Transgenic mice with p53-responsive *lacZ*: p53 activity varies dramatically during normal development and determines radiation and drug sensitivity *in vivo*

Elena A.Komarova, Mikhail V.Chernov¹,
Roberta Franks, Kaihua Wang,
Gabriella Armin, Carolyn R.Zelnick²,
Dot M.Chin², Sarah S.Bacus²,
George R.Stark¹ and Andrei V.Gudkov³

Department of Genetics, University of Illinois at Chicago, Chicago, IL 60607, ¹Cleveland Clinic Foundation, Cleveland, OH 44195 and

²Advanced Cellular Diagnostics, Inc., Elmhurst, IL 60126, USA

³Corresponding author

To analyze the involvement of p53-dependent transcriptional activation in normal development and in response to DNA damage *in vivo*, we created transgenic mice with a *lacZ* reporter gene under the control of a p53-responsive promoter. Five independent strains showed similar patterns of transgene expression. In untreated animals, *lacZ* expression was limited to the developing nervous system of embryos and newborn mice and was strongly decreased in the adult brain. γ -irradiation or adriamycin treatment induced *lacZ* expression in the majority of cells of early embryos and in the spleen, thymus and small intestine in adult mice. Transgene expression was p53 dependent and coincided with the sites of strong p53 accumulation. The *lacZ*-expressing tissues and early embryos, unlike other adult tissues and late embryos, are characterized by high levels of p53 mRNA expression and respond to DNA damage by massive apoptotic cell death. Analysis of p53-null mice showed that this apoptosis is p53 dependent. These data suggest that p53 activity, monitored by the reporter *lacZ* transgene, is the determinant of radiation and drug sensitivity *in vivo* and indicate the importance of tissue and stage specificity of p53 regulation at the level of mRNA expression.

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Introduction

p53 is a key modulator of stress response (reviewed by Gottlieb and Oren, 1996). The p53 protein turns over rapidly in normal cells and is stabilized under conditions of stress which include DNA damage (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991), disorganization of microtubules (Tishler *et al.*, 1995), hypoxia (Graeber *et al.*, 1996) and detachment of cells from their natural substrate (Nikiforov *et al.*, 1996). The normal function of p53 is required for control of cell aging (Atadja *et al.*, 1995). Depending on the cell type, p53 activation results in growth arrest at one of the cell cycle checkpoints or in apoptosis (Michalovitz *et al.*, 1990).

Functional inactivation of p53, which occurs in most

cancers and established cell lines (Vogelstein and Kinzler, 1992) is achieved by mutation (many generate dominant-negative variants of p53), deletion or the inhibitory activity of p53-binding proteins of cellular (mdm-2) or viral (SV40 large T-antigen, the E6 protein of papilloma viruses) origin (reviewed by Gottlieb and Oren, 1996). Lack of p53 in normal cells leads to loss of genomic stability (Yin *et al.*, 1992), spontaneous immortalization (Donehower *et al.*, 1992), abrogation of apoptosis (Clarke *et al.*, 1993; Lowe *et al.*, 1993) and, as a result, susceptibility to transformation by dominant oncogenes (Olson and Levine, 1994). Loss of p53 in tumor cells may be associated with a high level of resistance to chemo- and radiation therapy and the establishment of metastatic behavior (Lowe *et al.*, 1993, 1994; Nikiforov *et al.*, 1996). Although the lack of p53 does not interfere severely with the program of embryonal development in p53 knock-out mice, it does lead to a high incidence of spontaneous cancers (Donehower *et al.*, 1992; Jacks *et al.*, 1994).

At least some of its p53-mediated effects are determined by the ability of p53 to act as a transcription factor. After stabilization, p53 is translocated into the nucleus where it activates transcription of p53-responsive genes upon binding to specific DNA sequences in their promoters (Gottlieb and Oren, 1996). A number of p53-responsive genes [i.e. *p21/waf1/cip1/sdi1* (El-Deiry *et al.*, 1993), *gadd45* (Kastan *et al.*, 1992), *mdm2* (Wy *et al.*, 1993), *bax* (Miyashita and Reed, 1995), cyclin G (Okamoto and Beach, 1994), IGF-BP3 (Buckbinder *et al.*, 1995) and adenosine deaminase (Kondratov *et al.*, 1996)] have been identified. Their activity accounts in part for p53-mediated checkpoint control (*waf1*), apoptosis (*bax*) and feedback of p53 regulation activity (*mdm2*) (Gottlieb and Oren, 1996). In addition, p53 can suppress transcription from a number of promoters (Mack *et al.*, 1993) and directly inhibit DNA replication (Cox *et al.*, 1995).

