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Knock-in mice with a chimeric human/murine p53 gene develop normally and show wild-type p53 responses to DNA damaging agents: a new biomedical research tool

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The high prevalence and great diversity of p53 tumor suppressor gene mutations in human tumors call for development of therapeutic molecules that rescue function of aberrant p53 protein. P53 mutations also offer new approaches to the study of the origins of mutations in human cancer. An experimental mouse model with a genetically modified but normal functioning p53 gene harboring the human rather than the murine core domain, would be of considerable benefit to research on both cancer therapeutics and etiology; however, it is uncertain whether such mice would permit biological functions of p53 to be retained. Using a Cre/lox P gene-targeting approach, we have constructed a human p53 knock-in *(hupki)* mouse strain in which exons 4–9 of the endogenous mouse p53 allele were replaced with the homologous, normal human p53 gene sequence. The chimeric p53 allele ($p53^{KI}$) is properly spliced, transcribed in various tissues at levels equivalent to wild-type mice, and yields cDNA with the anticipated sequence, that is, with a core domain matching that of humans. The *hupki* p53 protein binds to p53 consensus sequences in gel mobility shift assays and accumulates in the nucleus of *hupki* fibroblasts in response to UV irradiation, as is characteristic of wild-type p53. Induction of various p53-regulated genes in spleen of γ-irradiated homozygous *hupki* mice ($p53^{KI/KI}$), and the kinetics of p53-dependent apoptosis in thymocytes are similar to results with wild-type ($p53^{+/+}$) mice, further indicating normal p53 pathway function in the *hupki* strain. The mice are phenotypically normal and do not develop spontaneous tumors at an early age, in contrast to knock-out ($p53^{-/-}$) strains with a defective p53 gene. The chimeric ($p53^{KI}$) allele thus appears to provide a biological equivalent to the endogenous murine ($p53^{+}$) gene. This strain is a unique tool for examining *in vivo* spontaneous and induced mutations in human p53 gene sequences for comparison with published human tumor p53 mutation spectra. In addition, the *hupki* strain paves the way for mouse models in pre-clinical testing of pharmaceuticals designed to modulate DNA-binding activity of *human* p53. *Oncogene* (2001) **20,** 320–328.

Keywords: p53 mouse model; gene targeting; mutagenesis

Introduction

The p53 protein is dysfunctional or absent in the majority of human tumors, primarily due to single missense point mutations in the gene (Vogelstein and Kinzler, 1992; Hainaut and Hollstein, 2000). Most of the 15 000 published human tumor mutations lie in the core (DNA-binding) domain (Harris and Hollstein, 1993; Lane, 1999), and reduce or abolish the ability of the p53 protein to bind to recognition sequences of p53-responsive genes and stimulate transcription. To investigate factors contributing to p53 tumor mutations in tumors, published p53 mutation spectra of human cancers can be compared with experimentally induced tumor p53 mutations in standard laboratory mouse strains (reviewed in Hollstein *et al*., 1999); however, there has been to date no practical approach to the experimental induction of mutations in the *human* p53 gene sequence *in vivo*. The core domains of the mouse and human p53 genes differ at 15% of base residues. Since mutation patterns are dictated in the first instance by the exact DNA base context, even subtle sequence differences can modify mutation spectra. Expectedly, specific carcinogen-induced p53 tumor mutations do not accumulate at precisely the same sites in the mouse and in humans (Dumaz *et al*., 1997). These considerations call for generation of mouse strain with human p53 sequences, which would be particularly relevant if the modified gene were transcribed at normal levels and encoded a functional protein.

A human p53 knock-in mouse strain of this nature would also lend itself to investigation of molecules designed to target the tumor suppressor protein. While mice with an unmodified endogenous p53 gene can be used to test the efficacy of drugs targeting the mouse

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p53 protein, human/mouse species sequence differences complicate assessment of experimental results for humans. Although the similarity between mouse and human p53 is remarkably high at the amino acid level (91% homology in the core domain), single residue differences may affect response to drugs designed either to restore DNA-binding activity of the common human tumor missense mutants (Foster *et al*., 1999), or conversely, to block wild-type protein function temporarily in order to reduce side effects of chemo- or radiation therapy (Komarov *et al*., 1999). Genetically engineered mice with a 'humanized' p53 gene thus would provide a new dimension to existing *in vivo* test systems not only for mutagenesis studies but also for pre-clinical testing of p53-modifying drugs; however, it remains to be shown whether in this chimeric context, p53 would continue to exercise its cellular activities.

Towards this end, we generated a mouse strain with p53 gene harboring the human core domain sequence. In these mice, referred to as *hupki* mice (for human p53 knock-in), exons 4–9 and intervening introns of the mouse allele have been replaced with the corresponding homologous segment of the normal human p53 gene, and transcription remains under endogenous control of the mouse locus. Biochemical and functional studies described here demonstrate that the genetically-modified p53 gene of homozygous *hupki* mice is expressed at physiological levels and can perform various cellular functions of normal p53.

Results

Generation of human p53 knock-in (hupki) mice

After identification of ES clones containing the recombinant allele ($p53^{KI}$) of p53 (Figure 1, upper panel), ES cells were injected into blastocytes to generate chimeric mice. Germline transmission of the knock-in allele originally derived from ES clone 3–16 was identified by Southern blotting after breeding chimeric mice with wild-type mice (Figure 1). F1 germline heterozygous offspring ($p53^{KI/+}$) were intercrossed to produce F2 homozygous *hupki* mice. Both heterozygous and homozygous *hupki* mice from such crosses were born at the expected frequency (Mendelian ratio), and homozygous *hupki* mice are phenotypically normal and fertile (data not shown), suggesting that the chimeric p53 protein is compatible with mouse development. Homozygous *hupki* mice developed no spontaneous tumors by 8 months of age, in contrast to p53 null mice which are highly susceptible to lymphomas and sarcomas at an early age (Donehower *et al*., 1992; Jacks *et al*., 1994).

