A new transgenic mouse line to image chemically induced p53 activation *in vivo*

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Monitoring p53 transcriptional activity to identify genotoxic damages induced by drugs has been proposed and validated *in vitro*. However, this methodology is by design limited to the cell line tested. In this study, we have fully validated a luciferase-based p53-reporter system *in vitro* and *in vivo*. We generated a mouse transgenic line to monitor non-invasively p53 activation in response to chemically induced DNA damage. Doxorubicin was used as a drug of known toxicity to validate our model. Reporter gene expression was measured using bioluminescence imaging. In females, a weak p53 luciferase activity driven by a p53-responsive promoter was detectable in the oral cavity region after doxorubicin treatment. In males, the signal increased in the lower abdominal region. Imaging of various organs revealed that the luciferase activity was mainly generated from the testes. Immunohistology demonstrated that the cells in the seminiferous tubules were damaged by the drug and confirmed that they were luciferase and p53 positive. Therefore, these transgenic mice could provide a powerful tool to predict, map and characterize at the organ and cellular levels the toxicity of compounds and help to develop new therapeutic agents in humans. (*Cancer Sci* 2008; 99: 683–688)

Exposure to certain chemicals is associated with the development of specific human cancers.[1,2] The methods used to identify human carcinogens involve *in vitro* tests such as mutagenesis in *Salmonella*, but the generally accepted definitive method of classification remains *in vivo* animal testing. Stimulated by a general interest in replacing these large-scale toxicological studies on animals, new *in vitro* tests based on the knowledge that has been accumulated over the last decade in molecular and cell biology are emerging. A promising strategy exploits p53.

The tumor suppressor p53 is normally kept at a low cellular level through its interaction with Murine double minute protein.[3] However, various cellular stresses including chemically induced DNA damage, lead to p53 stabilization and activation.[4,5] As a result, p53 modulates the expression of numerous target genes implicated in the regulation of the cellular cycle and apoptosis, through its interaction with specific DNA binding sites located within the promoters of these genes.[6,7] The detection of a p53 response has been proposed as a survey for genotoxins and potentially useful anticancer agents.[8–10] A reporter system using a p53 responsive promoter has been previously developed as an indicator of genotoxic damage *in vitro*.[11] However, *in vivo*, p53 action is tissue-specific.[12,13] Understanding its function generally requires invasive processes, the analysis relying on fixed tissue samples.[14–16] Thus a transgenic mouse model to visualize gene expression non-invasively on a whole body scale is of great interest.

In the current study, we have generated a new p53-responsive transgenic line to map p53 activation in response to chemically induced DNA damage. Such a model could provide unique information on the potential toxicity of new compounds and help to characterize specific tissue(s) and/or cell-types responding to a particular drug. Using bioluminescence imaging, we visualize p53 activation in response to doxorubicin and characterize the site of p53 activation by immunohistochemistry.

Materials and Methods

Cells, plasmids and transfection. Colon carcinoma cells HCT116 $p53^{-/-}$ and $p53^{+/+}$ were kindly provided by Pr. B. Vogelstein (Department of Pathology, Johns Hopkins Medical Institution, Baltimore, USA). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Calf Serum and were transfected using Lipofectamine 2000 (Invitrogen) with a plasmid containing a p53 reporter system previously described,[17] in which four copies of a p53-responsive element (p53RE) were cloned upstream of a minimal promoter. The resulting promoter is driving the expression of the Firefly Luciferase (Luc) cDNA. The transfected cells are noted HCT116-p53RE-Luc thereafter. To obtain stably transfected cells, HCT116 were cotransfected with an empty vector containing a Puromycin resistance gene and grown according to manufacturer's instructions. Resistant populations were selected to perform the *in vitro* experiments and to establish tumor xenografts *in vivo*.

