breast tumor progression seems to be associated with mutant *p53*, as illustrated by a higher frequency of *p53* mutations in patients with advanced disease. Furthermore, the prevalence of *p53* mutations is higher in recurrent tumors than in the primary ones (4). Finally, specific *p53* mutations are associated with resistance to doxorubicin therapy in breast cancer patients (5). Taken together, these observations imply that acquiring *p53* mutations in breast cancer predisposes to increased tumor malignancy. The role of *p53* mutations early in breast cancer is further supported by the observation that Li-Fraumeni syndrome patients, carrying germ line *p53* mutations, are predisposed to developing breast cancer at a relatively early age (6). Codons R175, R248, and R273 are the most common hotspots for mutations in both sporadic and hereditary *p53* associated human breast cancer (7).

Given the apparent important role of *p53* in preventing breast tumor development in humans, several attempts were made to generate mouse models with defects in *p53* to study mammary gland tumor development. *p53* knockout mice ($p53^{-/-}$) were generated and extensively studied (8). Although several important insights were obtained from these studies, *p53* knockout mice did not fully recapitulate the spectrum of tumors found in Li-Fraumeni patients (9). In particular, the breast tumors associated with germ line mutation of *p53* in humans did not arise in $p53^{-/-}$ mice, and were only observed in heterozygous animals in a specific genetic background (10, 11). This could be due to the fact that in humans, null mutations are rarely found; rather, 50% of human tumors harbor a point mutation in the *p53* gene. Mutated *p53* might have a completely different effect on (breast) tumorigenesis than loss of the gene. In fact, several *in vitro* studies suggest that mutant *p53* has dominant-negative or gain of function properties distinct from *p53* loss of function (12–15). Alternatively, early death due to lymphoma could be masking a phenotype in the mammary gland of $p53^{-/-}$ mice. Indeed, mammary gland transplantation studies involving *p53* knockout mice (10, 16) showed an increase in tumor burden and incidence after 7,12-dimethylbenz(*a*)anthracene (DMBA) treatment in combination with hormonal stimulation (16). In addition, epithelium-specific deletion of the *p53* gene in recently developed conditional mouse models resulted in spontaneous mammary tumor development (17, 18). Recently, several strains of mice were reported that harbor targeted mutations of *p53* in the endogenous gene locus (19–25). In particular, two different mouse models for Li-Fraumeni syndrome, with tumor-derived mutations in *p53*, were described (19, 20): *p53.R172H* and *p53.R270H*. In both cases, expression of mutant *p53* in the absence of wild-type *p53* resulted in a shift in tumor spectrum compared with $p53^{-/-}$ mice, indicative of an *in vivo* gain of function property

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of mutant *p53* (19). In addition, dominant-negative effects were observed in mouse embryonic fibroblasts and thymocytes, as was earlier found for heterozygous *p53.R270H* cells (26). *In vivo* expression of the mutation in all tissues was achieved using *Protamine-Cre* transgenic mice, resulting in a broad variety of tumors (19), but hardly any mammary tumors were found. Because one of the strengths of conditional mouse technology is the potential for tissue-specific analyses, we use the *p53.R270H* model in the present study to analyze the role of the *p53.R270H* mutation in spontaneous and carcinogen-induced mammary gland tumorigenesis. Expression of the mutation in mammary tissue was achieved by crossing *p53.R270H* mutant mice with mammary-specific *Cre* transgenic mice having *Cre* recombinase under the control of the hormone-inducible Whey Acidic Protein (*WAPCre* mice; ref. 27). We show that $p53^{R270H/+}$*WAPCre* mice develop both spontaneous as well as carcinogen-induced mammary tumors at high frequency, indicating that the R270H mutation in *p53* predisposes for mammary tumor development in mice.

Materials and Methods

Generation of *p53.R270HWAPCre* Mice

Conditional *p53.R270H* mice were generated by homologous recombination of mutant targeting vectors in J1 embryonic stem cells (19). Cloning of the *p53.R270H* mutant targeting vector and homologous recombination experiments in embryonic stem cells were described elsewhere (19, 26). The targeting vector with the stop cassette in intron 1 flanked by loxP sites is depicted in Fig. 1*A*. Genotyping of the mice was done by a PCR/digestion-based assay. The following primers were used to amplify the *p53* alleles: p53in7#2: 5′-TTGGGCTTAGGGACGTCTCTTATC-3′ (intron 7); p53in9#1: 5′-ATGCGACTCTCCAGCCTTGGTA-3′ (intron 9).

The resulting PCR product (486 bp) was digested with *Nla*III. The *p53.R270H* mutation results in a new *Nla*III site in the PCR product resulting in two products of 269 and 217 bp, discriminating the mutant from the wild-type allele.

Mice expressing *Cre* recombinase under the control of a Whey Acidic Protein promoter, *WAPCre* mice, were used to induce *Cre*-mediated deletion of the floxed stop cassette specifically in the mammary gland (26). *WAPCre* mice [B6129-TgN(WAP-Cre)11738Mam, in a mixed 129Sv/C57BL/6 background] were obtained from The Jackson Laboratory (Bar Harbor, ME). The presence of *Cre* recombinase was determined by PCR (product size, 676 bp) using the following primers: Cre 3, 5′-GCTGGC-TGGTGGCAGATGG-3′; Cre 5, 5′-GTTCAGGGATCGCCAGGCG-3′.

For all studies described, heterozygous *p53.R270H* mice and wild-type littermates in backcross generation F2-F3 ($129S_4$/SvJae to C57BL/6) were crossed to *WAPCre* mice to generate respectively $p53^{R270H/+}$*WAPCre* and $p53^{+/+}$*WAPCre* mice. Mice used in these experiments contained an estimated C57BL/6 allele contribution of 75% to 81.25%. Subsequent mating of female mice resulting in pregnancy and lactation of the litters was necessary for activation of the *WAP* gene promoter in the mammary gland of the dams, resulting in expression of *Cre* recombinase.

In Vivo Expression of the *p53.R270H* Mutant Allele

Female $p53^{R270H/+}$*WAPCre* mice (8-12 weeks old) were mated with $p53^{R270H/+}$ mice. After either one or two pregnancies, followed by lactation of the litters, the mammary gland of the dams was isolated, snap-frozen in liquid nitrogen, and stored at −80°C. RNA isolation of tissues was done following the procedure of the manufacturer (Qiagen, Valencia, CA). The following primers were used for cDNA synthesis and the first PCR reaction (Titan One Tube RT-PCR system; Roche, Indianapolis, IN), amplifying exons 7 to 11 of the *p53* gene: 1156, 5′-TTCGCCACAGCGTGGTGGTACC-3′; 1157, 5′-AGAAGGGACCGGGAGGATGTG-3′.