To examine whether the chimeric p53 is transcribed correctly *in vivo*, p53 cDNA prepared from *hupki* ($p53^{KI/KI}$) mouse spleen RNA was sequenced and shown to match precisely the mouse wild-type p53 gene sequence in exons 2, 3, 10 and 11, whereas exons 4–9 (codons 33–332) were identical to the normal human p53 sequence, as expected. The human polymorphic codon 72 encodes arginine in the $p53^{KI}$ gene. The cDNA sequence confirmed that the targeted chimeric gene is correctly spliced and transcribed in the spleen of *hupki* mice (data not shown).

Analysis of $p53^{KI}$ gene expression

Our objective was to generate a mouse with a human/mouse chimeric p53 gene at the endogenous locus that would remain under normal transcriptional regulation, and would maintain normal p53 tumor suppressor-associated functions. In view of the high divergence between mouse and human intron sequences, and their possible role in transcription regulation (Shamser and Montano, 1996), we first compared p53 mRNA levels in various tissues of 10-week-old wild-type ($p53^{+/+}$) and $p53^{KI/KI}$ mice, and explored possible transcription preferences of $p53^{+}$ and $p53^{KI}$ alleles by three different approaches: (i) semiquantitative RT–PCR: Duplex semiquantitative RT–PCR of RNA extracted from 10 hupki ($p53^{KI/KI}$) and wild-type ($p53^{+/+}$) tissues using β-actin (reference sequence) and p53- specific primer pairs indicated similar levels of p53 expression for the two genotypes in each tissue compared (Figure 2A); p53 expression is high in spleen, thymus, lung and kidney, relative to levels in brain; (ii) restriction patterns of p53 RT–PCR products: To corroborate that the $p53^{KI}$ transcript is present in various tissues and at levels comparable to wild-type mice, we took advantage of *Ava*I restriction sites present in exon 6 of the normal mouse p53 gene, but absent in the human exon 6 sequence. RT–PCR amplifications encompassing exon 6 and using template from $p53^{KI/+}$ heterozygotes, followed by enzymatic digestion with *Ava*I yielded comparable amounts of cleaved product (from the $p53^{+}$ allele) and uncleaved product (from the $p53^{KI}$ allele) for all 10 organs examined. (Results with liver and spleen are shown in Figure 2B; data not presented for the eight additional tissues referred to in Figure 2A); and (iii) Northern blotting: The presence of equivalent p53 mRNA levels in $p53^{KI/KI}$ and $p53^{+/+}$ mice was verified in thymus, spleen and brain (showing high, medium and low levels respectively) of 10-week-old mice by Northern blot analysis using exon 11 probe P (data not shown).

The presence of *hupki* p53 protein, and its accumulation following DNA damage by γ-irradiation were examined on immunoblots from primary embryonic fibroblast whole cell extracts using anti-mouse p53 polyclonal antibody CM5. As shown in Figure 3A, $p53^{KI}$ protein from *hupki* cells is expressed, and levels increase following 5 Gy γ-irradiation, as is seen with wild-type murine p53 ($p53^{+}$). With CM5 antiserum, p53 signal intensity on immunoblots is lower in *hupki* and in human neonatal fibroblast extracts than in extracts from wild-type mice (data not shown), as anticipated from Pepscan ELISA analysis of CM5 (Lane *et al*., 1996), so the issue of whether p53 protein levels are equivalent in wild-type and *hupki* cells is not addressed in this experiment. Electromobility shift assays (EMSA) with protein from irradiated primary