Biochemical assays. HCT116 were seeded at a density of 3.5×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and incubated for 24 h in medium containing 0–0.5 μM doxorubicin (Sigma-Aldrich). Cell viability was assessed using a tetrazolium salt (MTT) based assay as previously described,[18] and total proteins were quantified using the bicinchoninic acid (BCA) method (BCA Protein Assay, Pierce, Rockford, IL, USA). For Western blot analysis, cells were harvested with a scraper after being washed with ice-cold phosphate buffer saline (PBS) and lyzed in RIPA (1% NP40, 0.5% NaDeoxycolate, 0.1% SDS, protease inhibitor). Samples were fractioned in a Tris-HCl buffered 4–12% gradient polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and blotted to nitrocellulose membranes as previously described.[19] The membranes were probed for p53 (DO-1, Santa Cruz Biotechnology, USA), Ser^{15}-phosphorylated p53 (Calbiochem, EMD Biosciences, La Jolla, CA, USA) or Ku70 (C-19, Santa Cruz Biotechnology, USA) and then probed with secondary antibodies conjugated with horseradish peroxydase (Dako, Denmark) and visualized using chemiluminescence (ECL, GE Healthcare, UK). Finally, a luciferase assay was performed using a Promega kit following manufacturer's instruction. HCT116-p53RE-Luc lysates were analyzed for luciferase activity using a luminometer (Luminoskan Ascent, Thermo Labsystems).

Infections with adenoviruses. HCT116-p53RE-Luc cells were infected with recombinant replication incompetent adenoviruses expressing either the Green Fluorescent Protein (Ad-GFP) or p53 (Ad5-CMV-p53) at a multiplicity of infection of 50. A luciferase assay was performed 24 h later.

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Animal experiments. Experiments were conducted after appropriate ethical approval and licensing was obtained in accordance with the United Kingdom 'Guidance on the operation of animals (Scientific Procedure) Act 1986' (HMSO, London, United Kingdom, 1990).

Generation of s.c. tumor xenografts in nude mice. BALB/c nude mice were obtained from Harlan (Oxfordshire, United Kingdom) and kept in a germ-free environment with irradiated food and acidified water *ad libitum*. HCT116-p53RE-Luc wild type and $p53^{-/-}$ suspension (2×10^6 cells in 100 μL) in normal saline was injected subcutaneously in the left flank ($p53^{+/+}$) and the right flank ($p53^{-/-}$). Tumor-bearing animals were then scanned using bioluminescence imaging 24 h prior and 24 h after receiving an intraperitoneal injection of 1.5 mg/kg doxorubicin.

Transgenic animals. All animals were kept in a germ-free environment with irradiated food and acidified water *ad libitum*. The p53RE-luciferase construct was used to generate transgenic mice at Cancer Research UK Transgenic Services (Clare Hall Laboratories, UK) on a CBA/F1 background. Transgenic animals were characterized using standard polymerase chain reaction techniques with primers specific for the luciferase transgene (luciferase forward 5′-TGGATTCTAAAACGGATTACCAGGG-3′ and reverse 5′-CCAAAACAACAACGGCGGC-3′). A total of four distinct founder transgenic lines were obtained and a single founder was selected, which showed transgene expression using bioluminescence imaging (BLI). Animals were between ages 8–12 weeks at the time of experiments.

Bioluminescent imaging. Mice were anesthetized with a 2% Halothane/O_2/N_2O mixture and given a single intraperitoneal injection of 150 mg/kg D-Luciferin (Xenogen, Alameda, USA) in 0.2 mL normal saline, in a way that allows homogenous distribution of this substrate.[20] BLI was initiated 10 min after D-luciferin injection using a Xenogen Ivis Imaging Series 100 system. During image acquisition, halothane anesthesia was maintained by using a nose cone delivery system. Animal body temperature was regulated with a temperature-controlled stage. A gray-scale whole body image was collected (field of view: D; exposure, 0.2 second; binning: medium; and f/stop, 16) followed by acquisition and overlay of a pseudo color image representing the spatial distribution of the detected photons emitted from the animal (field of view D; exposure, 300 s; binning, large [high sensitivity] or medium [nude mice]; and f/stop, 1) with all luminescent data recorded as photons per second (total flux). Signal intensity was quantified as the sum of all detected photon counts within uniform-sized regions of interest that were manually placed during post data acquisition image analysis. To evaluate doxorubicin toxicity, transgenic mice were imaged 24 h before and 24 h after an intraperitoneal injection of doxorubicin (1.5 mg/mL). For analysis of organ-specific differences in bioluminescence, transgenic animals were killed by cervical dislocation 10 min after D-luciferin injection and quickly dissected. Individual organs were assessed by BLI and samples were fixed in 4% Formaldehyde.