Despite the importance of p53-mediated transcriptional activation, this function of p53 has been studied predominantly in cultured cells, most of which originate from tumors, and they have abnormal regulation of stress responses and proliferation. Studies of p53 *in vivo* or in primary cells are usually limited to analysis of the accumulation of p53 or induction of p53-responsive gene products in response to various stresses (Midgley *et al.*, 1995). However, the significance of these studies is limited because there are several levels of post-translational regulation and modification of p53 (reviewed by Meek, 1994), making it difficult to link p53 expression with its activity. The value of the cellular p53-responsive genes (such as *waf1*) for monitoring p53 activity is also limited since they can be expressed in a p53-independent manner (Macleod *et al.*, 1995).

In order to obtain an experimental tool for the analysis of p53 transcriptional activation *in vivo*, we created

transgenic mice carrying a bacterial β -galactosidase (*lacZ*) reporter cDNA under the control of a p53-responsive promoter. These mice allowed us to detect easily p53-directed transactivation by histochemical staining and to trace p53 activity through different stages of mouse development, under normal conditions and after damaging DNA. We found that p53-responsive *lacZ* expression is poor under normal conditions in most embryonal and adult tissues, with the exception of the developing nervous system. Adriamycin or γ -radiation treatment stimulated induction of the transgene in several mouse organs (spleen, thymus, intestine) and in most cells of early, but not late embryos, correlating with the p53 mRNA expression, degree of induction of p53 in these tissues, and the induction of apoptosis. Both *lacZ* expression and apoptosis induced by radiation or adriamycin in the spleen, thymus and in 8- to 9-day embryos were p53 dependent. Our data indicate the importance of tissue and stage-specific p53 gene expression, regulated at the level of mRNA, for radiation and drug sensitivity *in vivo*.

Results

Transgene construction and testing in transfected cells *in vitro*

We constructed a recombinant plasmid to express a bacterial β -galactosidase gene (*lacZ*) in response to p53 (Figure 1). In this plasmid, *lacZ* expression is controlled by a minimal heat shock (hsp70) promoter located downstream of two different p53-binding sequences. The upstream sequence ('fragment A') is a 46 bp fragment from a putative replication origin of the human ribosomal gene cluster, selected for p53 binding (Kern *et al.*, 1991). The downstream element, 19 bp long, represents a consensus p53-binding sequence (Funk *et al.*, 1992). Both sequences were previously shown to act as p53-dependent transcriptional activators (Funk *et al.*, 1992).

To test this construct, we transfected it into a clone of mouse Balb 3T3 cells, line 12-1 (a gift of Arnold Levine; Harvey and Levine, 1991), which is known to have wild-type p53. The resulting cell line (ConA) had a low background of *lacZ* expression under normal growth conditions and showed strong activation of the transgene after irradiation or drug treatment as revealed by bright blue X-gal staining *in situ* or by quantitative β -galactosidase assays in cell lysates (Figure 1). The intensity and kinetics of *lacZ* activation coincided with the stabilization of p53 in treated cells; the *lacZ* response was significantly suppressed in a population of ConA cells transduced by a dominant-negative mutant of p53 in retroviral vector (Ossovskaya *et al.*, 1996; data not shown). These characteristics strongly indicated that the construct is adequate to assay transactivation by p53 and can be used to create transgenic mice.

Creation of transgenic mice and expression of the transgene in the organs of adult animals

Linearized pConA plasmid DNA was injected into fertilized zygotes of CD1 mice. The presence of the transgene in the DNA of the mice that developed from the injected eggs was determined by Southern blot analysis of genomic DNA from the tails. Of the 86 mice tested, nine were positive. Five of the founders that transmitted the transgene

