The EMBO Journal Vol. 20 No. 13 pp. 3535–3543, 2001

Loss of poly(ADP-ribose) polymerase-1 causes increased tumour latency in p53-deficient mice

Carmen Conde, Manuel Mark[1], F.Javier Oliver[2], Aline Huber, Gilbert de Murcia and Josiane Ménissier-de Murcia[3]

UPR 9003 du CNRS, Laboratoire Conventionné avec le Commissariat à l'Energie Atomique, Ecole Supérieure de Biotechnologie, Université Louis Pasteur de Strasbourg, Boulevard Sébastien Brant, F-67400 Illkirch, [1]Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, BP 163, F-67400 Illkirch Cedex, France and [2]Unidad Mixta de Investigaciones Médicas, Hospital Clinico San Cecilio, Universidad de Granada, Spain

[3]Corresponding author
e-mail: josiane@esbs.u-strasbg.fr

PARP-1-deficient mice display a severe defect in the base excision repair pathway leading to radiosensitivity and genomic instability. They are protected against necrosis induced by massive oxidative stress in various inflammatory processes. Mice lacking p53 are highly predisposed to malignancy resulting from defective cell cycle checkpoints, resistance to DNA damage-induced apoptosis as well as from upregulation of the iNOS gene resulting in chronic oxidative stress. Here, we report the generation of doubly null mutant mice. We found that tumour-free survival of *parp-1*$^{-/-}$*p53*$^{-/-}$ mice increased by 50% compared with that of *parp-1*$^{+/+}$*p53*$^{-/-}$ mice. Tumour formation in nude mice injected with oncogenic *parp-1*$^{-/-}$*p53*$^{-/-}$ fibroblasts was significantly delayed compared with *parp-1*$^{+/+}$*p53*$^{-/-}$ cells. Upon γ-irradiation, a partial restoration of S-phase radiosensitivity was found in *parp-1*$^{-/-}$*p53*$^{-/-}$ primary fibroblasts compared with *parp-1*$^{+/+}$*p53*$^{-/-}$ cells. In addition, iNOS expression and nitrite release were dramatically reduced in the *parp-1*$^{-/-}$*p53*$^{-/-}$ mice compared with *parp-1*$^{+/+}$*p53*$^{-/-}$ mice. The abrogation of the oxydated status of *p53*$^{-/-}$ cells, due to the absence of *parp-1*, may be the cause of the delay in the onset of tumorigenesis in *parp-1*$^{-/-}$*p53*$^{-/-}$ mice.

Keywords: cell cycle/γ-irradiation/iNOS expression/knockout mice/nude mice

Introduction

Poly(ADP-ribose) polymerase (PARP-1) is a highly conserved constitutive factor of the DNA damage surveillance network developed by the eukaryotic cell to cope with various environmental and endogenous genotoxic agents (de Murcia and Ménissier de Murcia, 1994). At a site of DNA breakage, the immediate poly(ADP-ribosyl)ation of a limited number of nuclear proteins involved in chromatin architecture and DNA metabolism translates the presence of DNA interruptions into intracellular signals that modulate DNA repair and cell survival. PARP-1 is associated *in vivo* with XRCC1, a DNA repair protein involved, together with DNA polymerase β and DNA ligase III, in the base excision repair (BER) of DNA (Masson *et al*., 1998). PARP-1-deficient cells display a severe DNA repair defect that appears to be the primary cause of the observed genomic instability and the cytotoxicity of DNA damaging agents inducing BER (Trucco *et al*., 1998; Beneke *et al*., 2000; Dantzer *et al*., 2000). Accordingly, *parp-1*$^{-/-}$ mice are hypersensitive to alkylating agents or γ-irradiation (Ménissier de Murcia *et al*., 1997; Masutani *et al*., 2000), but paradoxically are not cancer prone.

The knockout strategy has revealed the unexpected instrumental role of PARP-1 in cell death after ischaemia–reperfusion injury (Eliasson *et al*., 1997), streptozotocin-induced diabetes (Burkart *et al*., 1999; Masutani *et al*., 1999) and in different pathologies associated with inflammation (Szabo and Dawson, 1998; Pieper *et al*., 1999).

We and others (Hassa and Hottiger, 1999; Oliver *et al*., 1999) have shown recently that *parp-1*-deficient cells are defective in NF-κB-dependent transcription activation, but not in its nuclear translocation, in response to tumour necrosis factor-α and to lipopolysaccharide (LPS), demonstrating a functional link, *in vivo*, between PARP-1 and NF-κB. Therefore the complete abrogation of the transcriptional activation of NF-κB leads to a spectacular improvement of the outcome of a systemic inflammatory process in *parp-1*$^{-/-}$ mice treated with LPS (Oliver *et al*., 1999).

DNA damage caused by exposure to ionizing radiation, UV light or exogenous chemical mutagens that result in DNA strand breakage, triggers the accumulation of the tumour suppressor p53, which transactivates a number of genes involved in either growth arrest, DNA repair or apoptosis (for review see Lakin and Jackson, 1999; Oren, 1999; Sionov and Haupt, 1999). Mice lacking p53 are prone, very early, to the spontaneous development of malignant lymphomas and sarcomas (Donehower *et al*., 1992; Jacks *et al*., 1994). The early carcinogenesis observed in these mice was recently related to a constitutive upregulation of inducible nitric oxide synthase (iNOS) (Ambs *et al*., 1998a,b).

p53 and PARP-1 are both involved in the cell's response to genotoxic stress but presumably at different steps. The functional interaction between these two proteins being unknown, here we explore the potential role of PARP-1 in the p53-deficient background. Various cohorts of the four possible homozygous genotypes of both genes were generated by breeding mice mutant for *parp-1* and *p53*. Upon γ-irradiation, a partial restoration of S-phase radiosensitivity was found in primary fibroblasts isolated from *parp-1*$^{-/-}$*p53*$^{-/-}$ mice compared with *parp-1*$^{+/+}$*p53*$^{-/-}$ mice. However, the most striking finding was the gain in tumour-free survival promoted by the genetic deletion of *parp-1* in *p53*-deficient mice. This significant delay in tumour

formation was confirmed in nude mice injected with *ras*-transformed fibroblasts from *parp-1*$^{-/-}$*p53*$^{-/-}$ mice. The retarded initiation in spontaneous tumour formation in double-mutant mice was inversely correlated with the cell's ability to activate iNOS protein expression and generation of NO-derived oxidative products.

Taken together, our results show that the PARP-1 status dramatically influences tumour latency and cell proliferation, which could lead to new therapeutic approaches for the treatment of human p53 mutant tumours.

Results

Genetic deletion of parp-1 in p53-deficient mice promotes a gain in tumour-free survival

Pregnant or nursing *parp-1*$^{+/+}$*p53*$^{-/-}$ females tend to develop tumours and attempts to use these mice to generate *parp-1*$^{+/+}$*p53*$^{-/-}$ mice have often failed. Therefore, *parp-1*$^{+/+}$*p53*$^{+/-}$ mice were intercrossed to generate *parp-1*$^{+/+}$*p5*$^{+/+}$ mice and *parp-1*$^{+/+}$*p53*$^{-/-}$ mice. Consistently, the *parp-1*$^{-/-}$*p53*$^{-/-}$ mice were generated from *parp-1*$^{-/-}$*p53*$^{+/-}$ intercrosses although *parp-1*$^{-/-}$*p53*$^{-/-}$ females are generally able to produce progeny. *parp-1*$^{-/-}$*p53*$^{-/-}$ mice were born at the predicted Mendelian frequency indicating that there was no prenatal death of the double mutant.

Mice of the four genotypes were observed concurrently to determine the spontaneous rate of tumour development. During the time of the study, no *parp-1*$^{+/+}$*p53*$^{+/+}$ or *parp-1*$^{-/-}$*p53*$^{+/+}$ mice died. We found that *parp-1*$^{-/-}$*p53*$^{-/-}$ mice had substantially longer lifespans compared with the *parp-1*$^{+/+}$*p53*$^{-/-}$ control mice (Figure 1A). The average age at which tumour formation occurred in the *parp-1*$^{+/+}$*p53*$^{-/-}$ mice was 15–16 weeks compared with 24 weeks for *parp-1*$^{-/-}$*p53*$^{-/-}$ mice. When they became moribund mice were killed and a detailed histological analysis of the tumour was conducted (Table I). Nearly all the mice had succumbed to tumours although some of them (two *parp-1*$^{+/+}$*p53*$^{-/-}$ and one *parp-1*$^{-/-}$*p53*$^{-/-}$ mice) presented unresolved infections. These cases were not included in Table I.

