

Rats deficient for *p53* are susceptible to spontaneous and carcinogen-induced tumorigenesis

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The *p53* tumor suppressor gene is highly mutated in human cancers. Individuals who inherit one *p53* mutant allele are susceptible to a wide range of tumor types, including breast cancer and sarcoma. We recently generated *p53* knockout rats through gene targeting in embryonic stem cells. Here we show that rats homozygous for the null allele are prone to early onset spontaneous sarcomas and lymphoma with high incidence of metastases. Heterozygous rats are also highly predisposed to cancer, but with a delayed onset and a wider spectrum of tumor types compared with homozygotes. Importantly, up to 20% of female heterozygotes developed breast cancer and about 70% of the tumors were positive for estrogen receptor. Exposing *p53*-deficient rats to a low dose of the carcinogen diethylnitrosamine dramatically decreased the latency for sarcoma development and survival time compared with equivalently treated wild-type rats. These unique features make this knockout line a valuable model for investigating human malignancy and *in vivo* carcinogenicity of chemicals and therapeutic compounds.

Introduction

Loss or mutation of the *p53* tumor suppressor gene is the most frequently observed genetic lesion in human cancers (1). Mutant *p53* alleles that encode no or dysfunctional *p53* protein arise both in somatic cells during sporadic human tumorigenesis and in the germline (Li–Fraumeni cancer syndrome) (2). A number of mouse models have been developed to investigate the role of *p53* in mammalian tumorigenesis (3). Constitutive inactivation of *p53* through gene targeting has allowed for *in vivo* analysis of the effects of *p53* activity loss. *p53*-null mice have been generated by several different groups, and, despite subtle differences in the targeting strategies employed, all strains exhibit very similar phenotypes with relatively uniform tumor spectra (4–6). Among the types of tumors observed in the *p53*-deficient mice, lymphomas, usually of the T-cell type, were most common, although *p53* is less frequently mutated in human lymphomas (7). Soft tissue sarcomas and osteosarcomas appear at a relatively lower frequency but malignant carcinomas are rarely observed in the *p53*-deficient mice. The spectrum of lesions differed from that predicted by the Li–Fraumeni syndrome, which has been linked to a variety of germline *p53* mutations. Li–Fraumeni families are prone to various types of tumors, with breast cancer, soft tissue sarcomas, osteosarcomas, brain tumors and leukemias being identified as the most frequently observed cancers (2). Interestingly, the high rate of mammary tumor development was found in the BALB/c background but not in other mouse strains (8). A notable absence of tumors of epithelial origin in most *p53*-deficient mice suggests that the mouse model may not be an exact mimic of the human syndrome, possibly due to species differences.

Abbreviations: DEN, diethylnitrosamine; ENU, N-ethyl-N-nitrosourea; ES, embryonic stem; HCC, hepatocellular carcinoma.

As rats are physiologically more similar to humans than are mice, rat models are more appropriate for studying human diseases and testing the pharmacodynamics and toxicity of potential therapeutic compounds (9). Gene targeting in mouse embryonic stem (ES) cells has been used to generate various mouse strains for the study of diverse human genetic diseases and cancer, but the inability to establish germline-competent ES-cell lines from other species had prevented broader application of this technology. Recently, we developed the 3i/2i culture system that enabled the derivation of germline-competent rat ES cells and generated a *p53* knockout rat *via* homologous recombination in rat ES cells (10). It was demonstrated that rats homozygous for a null *p53* allele were developmentally normal but highly susceptible to early onset of cancer. The most frequently observed tumor type in *p53*-null rats was hemangiosarcoma whereas existing *p53*-deficient mouse models predominantly develop lymphomas (11). A similar observation was made in another *p53* knockout rat generated *via* N-ethyl-N-nitrosourea (ENU)-mediated random mutagenesis (12). However, due to the low number of animals followed in that study, a complete tumor spectrum was not presented.