A second PCR reaction, amplifying exons 7 to 8, was done with nested primers: 1150: 5′-tgtaaaacgacggccagt CGTGGTGGTACCTTATGAGCCA-3′; 1045, 5′-caggaaacagctatgacc TCTCCATCAAGTGGTTTTTT-3′.

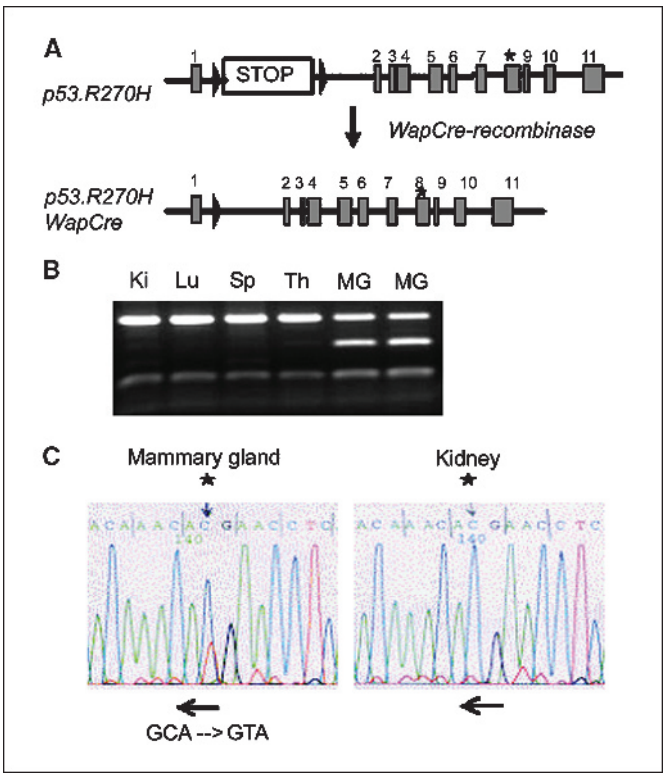

Figure 1. Characterization of mammary gland–specific *p53.R270H* expression in tissues of $p53^{R270H/+}$WAPCre females. *A,* schematic representation of the targeted conditional *p53* allele with the transcriptional stop cassette flanked by loxP sites in intron 1 and the R270H mutation in exon 8 (*asterisk*; ref. 19). Lower map is the floxed *p53* allele after excision of the stop cassette by *Cre* recombinase (*p53.R270HWAPCre*). *B,* RT-PCR analysis followed by *Nla*III digestion to determine *p53.R270H* expression in different tissues of $p53^{R270H/+}$*WAPCre* females after one pregnancy. *Lanes 1* to *4,Nla*III digestion products of wild-type *p53* allele in kidney, lung, spleen, and thymus. *Lanes 5* and *6,Nla*III digestion products of both wild-type *p53* and R270H mutated *p53* in mammary glands isolated from two individual mice (see Materials and Methods for fragment sizes). *C,* sequence analysis of mammary gland and kidney cDNA of a young adult female $p53^{R270H/+}$*WAPCre* mouse. *Asterisk,* C to T mutation in codon 270 of the *p53* gene in mammary gland but not in control kidney.

Expression of the *R270H* point mutation was determined in the obtained PCR product of 333 bp by digestion with *Nla*III. Digestion of wild-type *p53* resulted in four products of 239, 67, 18, and 9 bp, whereas in the presence of the *R270H* mutation, five products are generated (157, 82, 67, 18, and 9 bp).

Sequencing. After purification of the second PCR product using the QIAquick PCR purification kit (Qiagen), DNA concentrations were determined. PCR product, 3 to 10 ng, was amplified in a subsequent sequence PCR reaction with the BigDye Terminator Reaction kit (Applied Biosystems, Foster City, CA) and 3.2 pmol of primer 1150 or 1045 (described above) in the forward or reverse sequence reaction, respectively. Sequence analysis was done on a 3700 Genetic Analyzer (Applied Biosystems) using Sequencing Analysis 3.7 software.

Analysis of Spontaneous and 7,12-dimethylbenz(*a*)anthracene–induced Mammary Gland Tumor Development

Spontaneous mammary tumor development was determined in groups of female $p53^{R270H/+}$*WAPCre* mice and $p53^{+/+}$*WAPCre* littermates receiving 0.1 mL sunflower oil by gavage weekly for 6 weeks. Heterozygous $p53^{F2-10/+}$ mice (17), crossed with *WAPCre* mice to delete exons 2 to 10 of one *p53* allele specifically in the mammary gland, were exposed to the same protocol and used as control animals in this experiment.

In the DMBA exposure study, 4-week-old $p53^{R270H/+}$*WAPCre* mice and $p53^{+/+}$*WAPCre* littermates were treated once a week during 6 subsequent

weeks by gavage with 1 mg DMBA (Sigma, St. Louis, MO) dissolved in 0.1 mL sunflower oil. After 6 weeks of treatment, females were bred to activate the *WAPCre* gene and, consequently, the *p53.R270H* mutation. All mice were weighed weekly and checked for the development of tumors until the age of 78 weeks. Moribund animals or those with visible tumors were killed as well as the surviving mice at the end of the experiment. Tumors and tissues were collected and processed for histopathology and DNA/RNA isolation following standard procedures.

Histology and Immunohistochemistry

Collected tissues were preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde (10% neutral buffered formalin). The tissues were embedded in paraffin wax, sectioned at 5 μm, and stained with H&E for histopathologic evaluation. p53 protein accumulation was detected using the polyclonal CM5 antibody (1: 400; Novocastra Laboratories, Newcastle, United Kingdom), which recognizes several epitopes of both wild-type and mutant mouse p53 protein. CM5 immunostaining was done as described earlier (28) using a secondary goat anti-rabbit/biotin antibody (Vector Laboratories, Burlingame, CA) and subsequently a streptavidin-complex peroxidase Elite kit (Vector Laboratories). Expression of the estrogen receptor α was analyzed using the estrogen receptor α rabbit polyclonal antibody MC-20 (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) using the same protocol as described for CM5. For antigen retrieval, deparaffinized tissue sections were heated for 30 minutes in a 10 mmol/L citrate buffer (pH 6.0) at 95°C.

Molecular Analysis of Tumors and Tissues

Expression of the R270H point mutation. Expression of the point mutation was determined as described above in mammary glands, tumors, and control tissues of both unexposed and DMBA-exposed $p53^{R270H/+}$*WAPCre* females.