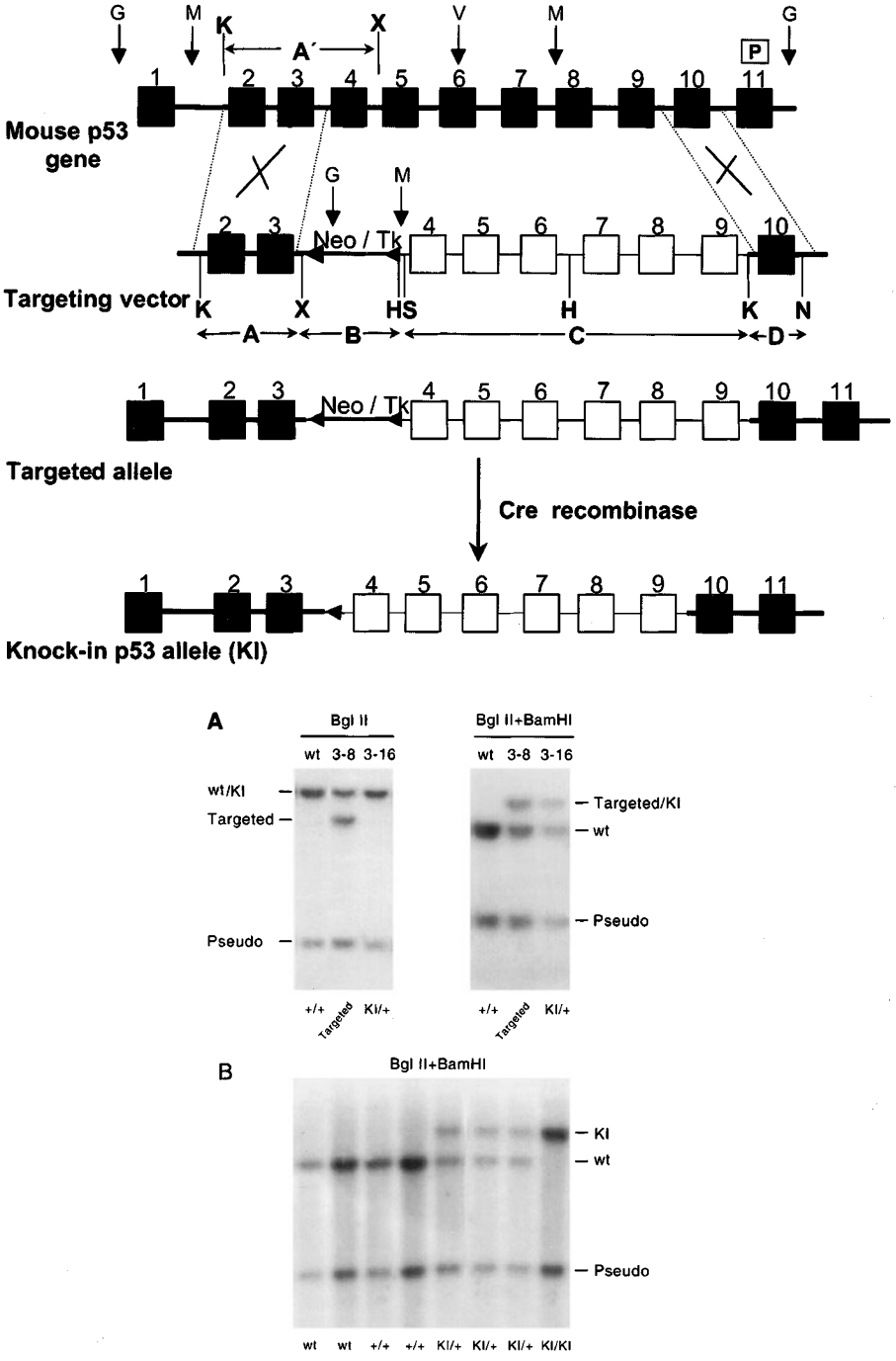

Figure 1 Generation of p53 *hupki* mice. **Upper panel**: Schematic diagram showing gene-targeting strategy and structure of the p53*hupki* allele. Not to scale. Fragment A′ (3.6 kb) and fragment D (1.4 kb), mouse genomic p53 sequences; Fragment B: selection cassette containing neo and TK genes; Fragment C, 2.8 kb human p53 segment encompassing human exons 4–9 (aa 33–332). Symbols: Solid squares: mouse exons; open squares: human exons; Open square 'P': exon 11 probe (outside recombination junction) used for Southern and Northern blot hybridizations; solid horizontal arrows: 34 bp loxP sequence; restriction enzyme sites used or generated by cloning strategy or used in genotyping include: K, *Kpn*I; X, *Xba*I; H, *Hind*III; S, *Spe*I, N, *Not*I; V, *Ava*I, G, *Bgl*II; M, *Bam*HI. **Lower panel**: Genotyping of ES clones and mice by Southern blot analyses. Genomic DNA was isolated from ES clones or tails of *hupki* mice, and digested by *Bgl*II alone, or by *Bgl*II and *Bam*HI, followed by hybridization to ^{32}P-labeled mouse exon 11 probe P (box marked 'P'). *Bgl*II digestion: the wild-type allele (+) and knock-in allele (KI) (after excision of the neo/TK selection cassette by Cre) generate a 13 kb fragment, whereas the targeted allele before cassette excision generates the smaller 9 kb fragment due to a *Bgl*II site in the cassette. *Bgl*II/*Bam*HI digestion: The untargeted endogenous p53 allele generates a 4.2 kb fragment, whereas both the targeted allele (before cassette excision), and the knock-in allele after cassette excision (KI), generate a 6.2 kb probe-hybridizing fragment. Note that probe P also hybridizes in all genotypes to a faster migrating fragment corresponding to the mouse p53 pseudogene. (**A**) ES cell genotyping: (**B**) mouse genotyping