The histological analysis showed that both groups of mice developed predominantly lymphomas, although sarcomas were also observed. Flow cytometric analysis of *parp-1*$^{+/+}$*p53*$^{+/-}$ and *parp-1*$^{-/-}$*p53*$^{+/-}$ thymic lymphomas revealed that they were exclusively of immature T-cell origin (data not shown), as described previously (Jacks *et al*., 1994). Although the vast majority of *p53*$^{-/-}$ thymic lymphomas are in the CD4^{+}/CD8^{+} double positive class, we found some double negative CD4^{-}/CD8^{-} thymic lymphomas in *parp-1*$^{-/-}$*p53*$^{-/-}$ mice. Therefore, the longer latency for tumour formation in the double-mutant animals might be a reflection of resistance of double-positive cells to transformation or tumour development. T-cell lymphomas were also described in PARP-deficient mice in SCID background (Morrison *et al*., 1997). The tumour spectrum observed in *parp-1*$^{-/-}$*p53*$^{-/-}$ mice is therefore similar to that seen in *parp-1*$^{+/+}$*p53*$^{-/-}$ mice, and is consistent with that reported previously (Jacks *et al*., 1994) in *p53*$^{-/-}$ mice. These results suggest that *parp-1* does not determine the cell type of the tumour but may contribute to the initiation step of tumorigenesis. To explore this hypothesis, we tested the ability of *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$-transformed primary fibroblasts to generate tumours in immunocompromised BALB/c nude mice.

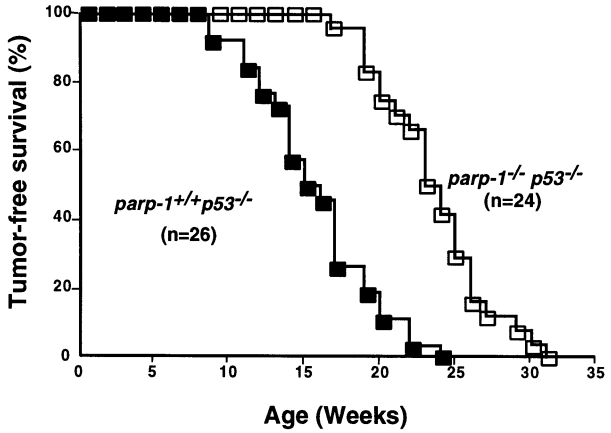

Fig. 1. Kaplan–Meier plot of tumour incidence in *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ mice. The percentage of mice remaining tumour free versus mice alive at the outset is plotted against age. The difference was highly significant ($p < 10^{-5}$ by Gehan's Wilcoxon test and $p < 10^{-5}$ by log-rank test). Note that during the time of study, no *parp-1*$^{+/+}$ *p53*$^{+/+}$ or *parp-1*$^{-/-}$*p53*$^{+/+}$ mice died.

Tumour formation is delayed in nude mice injected with ras-transformed fibroblasts from parp-1$^{-/-}$*p53*$^{-/-}$

Oncogenic *ras* efficiently transforms rodent primary cells deficient in *p53* or *p16* (Tanaka *et al*., 1994; Serrano *et al*., 1996). We have introduced an activated *ras* allele (H-*ras* V12) into *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ primary fibroblasts using recombinant replication-deficient retrovirus (Serrano *et al*., 1997). Before transformation, PARP-1 and p53 expression in mouse embryonic fibroblasts (MEFs) was analysed by western blotting. As shown in Figure 2A, PARP-1 expression was found in *parp-1*$^{+/+}$*p53*$^{+/+}$ and in *parp-1*$^{+/+}$*p53*$^{-/-}$ MEFs and 4 h after 5 Gy γ-irradiation, p53 was detectable in *parp-1*$^{+/+}$*p53*$^{+/+}$ and in *parp-1*$^{-/-}$*p53*$^{+/+}$ fibroblasts.

The presence of oncogenic *ras* detected by Southern blotting with a *ras* V12 DNA probe was used to determine the transduction efficiency in *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ primary fibroblasts. As shown in Figure 2B, oncogenic *ras* was detected in both primary cells. We then tested whether immortalization of both cell lines was achieved by the expression of the H-*ras* V12 oncogene. Changes in cell morphology were observed after retroviral infection and puromycin selection of pure populations. Both *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ cells adopted a prominent refractile morphology (Figure 2C) compared with parental lines. Furthermore, both cell lines continued to incorporate bromodeoxyuridine (BrdU) and to proliferate although the rate of proliferation of *parp-1*$^{-/-}$*p53*$^{-/-}$ cells was 25% less than that of *parp-1*$^{+/+}$*p53*$^{-/-}$cells (data not shown). The tumorigenicity of *ras*-transduced primary (passage 1) *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ embryonic fibroblasts was demonstrated by their ability to form colonies in soft agar, a measure of anchorage-independent growth, and to produce tumours when implanted subcutaneously (s.c.) into immunocompromised nude mice. When grown in soft agar, both *ras*-transduced fibroblasts formed colonies

Table I. Tumor spectrum of *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ mice

Case	Genotype	Histological site	Anatomical site
1	*parp-1*$^{+/+}$*p53*$^{-/-}$	teratoma	ovary
2	*parp-1*$^{+/+}$*p53*$^{-/-}$	lymphoma	thymus
3	*parp-1*$^{+/+}$*p53*$^{-/-}$	ND	stomach
4	*parp-1*$^{+/+}$*p53*$^{-/-}$	osteosarcoma	femur
5	*parp-1*$^{+/+}$*p53*$^{-/-}$	lymphoma	thymus
6	*parp-1*$^{+/+}$*p53*$^{-/-}$	lymphoma	thymus
7	*parp-1*$^{+/+}$*p53*$^{-/-}$	lymphoma	thymus
8	*parp-1*$^{+/+}$*p53*$^{-/-}$	lymphoma	hepatic metastasis
9	*parp-1*$^{+/+}$*p53*$^{-/-}$	lymphoma	thymus
10	*parp-1*$^{+/+}$*p53*$^{-/-}$	lymphoma	thymus
11	*parp-1*$^{+/+}$*p53*$^{-/-}$	lymphoma	thymus
1	*parp-1*$^{-/-}$*p53*$^{-/-}$	lymphoma	thymus
2	*parp-1*$^{-/-}$*p53*$^{-/-}$	lymphoma	thymus
3	*parp-1*$^{-/-}$*p53*$^{-/-}$	lymphoma	thymus
4	*parp-1*$^{-/-}$*p53*$^{-/-}$	fibrosarcoma	muscle
5	*parp-1*$^{-/-}$*p53*$^{-/-}$	osteosarcoma	femur
6	*parp-1*$^{-/-}$*p53*$^{-/-}$	adenosarcoma	lung
7	*parp-1*$^{-/-}$*p53*$^{-/-}$	lymphoma	thymus
8	*parp-1*$^{-/-}$*p53*$^{-/-}$	lymphoma	thymus
9	*parp-1*$^{-/-}$*p53*$^{-/-}$	lymphoma	thymus
10	*parp-1*$^{-/-}$*p53*$^{-/-}$	angiosarcoma	vessel
11	*parp-1*$^{-/-}$*p53*$^{-/-}$	lymphoma	thymus + ovarian metastasis
12	*parp-1*$^{-/-}$*p53*$^{-/-}$	epithelioma baso-cellular	skin
13	*parp-1*$^{-/-}$*p53*$^{-/-}$	lymphoma	hepatic metastasis
14	*parp-1*$^{-/-}$*p53*$^{-/-}$	ND	?
15	*parp-1*$^{-/-}$*p53*$^{-/-}$	lymphoma	thymus
16	*parp-1*$^{-/-}$*p53*$^{-/-}$	lymphoma	thymus

(Figure 2D). Notably, *parp-1*$^{-/-}$*p53*$^{-/-}$ colonies were smaller than *parp-1*$^{+/+}$*p53*$^{-/-}$ colonies, suggesting that transformed *parp-1*$^{-/-}$*p53*$^{-/-}$ cells need a more physiological environment to develop.