Determination of loss of the wild-type *p53* allele (loss of heterozygosity) in mammary tumors. Both PCR (as described above) and Southern blot analysis were done to detect loss of the wild-type *p53* allele. Genomic DNA was isolated from mammary tumors following standard procedures (Maniatis Laboratory Manual). After restriction with the *Msl*I restriction enzyme and electrophoresis, DNA was transferred to nylon membranes (Hybond-N+, Amersham, Piscataway, NJ). A cDNA probe of 343 bp (*Sac*II to *Kpn*I fragment) spanning exons 7 to 10 of the *p53* gene was randomly labeled with [^{32}P]dCTP and used for hybridization (29). The size of the fragment was 2,976 and 1,496 bp for the wild-type and mutated *p53* allele, respectively. Quantification of LOH was done by calculating the signal of the wild-type allele divided by the signal of the mutant allele using kidney of the same mice as internal control for hybridization efficiency.

Analysis of Gene Expression Profiles in Mammary Glands and Mammary Tumors

Macroarray analysis of gene expression profiles in normal mammary glands and mammary tumors was done using two different GEarray Q series kits (i.e., the Mouse Cell Cycle Gene Array and the Mouse p53 Signaling Pathway Gene Array kit (SuperArray Biosciences, Frederick, MD). For this analysis, three to four RNA samples of different tumors isolated from animals with the same genotype and treatment protocol were pooled.

Pooled total RNA (3.5 μg) was used as a template for [^{32}P]cDNA probe synthesis. Subsequently, the probe was hybridized overnight to membranes containing 96 gene-specific cDNA fragments supplemented with four house keeping genes (*GAPDH, cyclophilin A, RPLA13A*, and *actin*) as positive controls, and pUC18 plasmid DNA and blank spots as negative controls. Analysis of the spots was done by scanning the membranes on a PhosphorImager/Storm 860 (Molecular Dynamics, Sunnyvale, CA) and quantifying the spots using the *Total*Lab program version 2.00 (Nonlinear Dynamics, Durham, NC).

Statistical Analysis

Statistical analyses of tumor-free survival curves included calculation of Kaplan-Meier distributions of survival of two different treatment groups and comparison by a two-sided log-rank test (SPSS version 11).

Results

Mammary gland–specific expression of the *p53.R270H* point mutation. To test whether the induction of *WAPCre* leads to mammary gland–specific removal of the transcriptional stop cassette and subsequent expression of the *p53.R270H* mutation in the mammary gland (Fig. 1*A*), we determined expression of the mutation in the mammary gland and control tissue of different $p53^{R270H/+}$*WAPCre* female mice after one full-term pregnancy. The *p53.R270H* mutation is highly expressed in the mammary gland (Fig. 1*B*), whereas in other tissues of the same mouse (e.g., kidney, lung, spleen, and thymus), only the wild-type *p53* allele was detectable (Fig. 1*B*). These observations were underscored by a reduction in the presence of the transcriptional stop cassette in intron 1 in mammary gland tissue but not in control tissue of $p53^{R270H/+}$*WAPCre* mice after pregnancy, analyzed by PCR using genomic DNA (data not shown). In addition, the CGT→CAT mutation (in the reverse reaction GCA→GTA) was clearly present in the cDNA of the mammary gland, but was not detectable in the kidney (Fig. 1*C*). All mice analyzed showed the same expression pattern of the point mutation.

Immunohistochemical staining of $p53^{R270H/+}$*WAPCre* mammary glands with the CM5 clone revealed discrete positive cells in a young animal after one pregnancy (Fig. 2*A* and *B*), indicating that the *p53.R270H* point mutation is already detectable at the protein level in mammary glands of young $p53^{R270H/+}$*WAPCre* mice (i.e., 13 weeks). Furthermore, the number of *p53* mutant cells increased in time as normal (nonhyperplastic) mammary glands of older $p53^{R270H/+}$*WAPCre* females showed increased levels of CM5 staining (Fig. 2*C* and *D*), presumably indicative of clonally expanded mutant cells.

Effects of *p53.R270H* expression on spontaneous mammary tumorigenesis in mice. Female $p53^{R270H/+}$*WAPCre*, $p53^{F2-10/+}$*WAPCre*, and $p53^{+/+}$*WAPCre* littermates were subjected to one pregnancy to induce either expression of the *p53.R270H* mutation or deletion of exons 2 to 10, and subsequently followed for the

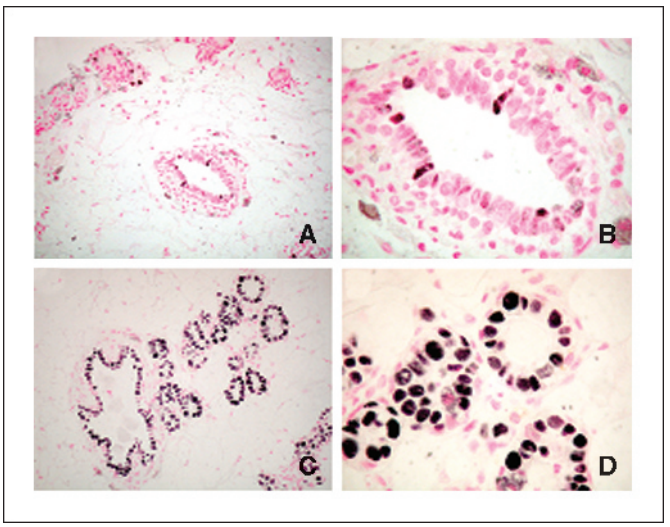

Figure 2. CM5 staining of normal mammary gland tissue to determine p53 protein expression after one pregnancy in young adult $p53^{R270H/+}$*WAPCre* female mice. *A* and *B*, mammary gland tissue of a 13-week-old female mouse at time of weaning of the litters (i.e., 3 weeks after expression of the *p53.R270H* point mutation). Some ductal cells stain positively. *C* and *D*, strong CM5 positive staining in mammary gland tissue 8 weeks after *p53.R270H* expression. Objective used is 20× (*A* and *C*) or 40× (*B* and *D*).