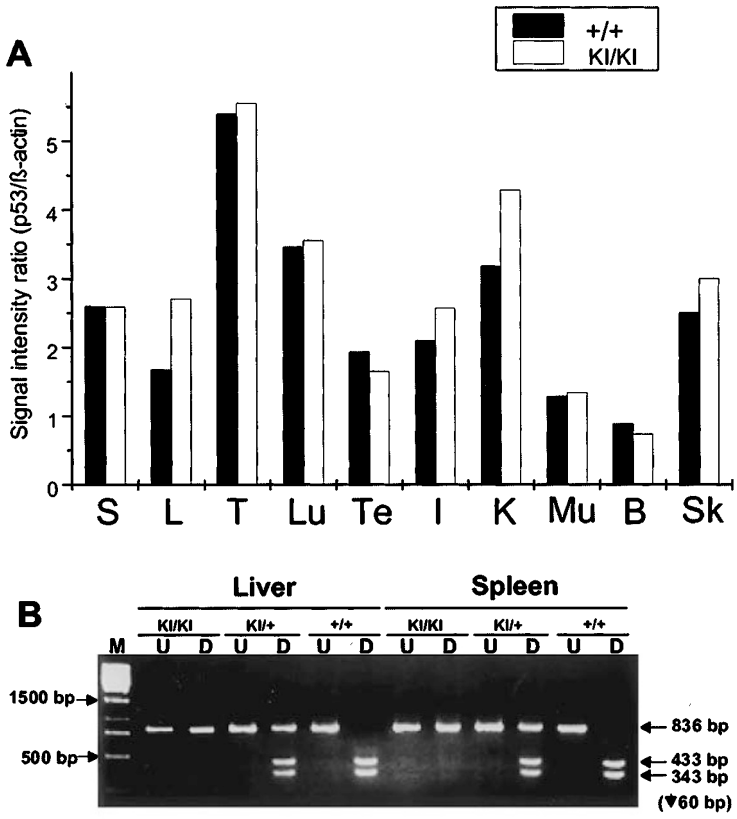

Figure 2 P53 transcript levels in various tissues. (**A**) RT–PCR analysis of p53 mRNA in tissues of wild-type ($p53^{+/+}$) and *hupki* ($p53^{KI/KI}$) mice. Semiquantitative duplex RT–PCR of p53 and β-actin messenger RNA was performed for 10 tissues of wild-type and *hupki* mice. Symbols: S, spleen, L, liver; T, thymus; Lu, lung; Te, testis; I, small intestine; K, kidney; Mu, muscle; B, brain; Sk, skin. Bar chart shows ratio of p53 and β-actin signals from densitometric scanning of RT–PCR products in ethidium bromide-stained agarose gels. (**B**) *Ava*I digestion of liver and spleen p53 RT–PCR product from wild-type ($p53^{+/+}$), and from *hupki* homozygous ($p53^{KI/KI}$) and heterozygous ($p53^{KI/+}$) mice, indicating equivalent levels of wild-type (with *Ava*I sites), and KI (no *Ava*I site) p53 transcripts. cDNA was amplified by PCR using p53 primers P42A and P10B to generate an 836 bp product. *Ava*I digestion in exon 6 cleaves this product when cDNA is derived from wild-type mouse tissues but not when from *hupki* tissues. cDNA of $p53^{KI/+}$ heterozygote shows both cleaved and uncleaved fragments of approximately equal intensities on ethidium bromide-stained agarose electrophoresis gels. Symbols: U, undigested PCR product (836 bp); D: PCR product incubated with *Ava*I restriction enzyme, yielding two major fragments from wild-type fragment: 433 and 343 bp, (and 60 bp; not visible on gel photo)

embryonic fibroblasts were performed to test whether the *hupki* protein is able to bind to p53 target DNA sequences, a pre-requisite for many tumor suppressor activities of p53. Experiments using p53 consensus oligonucleotide $p53^{CON}$ and anti-p53 antibody PAb421 revealed specific complexes with *hupki* cell extracts, as was observed with complexes from wild-type cell extracts (Figure 3B). Specific competition with non-radioactive consensus sequence abolished the signal, whereas a 20-fold excess of mutant consensus sequence, which does not bind p53 protein had no effect on signal intensity (Figure 3B).

P53 functional analysis

Transcriptional regulation of numerous genes is critical to p53-mediated cell cycle arrest and apoptosis following DNA damage by γ-irradiation. Specific transactivation of two well-studied genes, p21/Waf1/Cip1, encoding a cyclin-dependent kinase, and Bax, a regulator of programmed cell death, was initially observed by semi-quantitative RT–PCR in spleen of γ-irradiated (5 Gy) wild-type and homozygous *hupki* mice 6 and 24 h post treatment (data not shown). Irradiation induction of Bax and p21/Waf1 was verified in both genotypes by Northern blot analysis (Figure 4A). Gene expression profiling was performed with Clontech Mouse 1.2 Atlas™ membranes (>1000 distinct cDNA sequences) for spleen of homozygous *hupki* mice to investigate concordance with wild-type profiles. Expression patterns were highly similar in the two genotypes, both from untreated and from irradiated mice 24 h post treatment; *Hupki* spleen transcript levels of numerous genes involved in distinct cellular processes were induced by treatment, including p53-inducible genes Cyclin G, and Mdm2, consistent with expectations from the literature on p53 wild-type mice (Table 1).

Wild-type p53 protein in normal cells accumulates in nuclei in response to ultraviolet light (UV)-induced DNA damage to levels readily detected by immunocytochemistry. We investigated this response to UV

