Prostate-Specific Expression of p53R172L Differentially Regulates p21, Bax, and mdm2 to Inhibit Prostate Cancer Progression and Prolong Survival

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Abstract
Loss of heterozygosity or mutation at the p53 tumor suppressor gene locus is frequently associated with advanced human prostate cancer. Hence, replacement p53 gene therapy may prove to be efficacious for this disease. While many mutations result in p53 molecules with oncogenic properties, other variants may possess wild-type properties with increased tumor suppressor activity. We have chosen to investigate the activity of a naturally occurring variant p53 molecule, p53R172L, carrying an arginine-to-leucine mutation at codon 172. We demonstrate that p53R172L can differentially activate expression of genes involved in cell cycle control and apoptosis in vitro. Transgenic mice expressing a subphysiological level of a p53R172L minigene (PB-p53R172L) in the prostate epithelium were generated and bred to the transgenic adenocarcinoma mouse prostate (TRAMP) model of prostate cancer. While PB-p53R172L transgenic mice developed normally with no detectable prostate gland phenotype, we observed a significant increase in the apoptotic index in the prostate glands of TRAMP × PB-p53R172L F1 mice. We noted an increase in the expression of Bax in the bigenic mice concomitant with the reduced incidence and rate of tumor growth and increased survival. While low-level expression of the p53R172L variant had no obvious influence on normal prostate tissue, it was able to significantly inhibit prostate cancer progression in the context of a genetically predisposed model system. This suggests that additional tumor-related events specifically influence the ability of the variant p53R172L molecule to inhibit tumor growth. These studies support gene therapy strategies employing specific p53 variants.

Introduction
Prostate cancer is the second leading cause of cancer death in men in the United States with over 28,000 deaths predicted in 2003 (1). Several studies have demonstrated significant incidence of mutations in p53 in advanced prostate cancer (2–4), and p53 mutations have been suggested to occur frequently in early stages of prostate cancer (5). Loss and mutation of p53 has also been described for metastatic prostate cancer (6–8) and in association with the emergence of the androgen-independent phenotype (9–11). These data support the hypothesis that abrogation of a normal pattern of functional p53 expression is an important event in prostate cancer progression (12) and suggests that specific strategies could be designed to exploit the p53-independent nature of advanced prostate cancer (13).

The p53 tumor suppressor gene mediates cellular responses to events such as DNA damage, hypoxia, mitotic spindle damage, and expression of activated oncogenes (14–16) through the initiation of growth arrest or cell death pathways and is generally believed to prevent the accumulation and propagation of genetic aberrations (17). Functionally, p53 is a sequence-specific transcription factor that binds to the upstream regulatory region of genes such as the cyclin-dependent kinase inhibitor WAF-1/p21 (18), 14-3-3s (19), and Bax (20). In addition, p53 has been shown to down-regulate prostate-specific antigen, a marker used in the diagnosis of prostate cancer (21). One important p53 target gene is mdm2, which in turn binds to p53 and facilitates ubiquitin-mediated p53 degradation (22–24).

In human cancer, wild-type p53 expression is often abrogated by somatic mutation. In fact, over 50% of human tumors carry mutations in the p53 gene, with the majority being single-base substitutions. The majority of p53 mutations occur in the DNA-binding domain (25) with hotspots for mutation at codons 175, 248, and 282 resulting in alterations in DNA binding (26). In general, mutations in p53 abrogate normal function and extend the half-life of the protein. More
specifically, some p53 mutations act in a dominant-negative fashion to block normal p53 function (27) while other mutations may impart a gain of function (28) resulting in up-regulation of nonclassical p53-regulated genes such as multidrug resistance and epidermal growth factor receptor (reviewed in Ref. 29). Mutant p53 molecules often act as oncogenes, and p53 proteins carrying missense mutations may impart significant growth advantage or aggressive metastatic potential (6–8). Adding complexity to the spectrum of p53 mutation, individual mutations may result in specific cellular responses (30). Among the range of mutations, p53 carrying an arginine-to-histidine mutation at codon 175 (p53R175H) has been detected at high frequency in aggressive breast cancers and has been demonstrated to impart a predisposition to transformation in transgenic mice (31–34).