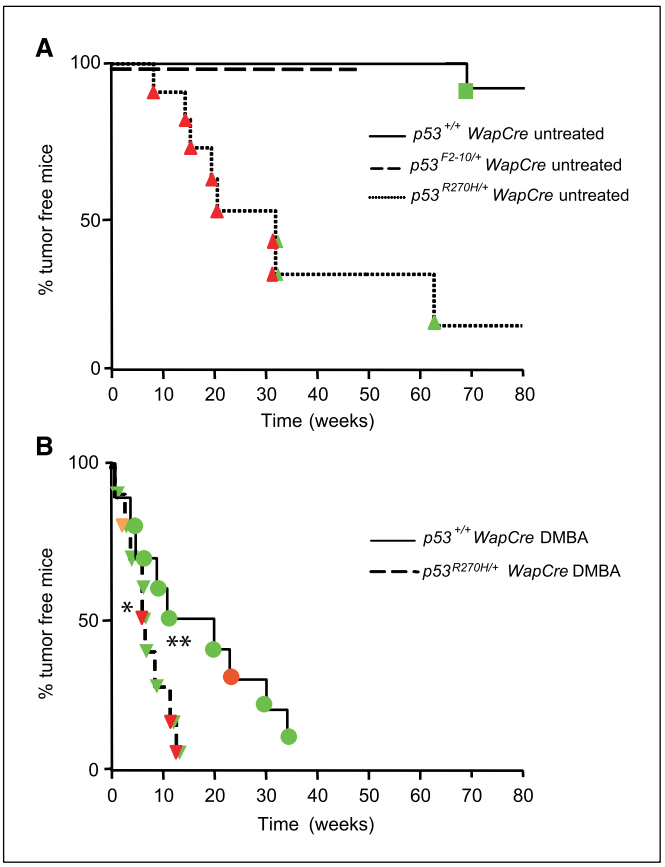

Figure 3. Effects of expression of the *p53.R270H* mutation on spontaneous and DMBA-induced mammary tumorigenesis. *A,* tumor-free survival curves of untreated $p53^{R270H/+}$*WAPCre,* $p53^{F2-10/+}$*WAPCre*, and $p53^{+/+}$*WAPCre* mice. Time is depicted in weeks after *p53.R270H* expression or exon 2 to 10 deletion, set at time of birth of the litters. Tumor types were classified into mammary tumors (*red*) and other tumors (*green*). *B,* tumor-free survival curves of DMBA-treated $p53^{R270H/+}$*WAPCre* and $p53^{+/+}$*WAPCre* mice. Time is depicted in weeks after *p53.R270H* expression, set at time of birth of the litters. Tumor types were classified into mammary tumors (*red*), mammary gland hyperplasia (*orange*), and other tumors (*green*). Survival curves were computed using the Kaplan-Meier product limit method. *, statistically significant reduction in tumor-free survival between untreated and DMBA-treated $p53^{R270H/+}$*WAPCre* mice ($P < 0.001$). **, statistically significant reduction in tumor-free survival between DMBA-treated $p53^{R270H/+}$*WAPCre* and $p53^{+/+}$*WAPCre* mice ($P < 0.05$).

development of mammary tumors for a total time period of 18 months. Figure 3*A* shows the percentage of tumor-free untreated $p53^{R270H/+}$*WAPCre,* $p53^{F2-10/+}$*WAPCre*, and $p53^{+/+}$*WAPCre* mice. Clearly, mice with a heterozygous *p53.R270H* mutation are highly susceptible for mammary tumor development. Seventy-three percent of the moribund $p53^{R270H/+}$*WAPCre* mice were tumor-bearing, with seven of eight mice developing one or more tumors of the mammary gland. The first mammary tumor in a $p53^{R270H/+}$ *WAPCre* female already appeared 8 weeks after induction of the *p53.R270H* mutation. The mean latency time for spontaneous mammary tumor development was 19.7 ± 8.6 weeks in this group (Table 1). Preliminary results with $p53^{F2-10/+}$*WAPCre* mice revealed no development of mammary tumors until 48 weeks after deletion of one functional *p53* allele (Fig. 3*A*), in line with recent results described by others (18). Only 2 of the 11 untreated $p53^{R270H/+}$ *WAPCre* mice survived the experimental period of 78 weeks, whereas 9 of 10 $p53^{+/+}$ littermates survived. Apart from the mammary tumors, some untreated *p53.R270H* mutant mice developed an additional sarcoma in the abdomen, lymphoma, or tumor of the lung. $p53^{+/+}$*WAPCre* control animals showed normal background pathology and, as such, no primary mammary tumors were found (Fig. 3*A*).

Development of 7,12-dimethylbenz(*a*)anthracene–induced mammary tumors in *p53.R270HWAPCre* mice. It is thought that malignant tumors in humans occur through interactions of multiple environmental and genetic factors. To investigate whether spontaneous mammary tumor development in $p53^{R270H/+}$*WAPCre* mice could be further accelerated or increased in incidence by exposure to a chemical compound, $p53^{R270H/+}$*WAPCre* female mice were exposed to a weekly oral dose of the chemical carcinogen DMBA for 6 subsequent weeks. Although none of the DMBA-treated $p53^{R270H/+}$*WAPCre* mice went through a full-term pregnancy, 40% of them did develop mammary tumors (Fig. 3*B*; Table 1), an incidence clearly lower compared with untreated $p53^{R270H/+}$ *WAPCre* females (64%). Nevertheless, the mean latency time for mammary tumor development was 8.5 ± 4.7 weeks and significantly shorter compared with untreated $p53^{R270H/+}$*WAPCre* mice (Kaplan-Meier, $P = 0.0046$). Evidently, exposure to DMBA at an early age accelerates mammary tumor development in $p53^{R270H/+}$ *WAPCre* mice. Other tumors that occurred in the DMBA-treated mice included lymphomas, ovary, lung, stomach, uterus, and skin tumors. A comparable DMBA-induced tumor spectrum was found in treated $p53^{+/+}$*WAPCre* mice (Fig. 3*B*), however, the latency time for (mammary) tumor development in DMBA-treated $p53^{+/+}$ *WAPCre* mice was significantly decelerated compared with treated $p53^{R270H/+}$*WAPCre* mice (Kaplan-Meier, $P = 0.047$).