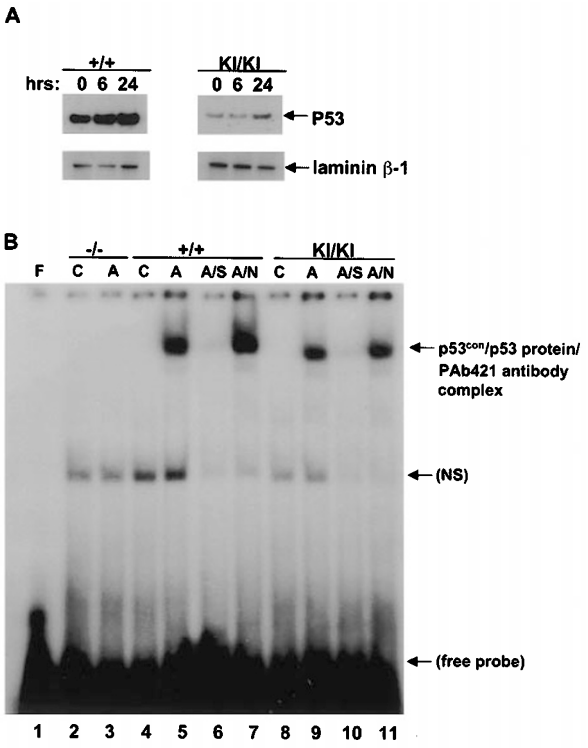

Figure 3 Accumulation of p53 *hupki* protein following DNA damage, and binding to p53 target consensus sequence. (**A**) Immunoblot analysis of p53 protein from primary embryonic fibroblasts. Membranes were incubated with polyclonal anti-p53 antiserum CM5, and duplicate blots with polyclonal anti-laminin β-1 (C-19) antiserum to control for sample loading (Santa Cruz Biotechnology). Primary embryonic fibroblasts of wild-type ($p53^{+/+}$) and *hupki* ($p53^{KI/KI}$) mice (2nd passage) were exposed to 5 Gy γ-irradiation, and cell extracts in RIPA buffer were prepared 0, 6 and 24 h post-irradiation. (**B**) EMSA with protein from γ-irradiated embryonic fibroblasts derived from $p53^{+/+}$ and *hupki* (p53 KI/KI) mice. Whole cell extracts were incubated with ^{32}P-labeled p53-binding oligonucleotide ($p53^{CON}$) (Santa Cruz Biotechnologies), in the presence (A) or absence (C) of anti-p53 antibody PAb421. Specific (S) or non-specific competitor (N) oligonucleotides were added to parallel incubations as indicated. Negative controls: reactions with extracts from p53 null ($p53^{-/-}$) fibroblasts are shown in lanes 2–3), or with free oligonucleotide (F) alone, in lane 1

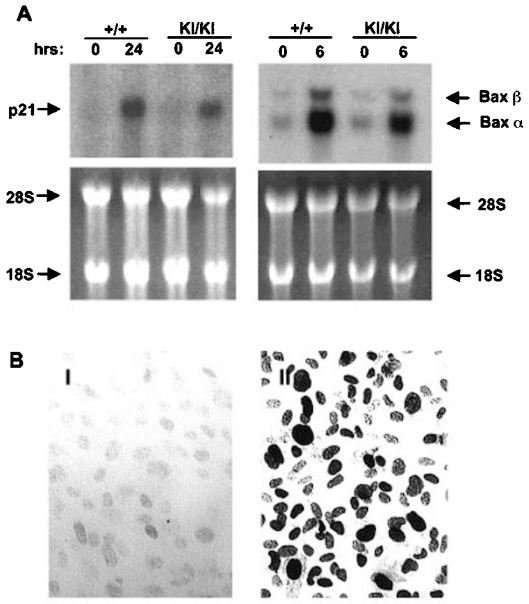

Figure 4 Induction of p21/Waf1 and Bax mRNA, and nuclear localization of p53 *hupki* protein following DNA damage. (**A**) Northern analysis of p21/Waf1 and Bax transcript levels in spleen of wild-type and *hupki* mice, untreated or γ-irradiated (5 Gy) as indicated. Ethidium bromide-stained agarose gels prior to blotting are shown in lower panels. (**B**) Accumulation and nuclear localization of p53 in embryonic fibroblast cell line MEF 42(B1) ($p53^{KI/KI}$) by UV irradiation. Semi-confluent monolayers were fixed in methanol and incubated with p53-specific antiserum CM1 and peroxidase-conjugated secondary antibody according to standard immunocytochemical procedures; photomicrographs (200× magnification) were prepared with Leica Quantimet 500 imaging software following immunocytochemical staining of cells. I. Untreated cells; II. UV-irradiated cells. (Contrast in I was enhanced to show nuclear contours.) Treated and untreated cells incubated with secondary antibody alone were negative (not shown)

irradiation in *hupki* cells. Nuclei of *hupki* ($p53^{KI/KI}$) embryonic fibroblasts exposed 24 h previously to 25 J/m^2 UV light (254 nm) revealed intense nuclear staining with polyclonal p53 antibody CM1, whereas nuclei of unirradiated control cell cultures showed faint to no signal with the same staining procedure (Figure 4b). This response is further indication that the *hupki* p53 protein is subject to this cellular regulation in a manner similar to wild-type p53.

P53-dependent and -independent apoptosis in thymocytes of hupki *mice*

To test whether the chimeric p53 protein would function in a p53-dependent biological process, we studied apoptosis induced by DNA damaging agents. Thymocytes were isolated from wild-type ($p53^{+/+}$), *hupki* ($p53^{KI/KI}$), and p53 null ($p53^{-/-}$) animals, and exposed to ionizing radiation (Figure 5), which induces apoptosis that is dependent on functional p53, or to dexamethasone, which induces p53-independent apoptosis in this cell type. After treatment with dexamethasone, thymocytes from all three genotypes exhibited apoptosis with similar kinetics (Figure 5A), indicating that this death pathway was not affected by the absence of wild-type p53 or the presence of *hupki* p53 protein. As expected, treatment with 5 Gy γ-irradiation induced apoptosis in wild-type cells, whereas thymocytes from $p53^{-/-}$ mice were profoundly resistant to the effects of ionizing radiation (Figure 5B), consistent with previously published results (Lowe *et al*., 1993; Clarke *et al*., 1993). Notably, thymocytes from *hupki* mice were susceptible to γ-irradiation-induced apoptosis at the same levels as those in wild-type thymocytes. In addition, in a dose-response experiment, *hupki* thymocytes exhibited apoptosis after various doses of γ-ray treatment with similar kinetics to wild-type cells at all doses tested (Figure 5C). These data indicate that the chimeric *hupki* p53 protein functions as efficiently as wild-type p53 with respect to its role in thymocyte apoptosis.