to their progeny were used to establish transgenic strains. Initial characterization of the transgenic mice included: (i) determination of the patterns of transgene expression in several independently derived strains, (ii) analysis of the association of transgene activity with the expression of p53, and (iii) analysis of correlations between transgene expression and tissue response after genotoxic stress. Heterozygous 6- to 8-week-old males from each founder were tested for transgene expression by X-gal staining of tissue samples. Three animals taken from each line were untreated, injected with adriamycin (10 μ g/g) or γ -irradiated (8 Gy). The mice were sacrificed 24 h after irradiation or drug injection and their tissues were tested for β -galactosidase activity by X-gal staining (Figure 2 and Table I). Of the organs tested, strong radiation-induced β -galactosidase activity was detected in the spleens and thymus glands of all transgenic strains which, however, differed in the degree of induction and in the background expression in unirradiated organs. Induction of the transgene by radiation was detected also in the small intestine, though a high endogenous background of β -galactosidase in this organ (starting several days before birth and gradually decreasing for several months) interfered with the analysis of transgene activity. Testis and kidney, which also displayed a high endogenous background of β -galactosidase, were excluded from further analysis. Two of the transgenic strains expressed *lacZ* in brain and one strain expressed it in skin, although not in an inducible fashion. The founder of this latter strain carried two independently inherited clusters of transgenes, although only one was associated with the skin expression. These two clusters were segregated in the progeny. None of the transgenic strains expressed *lacZ* in liver, lung, skeletal muscle or heart. It should be noted that γ -irradiation or injection of adriamycin led to similar patterns of *lacZ* activation and, therefore, only radiation-induced transgene expression is shown. Similarity, the tissue specificity of transgene expression in five independent transgenic lines indicates that the observed pattern reflects the properties of the transgene rather than a position effect which, however, may be responsible for skin expression in one strain.

The induction of *lacZ* expression was compared with p53 activation in irradiated tissues of transgenic mice. p53 expression was detected by immunohistochemical staining using anti-p53 polyclonal antibodies CM5 (Midgley *et al.*, 1995; provided by David Lane). These experiments (Figure 2) demonstrated strong activation of p53 in irradiated spleen, thymus and bones (also positive for transgene expression) with no or significantly less activation in muscles, heart, lungs, skin and liver (tissues with no detectable *lacZ* activity). Interestingly, *lacZ* expression was strong in red pulp and hardly detectable in white pulp of the spleen. Thus, inducibility of the transgene by γ -radiation or adriamycin correlates with p53 activation.

In order to determine whether *lacZ* transgene expression *in vivo* is in fact p53 dependent, we crossed our transgenic mice (line #61) with p53-null mice. The F1 generation heterozygous for both the transgene and wild-type p53 was back-crossed with p53^{-/-} mice. The progeny was genotyped and *lacZ*⁺ p53^{-/-} animals were analyzed for *lacZ* expression after whole-body γ -irradiation. Since no *lacZ* transgene expression was detected in p53^{-/-} animals (data

Fig. 1. Structure of the transgene and its activity in transfected Balb 3T3 cells, line ConA, *in vitro*. p53 protein detection was done by Western immunoblotting in control (c), UV light-irradiated (UV) and adriamycin-treated (Adr) cells. Bar diagram demonstrates the results of a quantitative assay for β -galactosidase activity in lysates of the cells treated as indicated and tested 24 h after treatment. In parallel, cells were fixed and stained *in situ* by X-gal.

not shown), we concluded that *lacZ* activation truly reflects p53 activity *in vivo*.

Transgene expression during normal development

Mice with p53-responsive *lacZ* were used to evaluate the activity of p53 in several stages of mouse development. We focused on analyzing two transgenic strains, #27 from

outbred CD1 mice, and #61 from inbred FVB/N mice. Both carried multiple copies of the transgene (100–200 per genome in #27 and 30–60 in #61), expression of which was strongly activated after γ -irradiation or adriamycin treatment in spleen, thymus and intestine of adult animals (see above). We tested transgene expression in embryos and newborn mice. In all experiments, we compared

Fig. 2. Expression of β -galactosidase in organs of transgenic adult mice, strain #27. Upper panel: X-gal staining of dissected tissue samples from control (–) and γ -irradiated (+) mice. Lower panels: tissue sections of the spleen and thymus from the same mice stained *in situ* by X-gal for β -galactosidase (lacZ), by anti-p53 antibodies (p53) and by TUNEL assay for apoptosis (TUNEL).

γ -irradiated transgenic mice with wild-type CD1 mice of similar age, allowing us to distinguish transgene expression from the endogenous β -galactosidase activity present in some mouse tissues. Typically, samples of tissues or whole embryos were imbedded in OCT, frozen, cut into 15–25 μ m sections, fixed and stained for transgene expression. Parallel sections were used to detect p53 expression. Seven- and nine-day transgenic embryos showed no

obvious *lacZ* expression (Figure 3A). However, in 11-day embryos, expression of the transgene was seen in the brain and was detected at all later stages of development analyzed (15-, 17-, 19-day embryos and newborn animals). In prenatal embryos and newborn mice, the most intensive *lacZ* expression was observed on the surface of the thalamic region, in the superficial part of the cerebral cortex, in the cerebellum and in the hypothalamus, though

Fig. 3. Expression of the transgene in untreated and γ -irradiated mice at different stages of development. (A) Transversal sections of 9-day transgenic embryos *in utero*: untreated (a) and γ -irradiated (b–d). Staining with: X-gal (a and b), hematoxylin (c) and TUNEL (d). (B) Parasagittal sections of untreated 17-day embryos from control (a–c) and transgenic (d–f) mice stained with X-gal. a, heads of the embryos; b, enlarged fragment of the midbrain; c, whisker pads. (C) tangential sections of the heads of untreated newborn control (a and b) and transgenic (c and d) mice stained with X-gal. b and d, magnified areas of the brain are indicated by frames in a and c.