In contrast to oncogenic mutations of p53, mutated forms of p53 with wild-type properties have also been identified in human tumors. A mutation in p53 resulting in an arginine-to-leucine substitution at codon 175 (p53R175L) has been identified in several breast cancer biopsies (35) as well as frequently in lung (36–38) and colon (39, 40) carcinoma. In contrast to the p53R175H mutation, p53R175L is not transforming (41, 42). In fact, p53R175L has many properties consistent with wild-type p53 including the ability to recognize p53-specific DNA-binding sequences, to modulate the expression of genes known to be regulated by wild-type p53, and to inhibit cell growth (43). Furthermore, transgenic mice that overexpress a murine p53R172L (analogous to human p53R172L) in the mammary gland exhibit increased apoptosis in the mammary gland and significant decrease in the incidence of 7,12-dimethylbenz(a)anthracene-induced mammary tumors (42).

Translational prostate cancer research and the ability to validate the efficacy of candidate therapeutic regimens in preclinical trials have been hampered by the paucity of well-defined relevant in vivo tumor models. The transgenic adenocarcinoma mouse prostate (TRAMP) model was developed using the minimal rat probasin promoter to express the SV40 early genes (T/t antigen: Tag) specifically in the epithelial cells of the mouse prostate (44, 45). Male TRAMP mice develop progressive prostate disease that histologically and pathologically mimics human disease with metastatic spread to distant sites (46, 47), making the TRAMP model a useful model for preclinical evaluation of therapeutic strategies.

We show in this study that p53R172L can discriminate among the intrinsic response elements of p21, mdm2, and Bax in vivo and can increase expression of WAF-1/p21 and mdm2 promoter-based reporter constructs 2–5-fold over wild-type p53. Based on these observations, we established transgenic mice that carry a p53R172L fusion minigene (PB-p53R172L) and demonstrate subphysiological level of p53R172L expression in prostate epithelium. The mice develop normally with no detectable changes in prostate gland cellular architecture, morphology, wet weight, proliferation, or apoptotic indices. However, when PB-p53R172L transgenic mice were crossed to TRAMP mice, there was a reduction in tumor progression and rate of tumor growth with a significant increase in survival associated with p21 expression and Bax-mediated tumor-specific apoptosis. Therefore, this study demonstrates that a specific variant p53 molecule does not perturb normal cell growth and development yet can dramatically inhibit tumor progression. Our data suggest that variants such as p53R172L may prove useful in treating prostate cancer.

**Results**

**Characterization of p53R172L.**

One of the major biochemical functions of wild-type p53 is the ability to act as a sequence-specific transcriptional factor (48). As mutant forms of p53 may differentially influence transcription of target genes (49), we investigated the abilities of wild-type p53 and p53R172L to activate expression of specific gene reporter constructs. For this analysis, expression plasmids carrying either wild-type p53 or p53R172L were introduced into p53-null fibroblasts (a generous gift of Dr. G. Lozano) along with WAF-1/p21-luc, Bax-luc, or mdm2-luc reporter plasmids (a generous gift of Dr. M. Oren). Luciferase activity was used to measure p53-mediated transcriptional activity, and immunoblotting was used to normalize transcription data to levels of expressed p53 protein. As the large tumor antigen (Tag) of SV40 can bind to and abrogate the function of wild-type p53 (50), transcriptional activities of both wild-type p53 and p53R172L were assayed in the presence of increasing amounts of Tag. As shown in Fig. 1, wild-type p53 was a better transcriptional activator of Bax-luc than p53R172L. The p53R172L variant was able to stimulate expression of the WAF-1/p21-luc and mdm2-luc reporter plasmids ~4- and 1.5-fold higher than wild-type p53, respectively. Transcriptional activity of p53R172L appeared to be more sensitive than wild-type p53 to the presence of Tag in the Bax-luc and whey acidic protein (WAP)/p21-luc transactivation assays. In general, mutated forms of p53 display half-lives greater than observed for wild-type p53. However, as shown in Fig. 1, the p53R172L variant was found by pulse-chase analysis to have a half-life of ~60 min in our system in contrast to the 135-min half-life of wild-type p53. This result is consistent with the observation that the variant p53R172L induced mdm2 transcription, a negative regulator of p53 expression, to a level greater than wild-type p53. In general, this biochemical evidence supports the hypothesis that the variant p53R172L possesses gain of function characteristics and increased transcriptional activity when assayed on classical p53-regulated promoters.