Here we report a complete assessment of tumor development in our *p53*-deficient rat models. We observed a more human-like tumor spectrum in our models, particularly in the *p53* heterozygous rats, than what has been reported in *p53*-deficient mice or ENU-generated *p53* knockout rats. Spontaneous breast cancer, which is frequently seen in Li–Fraumeni females but rarely in most *p53*-deficient mice, appear in some *p53* heterozygous rats, making them a better predisposition model to mimic the cognate human syndrome. Furthermore, *p53*-deficient rats were extremely susceptible to carcinogen-induced tumorigenesis. Thus, *p53*-deficient rats provide a sensitive model system for mechanistic study of cognate human syndromes and assessment of the carcinogenic potential of novel compounds.

Materials and methods

Animals

Generation of rats lacking one or both alleles of *p53* by gene targeting of DAC8 rat ES cells has been described previously (10). Rats that were *p53* heterozygous were intercrossed to produce rats with no, one or two *p53*-null alleles. These rats were designated wild-type, heterozygotes (*p53*^{+/-}) or homozygotes (*p53*^{-/-}), respectively, and were monitored for spontaneous and carcinogen-induced tumor development. These *p53*-mutant rats have been deposited to the Rat Resource and Research Center in Missouri. All procedures were performed according to investigator's protocols approved by the University of Southern California Institutional Animal Care and Use Committee.

Tumor analysis

Animals that died or were sacrificed were subjected to complete necropsy. Tumor samples were surgically removed and fixed in 10% neutral buffered formalin and processed for histological examination. The paraffin blocks were sectioned at 5 μ m, and the sections were stained with hematoxylin and eosin.

Immunohistochemistry

For immunohistochemical analysis, sections were mounted on charged slides, dried overnight at 55°C and deparaffinized. Rehydrated sections were subjected to antigen unmasking in 10 mM sodium citrate buffer, pH 6.0, at a subboiling temperature for 10 min. Once cooled, sections were exposed to 3% hydrogen peroxide, rinsed and blocked with 5% normal goat serum. Then, tissue sections were probed with antibodies specific for the following protein targets: Vimentin (Ab-2), Pancytokeratin (Ab-3), CD3 (SP7, T-cell marker), CD31/PECAM-1 (endothelial cell marker), CD68 (Ab-3, macrophage marker), and CD79a/mb-1 (Ab-1, B-cell marker). All antibodies were purchased from Lab Vision (Thermo Scientific, Kalamazoo, MI).

Carcinogen-induced tumorigenesis

Thirteen *p53* wild-type rats, 14 *p53* heterozygotes and 12 *p53* homozygotes were intraperitoneally injected with diethylnitrosamine (DEN) (20 mg/kg body weight; Sigma–Aldrich) weekly for 5 weeks. Rats were monitored daily over the course of the experiment. Thirteen wild-type rats, 16 *p53* heterozygotes and 11 *p53* homozygotes not treated with the carcinogen were monitored in parallel with the treated rats. Dead animals were examined by necropsy. Liver and lung tissues were fixed and stained as described above and examined for histopathologic lesions.

Results*Spontaneous tumorigenesis in p53-deficient rats*

Forty *p53* homozygous and 66 heterozygous animals were examined for tumors twice each week. Although initially healthy, rats homozygous for the *p53* deficiency were highly predisposed to malignancy. By the age of 2.5 months, approximately half of the homozygotes had developed tumors and almost all of these animals had died of tumors by 6 months of age. Compared with wild-type rats, the *p53* heterozygotes also had a greatly accelerated rate of tumorigenesis. By 8 months of age, half of the heterozygotes had developed tumors (33 out of 66) and most rats (90.9%) that survived to 12 months of age had succumbed to tumors or death (Figure 1A). All control littermates (38 rats) with wild-type *p53* alleles survived to the end of the study with the exception of two rats that were euthanized upon recognition of subcutaneous masses. Both of them had developed undifferentiated subcutaneous sarcoma on their flanks and were more than 10 months old, which is consistent with the observations of incidental tumor development in Sprague–Dawley rats of this age (13).