Table I. Expression of *lacZ* in organs of non-treated and γ -irradiated mice of five transgenic strains

Organ ^a	Transgenic strain (control/ γ -irradiated) ^b				
	#27	#71	#68	#21	#61
Liver	–/–	–/–	–/–	–/–	–/–
Spleen	–/+ +	+/+ +	+/+ +	+/+ +	+/+ +
Thymus	–/+ +	–/+ +	–/+ +	–/+ +	–/+ +
Brain	+/+	–/–	–/–	–/–	+/+
Lung	–/–	–/–	–/–	–/–	–/–
Bone	–/+	–/+	–/+	–/–	–/+
Skin	+/+	–/–	–/–	–/–	–/–
Heart	–/–	–/–	–/–	–/–	–/–

^aHigh levels of endogenous β -galactosidase expression were detected in kidney, testis and intestine; these organs were excluded from the analysis.

^bMice were γ -irradiated (8 Gy) and tested 24 h later. Similar results were obtained with adriamycin (10 μ g/g injected 24 h before testing).

not in all cells. In addition to the brain, strong expression of the transgene was observed in the whisker pads of untreated newborn mice (Figure 3B). No *lacZ* activity was found in the remaining tissues tested, including liver, muscle, lung and heart. Endogenous β -galactosidase expression in osteoblasts and in the gut, activated in late embryos, complicated the analysis of transgene expression in these organs. Nevertheless, β -galactosidase staining in bones of transgenic mice was significantly stronger than in wild-type animals, suggesting that it was caused in

part by the activity of the transgene. Extremely strong endogenous β -galactosidase expression did not allow us to determine the impact of transgene expression in the gut.

No *lacZ* expression was detected in most organs of adult transgenic mice. The strong expression seen in the brains of newborn animals was limited to rare sporadic cells in adult brains (data not shown). A small proportion of β -galactosidase-positive cells was found in the spleen and thymus (Figure 2).

Fig. 4. p53 mRNA expression is decreased during embryonic development. p53 mRNA in mouse embryos of different ages (indicated) was determined by Northern blot hybridization (upper panel); re-hybridization of the same membrane with the GAPDH probe is shown below.

Induction of transgene expression by γ -radiation correlates with p53 mRNA expression in mouse embryos

Whole-body irradiation (8 Gy) of the transgenic mice (newborn animals and pregnant females at different stages of gestation) was carried out to analyze the response to DNA damage at different stages of mouse development. Whole-body sections of embryos and newborn mice were stained with X-gal and compared with similar sections of unirradiated transgenic animals and wild-type mice.

In 8- to 9-day embryos, β -galactosidase activity was induced by irradiation in the majority of cells, though to different extents. The highest *lacZ* expression was observed in the neural tubes and visceral arches of 9-day embryos (Figure 3A). *lacZ* expression was strong 3 h after irradiation and reached its maximum after 6 h. Massive cell destruction starting at this time, due to radiation-induced apoptosis (see below) was accompanied by a gradual decrease in β -galactosidase staining, possibly due to the loss of *lacZ*-expressing cells. It was impossible to analyze embryos 24 h after irradiation since their cells had already lost their integrity. The *lacZ* transgene was constitutively expressed in the decidua layer of the uterus (Figure 3A). Unlike embryonal cells, *lacZ* staining increased after irradiation and remained bright for up to 24 h, with no trace of tissue degradation.

Unlike early embryos, where expression of the transgene was activated in most cells after irradiation, no significant differences in *lacZ* expression were found in γ -irradiated and untreated late embryos and newborn transgenic mice. Instead, γ -irradiation was accompanied by a slight increase in the intensity of staining without changing the pattern of tissue distribution of *lacZ*-positive cells (data not shown).

No *lacZ* induction was found in γ -irradiated *lacZ*⁺ p53⁻ embryos indicating that, as in adult animals (see above), transgene expression was p53 dependent (data not shown).