**Expression of p53R172L in Transgenic Mice**

To determine the consequence of enforced expression of p53R172L in prostate epithelial cells, transgenic mice carrying the −426/+28-bp PB promoter sequences fused to a mouse p53R172L minigene (Fig. 2A) were generated by pronuclear injection into FVB/N zygotes. Four independent founder mice were identified, and of these, line 7856 was found to express subphysiological levels of the transgene in a very tissue-restricted manner (Fig. 2B) and was therefore chosen for further study. In line 7856, transgene-specific transcripts could only be detected by reverse transcription-PCR (RT-PCR) followed by hybridization with radiolabeled probe and
overnight exposure to X-ray film (Fig. 2C). This is in contrast to the 5–10-min exposure required to detect endogenous p53 in parallel reactions despite similar primer efficiency (Fig. 2D). The ratio of PB-p53R172L transgene expression to endogenous p53 expression was determined to be ~1–50. It is important to note, however, that profound phenotypes have been observed in mice where the level of expression of a mutant p53 molecule was substantially lower than levels of wild-type p53 (51).

**PB-p53R172L Transgenic Mice Display Normal Prostate Development**

Histological examination of H&E sections of prostate tissues of 18-week-old mice was used to evaluate the consequence of PB-p53R172L expression in vivo. As shown in Fig. 3, enforced expression of p53R172L in prostate epithelium was not detrimental to normal prostate gland development. The histological features of the prostate glands in PB-p53R172L mice appeared identical to those of nontransgenic littermates at 18 weeks of age (Fig. 3, A and B). The prostatic acini in PB-p53R172L transgene mice were lined by columnar secretory epithelium surrounded by scattered basal epithelial cells and a fibromuscular stroma containing three to four cell layers of smooth muscle (Fig. 3, C and D).

It had been demonstrated previously that p53R172L could induce apoptosis when hyperexpressed in the mammary gland of transgenic mice (42); therefore, the level of apoptosis in prostate tissues from nontransgenic and PB-p53R172L transgenic mice was determined. As shown in Fig. 3, low numbers of apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) method in both control (Fig. 3E) and PB-p53R172L (Fig. 3H) mice. Because p53R172L could transactivate WAF-1/p21 reporter constructs at high levels in vitro, endogenous p21 expression was examined by immunohistochemistry. As demonstrated in Fig. 3, F and I, there was no apparent difference in p21 expression between PB-p53R172L mice (57%; Fig. 3I) and control animals (53%; Fig. 3F). There was also little change in the level of cellular proliferation between nontransgenic and PB-p53R172L mice determined by BrdUrd labeling (Fig. 3, G and J, respectively). These data demonstrate that in the context of an otherwise normal prostate gland, enforced subphysiological expression of p53R172L has little consequence on epithelial cell proliferation or apoptosis in vivo.

**PB-p53R172L Inhibits Prostate Cancer in Vivo**

Although there was no discernable phenotype in the prostate gland as a consequence of PB-p53R172L expression, it should be noted that p53R175L had been identified in tumor tissue and shown to inhibit lobuloalveolar development in proliferating mammary tissues. Hence, we postulated that expression of p53R172L could influence the development of prostate cancer in vivo. To test the hypothesis that p53R172L exhibited tumor-specific properties, we established a cross between the PB-p53R172L mice and the TRAMP model of prostate cancer. The 18-week-old PB-p53R172L, TRAMP, TRAMP x PB-p53R172L F1 bigenic mice, and nontransgenic littermates were examined at a gross level to characterize the consequence...
of p53R172L expression in the context of spontaneous and autochthonous prostate cancer. Note that all mice in this study were C57BL/6 × FVB F1, and each group contained 8–14 mice. As shown in Fig. 4, we noted a marked reduction in prostate tumor burden in the TRAMP/C2 PB-p53R172L bigenic mice. This was a very surprising result given the low level of expression of the PB-p53R172L transgene.