Discussion

We have generated a mouse strain in which human p53 gene sequences encompassing the p53 core domain are embedded in the endogenous p53 locus on mouse chromosome 11, and replace the corresponding mouse gene segment. We designed the targeting vector so that the entire core domain and flanking sequences would be homogeneous with respect to species origin, since an engineered human/murine chimeric core domain theoretically could present a sequence configuration that would render various subsequent somatic missense mutations innocuous by an intragenic suppressor mechanism (Brachmann *et al*., 1998). Expression and endogenous regulation of the modified p53 gene ($p53^{KI}$) have been achieved in homozygous *hupki* mice, despite the chimeric nature of the allele and the notorious sensitivity of p53 to minor sequence alterations not directed by evolution (Hainaut and Hollstein, 2000; Brachmann *et al*., 1998; Cho *et al*., 1994).

It has been shown recently that arginine at the common Arg/Pro polymorphic residue 72 of human p53 can enhance tumorigenicity of certain p53 mutants by compromising control of apoptosis by p73β, a binding partner of p53 (Marin *et al*., 2000). Since the *hupki* protein harbors the Arg72 residue, this raises the interesting possibility that presence of this residue will recapitulate the human situation by increasing tumorigenic potential of somatic mutations arising in mutagen-exposed *hupki* mice.

The biochemical and functional studies we have conducted thus far indicate we have achieved our aim to generate human p53 knock-in mice that retain *in vivo* physiological control of p53 and several of its functions. The *hupki* $p53^{KI/KI}$ mice exhibit normal development, fertility, and a p53 phenotype thus far corresponding to wild-type $p53^{+/+}$ mice of the same genetic background. Expression profiling with Affymetrix™ gene expression arrays is underway to explore potential differences between *hupki* and normal mice in the complex transcription patterns/induction kinetics of p53-downstream genes (Yu *et al*., 1999). Recent studies have demonstrated that distinct subsets of genes are regulated by p53 according to the nature of the inducing factor and cell type (Yu *et al*., 1999; Zhao *et al*., 2000). We

Table 1 Atlas™ mouse 1.2 array analysis of genes induced* by γ-irradiation in *hupki* mice

Cell cycle regulators, oncogenes and tumor suppressors:	
Cyclin G	(Z37110)**
Mdm2	(X58876)
Cyclin D1	(S78355)
Net/Ets-related	(Z32815)
Regulators of apoptosis or other biological processes:	
IGF-BP6	(X81579)
Bax	(L22472)
IRF-7	(U73037)
IGF-IA	(X04480)
Clustrin/MSGP-2	(L08235)
ICE	(L28095)
cathepsin D	(X53337)
RA-inducible protein E3	(U29539)

*Selected listing of Array cDNAs giving a >2.5-fold increase in signal; **GenBank Accession #

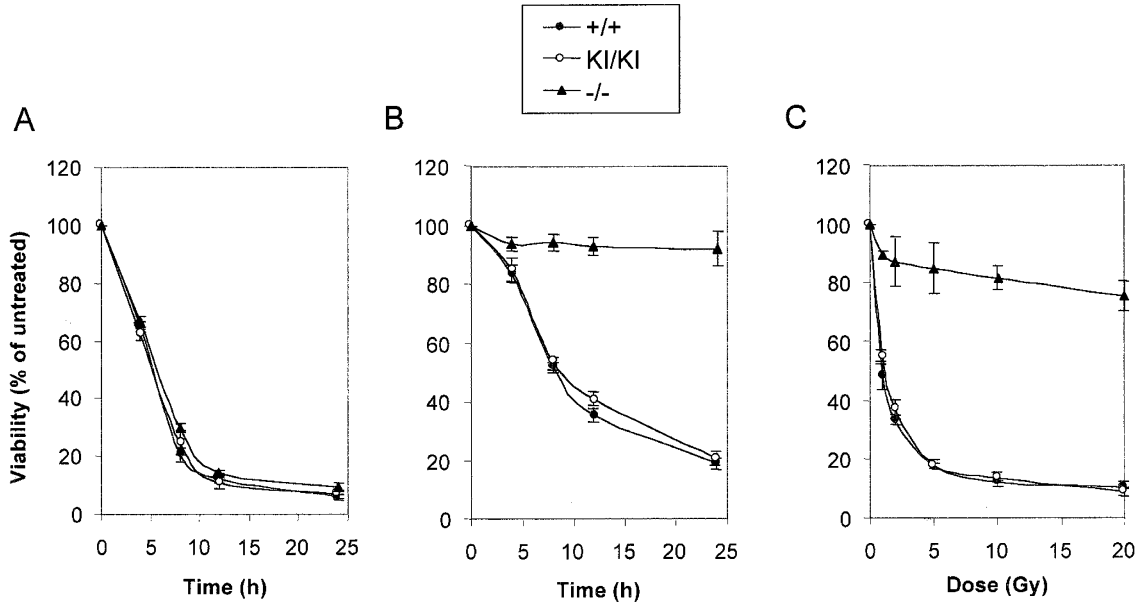

Figure 5 Function of *hupki* p53 protein in thymocyte apoptosis. Thymocytes were treated with 1 μM dexamethasone (**A**), 5 Gy ionizing radiation (**B**), and various doses of irradiation (**C**), as indicated. Viability was assessed at indicated time points after treatment. For the dose-response analysis, viability of thymocytes was measured as 20 h after γ-irradiation. Values represent average of triplicate assays and were normalized to the amount of viable cells remaining in untreated cultures derived from the same animals. One of two independent experiments is shown

chose spleen tissue, γ-irradiation, and 6 or 24 h post-treatment times for initial characterization of *hupki* mice; however, since a complexity of phosphorylation events and protein–protein interactions govern p53 function (Prives and Hall, 1999), further investigations using other parameters may reveal more subtle differences between *hupki* and wild-type mice in post-translational modification, biological function, or kinetics of activation. An important aspect of p53 function to address in future experiments is the ability of *hupki* p53 protein to perform similarly to wild-type p53 as participant in processes of DNA repair and recombination (Albrechtsen *et al*., 1999; Dudenhoffer *et al*., 1999). Paradoxically, a *hupki* mouse deficiency in p53 'guardian of the genome' activities could enhance fortuitously the usefulness of this mouse model by rendering the strain more susceptible to tumorigenesis when exposed to carcinogenic DNA damaging agents.