Histopathological examination revealed a significant difference in tumor spectra between the *p53* homozygous and heterozygous rats (Figure 1B and 1C). Almost 80% of *p53* homozygotes developed liver hemangiosarcoma and a large proportion thereof displayed pulmonary metastases. These hemangiosarcomas stained positive for factor VIIIra by immunohistochemistry, confirming an endothelial origin of these neoplastic cells (Figure 2A and 2B). Lymphoma, a dominant tumor type observed in *p53* homozygous mice, was found in 18% of homozygous mutant rats, and extensive CD3 or CD79a immunoreactivity indicated the lymphomas were of either T- or B-cell origin. One animal with hemangiosarcoma also had a lymphoma in its thymus. No carcinomas were observed among the homozygotes.

In contrast, the heterozygous rats showed a much higher rate of development of lymphoma than sarcoma, mainly of T-cell origin, accounting for 55% of the tumor incidence in heterozygous rats (Figure 2C). Enlarged spleens and livers in rats with lymphoma were frequently observed as a result of diffuse infiltration by malignant lymphocytes (Figure 2D). Interestingly, although lymphoma was the predominant type of spontaneous tumor in heterozygous rats, it was often observed in rats older than 7 months of age. About 25% of the heterozygotes developed hemangiosarcoma. Interestingly, among the tumors diagnosed in the heterozygous rats, 6 breast cancers were found in 31 female animals, and 4 of them showed immunoreactivity for estrogen receptor α (Figure 2E and F). Several osteosarcomas could be classified based on their histological appearance showing osteocytes within lacunae. Rhabdomyosarcomas that arose from the muscles of the legs and pelvis were positive for MyoD-1 (a skeleton marker) immunostaining and locally invasive. A large tumor was found in the region of the left adrenal gland in one heterozygous rat. The tumor cells were relatively uniform and organized along thin fibrovascular septa, as is typically observed in masses of endocrine origin. The animal also presented with hemangiosarcoma in its liver. The breast and endocrine cancers in combination with the hard and soft tissue sarcomas represent a spectrum of cancers similar to those of Li–Fraumeni syndrome in humans (14). Three heterozygous rats that died or were euthanized due to hind limb paralysis had no evidence of cancer based on macroscopic and histopathologic examination of tissues.

Carcinogen-induced tumorigenesis

To determine whether germline reduction of *p53* gene dosage confers higher sensitivity of rats to carcinogen, animals of all three *p53* genotypes were exposed to the carcinogen DEN, which is a