While the prostate gland and other male accessory organs were grossly similar in the nontransgenic and PB-p53R172L mice (Fig. 4, A and B), TRAMP mice typically presented with palpable and highly vascularized spherical tumors that measured from 1 to 5 cm in diameter (Fig. 4C). In stark contrast to the TRAMP mice, TRAMP/C2 PB-p53R172L bigenic mice did not develop palpable tumors by 18 weeks of age. At more advanced ages, the bigenic mice typically presented with irregular shaped prostates, and enlarged fluid-filled seminal vesicles were common (Fig. 4D). As shown in Fig. 4E, the mean wet weight of genitourinary (GU) tracts isolated from 18-week-old TRAMP mice exceeded that recorded for all other groups by 10–15-fold (P < 0.005). There was no statistical difference in GU tract wet weight between bigenic mice and PB-p53R172L transgenic mice (P < 0.26), although there was a statistically significant difference (P < 0.013) in GU tract wet weight between bigenic mice and nontransgenic mice. More importantly, only two bigenic mice (2/11; 18%) developed small tumors (0.5 and 0.9 g) and were confined to the ventral prostate.

Histological examination confirmed that all TRAMP mice (14/14; 100%) had prostate cancer by 18 weeks of age. The tumors ranged from well-differentiated adenocarcinoma (Fig. 5, FIGURE 2. Generation of PB-p53R172L transgenic mice. A. Representation of the PB-p53R172L transgene carrying the −426/+28-bp rat probasin promoter, the murine p53R172L genomic DNA (exons 2–11 and introns 2–9), and the SV40 polyadenylation signals. The arginine-to-leucine mutation is indicated at codon 172 of the minigene. Arrows, location of primer sets for screening and RT-PCR. B. RT-PCR analysis of PB-p53R172L transgene expression in different tissues. Total RNA (1 μg) extracted from tissues was subjected to 30-cycle RT-PCR and fractionated on 2% agarose gels. DNA was transferred to solid nylon support and was hybridized with a 666-bp AvaI restricted, 32P-labeled murine p53 cDNA probe. The tissues assayed are the following: pr, prostate gland; bl, bladder; ki, kidney; te, testis; lu, lung; br, brain; sp, spleen; he, heart; sv, seminal vesicle; li, liver; ap, coagulating gland. C. RT-PCR analysis of ventral (V) and dorsolateral (DL) prostate gland shows no p53R172L expression in normal mice (p53−/−). Expression of p53R172L is detected in bigenic mice (p53+/+T+) at levels well below endogenous p53. The exposure time for each Southern blot is indicated. The L19 internal RT-PCR control indicates equivalent amounts in each PCR reaction. D. The difference of RT-PCR detection of p53R172L versus endogenous p53 is not due to different primer efficiencies in the PCR reaction. Increasing amounts of the rat probasin promoter fused to p53 cDNA was used as a template in the RT-PCR reaction for each primer pair.
A and B) to poorly differentiated cancer according to our grading scheme (47). Of these mice, six (6/14; 43%) had palpable tumors ranging in size from 0.8 to 10.7 g. In contrast, there was no evidence of adenocarcinoma in the TRAMP × PB-p53R172L transgenic mice, although there was a profound increase in the cytoplasmic-to-nuclear ratio in the epithelial

FIGURE 3. Analysis of prostate tissues from nontransgenic and PB-p53R172L transgenic mice. A–D. Representative sections stained with H&E. A. Section through prostate of nontransgenic male mouse demonstrates acini composed of a single layer of columnar secretory epithelium with rounded basal nuclei (×10). B. Higher magnification of section shown in A demonstrates that three to four layers of smooth muscle cells and stromal fibroblasts (×40) surround each acinus. C. Section through prostate of a PB-p53R172L transgenic mouse demonstrates histology identical to that observed in A (×10). D. Higher magnification of section shown in C demonstrates that acini in PB-p53R172L transgenic mouse are surrounded by three to four layers of fibromuscular stroma, smooth muscle cells, and stromal fibroblasts (×40) similar to nontransgenic mice. E–G. Section from nontransgenic mice analyzed by TUNEL method (E), immunohistochemistry with anti-p21 antibody (F), and immunohistochemistry with anti-BrdUrd antibody (G; ×40). H–J. Sections from PB-p53R172L transgenic mice analyzed by TUNEL method (H), immunohistochemistry with anti-p21 antibody (I), and immunohistochemistry with anti-BrdUrd antibody (J; ×40). Methyl green was used as counterstain. Arrows, positive cells. Black bars, 5 μm.
TRAMP \times PB-p53^{R172L} Bigenic Mice Demonstrate Prolonged Survival