Histologic characterization of mammary gland tumors. Mammary tumors found in both untreated and DMBA-exposed $p53^{R270H/+}$*WAPCre* mice were heterogeneous, including the frequently found adenocarcinomas, solid carcinomas, papillary carcinomas, and adenosquamous carcinomas (as classified in

Table 1. Tumor spectrum in $p53^{R270H/+}$*WAPCre* mice

	Untreated	DMBA
Total number of mice analyzed	11	10
Tumor-bearing mice	8 (73%)	9 (90%)
Mammary gland tumor	7 (64%)	4 (40%)
Carcinoma*	3/7	2/4
Sarcoma/carcinosarcoma†	4/7	2/4
Intraepithelial neoplasia	0	0
Mammary gland hyperplasia	2	1
Lymphoma	1	6
Ovary/uterus	0	1
Other‡	2	3
Mean mammary gland tumor latency (wk)§	19.7 ± 8.6	8.5 ± 4.7*

*Carcinomas could be classified in adenocarcinomas, solid carcinomas, papillary carcinomas, and adenosquamous carcinomas.
†Sarcomas found consisted of carcinosarcomas and fibrosarcomas.
‡Other tumors were found in lung (bronchioalveolar carcinoma), abdominal cavity (sarcoma), stomach (squamous cell papilloma and carcinoma), and skin (sebaceous cell adenoma, squamous cell papilloma, and carcinoma).
§ Mean latency time is depicted as the number of weeks after expression of the *p53.R270H* point mutation ($t = 0$ at birth of the litters) was statistically significantly reduced when compared with untreated mice (Kaplan-Meier analysis, $P = 0.0046$).

ref. 30). Carcinosarcomas and sarcomas of the mammary gland were also found (Fig. 4*A-F*). In the majority of mammary tumors, the number of *p53* positive cells was greatly increased compared with normal mammary glands of young *p53* mutant mice (Fig. 4*G-I*), suggesting these tumors originated from a clonally expanded *p53.R270H* mutant epithelial cell. These results were confirmed by molecular analysis of mammary tumors using reverse transcription-PCR (RT-PCR). All, except one, mammary tumors were found to express the point mutant *p53* allele (data not shown), indicating that *WAPCre*-induced expression of the R270H mutant protein is linked to mammary tumor development.

Estrogen is thought to be play an important role in the pathogenesis of human breast tumors, with the estrogen receptor α acting as mediator of estrogen responsiveness in human breast cancer (31). Unfortunately, most mouse models of breast cancer exclusively develop estrogen receptor α–negative tumors (32). However, in our study, 67% (10 of 15) of the mammary tumors obtained from untreated as well as DMBA-treated $p53^{R270H/+}$ *WAPCre* mice appeared estrogen receptor α positive to varying degrees (Fig. 4*J-L*). The group of estrogen receptor α–positive tumors consisted of both carcinomas as well as sarcomas, and the grade of estrogen receptor α positivity seemed to be not associated with a specific mammary tumor or cell type.

Molecular analysis of spontaneous and 7,12-dimethylbenz(*a*)anthracene–treated tumors. In human breast cancer, *p53* mutations are frequently accompanied by LOH (3). Here, the majority (6 of 7, 86%) of mammary tumors from $p53^{R270H/+}$*WAPCre* mice showed evidence of LOH at the *p53* locus (Fig. 5*A*), ranging from 8% (*lane 3*) to 46% loss (*lane 8*). Samples with only a partial loss of the wild-type allele may result either from LOH in a subset of tumor cells or from infiltration of the tumor with wild-type stromal cells. Only tumor #3 almost completely retained the wild-type fragment (8% loss). These observations indicate a selective advantage for *p53* LOH (late) in mammary tumor development in this model.

In humans, various breast tumor subtypes harbor distinct profiles of gene expression that are likely to reflect basic differences in the cell biology of the tumors (33, 34). Here, differences in gene expression profiles between normal mammary gland versus mammary tumors and spontaneous versus DMBA-induced mammary gland tumors were analyzed by macroarray analyses, focusing on cell cycle and *p53*-signaling genes. A selection of the analyzed genes is shown in Fig. 5*B*. Clearly, gene expression profiles change during mammary tumor development in both DMBA-treated as well as untreated animals, with a high number of genes down-regulated in mammary tumors. For instance, *cyclin G, p57Kip2*, and *cyclin E* levels are significantly lower in tumors of both untreated as well as DMBA-exposed $p53^{R270H/+}$*WAPCre* mice compared with normal mammary glands. Up-regulation in tumors is detected for the cell proliferative marker *Ki67*, and *cyclin D1*, a breast tumor-related oncogene, abnormally accumulated in up to 35% of human breast cancer cases (35). For these and other genes like *GADD45, cyclin D3*, and *Noxa*, differences in gene expression are dependent on DMBA treatment (Fig. 5*B*), indicating that differences exist in gene expression profiles of mammary gland tumorigenesis in untreated and DMBA-treated $p53^{R270H/+}$*WAPCre* mice.

Discussion

In the present study, we analyzed development of mammary tumors in a conditional mouse model with the *p53.R270H* missense mutation, equivalent to human R273H, targeted into the genome of the mouse. Importantly, the wild-type and mutant p53 proteins are