High expression of the transgene in response to DNA damage correlated with high levels of p53 mRNA expression in early embryos. A dramatic reduction of p53 mRNA content, occurring between the 13th and 16th days of development (Figure 4), was accompanied by the loss of transgene induction. Similarly, strong induction of the transgene in the spleen and thymus of adult mice correlated with high levels of p53 mRNA expression (data not shown) and p53 accumulation (see above) in these organs. Our data on tissue and stage specificity of p53 mRNA

expression confirm the earlier observations of Rogel *et al.* (1985) and Schmidt *et al.* (1991).

p53 dependence of radiation-induced apoptosis in the mouse

The expression of the *lacZ* transgene and p53 accumulation were compared with the induction of apoptosis in tissues of irradiated mice. Apoptosis was monitored by the appearance of degraded DNA, using TUNEL staining and gel electrophoresis. These experiments (Figure 2) revealed that strong activation of the transgene and accumulation of p53 accompanied apoptosis in spleen, thymus and small intestine, with no or significantly less activation in tissues with no detectable *lacZ* activity (muscle, lung and liver). Interestingly, apoptosis and p53 activation occurred in both red and white pulp of the spleen, though transgene induction was limited to the red pulp (Figure 2). Hence, the level and cellular distribution of transgene induction does not necessarily correlate with the level of p53 activation and apoptosis.

Thus, transactivation of the transgene by radiation in adult mice occurred predominantly in the organs responding to γ -radiation by inducing apoptosis and known to be primary targets for radiation and drug toxicity in mammalian organisms. The sensitivity to γ -radiation changes dramatically during embryonal development: late embryos are significantly more resistant to γ -radiation than early embryos (Hall, 1994), consistent with the lack of apoptosis in late embryonal tissues as well as the absence of transgene induction (data not shown). In the early embryos, which are extremely sensitive to γ -radiation (Hall, 1994), there was complete apoptosis in all the cells, as determined by TUNEL staining, accompanied by strong induction of the transgene (Figure 5). Coincidence of the transgene activation with the sites of apoptosis suggested that p53 is the determinant of radiation and drug sensitivity *in vivo*. In fact, the p53 dependence of radiation- and drug-induced apoptosis in normal thymocytes has been demonstrated before (Clarke *et al.*, 1993; Lowe *et al.*, 1993) providing an additional argument in favor of this hypothesis. To address it, we compared the apoptotic response to γ -irradiation in tissues of p53-null mice and heterozygous animals carrying one wild-type allele of p53.

Both spleen and thymus of p53^{+/-} mice respond strongly to γ -irradiation resulting, at the macroscopic level, in a dramatic reduction in the size of these organs shortly after treatment. For example, the mass of the spleen is reduced almost 2-fold as early as 10 h after irradiation. At the microscopic level, irradiated spleen tissue is characterized by the appearance of numerous morphologically apoptotic cells, positively stained by TUNEL (Figure 5). Most of the DNA isolated from the spleen at this time is degraded, showing an 'apoptotic DNA ladder' and similar events occur in irradiated thymus glands of p53^{+/-} animals (data not shown). However, none of the above were observed in the spleens or thymus glands of p53-null mice (Figure 5). Thus, massive radiation-induced apoptosis in lymphoid organs *in vivo* is a p53-dependent process.

In order to study the p53 dependence of radiation apoptosis in early mouse embryos, p53^{+/-} females, mated with p53^{-/-} males, were irradiated during the 9th day of gestation. Six hours later, the animals were sacrificed and each embryo was used to prepare tissue sections, which

Fig. 5. p53 dependence of radiation-induced apoptosis in the spleen and in 9-day embryos. Upper panel: comparison of the sizes of untreated and γ -irradiated spleens of $p53^{+/-}$ and $p53^{-/-}$ mice (males of similar age) 24 h after irradiation. TUNEL staining of the sections of irradiated spleens 6 h after irradiation is shown at the right. Middle panel: comparison of radiation-induced DNA degradation in individual $p53^{+/-}$ and $p53^{-/-}$ embryos (a–k) isolated from one female. Upper gel shows the results of genotyping of embryos using PCR: a single band for $p53^{-/-}$ (b, d, f, h and k) and two bands for $p53^{+/-}$ embryos (a, c, e and g); l–n, control DNA samples from $p53^{-/-}$, $p53^{+/-}$ and $p53^{wt}$ mice respectively, used as PCR control (not loaded in the bottom gel). Lower gel contains total genomic DNA from individual embryos. DNA degradation occurs in $p53^{+/-}$ but not in $p53^{-/-}$ embryos. Lower panel: TUNEL staining of the sections of two indicated embryos ($p53^{+/-}$ and $p53^{-/-}$).