To study the impact of PB-p53^{R172L} on survival in the context of TRAMP, four cohorts of mice, control, PB-p53^{R172L}, TRAMP, and TRAMP \times PB-p53^{R172L} F1 bigenic, were established by random assignment of 22 appropriately genotyped mice to each cohort. All mice were C57BL/6 × FVB F1 littersmates. The mice were followed to death or until their tumors became obviously palpable at which time they were sacrificed. Consistent with previous observations, no deaths were recorded in the control and PB-p53^{R172L} cohorts before these mice reached 50 weeks of age. TRAMP mice began to develop palpable tumors at \sim 15 weeks of age and all mice were sacrificed by 30 weeks of age (Fig. 8). In contrast, none of the bigenic mice were sacrificed until \sim 28 weeks of age and the last of the bigenic mice was sacrificed at 48 weeks of age. This represents a 54% increase in tumor latency and a 63% increase in survival, respectively, as a consequence of p53^{R172L} expression.

Discussion

Loss of heterozygosity or mutation at the p53 tumor suppressor gene locus is associated with advanced human prostate cancer. The loss of wild-type p53 function has been implicated as a molecular mechanism contributing to the failure of conventional chemotherapy and radiotherapy, and replacement p53 gene therapy may therefore prove efficacious for this disease. We have explored the activity of a variant p53
FIGURE 5. Histological analysis of prostate tissues from TRAMP and TRAMP × PB-p53R172L bigenic mice. A–D. Representative sections stained with H&E. A. Section through prostate of TRAMP male mouse demonstrating well-differentiated adenocarcinoma. B. Higher magnification of section shown in A demonstrates that individual acini contain multiple smaller glands and are surrounded by multiple layers of smooth muscle cells and stromal fibroblasts (×40). C. Section through prostate of a TRAMP × PB-p53R172L transgenic mouse shows regions with similar histology as that observed in A (×10). D. Higher magnification of section shown in C demonstrates that acini show papillary projections of epithelial cells characteristic of prostatic intraepithelial neoplasia. Individual acini surrounded by three to four layers of fibromuscular stroma, smooth muscle cells, and stromal fibroblasts (×40). E–G. Section from TRAMP mice analyzed by TUNEL method (E), immunohistochemistry with anti-p21 antibody (F), and immunohistochemistry with anti-BrdUrd antibody (G; ×40). H–J. Sections from TRAMP × PB-p53R172L transgenic mice analyzed by TUNEL method (H), immunohistochemistry with anti-p21 antibody (I), and immunohistochemistry with anti-BrdUrd antibody (J; ×40). Methyl green was used as counterstain. Arrows, positive cells. Black bars, 5 μm.
molecule carrying an arginine-to-leucine mutation at codon 172, p53R172L. We demonstrate that murine p53R172L, equivalent to human p53R175L, exhibits properties characteristic of a hypermutifer positive function p53 in both cultured cells and autochthonous animal models.

In p53-null fibroblasts, the p53R172L construct was observed to induce transcription of mdm2 and WAF-1/p21 reporter constructs to levels between 2- and 5-fold over that of wild-type p53. This was not a general phenomenon related to the transfection protocol, as the variant was able to discriminate between the various p53-responsive elements. It is interesting to note that the arginine 175 residue resides within the L2 loop of p53 near the zinc-binding site (52), which is known to influence the conformation of the p53 DNA-binding domain, and p53R172L may possess a certain degree of freedom to bind to or activate WAF-1/p21 and mdm2 promoters more efficiently than wild-type p53. Furthermore, the inherent differences in the p21, mdm2, and Bax promoters likely contribute to the differential activation. It should be noted, however, that the transfection data did not predict the activation of Bax observed in the transgenic studies.