To avoid secondary mutations associated with culture passage, we injected s.c. into BALB/c nude mice primary fibroblasts at passage 1, 36 h after retroviral infection. Eight BALB/c nude mice 6–8 weeks old were injected with 0.5×10^6 transformed *parp-1*$^{+/+}$*p53*$^{-/-}$ cells in the right thigh and the same number of transformed *parp-1*$^{-/-}$*p53*$^{-/-}$ cells in the left thigh. Their tumour-forming ability was evaluated by measurements of tumour growth twice weekly. All the sites injected with transformed *parp-1*$^{+/+}$*p53*$^{-/-}$ developed tumours (8/8), which were visible 15 days after injection. The mice were killed 22–25 days after injection, when they became severely ill. At that time, no tumour (0/8) was detected in the sites injected with transformed *parp-1*$^{-/-}$*p53*$^{-/-}$ cells (Figure 3A).

To test whether the modulation of the tumour microenvironment could affect the efficiency of tumour formation in *parp-1*$^{-/-}$*p53*$^{-/-}$ cells, 2×10^6 transformed fibroblasts were mixed with Matrigel before implantation into nude mice. Tumour formations were observed in all cases (6/6), indicating that the stromal microenvironment has significant influence on *parp-1*$^{-/-}$*p53*$^{-/-}$ tumour growth. Although *parp-1*$^{+/+}$*p53*$^{-/-}$ cells formed tumours rapidly, a significant delay was observed in the initiation of *parp-1*$^{-/-}$*p53*$^{-/-}$ tumour formation (Figure 3B), indicating that the loss of PARP-1 expression in a p53$^{-/-}$ background affects the efficiency of tumour formation. We reasoned that the tumour cells might have quite different cell cycle

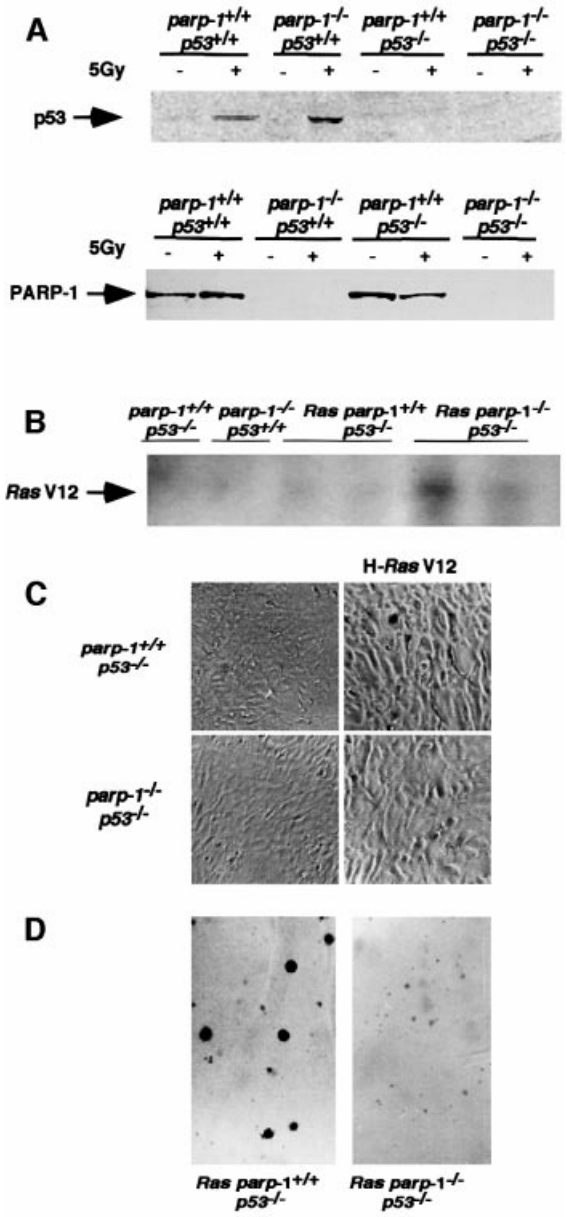

Fig. 2. (**A**) Western blot analysis of PARP-1 and p53 expression in nuclear (p53) or crude (PARP-1) extracts of MEFs of the indicated genotypes. p53 protein was induced after 5 Gy irradiation. (**B**) Southern blot of *Hin*dIII-digested DNA from *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ MEFs infected or not with H-*ras* V12 retrovirus, using the *ras* V12 probe. (**C**) Morphology of primary and transformed *parp-1*$^{+/+}$*p53*$^{+/+}$ and *parp-1*$^{-/-}$*p53* fibroblasts. (**D**) Anchorage-independent growth of transformed *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ fibroblasts.

parameters when introduced into a s.c. site. To test this, we isolated cells of explanted tumours from both genotypes and analysed the percentage of cells in S phase following BrdU incorporation. We found that *parp-1*$^{+/+}$*p53*$^{-/-}$ cells proliferate slightly more rapidly than *parp*$^{-/-}$*p53*$^{-/-}$ cells (S phase: $25.9 \pm 7\%$ and $21.0 \pm 0.4\%$ for *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ cells, respectively. This difference could account for reduced progression of the tumour. Moreover, histological analysis of tumours of both genotypes showed poorly differentiated cells without any detectable apoptotic event (Figure 3C).

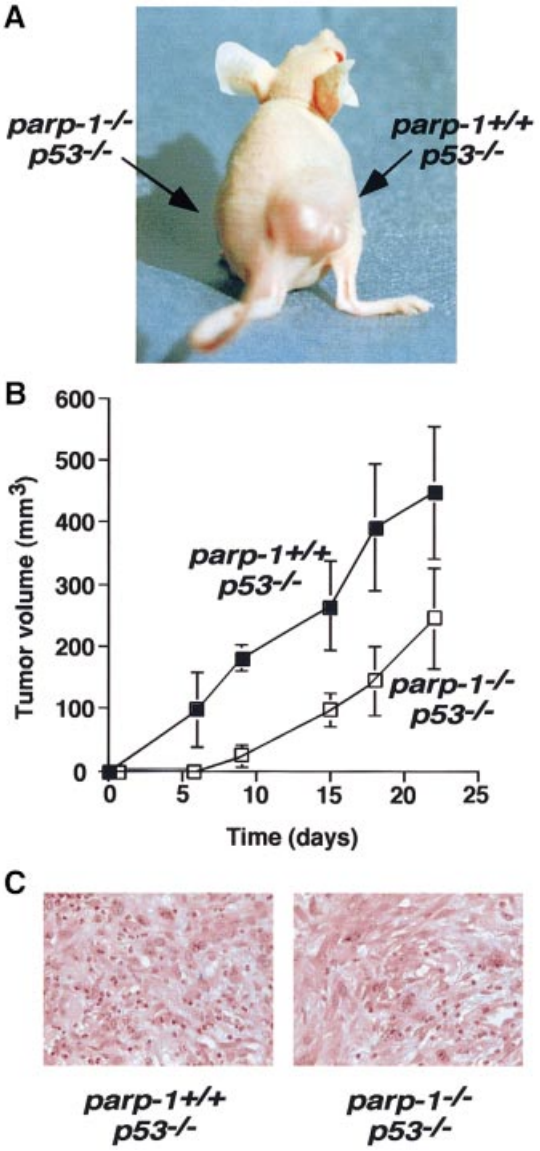

Fig. 3. (**A**) Tumour formation in BALB/c nude mice. This representative mouse was inoculated with transformed *parp-1*$^{+/+}$*p53*$^{-/-}$ fibroblasts in the right thigh and transformed *parp-1*$^{-/-}$*p53*$^{-/-}$ fibroblasts in the left thigh. (**B**) Tumour volume plot after s.c. injection of *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ transformed fibroblasts mixed in equal volume of Matrigel into Balb/c nude mice. For each genotype, six sites were injected. Each point represents the mean ± SD of measurements. The difference was significant at all data points (p <0.006, Mann–Whitney U-test).