were analyzed for apoptosis by TUNEL staining. DNA was also isolated from the tissues of each embryo and analyzed by agarose gel electrophoresis to monitor the DNA degradation associated with apoptosis. The genotype of each embryo was determined by PCR (Figure 5). The results obtained showed a complete coincidence between the p53 status of the embryo and the induction of apoptosis: in all $p53^{+/-}$ embryos strong DNA fragmentation, accompanied by the morphological appearance of apoptosis, was detected by both methods, while in $p53$ -null embryos both DNA and tissue morphology remained unchanged. These results strongly indicate that the high sensitivity of early mouse (and probably other mammalian) embryos to radiation is determined by p53, which is necessary to induce apoptosis in the irradiated cells.

Discussion

Our goal was to generate an animal model for monitoring p53 transcriptional activation *in vivo*, a major property of p53, which mediates many p53-related effects. We created transgenic mice carrying a reporter β -galactosidase gene under the control of a p53-responsive promoter with two different p53-binding sequences. The choice of the construct was based on its efficient and specific p53-dependent transcriptional activation, described by Funk *et al.* (1992) and confirmed during our *in vitro* testing of transfected cells. We planned to use these mice to determine p53 activity in embryonic and adult tissues under normal and under stress conditions. We hoped that such observations would help in understanding the role of p53 in normal development and in the responses of different cell types and tissues to cytotoxic compounds and physical shock.

The value of the transgenic animals as a tool to monitor p53 transcriptional activation *in vivo* depends directly on the p53 specificity of transgene expression. Considering that this aspect is crucial for the interpretation of our results and for future applications of the transgenic animals, we analyzed the transgene activity (i) in several independent transgenic lines of mice, (ii) under conditions known to affect p53 transcriptional activation (DNA damage), (iii) on a p53-null genetic background and (iv) in a comparison of transgene and p53 expression. Taken together, the results of these studies demonstrated that transgene expression is p53 dependent, correlates with p53 induction in response to DNA-damaging treatments and truly reflects p53 activity as a transcription factor *in vivo*. A similar conclusion was reached by E.Gottlieb, R.Haffner, A.King, G.Asher, P.Gruss, P.Lonai and M.Oren (submitted) and by McCallum *et al.* (1997) who created transgenic mice expressing *lacZ* from different p53-responsive promoters. All the above gives us confidence to interpret the results obtained by the analysis of *lacZ* staining as a reflection of the p53 activity *in vivo*.

In untreated animals, *lacZ* expression was limited to the nervous system of embryos and newborn mice. This observation is consistent with the possible involvement of p53 in certain stages of nervous system development. It should be mentioned that the only known developmental defect associated with p53 deficiency in mice is exencephaly, associated with lethality in up to 16% of female embryos (Sah *et al.*, 1995). Additional studies are needed to determine whether there is a mechanistic link between transgene activation and the occurrence of these lesions. The detailed characterization of the topography and dynamics of *lacZ* expression in different brain areas is the subject of future analysis. However, it is already clear that the neurons and neuroblasts stained positive for β -galactosidase activity were clustered in several specific areas, with no correlation with the TUNEL-detectable apoptosis normally ongoing in the developing nervous system (Raff *et al.*, 1993).

lacZ transgene expression was induced in several sites in transgenic mice after γ -irradiation or adriamycin treatment. The response was found in early embryos and in the spleen, thymus and small intestine of adults. Other adult organs as well as cells of late embryos did not show any *lacZ* induction. What is common among all these sites of

the transgene induction? They are all characterized by: (i) very high levels of p53 mRNA expression (Figure 5; Rogel *et al.*, 1985; Schmidt *et al.*, 1991), (ii) strong accumulation of p53 after DNA damage (Midgley *et al.*, 1995; McCallum *et al.*, 1997; present work) and (iii) a rapid apoptotic response to irradiation. The correlation among high transgene activation, p53 expression and apoptosis in these organs suggested that their high radiosensitivity was p53 dependent. In fact, no apoptosis or morphological changes were induced by radiation in the spleens or thymus glands of p53-null mice, showing that the radiosensitivity of these tissues is indeed p53 dependent. p53 dependence of apoptosis in the thymus is in agreement with the earlier observations by Clarke *et al.* (1993) and Lowe *et al.* (1993) showing p53-dependent apoptosis in normal thymocytes *in vitro* caused by DNA damage.