Having characterized the ability of the variant p53R172L to act as a positive sequence-specific transcription factor in cells in culture, the consequence of p53R172L expression was examined in vivo. To this end, transgenic mice were generated with a construct carrying the −426/+128-bp rat minimal probasin promoter fused to a murine p53R172L minigene. As demonstrated above, the PB-p53R172L transgene was expressed at low levels in the prostate gland of line 7856. This low-level expression was consistent with our previous observations using the minimal probasin promoter (44, 53). Although maximal expression of probasin-directed transgenes is normally achieved by 12 weeks of age (44, 53), the morphology of the normally quiescent prostatic epithelium was not altered by 18 weeks of age in the PB-p53R172L mice. The lack of an increase in cell proliferation or apoptosis in the prostate as a consequence of p53R172L expression is likely related to the level of transgene expression. In contrast, when the lactation-regulated WAP promoter was used to direct p53R172L expression in transgenic mice (41), there was a marked increase in apoptosis that altered development of the mammary gland. It should be noted that in this mammary gland model, the inhibition of normal lobuloalveolar development occurred during late pregnancy, a time of rapid cellular proliferation in the mammary gland. Consistent with the argument that high-level expression of p53R172L is cytotoxic, our attempts to isolate stable clones of prostate epithelial cells that express high levels of p53R172L following transfection have been unsuccessful (data not shown). Based on the observations with the PB-p53R172L mice and the WAP-p53R172L mice, we tend to favor the explanation that activation of the p53R172L mutant is restricted to cells undergoing rapid division and differentiation. That raised the intriguing possibility that p53R172L might exert an enhanced tumor suppressor phenotype in cells undergoing neoplastic transformation. Data supporting this interpretation come from studies showing that radiation-induced p53-mediated apoptosis can be correlated with the proliferative state of the mammary gland (54).

To test our hypothesis that p53R172L might act in a dominant manner to abrogate tumor progression in vivo, the PB-p53R172L

![Figure 6](image6.png)

**FIGURE 6.** Reduced tumor burden as a function of PB-p53R172L-directed apoptosis. The apoptotic and proliferation indices for nontransgenic controls, PB-p53R172L, TRAMP, and TRAMP × PB-p53R172L bigenic mice were estimated by counting TUNEL-positive cells and BrdUrd-labeled cells, respectively. Twenty high-power fields were counted for each of five or six paraffin sections for each of the 22 mice/cohort. Columns, mean percent positive cells; bars, SE.

![Figure 7](image7.png)

**FIGURE 7.** Increased expression of Bax in vivo as a function of PB-p53R172L expression. Expression of Bax protein was evaluated by immunoblotting in extracts prepared from ventral prostates of 12 individual mice. *Lanes 1, 5, and 9, nontransgenic mice (N); lanes 2, 6, and 10, PB-p53R172L mice (P); lanes 3, 7, and 11, TRAMP × PB-p53R172L bigenic mice (PT); and lanes 4, 8, and 12, TRAMP mice (T)*. Arrows, relative migration of the Bax protein. Blots were probed with antibody specific for β-actin to demonstrate equal loading in all lanes.
transgenic mice were crossed to the TRAMP mice. Remarkably, this resulted in a dramatic reduction in the gross wet weight of the GU tissue and an increased apoptotic index and a corresponding decrease in tumor incidence in TRAMP/C2 PB-p53R172L bigenic mice. Because both p21 and Bax were induced in vivo, we now propose that p53R172L has a tumor-specific function that is independent of the Tag oncoprotein. Presumably, p53R172L could also contribute to apoptosis through a p21-independent and Bax-independent mechanism, as only a slight shift in the proliferation-to-apoptosis ratio may be required to facilitate the protective phenotype. In fact, Noxa, a BH3 domain-only member of the Bcl-2 family (55), as well as the FAS receptor (56) may be mediators of p53-induced, apoptosis. Clearly, additional studies will be necessary to determine if p53R172L can induce expression of these genes. Furthermore, p53 has been suggested to have apoptotic functions that are independent of transcription (57). While the expression of p53R172L in prostate could extend the survival of bigenic mice, as shown in Fig. 8, these mice were found to eventually succumb to prostate cancer. Despite the aggressive nature of the TRAMP system, the low level of PB-p53R172L expression in the bigenic mice was sufficient to increase survival by as much as 60%. In clinical terms, application of p53R172L in a therapeutic strategy might translate into significant reductions in mortality and associated morbidity.

In conclusion, we undertook in vitro and in vivo studies to address the activity of a variant p53 molecule, p53R172L. Our in vitro studies indicated p53R172L possesses promoter-specific properties. While in vivo, expression of low-level expression p53R172L in otherwise normal prostate epithelial cells resulted in no observable abnormalities. When expressed in combination with the autochthonous TRAMP model, the subphysiological expression of p53R172L significantly decreased tumor incidence and tumor growth. The observation that low-level expression of p53R172L could specifically induce significant apoptosis in tumor cells in vivo suggests that p53R172L may be a good candidate for gene therapy.