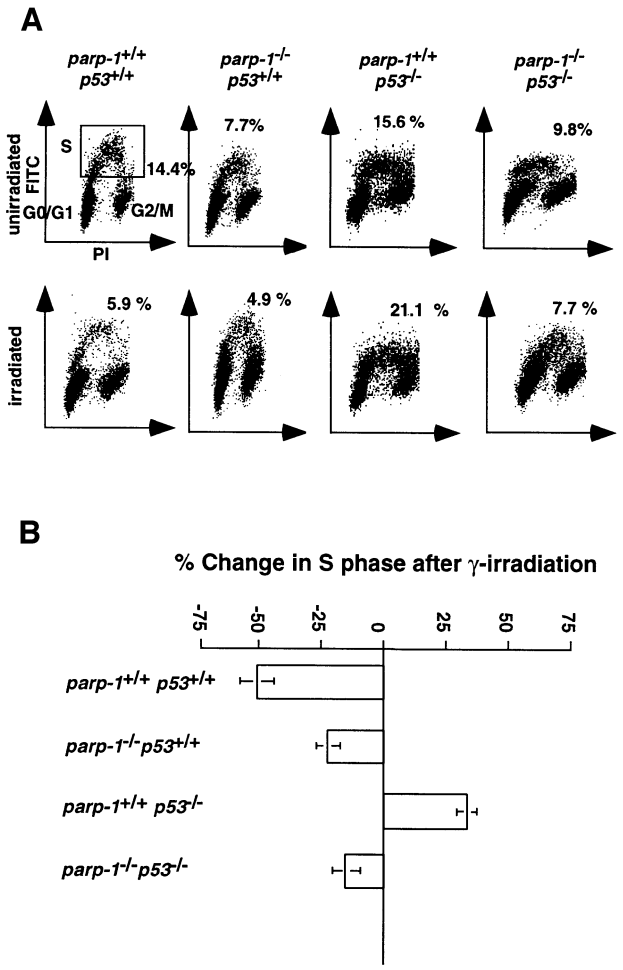

Fig. 4. (**A**) Representative flow cytometry scatter plots of MEFs nuclei 18 h after irradiation (5 Gy), plotted as increasing fluorescence of propidium iodide (PI; x-axis) versus increasing FITC fluorescence obtained with an anti-BrdU–FITC-conjugated antibody (y-axis). Gating for S-phase cells is shown in the panel from unirradiated wild-type mice (upper left). (**B**) Graph representing the mean ± SD change in the percentage of S-phase cells after irradiation (n = 3–6).

Partial restoration of the cell cycle G_1 arrest following γ-irradiation in double-mutant cells

Given the importance of controlling the checkpoints for the prevention of neoplasia, we measured the cell cycle progression through the S phase of asynchronous primary MEFs of the four genotypes 18 h after γ-irradiation (5 Gy). The percentages of S-phase cells were assayed by flow cytometry (Figures 3B and 4A). The p53 protein is a regulator of the G_1 checkpoint (Levine, 1997). Irradiation exposure of wild-type as well as *parp-1*$^{-/-}$*p53*$^{+/+}$ MEFs resulted in a drastic reduction (50.2 ± 6.7 and 25.2 ± 7.4%, respectively) of the number of BrdU-positive S-phase cells. In contrast, the G_1/S-phase checkpoint in *parp-1*$^{+/+}$*p53*$^{-/-}$ cells was defective, resulting in an increase (32.8 ± 3.5%) in BrdU-positive cells, as reported previously (Kastan *et al*., 1992). There was also a marked reduction (14.8 ± 5.6%) in BrdU-positive *parp-1*$^{-/-}$*p53*$^{-/-}$ MEFs after irradiation. Thus, in the absence of PARP-1, a partial restoration of the G_1/S-phase checkpoint occurs in p53$^{-/-}$ MEFs that might account for the observed delay in tumorigenesis.

Increased genomic instability in primary fibroblasts from double-mutant mice

Genomic instability often correlates with a susceptibility to mutation and consequently with a propensity to carcinogenesis. It has been reported previously that both PARP-1 (Ménissier de Murcia *et al*., 1997; Wang *et al*., 1997; Trucco *et al*., 1998; Simbulan-Rosenthal *et al*., 1999) and p53 act to prevent genomic instability (Harvey *et al*., 1993; Lee *et al*., 1994). During their *in vitro* passaging, the tumorigenic *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ MEFs expressing H-*ras* V12 might have sustained genetic changes such as chromosomal abnormalities. This genomic instability might well predispose to

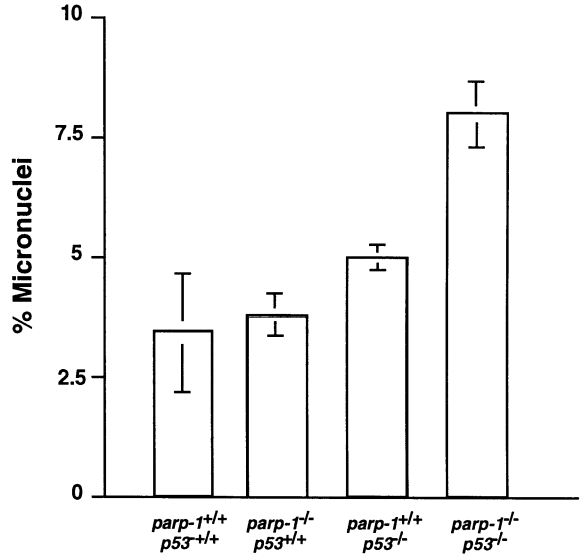

Fig. 5. Micronuclei formation in primary MEFs at passage 2 isolated from mice of the indicated genotypes. A total of 700–1000 binucleated cells in each genotype was counted.

carcinogenesis. Both primary *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ populations contained a mixture of near-diploid (65%) and near-tetraploid (35%) cells. The majority (70%) of tumorigenic *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ cells expressing H-*ras* V12 are near tetraploid. Since micronuclei represent extranuclear chromosomal fragments that are not incorporated into the nucleus during mitosis, they are considered as biomarkers of chromosomal instability. Spontaneous primary binucleated fibroblasts containing micronuclei were enumerated from four different genotypes. As shown in Figure 5, the percentage of *parp-1*$^{-/-}$*p53*$^{+/+}$ (3.3 ± 0.4%) and *parp*$^{+/+}$*p53*$^{-/-}$ (4.5 ± 0.1%) MEFs containing micronuclei was increased by a factor of 1.4–1.9 compared with the wild-type cells (2.3 ± 1.3%), whereas double-mutant MEFs containing micronuclei (7.1 ± 2.1%) increased 3-fold in comparison with wild-type controls ($p \leq 0.005$). These results further indicated that (i) the lack of both PARP-1 and p53 had a synergic effect on genomic stability and (ii) the observed tumorigenicity did not seem to be correlated with the level of spontaneous genomic instability.