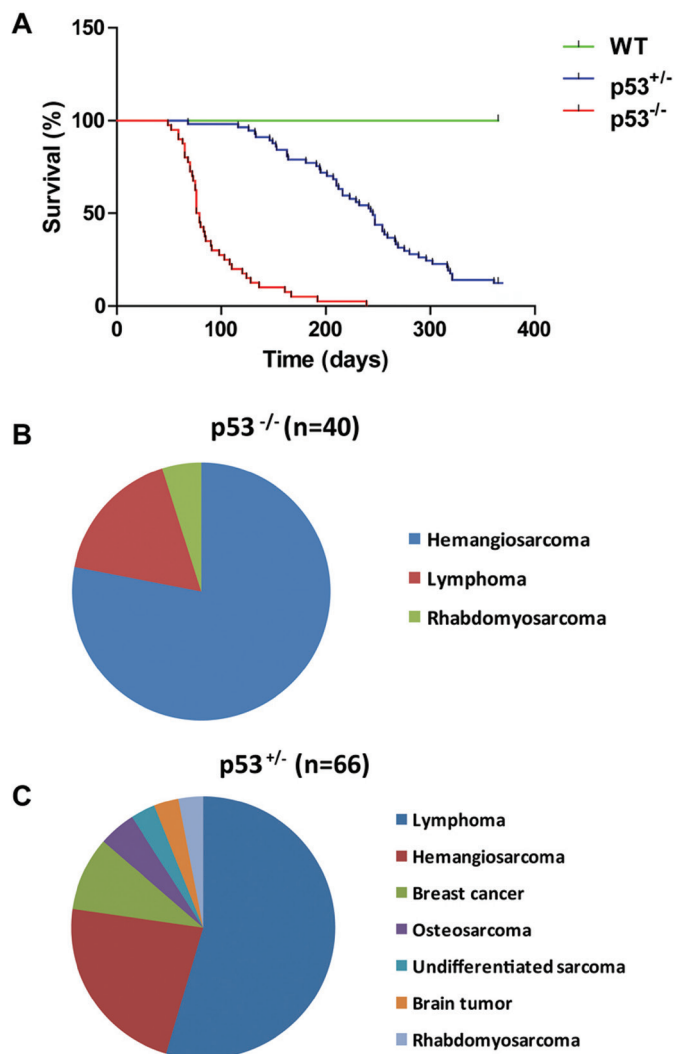


Fig. 1. Tumor incidence in *p53*-deficient rats. (A) Survival curves of wild-type, *p53* heterozygous (*p53*^{+/-}) and *p53* homozygous (*p53*^{-/-}) rats. Forty *p53*^{-/-} homozygous rats, 66 *p53*^{+/-} heterozygous rats and 38 wild-type littermate controls were monitored twice weekly for the development of tumors over a period of 365 days. Moribund rats or rats with obvious tumors were euthanized, necropsied and the tumor tissue was examined by histopathology. Kaplan–Meier analysis, $P < 0.0001$. (B and C) Pie charts of the tumor types identified in homozygous (B) and heterozygous (C) mutant animals. Numbers in parentheses indicate the total number of tumors analysed.

particularly potent inducer of liver hemangiosarcoma and hepatocellular carcinoma (HCC). Low dose levels of DEN (20 mg/kg) were administered weekly for 5 weeks to 13 wild-type rats, 14 heterozygotes and 12 homozygotes, beginning at 6 weeks of age. The animals were observed until moribund. The *p53*-deficient rats were dramatically more susceptible to the effects of DEN than were wild-type controls (Figure 3A). All homozygous rats treated with DEN died of liver hemangiosarcoma within 3 months of initial exposure. The untreated homozygotes survived significantly longer, although most of them developed spontaneous hemangiosarcoma at the end of the observation period (Figure 3B). Heterozygous rats also displayed significantly greater susceptibility to DEN-induced hemangiosarcoma and shorter survival time than did untreated controls. Only 4 of 16 untreated heterozygotes displayed obvious liver hemangiosarcoma, whereas 9 of 14 DEN-treated heterozygotes died of the disease (Figure 3C). Interestingly, only the latency