Materials and Methods

Plasmids

For transfection studies, the p53 expression plasmids consist of either murine wild-type p53 or mutant p53R172L minigene (41) fused between the cytomegalovirus promoter and SV40 cleavage and the polyadenylic acid signals in pBluescript SK- (Stratagene, La Jolla, CA). The Bax-luc, p21-luc, and mdm2-luc constructs (a generous gift of Dr. M. Oren) contain the p53 response elements as described previously (20, 58, 59).

Cell Culture

The p53-null fibroblasts were established from a p53−/− mouse embryo (a generous gift of Dr. G. Lozano) and grown in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 25 units/ml of penicillin and streptomycin (Life Technologies).

Half-life Measurement

Cells were seeded into 100-mm plates and grown to 60% confluence. Transfections were performed with SuperFect method (Qiagen, Inc., Valencia, CA) with 5 μg of either wild-type p53 or p53R172L expression plasmids for 24 h. Protein half-life was determined by radioactive pulse labeling with 200 μCi/ml of Trans-[35S] Label (1175 Ci/mmole; ICN Radiochemicals,
Irvine, CA) in methionine- and cysteine-free DMEM (ICN) containing 2% dialyzed fetal bovine serum (Hyclone) for 15 min and chased in DMEM containing 10% fetal bovine serum and 2 mM unlabeled methionine and cysteine for 0, 1, 2, and 3 h. The cells were harvested in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM polymethylsulfonyl fluoride], and the p53 proteins were immunoprecipitated with the polyclonal antibody FL-393 (Santa Cruz Biotechnology, Santa Cruz, CA). Precipitates were resolved by electrophoresis through 10% SDS-polyacrylamide gels. Quantification of labeled protein was performed with a STORM PhosphorImager (Amersham Biosciences, Piscataway, NJ).

Transactivation Assays

For transactivation assays, 7.5 × 10^5 cells were plated in six-well plates for 24 h. Transfections were carried out with 0.5 μg of wild-type p53 or p53R172L expression plasmids and 0.1 μg of reporter plasmid using the SuperFect method as described above. Cells were washed in DMEM and harvested 28 h later in lysis buffer [0.2 mM Tris (pH 8.0), 0.1% Triton X-100]. Luciferase activity was determined by mixing a 20 μl aliquot of the cell lysate with 100 μl of luciferase assay reagent [20 mM Tris (pH 8.0), 4 mM MgSO4, 0.1 mM EDTA, 30 mM dithiothreitol, 0.25 mM acetyl-CoA] and each reaction was monitored for 10 s in a luminometer.

Western Blotting

Aliquots of cell lysates prepared for transactivation assays were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA). Membranes were washed in TBST [10 mM Tris (pH 7.4), 150 mM NaCl, 0.05% Tween 20] and blocked by incubation with 5% Carnation milk in TBST at room temperature for 1 h. Blots were incubated overnight at 4°C with antibodies in TBST containing 3% Carnation nonfat dry milk. The antibodies were used at a dilution of 1:750 for the anti-p53 antibody (FL-393; Santa Cruz Biotechnology, Santa Cruz, CA). Precipitates were resolved by electrophoresis through 10% SDS-polyacrylamide gels. Immunoreactive bands were quantified using a horseradish peroxidase membrane (Millipore, Billerica, MA). Membranes were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA). Membranes were washed in TBST [10 mM Tris (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% Triton X-100] and blocked by incubation with 5% Carnation milk in TBST at room temperature for 1 h. Blots were incubated overnight at 4°C with antibodies in TBST containing 3% Carnation nonfat dry milk. The antibodies were used at a dilution of 1:750 for the anti-p53 antibody (FL-393; Santa Cruz Biotechnology, Santa Cruz, CA). Precipitates were resolved by electrophoresis through 10% SDS-polyacrylamide gels. Immunoreactive bands were quantified using a horseradish peroxidase membrane (Millipore, Billerica, MA). Membranes were subjected to SDS-PAGE and transferred to polyvinylidene chloride substrate development (Pierce Chemical Co., Rockford, IL). Immunoreactive bands were quantified using Alphalnager 2000 (3.3) from Alpha Innotech Corporation (San Leandro, CA).