Reduction of iNOS expression and nitrite, and nitrate release in double-mutant mice

iNOS expression is deregulated in both mouse models: downregulation in *parp-1*$^{-/-}$ mice (Oliver *et al*., 1999) and upregulation in *p53*$^{-/-}$ mice (Ambs *et al*., 1998a,b). Macrophages being the main source of iNOS, we determined the basal and LPS-induced iNOS expression and NO release in macrophages of the four genotypes. Mice were treated with LPS (20 mg/kg i.p.) and 18 h later, the iNOS protein levels were determined in peritoneal macrophages by western blotting (Figure 5A). Following LPS treatment, iNOS expression was increased in macrophages taken from *parp-1*$^{+/+}$*p53*$^{+/+}$mice, but not from *parp-1*$^{-/-}$*p53*$^{+/+}$ mice, as expected (Oliver *et al*., 1999). In contrast, iNOS protein levels were increased in *parp-1*$^{+/+}$

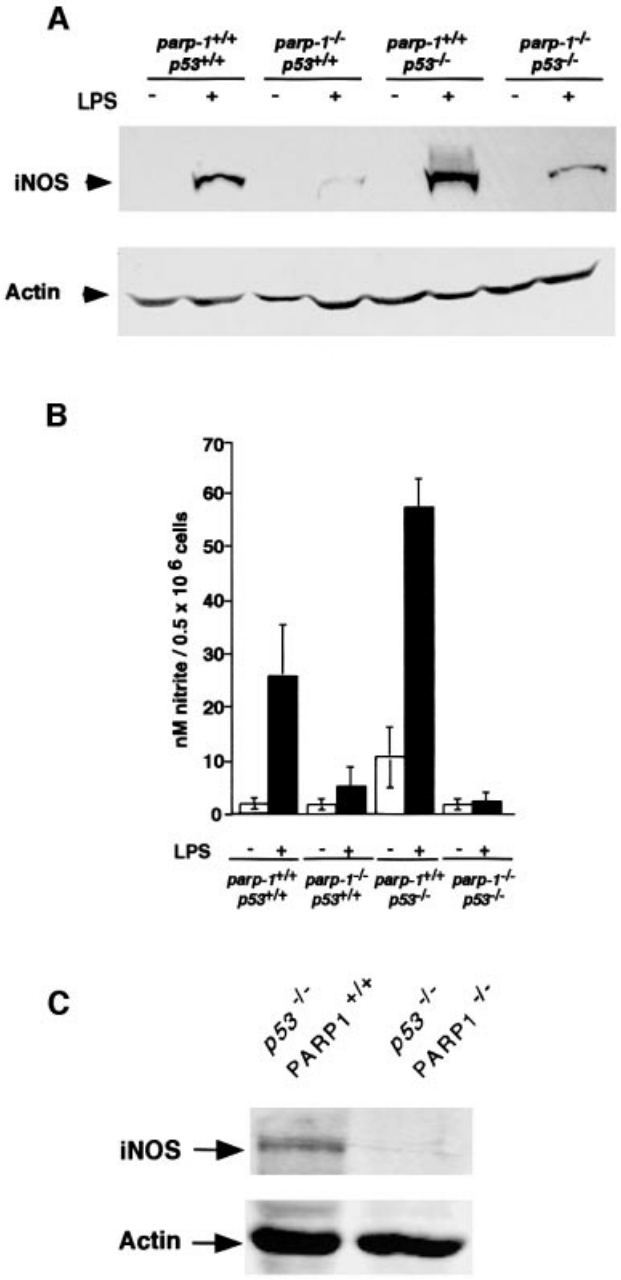

Fig. 6. (**A**) Western blot analysis of iNOS expression in crude extracts of peritoneal macrophages from mice of the genotypes indicated, 18 h after LPS treatment. Expression of actin is shown as a loading control. (**B**) Nitrite release in primary cultured murine macrophages from mice of the indicated genotype treated or not with 5 μg/ml LPS for 24 h. Each value represents the mean ± SD of three independent experiments. (**C**) iNOS expression in crude extracts of splenocytes derived from *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ mice.

p53$^{-/-}$ cells, as reported previously (Ambs *et al*., 1998b). A dramatic reduction of iNOS expression was also observed in *parp-1*$^{-/-}$*p53*$^{-/-}$ cells. This differential expression of iNOS in all four genotypes, was reflected in measurements of nitrite plus nitrate release in the supernatant of cultured primary macrophages stimulated with LPS (5 μg/ml) (Figure 6B). Because *parp-1*$^{+/+}$ *p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ mice spontaneously developed predominantly thymic lymphomas, we sought to determine whether the deregulated iNOS expression was

specific to macrophages or whether this occurred in thymocytes as well. To test this hypothesis, we performed western blot analysis on cellular extracts from *parp-1*$^{+/+}$ *p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ thymus. The results showed that iNOS is downregulated in *parp-1*$^{-/-}$*p53*$^{-/-}$ compared with *parp-1*$^{+/+}$*p53*$^{-/-}$ thymocytes (Figure 6C). Therefore, the downregulation of iNOS in *parp-1*$^{-/-}$*p53*$^{-/-}$ mice, accompanied by a drop in the oxidative status, may contribute to the delayed tumour initiation observed in double-mutant mice, by decreasing the mutagenic potential of NO (Caulfield *et al*., 1998).

Discussion

PARP-1 activity now appears to be a pivotal determinant in the mechanism of oxidant-induced cell death (Ha and Snyder, 1999). Recent studies from several laboratories have implicated PARP-1 in the pathogenesis of various diseases, such as myocardial ischaemia–reperfusion injury (Thiemermann *et al*., 1997; Zingarelli *et al*., 1998; Pieper *et al*., 2000), endotoxic shock (Hassa and Hottiger, 1999; Oliver *et al*., 1999), arthritis (Szabo *et al*., 1998), type-1 diabetes (Burkart *et al*., 1999; Masutani *et al*., 1999) and brain ischaemia (Eliasson *et al*., 1997). The inactivation of *parp-1* in genetically engineered animals confers protection against the diseases mentioned above. Significant protection against oxidant-induced tissue damage can also be achieved with pharmacological PARP-1 inhibitors (Szabo and Dawson, 1998; Szabo *et al*., 1998). Various mechanisms have been proposed to explain the role of PARP-1 in the pathogenicity of these diseases. The most prevalent explanation is the so-called 'PARP suicide pathway': the oxygen-derived free radicals, NO and peroxynitrite are generated during inflammation and induce DNA single-stranded breaks, which in turn overactivate PARP-1 (Berger, 1985). This excessive activation leads to intracellular NAD^+ and ATP depletion resulting in mitochondrial free radical generation and cell necrosis (Ha and Snyder, 1999; Pieper *et al*., 1999). We and others have reported previously a functional link between PARP-1 and the transcription factor NF-κB in a model of endotoxic shock (Hassa and Hottiger, 1999; Oliver *et al*., 1999). PARP-1-deficient mice are extremely resistant to death induced by LPS because NF-κB-dependent transcription is impaired, consequently the *in vivo* release of inflammatory mediators as well as iNOS expression are downregulated during endotoxic shock. PARP-1 appears to promote inflammation by co-activativation of NF-κB-dependent transcription and by mediating the cytotoxicity of NO derivatives.

The data presented in this study reveal a new role for PARP-1 in tumorigenesis that, again, appears to be linked to the regulatory properties of the cell's oxidative status. We have demonstrated that spontaneous tumour development in *p53*$^{-/-}$ mice is significantly delayed in the absence of PARP-1, resulting in increased survival of *parp-1*$^{-/-}$ *p53*$^{-/-}$ mice. Furthermore, tumour formation in nude mice showed that PARP-1 inactivation interfered with the initiation step of tumorigenesis. While the injection of 0.5×10^6 *ras*-transformed cells generated tumours at 15 days post-injection, *parp-1*$^{-/-}$*p53*$^{-/-}$ *ras*-transformed cells failed to generate any visible tumour, except when mixed with Matrigel. Even then tumour initiation was clearly delayed compared with that generated with single *p53* mutant cells (Figure 2C). Matrigel is a solubilized basement membrane preparation whose major components are laminin, collagen IV, heparan sulfate, proteoglycans and multiple growth factors. Matrigel could supply effective support for the attachment of transformed cells and a higher concentration of survival factors providing optimal conditions for the proliferation of *ras*-transformed *parp-1*$^{-/-}$*p53*$^{-/-}$ cells. It is conceivable that in the absence of PARP-1, *ras*-transformed *p53*$^{-/-}$ cells were unable to proliferate and develop tumours, explaining the observed delay in tumorigenesis in double-mutant mice.

Although the double-mutant mice exhibited more spontaneous genomic instability compared with *parp-1*$^{+/+}$ *p53*$^{-/-}$ mice, they did not exhibit an acceleration of tumour formation. In contrast, genomic instability inversely correlates with tumour development maybe through the induction of various cell cycle checkpoints. Consistent with these results, PARP-1-deficient mice did not show any particular predisposition to develop tumours but displayed genomic instability (Figure 5; Ménissier de Murcia *et al*., 1997; Wang *et al*., 1997; Trucco *et al*., 1998; Simbulan-Rosenthal *et al*., 1999). Genomic instability *per se* may therefore not be sufficient for tumorigenesis.