It was appreciated long ago that early mammalian embryos are characterized by a hypersensitivity to γ -radiation that gradually decreases during organogenesis (8–13 days) in mice. After the 13th day of development, the embryos are no longer hypersensitive to radiation (Hall, 1994). Our data elucidate the mechanism of this long-known phenomenon. We noticed that both transgene induction and apoptosis are dramatically decreased in the middle of embryonic development, consistent with the known switch in radiosensitivity. The results of comparing the p53^{-/-} and p53^{+/-} embryos (Figure 5) provided a clear argument that the radiosensitivity of early embryos is determined by p53-mediated apoptosis. The significant decrease in p53 response to DNA damage may explain the lack of both p53-mediated transcriptional activation and p53-mediated apoptosis in late embryos. The dramatic decrease in p53 mRNA expression during embryonal development (Figure 4; Rogel *et al.*, 1985; Schmidt *et al.*, 1991) is likely to be the basis for this effect. This indicates that developmental and tissue-specific p53 gene regulation at the level of mRNA may play a critical role in the determination of p53 effects *in vivo*. However, p53 regulation at the RNA level has not historically been considered important and remains poorly understood. The importance of gene dosage for p53-mediated effects *in vivo* also follows from the observation described in E.Gottlieb, R.Haffner, A.King, G.Asher, P.Gruss, P.Lonai and M.Oren (submitted) that shows a dramatic decrease in transcriptional activation by p53 in mice with a single functional allele of p53.

It is unclear whether p53-mediated transcriptional activation plays an active role in determining radiation sensitivity and apoptosis *in vivo* or whether it is just a marker indicating where p53 is active. In fact, p53 transactivation is dispensable for the induction of apoptosis in some cells (Caelles *et al.*, 1994; Wagner *et al.*, 1994; Haupt *et al.*, 1995). This may be the case in the spleen, where the red and white pulps strongly differ in transgene activity but induce p53 expression and apoptosis similarly after irradiation.

A much greater effect of p53 in early embryos (before the end of organogenesis) than at later stages of development supports the model that p53 acts to control the elimination of cells with genetic abnormalities during the early stages of embryogenesis by inducing p53-mediated apoptosis (Norimura *et al.*, 1996). The mechanism of

extreme sensitivity of the cells of early embryos to apoptosis requires efficient induction of p53 in response to DNA damage. Such efficient induction is made possible by very high levels of p53 transcription observed during the first half of embryonic development (Rogel *et al.*, 1985; Schmidt *et al.*, 1991; present work). Large amounts of p53 mRNA maintain high levels of p53 protein synthesis and allow for quick accumulation of p53 by protein stabilization in response to DNA damage. p53 mRNA expression in embryos declines rapidly in the middle of gestation (Rogel *et al.*, 1985; Schmidt *et al.*, 1991; present work) accompanied by the loss of sensitivity of embryonal cells to p53-mediated apoptosis. The level of p53 mRNA expression remains very low in the majority of adult tissues, with the exception of organs such as spleen, thymus and intestine which maintain embryonal levels of p53 expression and high sensitivity to apoptosis.

It should be mentioned that a return to the embryonal program of gene regulation is a common characteristic of neoplastic tissues (Abelev, 1989). If up-regulation of the p53 gene occurs as a result of this switch, it could determine high radiation and drug sensitivity of tumor cells used as a basis for anti-cancer therapy. Monitoring of p53 activity in the tumors induced in our transgenic mice may provide an experimental system to address this hypothesis.

Materials and methods

Vectors and cell lines

A recombinant plasmid with a p53-responsive *lacZ* gene was constructed from the vector pCH100 (Pharmacia) as described in Results. A derivative of Balb 3T3 cells, line 12-1, provided by Arnold Levine (Princeton University, NJ; Harvey and Levine, 1991), was transfected with the pConA-*lacZ* plasmid using a standard Ca-phosphate procedure and one of the resulting clones, ConA, which demonstrated the highest inducibility of *lacZ* in response to UV irradiation or adriamycin, was used for further studies. The ecotropic retroviral packaging cell line BOSC23 (Pear *et al.*, 1993), was kindly provided by Warren Pear and David Baltimore (Massachusetts Institute of Technology). All cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum supplemented with penicillin and streptomycin.