Transgenic Mice

To target expression of p53R172L to mouse prostate epithelium, the hybrid minigene (41) was placed downstream of the minimal −426/+28 rat probasin promoter modified to carry flanking SalI restriction sites. The restriction sites were introduced by 20 cycles of PCR using primers:

PB1: CCGGTGCACCAGGCTGTAGGTATCTGGACCT-
TTA and

PB2: CCGGTGCACCGGCTGTAGGTATCTGGACCT-
CAC.

The integrity of the PCR fragment was confirmed by DNA sequencing and subcloned into the unique SalI site upstream of the murine p53R172L minigene in plasmid pBL103 (41). A 5-kb BssHII DNA fragment carrying the transgene free of vector sequence was purified by adsorption to Qiagen beads and introduced into FVB embryos by microinjection. Founders carrying the PB-p53R172L transgene were identified by PCR screening (60) and primers:

P1: CCGGTGCACCAGGCTGTAGGTATCTGGACCT-
TTA and
P2: GCCTGAAAATGTCTCCTGGCTCAGAGGG

(Fig. 3A).

TRAMP mice were maintained in a pure C57BL/6 background and screened as described previously (44). The bigenic C57BL/6 × FVB F1 mice were generated by a cross of PB-p53R172L and TRAMP mice and were identified by multiplex PCR by the method described above. All mice in this study carrying PB-SV40 early genes (TRAMP) or the PB-p53R172L constructs were obtained as C57BL/6 × FVB F1 littermates. Nontransgenic C57BL/6 × FVB F1 littermates were used as controls in all studies.

RNA Extraction and Analysis

Prostate RNA samples were prepared as described (46). Total RNA was isolated from all other mouse tissues as described previously (61). For RT-PCR analysis, 1 μg of total RNA was reverse transcribed as described previously (44), except the RT reaction temperature was 42°C and the annealing temperature during PCR was 68°C. Expression of the p53 transgene was detected with primers:

P3: CTGGTCTAGTGAGTGCCAGATACCCAG and
P5: GTGGGGAGAGCTCTACAGCC.

Expression of endogenous p53 was detected with primer P4: ATGACTGCCATGGAGGAGTCACAGT and primer P5. RT-PCR products were fractionated on 1.2% agarose gels transferred to Zetaprobe membranes and probed with an [α]32P-dCTP-labeled 500-bp AvaI fragment of mouse p53 cDNA.

Measurement of Prostate Wet Weight

The GU tracts of transgenic and control mice were removed at necropsy according to the method described by Gingrich et al. (46). Following resection of seminal vesicles and coagulating gland, remaining bladder, urethra, and prostatic tissues were removed by microdissection, and weights were determined on a digital microbalance (Fisher Scientific, Pittsburgh, PA). Statistical analysis was performed using the Student’s t test and Mann-Whitney methods (StatView 4.5, Abacus Concepts, Inc., Berkeley, CA) on a Macintosh Power PC computer (Apple Computer, Cupertino, CA). Results were plotted using DeltaGraph Professional.
Histology and Immunocytochemistry

Prostate glands and other organs were surgically removed at necropsy, fixed in 10% neutral buffered formalin for 6 h, placed in 70% ethanol overnight, and processed and embedded in paraffin as described previously (44). Sections cut at 4 μm were placed on Probe-on Plus slides (Fisher Scientific) and every 10th slide was stained with H&E. The TUNEL method used to visualize apoptotic cells and the BrdUrd incorporation employed to visualize proliferating cells were performed as described previously (32, 62). Briefly, to detect p21, sections were deparaffinized and incubated with 20 μg/ml proteinase K for 5 min at room temperature. Endogenous peroxidases were quenched using 2% H2O2, and nonspecific binding sites were blocked with 5% goat serum for 30 min. Sections were incubated with a rabbit polyclonal antibody against p21 (generously provided by Dr. Wade Harper, Baylor College of Medicine) at a 1:400 dilution overnight at 4°C. The sections were washed, incubated with secondary antibody, and visualized using avidin-biotin complex immunoperoxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA).

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References


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Prostate-Specific Expression of \( p53^{R172L} \) Differentially Regulates \( p21 \), \( Bax \), and \( mdm2 \) to Inhibit Prostate Cancer Progression and Prolong Survival. National Cancer Institute awards CA58206, CA64851, and CA884296 and a CaP CURE award to N. M. G.

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