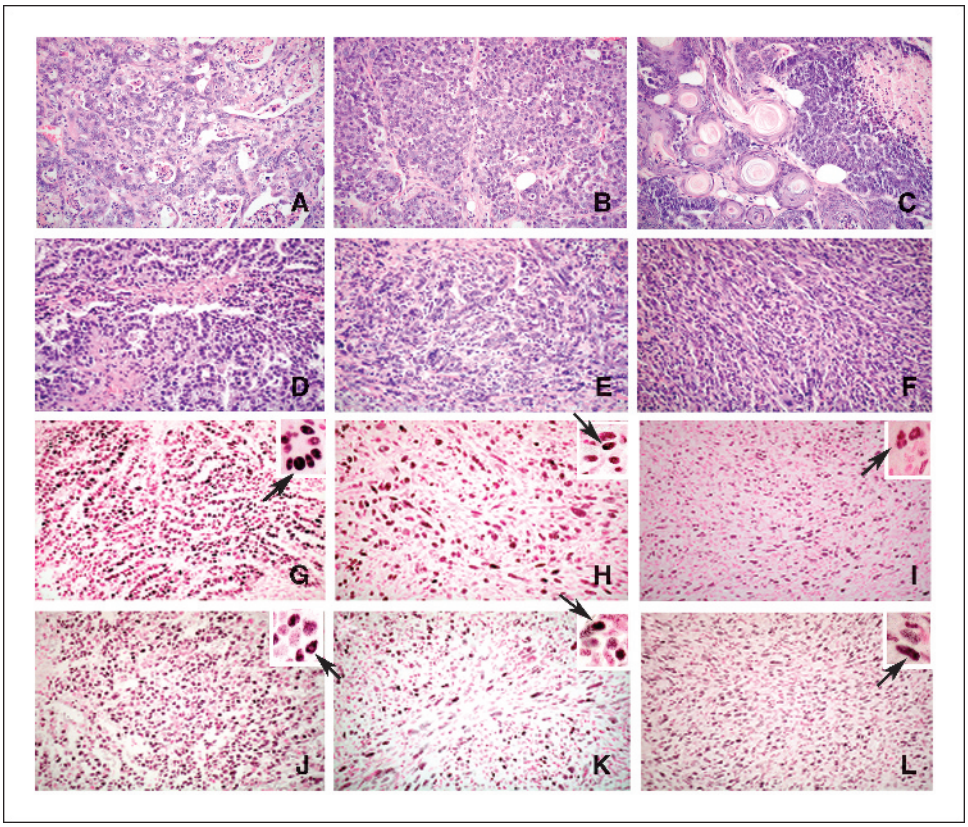

Figure 4. Histologic characterization of mammary tumors. Primary mammary tumors from $p53^{R270H/+}$*WAPCre* mice show various histopathologic features, including acinar carcinoma (*A*), adenocarcinoma (*B*), adenosquamous carcinoma (*C*), papillary carcinoma (*D*), carcinosarcoma (*E*), and sarcoma (*F*). Carcinosarcomas were characterized by both malignant epithelial components and poorly defined malignant stromal components. The sarcomas found were poorly differentiated tumors of mesenchymal origin. Molecular phenotypes of mammary tumors were determined using CM5 (p53) and MC-20 (estrogen receptor α) stainings. Examples of p53- and estrogen receptor α–positive tumors are a papillary carcinoma showing positive epithelial lining (*G* and *I*), carcinosarcoma with positive epithelial and mesenchymal staining (*H* and *K*), and sarcoma (*I* and *L*) with positive mesenchymal cells. Objective used for all pictures is 20×. *Insets,* stained cells (*arrows*) at a higher magnification (40×).

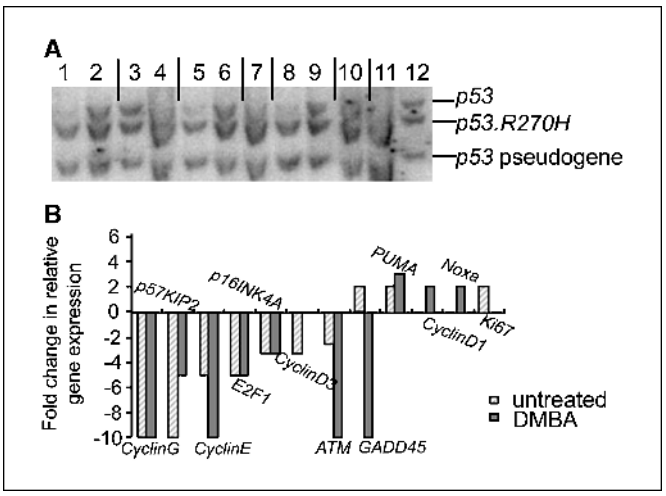

Figure 5. Molecular analysis of spontaneous and DMBA-induced mammary tumors. *A,* LOH analysis by Southern blotting in spontaneous mammary tumors (*1, 3, 5, 7, 8, 10*, and *11*) of $p53^{R270H/+}$*WAPCre* mice. *Mse*I-restricted tumor DNA was hybridized with a *p53* cDNA probe. Kidney DNA of the same mouse was used as control DNA (*2, 4, 6, 9*, and *12*). LOH was quantified by calculating the percent loss of the wild-type allele in a mammary tumor by using kidney as an internal control for hybridization efficiency (mean loss in kidney, 34.7%). Percent LOH in mammary tumors is 40% (*lane 1*), 8% (*lane 3*), 40% (*lane 5*), 46% (*lane 7*), 43% (*lane 9*), 23% (*lane 10*), and 41% (*lane 11*). *B,* RNA expression levels of cell cycle– and *p53* signaling–related genes were determined in mammary glands and mammary tumors from untreated and DMBA-treated mice. Changes in the relative expression level of the different genes were determined in tumors and normalized to the expression level in normal mammary glands. A selection of down-regulated and up-regulated genes is depicted in bars for both spontaneous (*striped bars*) and DMBA-induced tumors (*filled bars*).

expressed at physiologic levels (19). Heterozygous $p53^{R270H/+}$ mice developed a high frequency of spontaneous mammary tumors after tissue-specific expression of the *p53.R270H* mutation using *WAPCre* mice. The mean latency time for tumor development in $p53^{R270H/+}$ *WAPCre* mice was strongly reduced when compared with previous studies with tissue-specific transplantation models (16), even though the BALB/c mouse strain used in the earlier studies is much more sensitive for mammary tumor development than C57BL/6 / 129 mice (11, 36), the genetic background of mice used here.

We here show that heterozygous loss of *p53* in $p53^{F2-10/+}$ *WAPCre* mice does not predispose for spontaneous mammary tumors up to 48 weeks after *Cre* expression, whereas, at this time point, 64% of $p53^{R270H/+}$*WAPCre* mice had developed one or more mammary gland tumors (Fig. 3*A*). These observations clearly show that the *p53.R270H* mutation may have dominant-negative properties. In addition, comparison of mammary tumor development in $p53^{R270H/+}$*WAPCre* mice with $p53^{R270H/F2-10}$ and $p53^{F2-10/F2-10}$ mice[5] reveals that latency times are not significantly different between the three genotypes (P = 0.86). These results point towards a lack of gain of function mechanisms of the *p53.R270H* mutant in the mammary gland. Interestingly, because in a previous study Olive et al. (19) did show gain of function properties of this *p53* mutant allele mainly in the lung using the same mouse model, gain of function properties of *p53* apparently are tissue specific. In summary, mammary tumor development in $p53^{R270H/+}$*WAPCre* mice is evidently not simply caused by functional inactivation of one *p53* allele, but rather shows functional inactivation of both alleles through dominant-negative action of mutant *p53*, as also described previously for these mice (19). Further indication for this was provided by LOH analysis of spontaneous $p53^{R270H/+}$*WAPCre* tumors. Although we observed the majority of mammary tumors (partly) losing the wild-type *p53* allele, the wild-type fragment could still be detected, indicating that a proportion of tumor cells retained the wild-type *p53* allele. We therefore hypothesize that the *p53.R270H* mutation has a dominant-negative effect in early stages of mammary tumorigenesis, resulting in overall genome instability, followed by LOH later in tumor development. However, to address this, further studies are needed in analyzing LOH in preneoplastic stages of mammary gland development in $p53^{R270H/+}$*WAPCre* mice.

Tumor types found were adenocarcinomas and (carcino)sarcomas, the former also frequently found in human breast cancer patients, the latter uncommon in mammary glands of mice and man. In general, sarcomas are often found in $p53^{+/-}$ mice (8) and Li-Fraumeni syndrome patients (9), but no sarcomas of the mammary gland were reported thus far. However, (carcino)sarcomas of the mammary gland were found recently in conditional $p53^{F2-10/F2-10}$*K14Cre* mice, with homozygous *p53* deletion of exons 2 to 10 in epithelia,[6] pointing towards a role for defective *p53* in mammary sarcoma development.