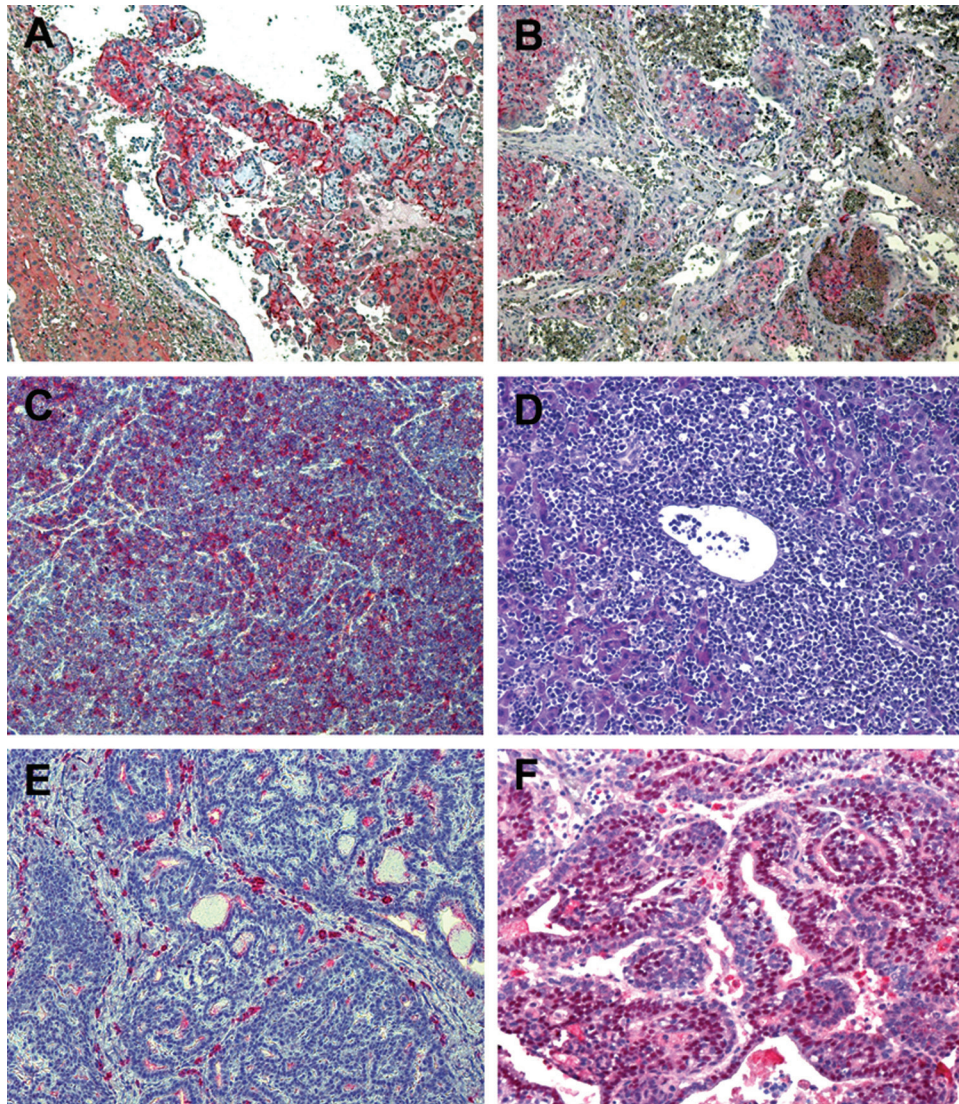


Fig. 2. Histopathology of representative tumors from *p53*-deficient rats. (A) Liver hemangiosarcoma stained with endothelium marker CD31/PECAM-1 showing tumor tissue of endothelial origin and large blood-filled spaces. (B) Immunoreactivity of CD31/PECAM-1 in intravascular pulmonary metastasis of liver hemangiosarcoma. (C) Thymic lymphoma from a *p53* heterozygous rat with monotonous tumor cells positive for T-cell marker CD3. (D) Extensive infiltration of malignant lymphocytes into the liver of a rat with thymic lymphoma. (E) Ductal breast carcinoma in a heterozygous *p53*-mutant rat is stained negative for estrogen receptor. (F) Breast carcinoma showing strong nuclear estrogen receptor immunopositivity.

of hemangiosarcoma development was significantly reduced in DEN-treated *p53*-deficient rats, consistent with the selective carcinogenic effects of DEN. In contrast, none of the wild-type rats developed liver hemangiosarcoma during the observation period of DEN treatment. With the dose of 20 mg/ml and 5-week treatment protocol, which efficiently induced liver hemangiosarcoma, no HCCs were found in the three *p53* genotypes at the end of the observation period.

The major cause of death in rats with hemangiosarcoma is tumor rupture and hemorrhagic shock. Multiple and large space-occupying hemangiosarcomas were found in both homozygous and heterozygous animals. Their body cavities were often filled with uncoagulated blood and lung metastasis was frequently evident. DEN-treated heterozygous liver displayed toxic lesions consisting of fatty change, coagulative necrosis, anisokaryosis of hepatocyte nuclei, chronic hepatic inflammation and fibrosis. In contrast, the wild-type rats equivalently treated with DEN showed only a similar degree of liver toxicity with no evidence of hemangiosarcoma development.