Among alternative mechanisms that could be responsible for the delay in tumour formation in *p53*-deficient mice, we examined the epistatic relationship between PARP-1 and p53 in response to γ-irradiation through S-phase progression. Radioresistant-DNA synthesis is one of the *p53*$^{-/-}$ fibroblast phenotypes (Harvey *et al*., 1993), whereas *parp-1*$^{-/-}$ fibroblasts exhibit normal G_1/S checkpoint. *parp-1*$^{-/-}$*p53*$^{-/-}$ cells showed a reproducible partial restoration of the G_1/S checkpoint that might account for the delay in tumorigenesis observed *in vivo*. However, the molecular mechanism underlying this restoration remains unknown.

NO is synthesized from L-arginine by a family of enzymes called NO synthases (NOSs). The inducible isoform of NOS (iNOS or NOS2) generates NO for longer periods of time and at rates several orders of magnitude greater than the constitutive isoforms (Nicolson *et al*., 1993). High levels of NO and peroxynitrite formed from their interaction with the superoxide anion have been shown to induce damage to membranes, oxidation of intracellular proteins, DNA single-stranded breaks, nitrosative deamination and DNA base oxidative damage. High NO concentrations are elevated in ulcerative colitis (Singer *et al*., 1996) and other chronic inflammatory conditions, such as chronic hepatitis and *Helicobacter pylori* gastritis (Mannick *et al*., 1996), that predispose individuals to cancer. An abundant expression of NOS, as well as NOS activity, has been positively correlated with the degree of malignancy in human ovarian and uterine cancers (Thomsen *et al*., 1994), central nervous system tumours (Cobbs *et al*., 1995) and breast cancer (Thomsen *et al*., 1995). Numerous studies in animal models have also provided direct evidence for a stimulatory role of NO in tumour progression. In a rat colonic adenocarcinoma model showing iNOS expression in the tumour vasculature, treatment with N^G-nitro-L-arginine methyl ester (L-NAME), a potent NOS inhibitor, reduces NO production and tumour growth (Kennovin *et al*., 1994). Similarly, engineered expression of murine iNOS in a human colonic

adenocarcinoma cell line resulting in continuous, moderate levels of NO production *in vitro*, was associated with increased tumour growth and vascularity *in vivo* following transplantation in nude mice (Jenkins *et al*., 1995). The exposure of cells to high concentrations of NO results in wild-type p53 accumulation, which in turn mediates a *trans*-repression of iNOS gene expression in a regulatory negative feedback loop (Forrester *et al*., 1996). In line with this, *p53*$^{-/-}$ constitutively upregulates the iNOS gene, leading to sustained production of NO in some cell types (Ambs *et al*., 1998b). This excessive NO production leading to radical-induced DNA damage as well as inhibition of DNA repair enzymes, may promote the lymphomagenesis observed in these mice. The data presented here show a dramatic reduction in iNOS expression and NO production in the macrophages of *parp-1*$^{-/-}$*p53*$^{-/-}$ mice upon LPS stimulation. Thus, the absence of PARP-1 downregulates the iNOS gene expression in *p53*-deficient mice, reducing the high levels of NO observed in these mice. The decreased carcinogenic potential of NO in *parp-1*$^{-/-}$*p53*$^{-/-}$ mice could therefore explain the delay in tumorigenicity. In line with this, it is interesting to notice that the disruption of the gene encoding p66^{shc}, an enzyme involved in oxidative stress response, confers a heightened cellular resistance to agents that cause oxidative damage and a 30% increase in lifespan (Migliaccio *et al*., 1999).

These and previous results (Oliver *et al*., 1999) from this laboratory are in full agreement with previously published data showing the attenuation or abrogation of established inflammatory diseases (Thiemermann *et al*., 1997; Szabo and Dawson, 1998; Pieper *et al*., 1999; Jijon *et al*., 2000) and a lack of tumorigenicity caused by the pharmacological inactivation of PARP-1 *in vivo* (Tseng *et al*., 1987; Bauer *et al*., 1996). It has also been shown recently that inhibition of PARP-1 by overexpression in HeLa cells of a *trans*-dominant-negative mutant dramatically reduces tumour formation of these cells injected into nude mice (Hans *et al*., 1999). The suggested mechanism was an increase in apoptosis due to the expression of a dominant-negative PARP mutant. In our model, this increase in apoptosis did not occur. The absence of PARP-1 failed to activate a p53-independent apoptotic pathway, suggesting that other mechanisms could be implicated in the observed interference with tumorigenesis.

The present results suggest a possible therapeutic approach to human cancers, most of which are *p53* mutated. It is possible that in specific tumours expressing functional p53, inactivation of PARP-1 may sensitize tumour cells to therapeutic irradiation. In addition, therapeutic targeting of PARP-1 may improve the clinical outcome by retarding the growth of tumours expressing mutant *p53*. Such an approach would have to be carefully tailored in order to avoid toxicity derived from DNA repair inhibition in surrounding tissues.

Materials and methods

Generation of parp-1$^{-/-}$*p53*$^{-/-}$ *mice*

parp-1$^{-/-}$ and *p53*$^{-/-}$ mice were described previously (Jacks *et al*., 1994; Ménissier de Murcia *et al*., 1997) and both were in the mixed genetic background C57BL/6 × 129Sv. *parp-1*$^{-/-}$ females were bred with a *p53*$^{+/-}$ male to generate *parp-1*$^{+/-}$*p53*$^{+/-}$ mice, which were intercrossed to generate *parp-1*$^{+/+}$*p53*$^{+/-}$ and *parp-1*$^{-/-}$*p53*$^{+/-}$ progeny. *parp-1*$^{+/+}$*p53*$^{+/-}$ and *parp-1*$^{-/-}$*p53*$^{+/-}$ mice were then intercrossed to generate mice of the four genotypes: *parp-1*$^{+/+}$*p53*$^{+/+}$, *parp-1*$^{-/-}$*p53*$^{+/+}$, *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$.

Cell culture and cell cycle analysis

MEFs were derived from day 13.5 embryos as described (Serrano *et al*., 1997). *parp-1*$^{+/+}$*p53*$^{-/-}$ and *parp-1*$^{-/-}$*p53*$^{-/-}$ were obtained from intercrosses of *parp-1*$^{+/+}$*p53*$^{+/-}$ and *parp-1*$^{-/-}$*p53*$^{+/-}$, respectively. MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal calf serum (FCS) and 0.05 mg/ml gentamicin at 37°C with 5% CO_2. For cell cycle analysis, primary MEFs at passage 2 were grown in 75 cm^2 flasks at a density of 10^6 cells/flask in normal DMEM, irradiated with a ^{60}Co γ-ray (5 Gy) source and cultured for 18 h. Then, the medium of the irradiated and non-irradiated MEFs was supplemented with 10 μM BrdU. After 30 min of BrdU labelling, cells were harvested, fixed in 70% ethanol, and analysed using a FACScan and the Cellquest program as described previously (Ménissier de Murcia *et al*., 1997). DNA content was revealed using propidium iodide and DNA synthesis by staining with V-fluorescein-5-isothiocyanate (FITC)-conjugated anti-BrdU antibody (Pharmingen). At least 10^4 cells were analysed per sample.

Tumorigenicity assay

Passage-1 MEFs were infected with an H-*ras* V12 oncogene-expressing retrovirus (kindly provided by M.Serrano, CNBC, CSIC, Madrid, Spain) as described previously (Serrano *et al*., 1997). Thirty-six hours after infection, cells were trypsinized, washed twice in phosphate-buffered saline (PBS) and 0.5 × 10^6 cells were injected s.c. into 6- to 8-week-old female BALB/c nude mice (Iffa-Credo, L'Arbresle, France). In some experiments, 2 × 10^6 transformed cells mixed in equal volume of cold Matrigel (Becton Dickinson, Bedford, MA, USA) were injected. Tumour volume (V) was determined twice a week by calliper measurement of the length (L) and width (W) as $V = (L \times W^2)/2$ (Soengas *et al*., 1999). Tumour cells were re-isolated by mincing the tumour and incubation in collagenase for 4 h. After washing in PBS, the cells were replated in DMEM supplemented with 10% FCS.