Transgenic mice

Gel-purified linearized DNA (4 ng/ μ l in 10 mM Tris-HCl pH 7.4, 0.25 mM EDTA) of the pConA plasmid was introduced by pronuclear injection into fertilized zygotes of outbred CD1 and inbred FVB/N mice. F1 heterozygous transgenic animals were subsequently cross-bred to generate homozygous transgenic lines. The presence of the transgene in the DNA of the mice that developed from the injected eggs was determined by preparing genomic DNA from their tails and analyzing it by PCR, using primers for the β -galactosidase gene, and by Southern analysis using the plasmid as a probe.

p53 knock-out mice

p53-null mice were kindly provided by Tyler Jacks (Massachusetts Institute of Technology, Boston, MA) (Jacks *et al.*, 1994). The genotype of the progeny obtained by crossing the p53-null mice with wild-type or transgenic animals was determined by PCR, to distinguish between the wild-type and knock-out copies of p53. A mixture of three primers was used, including two sense-oriented primers from exon 6 of p53 (5' ACA GCG TGG TGG TAC CTT AT 3'), the neomycin gene (5' TCC TCG TGC TTT ACG GTA TC 3') and an antisense primer from exon 7 of p53 (5' TAT ACT CAG AGC CGG CCT 3').

Irradiation of mice

Adult males, pregnant females and newborn mice were given whole-body γ -irradiation (8 Gy) from a G.L.Shepherd and Associates Model 143-68 Cs137 source at a dose rate of 4 Gy per min. The animals were

sacrificed later and their tissues were analyzed for transgene activity, p53 expression and apoptosis.

Immunohistochemical detection of p53

Five micron frozen tissue sections were fixed for 10 min in 10% neutral buffered formalin at room temperature, followed by 3 min in -20°C acetone. The sections were blocked with 10% rabbit serum to which Triton X-100 (0.01%) was added. The sections were incubated subsequently with primary polyclonal rabbit antibodies against p53 (Ab-CM5, provided by David Lane; Midgley *et al.*, 1995) diluted 1:1000 in PBS, with secondary antibody (rabbit anti-mouse IgG1, Jackson Labs, West Grove, PA); and tertiary ABC complex (Vector Labs, Burlington, CA) for 30, 20 or 15 min respectively, at 37°C , with PBS washes between the incubations. Detection was achieved by using DAB in citrate-phosphate buffer with sodium perborate. Counterstaining was performed with the CAS ethyl green (Becton Dickinson Cellular Imaging Systems, San Jose, CA). Detection of p53 in cell lysates by Western analysis was performed as described earlier (Nikiforov *et al.*, 1996).

Apoptosis assay (TUNEL)

The method we used is a modification of a previously described technique (Gavrieli *et al.*, 1992). Briefly, the endogenous peroxidase activity in tissue sections was quenched with 0.2% H_2O_2 . The terminal deoxynucleotidyl transferase reaction was performed *in situ* with digoxigenin-conjugated nucleotides, with subsequent treatment with peroxidase-conjugated anti-digoxigenin Fab fragments (all reagents from Boehringer Mannheim). Labeled nuclei were then visualized by treating the sections with 10 mg diaminobenzidine tetrahydrochloride in 20 ml of Tris-HCl pH 7.6, plus 20 μl of H_2O_2 . Counterstaining was done using a modified ethyl green procedure.

β -galactosidase assay

β -galactosidase staining was performed as described (Hogan *et al.*, 1994) with some modifications. In brief, frozen sections or PBS-washed cultured cells on plates were fixed (15–30 min in ice-cold 0.5% glutaraldehyde in PBS pH 7.4, with 1 mM MgCl_2) washed in cold PBS with 1 mM MgCl_2 , and stained in 0.2% X-gal, 3.3 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 3.3 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 0.02% NP-40 in PBS in the dark at 37°C overnight. For qualitative color reaction in solution, we placed the dissected 10 mg fragments of tissues directly into 1 ml of X-gal staining solution and incubated overnight at 37°C or at room temperature.

Quantitative β -gal assay was carried out using the Promega β -Galactosidase Enzyme Assay System Kit. The amount of protein in cell lysates was determined by the Bradford protein quantitation method (Bio-Rad). Absorbance of protein at 525 nm and the product of the β -galactosidase color reaction were detected by spectrophotometry at 420 nm.

Retroviral infection

Ecotropic packaging cells BOSC23 (2×10^6 per 60 mm plate) were transfected using the standard calcium phosphate method with 5 μg of the desired plasmids 1 day before first infection. The medium from transfected cells supplemented with polybrene (4 $\mu\text{g}/\text{ml}$) was diluted twice with fresh medium and applied to the recipient cells once a day for 2 days. The efficiency of infection was judged by the use of the control virus expressing human placental alkaline phosphatase.

Adriamycin treatment *in vivo* and *in vitro*

Cells in culture were treated for 24 h by 200 ng/ml of adriamycin. Ten micrograms of adriamycin per gram of body weight were injected intraperitoneally into mice as a 0.5 mg/ml solution of PBS. Animals were sacrificed 24 h after injection and analyzed as described in Results.

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