Apart from mammary gland tumors, some untreated *p53.R270H* mutant mice developed additional tumors. Expression of the *p53.R270H* mutation seemed to be not exclusively restricted to the mammary gland, but was also visible in some tumors and kidneys of older mice. This is most likely caused by some *Cre* expression in other tissues than the mammary gland, as was also described by others (18, 27). Another possibility is minor leakiness of the stop cassette, resulting in low levels of transcription of the mutant *p53* allele in some tissues when mice age.

Steroid status is one of the main differentiating characteristics of human breast cancer. About 70% of human breast tumors are estrogen receptor α positive and estrogen receptor dependent; however, mouse models rarely produce estrogen receptor α–positive mammary tumors (32). Therefore, it would be highly valuable to have mouse models developing estrogen receptor α–positive as well as estrogen receptor α–negative mammary tumors to study the factors that control estrogen receptor α expression and the effect of therapeutics. In the present study, mammary tumors of both estrogen receptor types were found in $p53^{R270H/+}$ *WAPCre* mice, with the pattern of estrogen receptor α expression (groups of contiguous cells) similar to that found in human breast carcinomas (37). These results resemble those reported recently (18), where homozygous conditional deletion of mouse *p53* in mammary epithelial cells by *WAPCre* also led to estrogen receptor α–positive mammary tumors in mice. Apparently, inactivation of *p53* induced by *WAPCre* expression, either through complete loss of both alleles or expression of mutant variants, directs estrogen receptor α–positive tumor development.

It is generally agreed that environmental factors and somatic genetic events are the predominant contributors to the development of sporadic cancer. Whether exposure to environmental compounds also has a significant effect on cancer development in the presence of an inherited, dominant mutant *p53* allele was examined by exposing $p53^{R270H/+}$*WAPCre* mice to the mammary carcinogen DMBA. Others have shown cooperation of DMBA with

[5] S.W.P. Wijnhoven et al., preliminary results.

[6] Jos Jonkers, personal communication.

p53 mutation or *p53* deficiency in several studies (11, 16). Here, latency time of DMBA-induced mammary tumors in $p53^{R270H/+}$*WAPCre* mice was shortened compared with untreated mice when mice were treated at a very young age (28-70 days), a period encompassing mammary gland development and terminal end bud proliferation and maturation. Interestingly, the latency time for mammary tumor development in older (98-140 days) DMBA-treated $p53^{R270H/+}$*WAPCre* mice is similar to untreated $p53^{R270H/+}$*WAPCre* mice (data not shown), indicating that age at the time of exposure is a significant factor in mammary tumor development. No histologic differences were observed between spontaneous and DMBA-induced mammary tumors, with estrogen receptor α–positive and –negative tumors found in all groups. A small difference was found in the grade of estrogen receptor α positivity: DMBA-induced tumors stained slightly more positive (data not shown).

Expression profiles of specific genes seemed to change during mammary tumor development in both DMBA-treated as well as untreated mice. Interestingly, the breast tumor–related oncogene *cyclin D1* is specifically induced in DMBA-treated tumors, in line with previous results obtained with DMBA-treated rats (38, 39). In contrast, other cyclins are down-regulated, which was unexpected because expression levels of these genes are frequently induced in mouse mammary tumors (40). The clearest example for this is *cyclin G*. Presumably, this reflects the *p53* dependence of this gene because we showed before that *p53.R270H* embryonic stem cells display diminished activation of *cyclin G* after γ-irradiation compared with wild-type cells (26). As a result, in mammary gland tumors solely consisting of *p53* mutant cells, levels of *cyclin G* will be much lower compared with noncancerous mammary glands also containing cells that do not express the R270H mutant protein. Indeed, comparing expression profiles of *p53*-mutant mammary glands with those of wild-type littermates reveals *cyclin G* and other genes differentially expressed, underscoring the *p53* dependency of these genes and the disturbance of RNA expression levels in R270H mutant mammary glands.

Differences exist in gene expression profiles in tumors obtained from untreated and DMBA-treated $p53^{R270H/+}$*WAPCre* mice, limited to a few selected genes like *GADD45, cyclin D1* and *cyclin D3, Ki67*, and *Noxa.* The levels of *GADD45*, a well-known DNA damage and *p53* responsive gene, were slightly induced in untreated tumors, whereas in DMBA-induced tumors levels were decreased. This difference may well be explained by the role *GADD45* is playing in nucleotide excision repair, a repair system recognizing DMBA-induced adducts (41). Levels of *GADD45* in normal mammary glands might be higher in DMBA-treated than in untreated mice to facilitate DNA repair in response to the introduction of DNA damage. Indeed, expression levels of *GADD45* are twice higher in mammary glands of DMBA-treated mice compared with those of untreated $p53^{R270H/+}$*WAPCre* mice (data not shown), explaining the relative difference of *GADD45* levels in tumors. Another explanation could be the selective proliferation of cells with low repair capacity into preneoplastic lesions. Clearly, these primary expression analyses reveal unique signatures for *p53* mutant and carcinogen-induced mammary tumors. In addition, we found some human relevant breast cancer genes up-regulated in mammary tumors of $p53^{R270H/+}$*WAPCre* mice (i.e., *cyclin D1*, a breast tumor related oncogene found up-regulated in 35% of human breast cancers, and *Ki67*, frequently coexpressed with estrogen receptor α in estrogen receptor α–positive human tumors). These results, although the number of genes analyzed is low, are interesting, in that they show that molecular events underlying mammary gland tumor development in the $p53^{R270H/+}$*WAPCre* mouse model may be, at least to some extent, similar to those occurring in human breast cancer development. However, to identify the cooperating oncogenic events in the development of mammary tumors in $p53^{R270H/+}$*WAPCre* mice, a genome-wide analysis of both spontaneous and DMBA-induced tumors needs to be done.

In conclusion, conditional knock-in mouse models (as the *p53.R270H* mutant), with mutations equivalent to those found in humans targeted into the mouse genome, show tumor responses and tumor types highly comparable to human cancer. As such, these models are very suitable to establish precise genotype-phenotype relationships between *p53* hotspot mutations found in humans and tumorigenesis in specific tissues like the breast. Ultimately, these highly human relevant mouse models can be used to study the effectiveness of novel cancer therapies.