Soft agar assays

A bottom layer of 0.8% agar in DMEM was first placed on to 6 cm dishes. Ras-transformed *parp-1*$^{+/+}$*p53*$^{+/-}$ and *parp-1*$^{-/-}$*p53*$^{+/-}$ fibroblasts (5 × 10^3 cells) were seeded in 0.3% top agar containing DMEM and 30% FCS. Colonies were counted after 3 weeks.

Micronuclei assay

Primary MEFs at passage 2 were seeded on coverslips the day before treatment with cytochalasin B (6 μg/ml) (Fenech and Morley, 1985), and 24 h later micronuclei were determined as described previously (Trucco *et al*., 1998).

Western blot analysis

Total or nuclear (Velasco *et al*., 1997) proteins were separated by 10% SDS–PAGE, transferred to a nitrocellulose membrane and probed with either anti-iNOS (Sigma), anti-p53 (pAb 421) or anti-PARP-1 (VIC-5) antibodies. Bound antibodies were revealed with goat anti-rabbit or sheep anti-mouse–horseradish peroxidase and blots were developed using the Renaissance blotting detection system (New England Nuclear, Boston, MA).

Southern blot analysis

Genomic DNA (15 μg) from retroviral-infected MEFs was restricted with *Hin*dIII, electrophoresed on a 1% agarose gel and transferred to a nitrocellulose sheet (Amersham) according to the manufacturer's protocol. The radiolabelled mouse oncogenic *ras* cDNA excised by *Hin*dIII from the retroviral pLPCX *ras* plasmid (Morgenstern and Land, 1990; Serrano *et al*., 1997) was used as a probe to hybridize the membrane. The hybridized filter was exposed to X-ray film.

Nitrite release

Nitrite release in the culture medium of murine macrophages was determined by the Griess reaction as described previously (Szabo *et al*., 1994), following stimulation *in vitro* with 1 μg/ml LPS for 24 h.

Acknowledgements

We are indebted to C.Waltzinger for FACScan assistance, to Professor P.Chambon for constant support, to the Centre Paul Strauss (Strasbourg) for radiation experiments and to Dr S.Natarajan-Amé for critical reading of the manuscript. C.C. was supported by a postdoctoral fellowship from EGIDE and by the Centre National de la Recherche Scientifique (CNRS). This work was supported by grants from Association pour la Recherche contre le Cancer, Ligue contre le Cancer, Electricité de France, Commissariat à l'Energie Atomique and Fondation pour la Recherche Médicale, and to F.J.O. from Fondo de Investigaciones Sanitarias 00/0948, Spanish Ministry of Health.

References

Ambs,S. *et al*. (1998a) p53 and vascular endothelial growth factor regulate tumor growth of NOS2-expressing human carcinoma cells. *Nature Med.*, **4**, 1371–1376.

Ambs,S., Ogunfusika,M.O., Merriam,W.G., Bennett,W.P., Billiar,T.R. and Harris,C.C. (1998b) Up-regulation of inducible nitric oxide synthase expression in cancer-prone p53 knockout mice. *Proc. Natl Acad. Sci. USA*, **95**, 8823–8828.

Bauer,P.I. *et al.* (1996) Modification of growth related enzymatic pathways and apparent loss of tumorigenicity of a *ras*-transformed bovine endothelial cell line by treatment with 5-iodo-6-amino-1,2-benzopyrone (INH_2BP). *Int. J. Oncol.*, **8**, 239–252.

Beneke,R., Geisen,C., Zevnik,B., Bauch,T., Muller,W.U., Kupper,J.H. and Moroy,T. (2000) DNA excision repair and DNA damage-induced apoptosis are linked to poly(ADP-ribosyl)ation but have different requirements for p53. *Mol. Cell. Biol.*, **20**, 6695–6703.

Berger,N.A. (1985) Poly(ADP-ribose) in the cellular response to DNA damage. *Radiat. Res.*, **101**, 4–15.

Burkart,V., Wang,Z.Q., Radons,J., Heller,B., Herceg,Z., Stingl,L., Wagner,E.F. and Kolb,H. (1999) Mice lacking the poly(ADP-ribose) polymerase gene are resistant to pancreatic β-cell destruction and diabetes development induced by streptozocin. *Nature Med.*, **5**, 314–319.

Caulfield,J.L., Wishnok,J.S. and Tannenbaum,S.R. (1998) Nitric oxide-induced deamination of cytosine and guanine in deoxynucleosides and oligonucleotides. *J. Biol. Chem.*, **273**, 12689–12695.

Cobbs,C.S., Brenman,J.E., Aldape,K.D., Bredt,D.S. and Israel,M.A. (1995) Expression of nitric oxide synthase in human central nervous system tumors. *Cancer Res.*, **55**, 727–730.

Dantzer,F., de la Rubia,G., Ménissier-de Murcia,J., Hostomsky,Z., de Murcia,G. and Schreiber,V. (2000) Base excision repair is impaired in mammalian cells lacking poly(ADP-ribose) polymerase-1. *Biochemistry*, **39**, 7559–7569.

de Murcia,G. and Ménissier de Murcia,J. (1994) Poly(ADP-ribose) polymerase: a molecular nick-sensor. *Trends Biochem. Sci.*, **19**, 172–176.

Donehower,L.A., Harvey,M., Slagle,B.L., McArthur,M.J., Montgomery,C.A.,Jr, Butel,J.S. and Bradley,A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, **356**, 215–221.

Eliasson,M.J. *et al.* (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. *Nature Med.*, **3**, 1089–1095.

Fenech,M. and Morley,A.A. (1985) Measurement of micronuclei in lymphocytes. *Mutat. Res.*, **147**, 29–36.

Forrester,K. *et al.* (1996) Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase expression by wild-type p53. *Proc. Natl Acad. Sci. USA*, **93**, 2442–2447.

Ha,H.C. and Snyder,S.H. (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proc. Natl Acad. Sci. USA*, **96**, 13978–13982.

Hans,M.A., Muller,M., Meyer-Ficca,M., Burkle,A. and Kupper,J.H. (1999) Overexpression of dominant negative PARP interferes with tumor formation of HeLa cells in nude mice: evidence for increased tumor cell apoptosis *in vivo*. *Oncogene*, **18**, 7010–7015.

Harvey,M. *et al*. (1993) *In vitro* growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene*, **8**, 2457–2467.

Hassa,P.O. and Hottiger,M.O. (1999) A role of poly(ADP-ribose) polymerase in NF-κB transcriptional activation. *Biol. Chem.*, **380**, 953–959.

Jacks,T., Remington,L., Williams,B.O., Schmitt,E.M., Halachmi,S., Bronson,R.T. and Weinberg,R.A. (1994) Tumor spectrum analysis in p53-mutant mice. *Curr. Biol.*, **4**, 1–7.

Jenkins,D.C. *et al*. (1995) Roles of nitric oxide in tumor growth. *Proc. Natl Acad. Sci. USA*, **92**, 4392–4396.

Jijon,H.B., Churchill,T., Malfair,D., Wessler,A., Jewell,L.D., Parsons,H.G. and Madsen,K.L. (2000) Inhibition of poly(ADP-ribose) polymerase attenuates inflammation in a model of chronic colitis. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **279**, G641–G651.

Kastan,M.B., Zhan,Q., el-Deiry,W.S., Carrier,F., Jacks,T., Walsh,W.V., Plunkett,B.S., Vogelstein,B. and Fornace,A.J.,Jr (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*, **71**, 587–597.

Kennovin,G.D., Stratford,M.R.L. and Flitney,F.W. (1994) Inducible nitric oxide synthase is expressed in tumor-associated vasculature: inhibition retards tumor growth *in vivo*. In Moncada S., Feelisch,M., Busse,R. and Higgs,E.A. (eds), *Biology of Nitric Oxide*, *Part 4: Enzymology*, *Biochemistry and Immunology*. Portland Press, London, UK, pp. 473–479.