Discussion

The rat is the preferred model organism in many fields of biomedicine, including cancer research, due to its having a physiopathology similarity to that of humans. Although mouse knockout models have been extremely powerful tools for identifying important cancer-related genes, there are discrepancies between the human disease phenotypes and those observed in mouse models. For example, mouse *Apc* knockouts mostly develop tumors in the small intestine whereas humans carrying *Apc* mutation suffer from familial colon cancer. Interestingly, rats with *Apc* deficiency develop colon cancer (15). Furthermore, mouse models that lack the same gene but in a different strain background display important differences, emphasizing the need for complementary mammalian mutant models in different species to enable *in vivo* phenotypic comparison. The higher tolerance of rats to genetic modification enables researchers to knockout genes that used to cause embryonic lethality in mice. For example, although knockout of the tumor suppressor gene *Brca2* was limited by low viability in mice, the *Brca2*^{-/-} rats are viable and the vast

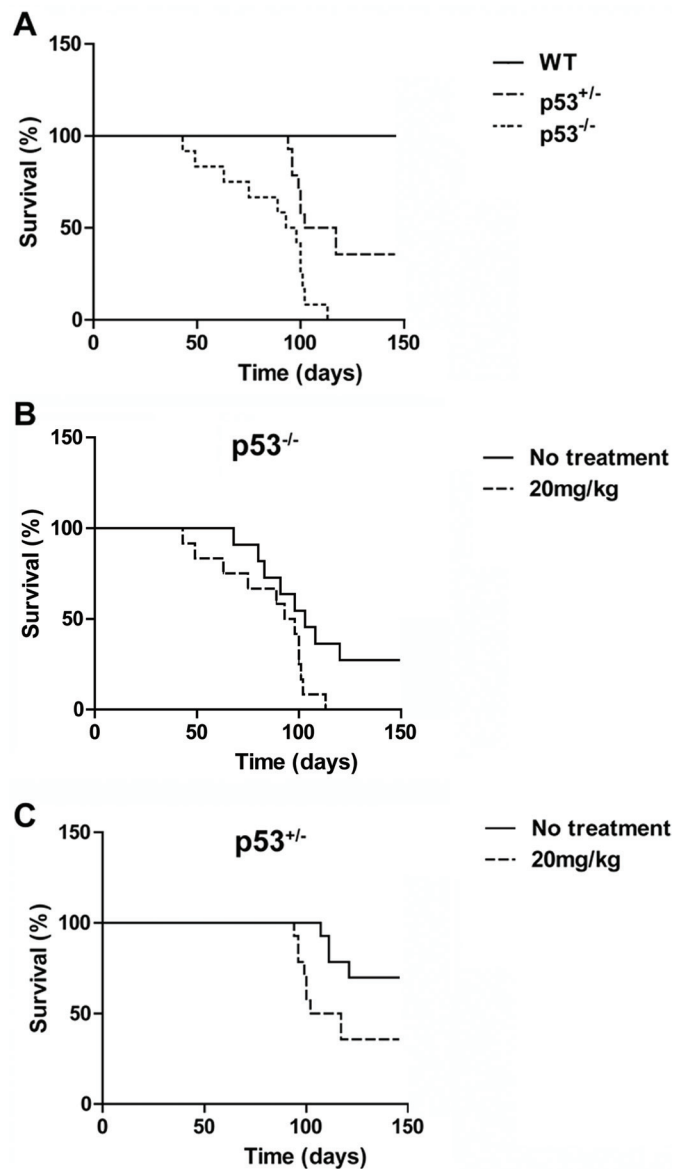


Fig. 3. Tumor development in carcinogen-treated $p53$ -deficient rats. (A) 13 wild-type, 14 heterozygous and 12 homozygous rats were intraperitoneally administered with DEN (20mg/kg) for 5 weeks. Comparison of the survival times of the three groups revealed an obvious difference between wild-type and $p53$ -deficient animals. Kaplan–Meier analysis, $P < 0.0001$. (B) Comparison of the survival times between non-treated and DEN-treated $p53$ homozygous rats. Kaplan–Meier analysis, $P < 0.05$. (C) Comparison of the survival times between non-treated and DEN-treated $p53$ heterozygous rats. Kaplan–Meier analysis, $P < 0.01$.

majority survive past 1 year of age (16). With their relatively large size, which facilitates experimental and surgical interventions, these genetically modified rats may serve as more physiologically appropriate models for the study of human cancer biology.