Mutation spectra

In principle, hypotheses regarding mutation induction by specific cancer risk factors may be tested by comparing p53 tumor mutations typical of a defined high-risk patient group with mutations generated experimentally in rodents, or even in simple cell systems such as yeast (Hainaut and Hollstein, 2000, Dumaz *et al*., 1997; Flaman *et al*., 1994). In practice, fundamental questions remain unanswered that require additional experimental models: *In vivo* 'spontaneous' p53 mutation patterns in humans in different cell/tissue types, in inflamed tissues, in regenerating tissues following toxicity or injury, or induced by oxidative stress, are ill-defined (Hollstein *et al*,. 1998; Beckman and Ames, 1998). These are key issues, since for a number of common cancers it is unlikely that chemical carcinogens are a major contributor to the tumor p53 mutations observed (Hollstein *et al*., 1998; Baker *et al*., 1991).

The *hupki* mouse offers a new refinement to p53 mutagenesis studies in rodents because it provides an *in vivo* rodent test model, yet with the precise human p53 sequence as mutation target. Various applications are open to investigators, such as detection of unselected mutations in normal tissues using sensitive PCR-based protocols (Aguilar *et al*., 1993), mapping of DNA damage sites in the p53 gene to show co-localization of damage sites with human tumor mutation hotspots in the p53 gene (Denissenko *et al*., 1996), or screening of neoplastic lesions and carcinomas in *hupki* mice for p53 core domain mutations with the Affymetrix p53GeneChip™ oligonucleotide array, an automated method applicable to *hupki* mouse samples because the p53 sequence is identical to the human sequence in exons 4–9. Tumor mutation spectra from experiments in *hupki* mice can be compared directly to the published human tumor p53 mutation data, unlike p53 mutations profiles that have been generated from examining tumors in wild-type mice.

Clinical implications

We anticipate that the *hupki* strain or its derivatives will pave the way for pre-clinical *in vivo* testing of new pharmaceuticals designed to modulate human p53 tumor suppressor DNA binding/transcription activation functions. The *hupki* strain facilitates further genetic refinement involving, for example, recombinational exchange of amino or carboxy terminal p53 sequences, which also are of keen interest in the development of new therapeutic strategies (Hupp *et al*., 1995; Selivanova *et al*., 1997; Rodriguez *et al*., 1999). *Hupki* strain derivatives carrying single point mutations discovered in Li-Fraumeni syndrome cancer-prone families (Kleihues *et al*., 1997) would provide a new mouse model with an allelic configuration that parallels the human situation, and complement existing animal models with mutant mouse p53 transgenes or p53-deficient genotypes (Donehower *et al*., 1992; Jacks *et al*., 1994; Hurstings *et al*., 1995; Zhang *et al*., 2000; Liu *et al*., 2000).

Materials and methods

Construction of the gene-targeting vector

DNA fragments A, C, and D of the gene targeting vector (Figure 1, upper panel) were obtained as follows: Fragments A′ and D were cloned from a 129/Sv genomic library. PCR-generated fragments (A, C), from template A′ and normal human genomic DNA respectively, were cloned into pGEM (Promega) vectors and sequenced using Big Dye™ (Applied Biosystems International, ABI) dideoxy chain terminators and an ABI Gene Analyzer Model 310 to confirm that no mutations were introduced by Pfu polymerase into coding or splice site sequences. Fragment B, comprising loxP-flanked HSV-thymidine kinase (TK) and neomycin phosphotransferase (neo) gene sequences, was obtained from *Xba*I/*Hind*III digestion of plasmid pHR-1. Fragments A–D then were introduced step-wise into a pBluescript KS II (Stratagene) plasmid to generate the targeting vector (Figure 1, upper panel).

Gene targeting in embryonic stem (ES) cells and generation of hupki *mice*

After electroporation of the targeting vector into ES cells (E14.1) and selection, ES clones with homologous recombination events at the endogenous p53 locus were identified by both PCR and Southern blotting analysis (Figure 1, lower panel). The correctly targeted ES clones were subjected to transfection with a Cre-expressing vector (pMC-Cre) which deletes the neomycin/TK cassette flanked by loxP sites to generate the knock-in allele (abbreviated KI in Figures 1–5). Clones with recombinant mouse–human p53 sequences were identified, total RNA was isolated, and the p53 cDNA sequence verified by dideoxy sequencing. Mouse-specific primers for sequencing, PCR or RT–PCR are from Ushijima *et al*. (1995) unless otherwise indicated. Sequences of primers for human p53 segments are from Lehman *et al*. (1991) and from the Affymetrix p53 GeneChip™ protocol. ES clones were injected into blastocytes to generate chimeric mice. Germline offspring were identified by Southern blotting

analysis after breeding of chimeric mice with C57/BL/6 or 129/Sv wild-type mice (Figure 1, lower panel). All experiments were performed using mice of mixed genetic background (129/Sv × C57BL/6).