Lakin,N.D. and Jackson,S.P. (1999) Regulation of p53 in response to DNA damage. *Oncogene*, **18**, 7644–7655.

Lee,J.M., Abrahamson,J.L., Kandel,R., Donehower,L.A. and Bernstein,A. (1994) Susceptibility to radiation-carcinogenesis and accumulation of chromosomal breakage in p53 deficient mice. *Oncogene*, **9**, 3731–3736.

Levine,A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.

Mannick,E.E. *et al*. (1996) Inducible nitric oxide synthase, nitrotyrosine and apoptosis in *Helicobacter pylori* gastritis: effect of antibiotics and antioxidants. *Cancer Res.*, **56**, 3238–3243.

Masson,M., Niedergang,C., Schreiber,V., Muller,S., Menissier-de Murcia,J. and de Murcia,G. (1998) XRCC1 is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage. *Mol. Cell. Biol.*, **18**, 3563–3571.

Masutani,M. *et al.* (1999) Poly(ADP-ribose) polymerase gene disruption conferred mice resistant to streptozotocin-induced diabetes. *Proc. Natl Acad. Sci. USA*, **96**, 2301–2304.

Masutani,M., Nozaki,T., Nakamoto,K., Nakagama,H., Suzuki,H., Kusuoka,O., Tsutsumi,M. and Sugimura,T. (2000) The response of Parp knockout mice against DNA damaging agents. *Mutat. Res.*, **462**, 159–166.

Ménissier de Murcia,J. *et al.* (1997) Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. *Proc. Natl Acad. Sci. USA*, **94**, 7303–7307.

Migliaccio,E., Giorgio,M., Mele,S., Pelicci,G., Reboldi,P., Pandolfi,P.P., Lanfrancone,L. and Pelicci,P.G. (1999) The $p66^{shc}$ adaptor protein controls oxidative stress response and life span in mammals. *Nature*, **402**, 309–313.

Morgenstern,J.P. and Land,H. (1990) A series of mammalian expression vectors and characterisation of their expression of a reporter gene in stably and transiently transfected cells. *Nucleic Acids Res.*, **18**, 1068.

Morrison,C., Smith,G.C., Stingl,L., Jackson,S.P., Wagner,E.F. and Wang,Z.Q. (1997) Genetic interaction between PARP and DNA-PK in V(D)J recombination and tumorigenesis. *Nature Genet.*, **17**, 479–482.

Nicolson,A.G., Haites,N.E., McKay,N.G., Wilson,H.M., MacLeod,A.M. and Benjamin,N. (1993) Induction of nitric oxide synthase in human mesangial cells. *Biochem. Biophys. Res. Commun.*, **193**, 1269–1274.

Oliver,F.J., Ménissier-de Murcia,J., Nacci,C., Decker,P., Andriantsitohaina,R., Muller,S., de la Rubia,G., Stoclet,J.C. and de Murcia,G. (1999) Resistance to endotoxic shock as a consequence of defective NF-κB activation in poly(ADP-ribose) polymerase-1 deficient mice. *EMBO J.*, **18**, 4446–4454.

Oren,M. (1999) Regulation of the p53 tumor suppressor protein. *J. Biol. Chem.*, **274**, 36031–36034.

Pieper,A.A., Verma,A., Zhang,J. and Snyder,S.H. (1999) Poly(ADP-ribose) polymerase, nitric oxide and cell death. *Trends Pharmacol. Sci.*, **20**, 171–181.

Pieper,A.A., Walles,T., Wei,G., Clements,E.E., Verma,A., Snyder,S.H. and Zweier,J.L. (2000) Myocardial postischemic injury is reduced by polyADPribose polymerase-1 gene disruption. *Mol. Med.*, **6**, 271–282.

Serrano,M., Lee,H., Chin,L., Cordon-Cardo,C., Beach,D. and DePinho,R.A. (1996) Role of the INK4a locus in tumor suppression and cell mortality. *Cell*, **85**, 27–37.

Serrano,M., Lin,A.W., McCurrach,M.E., Beach,D. and Lowe,S.W. (1997) Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and $p16^{INK4a}$. *Cell*, **88**, 593–602.

Simbulan-Rosenthal,C.M. *et al*. (1999) Chromosomal aberrations in

PARP(–/–) mice: genome stabilization in immortalized cells by reintroduction of poly(ADP-ribose) polymerase cDNA. *Proc. Natl Acad. Sci. USA*, **96**, 13191–13196.

Singer,I.I., Kawka,D.W., Scott,S., Weidner,J.R., Mumford,R.A., Riehl,T.E. and Stenson,W.F. (1996) Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology*, **111**, 871–885.

Sionov,R.V. and Haupt,Y. (1999) The cellular response to p53: the decision between life and death. *Oncogene*, **18**, 6145–6157.

Soengas,M.S., Alarcon,R.M., Yoshida,H., Giaccia,A.J., Hakem,R., Mak,T.W. and Lowe,S.W. (1999) Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science*, **284**, 156–159.

Szabo,C. and Dawson,V.L. (1998) Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion. *Trends Pharmacol. Sci.*, **19**, 287–298.

Szabo,C., Southan,G.J. and Thiemermann,C. (1994) Beneficial effects and improved survival in rodent models of septic shock with *S*-methylisothiourea sulfate, a potent and selective inhibitor of inducible nitric oxide synthase. *Proc. Natl Acad. Sci. USA*, **91**, 12472–12476.

Szabo,C., Virag,L., Cuzzocrea,S., Scott,G.S., Hake,P., O'Connor,M.P., Zingarelli,B., Salzman,A. and Kun,E. (1998) Protection against peroxynitrite-induced fibroblast injury and arthritis development by inhibition of poly(ADP-ribose) synthase. *Proc. Natl Acad. Sci. USA*, **95**, 3867–3872.

Tanaka,N. *et al*. (1994) Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. *Cell*, **77**, 829–839.

Thiemermann,C., Bowes,J., Myint,F.P. and Vane,J.R. (1997) Inhibition of the activity of poly(ADP ribose) synthetase reduces ischemia-reperfusion injury in the heart and skeletal muscle. *Proc. Natl Acad. Sci. USA*, **94**, 679–683.

Thomsen,L.L., Lawton,F.G., Knowles,R.G., Beesley,J.E., Riveros-Moreno,V. and Moncada,S. (1994) Nitric oxide synthase activity in human gynecological cancer. *Cancer Res.*, **54**, 1352–1354.

Thomsen,L.L., Miles,D.W., Happerfield,L., Bobrow,L.G., Knowles,R.G. and Moncada,S. (1995) Nitric oxide synthase activity in human breast cancer. *Br. J. Cancer*, **72**, 41–44.

Trucco,C., Oliver,F.J., de Murcia,G. and Ménissier-de Murcia,J. (1998) DNA repair defect in poly(ADP-ribose) polymerase-deficient cell lines. *Nucleic Acids Res.*, **26**, 2644–2649.

Tseng,A.,Jr, Lee,W.M., Jakobovits,E.B., Kirsten,E., Hakam,A., McLick,J., Buki,K. and Kun,E. (1987) Prevention of tumorigenesis of oncogene-transformed rat fibroblasts with DNA site inhibitors of poly(ADP ribose) polymerase. *Proc. Natl Acad. Sci. USA*, **84**, 1107–1111.

Velasco,M., Diaz-Guerra,M.J., Martin-Sanz,P., Alvarez,A. and Bosca,L. (1997) Rapid up-regulation of IκBβ and abrogation of NF-κB activity in peritoneal macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.*, **272**, 23025–23030.

Wang,Z.Q., Stingl,L., Morrison,C., Jantsch,M., Los,M., Schulze-Osthoff,K. and Wagner,E.F. (1997) PARP is important for genomic stability but dispensable in apoptosis. *Genes Dev.*, **11**, 2347–2358.

Zingarelli,B., Salzman,A.L. and Szabo,C. (1998) Genetic disruption of poly(ADP-ribose) synthetase inhibits the expression of P-selectin and intercellular adhesion molecule-1 in myocardial ischemia/reperfusion injury. *Circ. Res.*, **83**, 85–94.

Received November 7, 2000; revised April 27, 2001;
accepted May 2, 2001