Most techniques for genetic manipulation in mice, including homologous recombination and conditional mutations, depend upon the culture and manipulation of ES cells. Before the development of germline-competent rat ES-cell lines in 2008 (17), several technologies had already been devised to enable generation of rat models of human disease, including chemical mutagenesis using ENU, mobile DNA technology and nuclease-based technology. Despite the high mutagenicity of ENU and transposon-based mobile DNA technology, mapping mutations responsible for a particular

phenotype and distinguishing neutral mutations from causative mutations are typically difficult and time-consuming (18,19). The development of nuclease technology with the use of Zinc finger nucleases and Transcription Activator-Like Effector Nucleases enabled the transition from random to targeted mutagenesis in generating knockout rats. However, validating constructs encoding specific Zinc finger nucleases, as well as systematically archiving the mutant lines, remains a challenge. By using a specific culture medium containing 3 or 2 differentiation inhibitors (3i or 2i medium), we recently showed that true pluripotent rat ES cells could be isolated and propagated *in vitro*. Furthermore, successful gene targeting by homologous recombination in these cells results in the generation of a targeted $p53$ gene knockout rat (10). Gene targeting in ES cells *via* homologous recombination offers the unique advantage of a low rate of non-specific mutation in the genome, which seems to occur with relatively high frequency in Zinc finger nuclease-targeted animals and even more in ENU-mutagenesis, in which more than 1000 mutations per genome need to be introduced in order to identify the gene of interest (20).

Similar to $p53$ knockout mice, our rat model exhibited accelerated cancer rates and developed a large number of sarcomas and lymphomas. Interestingly, whereas $p53$ homozygous mice predominantly develop lymphoma, our $p53$ homozygous rats predominantly developed metastasizing hemangiosarcoma, similar to the ENU-mutated $p53$ homozygous rats. The majority of spontaneous tumors developed in our heterozygous rats were lymphomas, whereas osteosarcomas predominated in $p53$ heterozygous mice. Lymphoma is infrequently observed in ENU-mutated $p53$ heterozygous rats, possibly due to the low numbers of animals followed in that study (9 versus 62 in this study). In addition, when compared with $p53$ knockout mouse models, heterozygous rats had a more-rapid rate of tumor onset. These differences reflect the species-specific phenotypes in different rodent models. The tumor spectrum of the $p53$ knockout mice is similar to the incidence of spontaneous tumors in mice. Accordingly, in Harlan Sprague–Dawley and Wistar Han rats, the common spontaneous tumors are lymphoma, sarcoma and mammary tumors, which is consistent with the tumor spectrum observed in our $p53$ knockout rat models (21,22). A unique feature of our model is that the tumors originated from diverse tissues, including epithelial organs. As breast tumors are important component tumors of Li–Fraumeni syndrome but are infrequently observed in $p53$ knockout mice, the $p53$ knockout rats might serve as a better model for this disease.

The delayed latency for tumorigenesis in $p53$ heterozygous rats suggested that they might be used as a platform to assess the carcinogenic potential of chemicals. We tested their response to a low, chronic dosing of the carcinogen DEN. Compared with another hepatic carcinogen, dimethylnitrosamine, which induces high incidences of liver hemangiosarcoma, DEN mainly causes HCC when high doses (70mg/kg) were chronically administered to rats. Treatment of three genotypes of rats with a low dose of DEN (20mg/kg) failed to induce HCC development during the observation period. Instead, $p53$ heterozygous animals showed a dramatically accelerated development of liver hemangiosarcoma, whereas none of the wild-type rats displayed an observable tumor at the end of the study. These results indicate that the effects of a carcinogen could be readily monitored against $p53$ -deficient background of spontaneous tumors and that these rats might be a sensitive test animal for some carcinogenesis procedures.

In summary, our results demonstrate that the $p53$ -deficient rats are highly cancer prone, which is compatible with existing mouse models, as well as rat models developed with alternative targeting techniques in this species. Thanks to a wealth of detailed physiological and pharmacological phenotypic knowledge for rats, the value of $p53$ knockout rats extends beyond analysis of their particular tumor phenotypes to mapping of these traits to signaling contexts. These rats can be bred to other transgenic rat strains to examine the cooperative effects of $p53$ deficiency and other oncogenic mutations which might not have been fully appreciated in knockout mouse models.

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