Isolation and culture of primary mouse embryonic fibroblasts (MEFs)

Embryonic fibroblasts were isolated from embryos of intercrosses of $p53^{KI/+}$ mice at E13.5 mid-gestation and cultured as previously described (Wang *et al*., 1995): Fetuses were dissected from uteri and digested five times, 5 min each, with 0.25% trypsin (Gibco-BRL) at 37°C. Cells were then transferred to tissue culture flasks and cultured in standard DMEM medium supplemented with 10% fetal calf serum. Experiments were carried out with primary fibroblasts of each genotype (passages 2–3), derived from embryos of the same litter.

RNA analysis by semiquantitative RT–PCR, Northern blots, and Clontech cDNA arrays

Total RNA was extracted for RT–PCR or Northern analysis with an RNeasy Kit (Qiagen) according to supplier's instructions. First strand cDNA synthesis was accomplished with a Promega™ RT Kit. Duplex RT–PCR of tissue RNA with primers specific for p53 (P42A, P52A, P10B, Ushijima *et al*., 1995), p21/Waf1 (5′-CGGTCCCG TGGACAGTGAGC-3′; 5′-AAATCTGTCAGGC TGGTCTGCC-3′), Bax (5′-GCGT-CCA CCAAGAAGCTGAG-3′; 5′-CCAC CCTGGTCTTG-GATCCA-3′), β-tubulin (5′-GACAGTGTGGCAACCA-GATCG-3′, 5′-GTACGGAAGCAGATGTCGTAG-3′) and β-actin (5′-TGTGATGGTGGGAATGGGTCAG-3′; 5′-TTT-GATGTCACGCACGATTTC-3′) was performed initially in 50 μl reaction volumes over a range of cycles (20–35) in a thermal cycler using the following cycling conditions: 95°C, 1 min; 60°C, 1 min; 72°C, 45 s. For Northern analysis, 10–15 μg total RNA per lane were electrophoresed on formaldehyde-containing gels, transferred by capillary blot onto GeneScreenPlus™ (DuPont) nylon membranes and hybridized to specific probes; probe P was generated by PCR with 129/Sv mouse genomic DNA template and primers P11A (5′-ACCAAGAAGGGCCAGTCTAC-3′) and P11B (5′-TGGAG-GATATGG ACCCTATG-3′); Bax-, p21- or β-actin (control) probes were generated using primers as given above for RT–PCR. Washed blots were exposed to Amersham X-ray film for 1–7 days. cDNA Arrays: Procedures for obtaining RNA, cDNA and hybridization protocols for Clontech Atlas™ Mouse 1.2 Microarrays were as recommended by the manufacturer. Hybridized arrays were exposed to X-ray film (^{32}P) for 3–7 days, or to FujiImage™ plates (^{33}P) for PhosphorImager visualization. Data were evaluated by ArrayVision™ software (Imaging Research Inc., Canada).

Protein analysis by immunoblot and electromobility shift assay (EMSA)

P53 consensus sequence $p53^{CON}$, and p53 monoclonal antibody PAb421 were purchased from Santa Cruz Biotechnology. The assay was performed according to supplier's instructions, with 20 μg whole cell extracts from γ-irradiated (5 Gy) fibroblasts, 6 h post-treatment. Unlabeled $p53^{CON}$ (20×) were used as specific competitor, and the p53 mutant oligonucleotide (20×) (Santa Cruz) as non-specific competitor. Proteins for immunoblots were extracted from embryonic fibroblasts in RIPA buffer (25 mM Tris pH 8.2, 50 mM NaCl, 0.1% SDS, 0.5% Nonidet P-40, 0.5% deoxycholate) containing Complete™ proteinase inhibitor cocktail (Boehringer Mannheim), followed by centrifugation through Qiagen 'shredders'. Twelve μg protein per lane were loaded onto SDS–PAGE gels, electrophoresed, and gels were blotted onto PVDF membranes. Primary antibodies or antisera used were CM5 (anti-p53, Novacastra, Newcastle upon Tyne, UK) or anti-laminin-β1 (C-19, Santa Cruz Biotechnology, CA, USA). Protein from the AP43a ($p53^{-/-}$) mouse embryo fibroblast cell line (neg. control), and from neonatal primary human foreskin fibroblasts (positive control) were examined in parallel.

Irradiation of whole mice and embryonic fibroblasts

Whole body γ-irradiation (5 Gy) was performed on 10-week-old $p53^{+/+}$, $p53^{KI/+}$ and $p53^{KI/KI}$ mice. Mice were sacrificed 6 or 24 h post-irradiation. Fibroblasts from embryos were obtained according to our previously published protocol (Wang *et al*., 1995). Primary embryonic fibroblast monolayers were irradiated (5 Gy), and cultured an additional 6 or 24 h before extraction of RNA or protein. Embryonic fibroblast cell line MEF 42(B1) derived from $p53^{KI/KI}$ embryos was irradiated with 25 J/m^2 in a Stratagene Stratalinker™ and examined for p53 protein nuclear accumulation at 24 h post treatment by immunocytochemistry with CM1 antiserum.

Apoptosis in thymocytes treated with DNA damaging agents

Thymocytes were isolated from wild-type, *hupki*, and p53 null mice at 6 weeks of age as previously described (Wang *et al*., 1995), and adjusted to a density of 1×10^6 cells per ml. At time zero, cultures were treated (see legend to Figure 5), distributed into 35 mm wells (1×10^6 per well) and incubated at 37°C. Apoptotic cells were stained with ApoAlert Annexin V Kit (Clontech, Palo Alto, CA, USA) reagents, and analysed with FACScan (Becton Dickinson).

Abbreviations

hupki, human p53 knock-in mice; KI, knock-in allele

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