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References

1. Ferlay J, Bray F, Pisane P, Parkin DM. Cancer incidence, mortality and prevalence worldwide. Globocan 2000 (CD-ROM) IARC Press.
2. Coles C, Condie A, Chetty U, Steel C, Evans H, Prosser J. p53 mutations in breast cancer. Cancer Res 1992;52:5291–8.
3. Børresen-Dale A-L. TP53 and breast cancer. Hum Mutat 2003;21:292–300.
4. Norberg T, Klaar S, Karf G, Nordgen H, Holmberg L, Bergh J. Increased p53 mutation frequency during tumor progression—results from a breast cancer cohort. Cancer Res 2000;61:8317–21.
5. Aas T, Børresen A-L, Geisler S, et al. Specific p53 mutations are associated with *de novo* resistance to doxorubicin in breast cancer patients. Nat Med 1996;2:811–4.
6. Varley JM, Evans DGR, Birch JM. Li-Fraumeni syndrome—a molecular and clinical review. Br J Cancer 1997;76:1–14.
7. Soussi T, Beroud C. Assesing Tp53 status in human tumours to evualte clinical outcome. Nat Rev Cancer 2001;1:233–40.
8. Attardi LD, Jacks T. The role of p53 in tumour suppression: lessons from mouse models. Cell Mol Life Sci 1999;55:48–63.
9. Malkin D. p53 and the Li-Fraumeni syndrome. Biochim Biophys Acta 1994;1198:197–213.
10. Kuperwasser C, Hurlbut GD, Kittrell FS, et al. Development of spontaneous mammary tumors in BALB/c p53 heterozygous mice. A model for the Li-Fraumeni syndrome? Am J Pathol 2000;157:2151–9.
11. Blackburn AC, Jerry DJ. Knockout and transgenic mice of Trp53: what have we learned about p53 in breast cancer? Breast Cancer Res 2002;4:101–11.
12. Sigal A, Rotter V. Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer Res 2000;63:6788–93.
13. Dittmer D, Pati S, Zambetti G, et al. Gain of function mutations in p53. Nat Genet 1993;4:42–6.
14. Lin J, Teresky AK, Levine AJ. Two critical hydrophobic amino acids in the N-terminal domain of the p53 protein are required for the gain of function phenotypes of human p53 mutants. Oncogene 1995;10:2387–90.
15. Deb S, Jackson CT, Subler MA, Martin DW. Modulation of cellular and viral promoters by mutant human p53 proteins found in tumor cells. J Virol 1992;66:6164–70.
16. Jerry DJ, Kittrell FS, Kuperwasser C, et al. A mammary-specific model demonstrates the role of the p53 tumor suppressor gene in tumor development. Oncogene 2000;19:1052–8.
17. Jonkers J, Meuwissen R, van der Gulden H, Peterse H, van der Valk M, Berns A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet 2001;29:418–25.
18. Lin S-CJ, Lee K-F, Nikitin AY, et al. Somatic mutation of p53 leads to estrogen receptor α-positive and -negative mouse mammary tumors with high frequency of metastasis. Cancer Res 2004;64:3525–32.

19. Olive KP, Tuveson DA, Ruhe ZC, et al. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. Cell 2004;119:847–60.

20. Lang GA, Iwakuma T, Suh YA, et al. Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell 2004;17:861–72.

21. Johnson TM, Hammond EM, Giaccia A, Attardi LD. The p53QS transactivation-deficient mutant shows stress-specific apoptotic activity and induces embryonic lethality. Nat Genet 2005;37:145–52.

22. Wu Z, Earle J, Saito S, Anderson CW, Apella E, Xu Y. Mutation of mouse p53 Ser23 and the response to DNA damage. Mol Cell Biol 2002;22:2441–9.

23. Liu GL, Parant JM, Lang G, et al. Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. Nat Genet 2004;36:63–8.

24. MacPherson D, Kim J, Kim K, et al. Defective apoptosis and B-cell lymphomas in mice with p53 point mutation at Ser 23. EMBO J 2004;23:3689–99.

25. Bruins W, Zwart E, Attardi LD, et al. Increased sensitivity to UV radiation in mice with a p53 point mutation at Ser389. Mol Cell Biol 2004;24:8884–94.

26. De Vries A, Flores ER, Miranda B, et al. Targeted point mutations of p53 lead to dominant-negative inhibition of wild-type p53 function. Proc Natl Acad Sci U S A 2002;99:2948–53.

27. Wagner K-U, Wall RJ, St-Onge L, et al. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res 1997;25:4323–30.

28. Hoogervorst EM, van Oostrom CTM, Beems RB, et al. p53 heterozygosity results in an increased 2-acetylaminofluorene-induced urinary bladder but not liver tumor response in DNA repair-deficient Xpa mice. Cancer Res 2004;64:5118–26.

29. Jacks T, Remington L, Williams BO, et al. Tumor spectrum analysis in p53-mutant mice. Curr Biol 1994;4:1–7.

30. Cardiff RD, Anver MR, Gusterson BA, et al. The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. Oncogene 2000;19:968–88.

31. Shoker BS, Jarvis C, Sibson DR, Walker C, Sloane JP. Oestrogen receptor expression in the normal and precancerous breast. J Pathol 1999;188:237–44.

32. Nandi S, Guzman RC, Yang J. Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. Proc Natl Acad Sci U S A 1995;92:3650–7.

33. Van't Veer LJ, Dai H, van de Vijver, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530–6.

34. Hedenfalk I, Duggan D, Chen Y, et al. Gene-expression profiles in hereditary breast cancer. N Engl J Med 2001;8:539–48.

35. Gillett C, Fantl V, Smith R, et al. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res 1994;54:1812–7.

36. Näf D, Krupke DM, Sundberg JP, Eppig JT, Bult CJ. The Mouse Tumor Biology Database: a public resource for cancer genetics and pathology of the mouse. Cancer Res 2002;62:1235–40.

37. Shoker BS, Jarvis C, Clarke RB, et al. Estrogen receptor-positive proliferating cells in the normal and precancerous breast. Am J Path 1999;155:1811–5.

38. Shan L, He M, Yu M, et al. cDNA microarray profiling of rat mammary gland carcinomas induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and 7,12-dimethylbenz[*a*]anthracene. Carcinogenesis 2002;23:1561–8.

39. Jang TJ, Kang MS, Kim H, Lee JI, Kim JR. Increased expression of cyclin D1, cyclin E and p21(Cip1) associated with decreased expression of p27(Kip1) in chemically induced rat mammary carcinogenesis. Jpn J Cancer Res 2000;91:1222–32.

40. Said TK, Medina D. Cell cycle genes in a mouse mammary hyperplasia model. J Mammary Gland Biol Neoplasia 2004;1:81–93.

41. Hartman AR, Ford JM. BRCA1 induces DNA damage recognition factors and enhances nucleotide excision repair. Nat Genet 2002;32:180–4.