Immunohistology. Fixed transgenic and non-transgenic mouse testes were embedded in paraffin. Haematoxylin and Eosin sections were obtained as previously described.[21] Immunohistology analysis was conducted using the automated Ventana Discovery platform (Ventana Discovery Systems, Tuscon, AZ, USA). 4 μm sections were incubated for 30 min in Bovine serum albumin, 1% BSA, 1.5% normal goat serum (Dako, Denmark) and 3% normal donkey serum (Gene Tex, Inc., San Antonio, TX, USA), then incubated for 1 h with an antiluciferase antibody 1:50 (Promega, UK), washed three times with PBS and incubated for 45 min with the secondary antibody 1:500, AlexaFluor 488 donkey antigoat (Invitrogen). p53 was detected by immunohistochemistry using a polyclonal antibody (DO-1, obtained from Cancer Research UK) at a 1:1000 dilution and revealed by immunoperoxydase staining.

Statistics. All statistical analysis was done using GraphPad Prism (version 3.0c for Macintosh, GraphPad Software, San Diego, CA, USA). Differences between groups with continuous variables were done using student's *t*-test (two-tailed, unequal variances) with a $P < 0.05$ considered statistically significant.

Results

Doxorubicin reduces cell survival and activates p53 on HCT116 cells *in vitro*. HCT116 cells were incubated with various doses of doxorubicin. Twenty-four hours later, cells were subjected to a viability assay (MTT). The results demonstrate that a clear loss of viability is detectable at a concentration above 0.5 μM (data not shown). This toxicity is accompanied by an activation of p53 that shows that doxorubicin treatment significantly increases both phosphorylated p53 (Ser 15) and total p53 expression in a dose-dependent manner. As expected, no p53 signal is observed in HCT116 p53-negative cells after doxorubicin treatment (data not shown).

Validation of the p53-responsive expression cassette *in vitro*. HCT116-p53RE-Luc (either $p53^{+/+}$ or $p53^{-/-}$) were incubated with increasing doses of doxorubicin for 24 h. Figure 1a shows

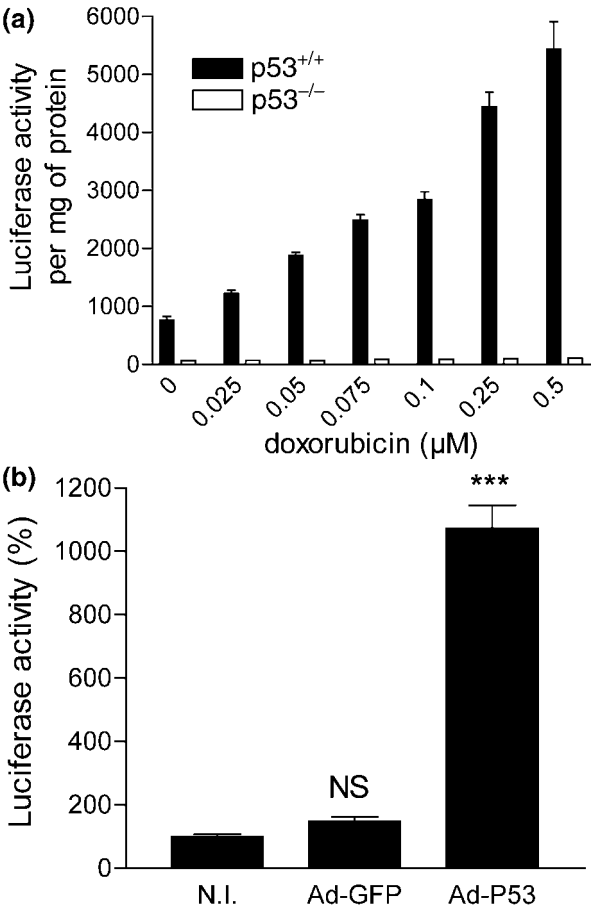

Fig. 1. Doxorubicin induces p53-dependant luciferase expression in HCT116-p53RE-Luciferase cells in a dose-dependant manner. (a) Stably transfected $p53^{+/+}$ and $p53^{-/-}$ cells were incubated with various doses of doxorubicin and luciferase activity was measured after 24 h. (b) HCT116-p53RE-Luciferase $p53^{-/-}$ were infected with either Ad-CMV-p53 or Ad-GFP (control) at a multiplicity of infection of 50. Luciferase expression was determined 24 h later. (NI: non-infected cells). *Columns*: averaged luciferase activity (4 wells per condition); *bars*: standard error of the mean. Luciferase activity in Ad-CMV-p53 is compared to the non-infected cells ($P < 0.0001$). NS: non-significant.

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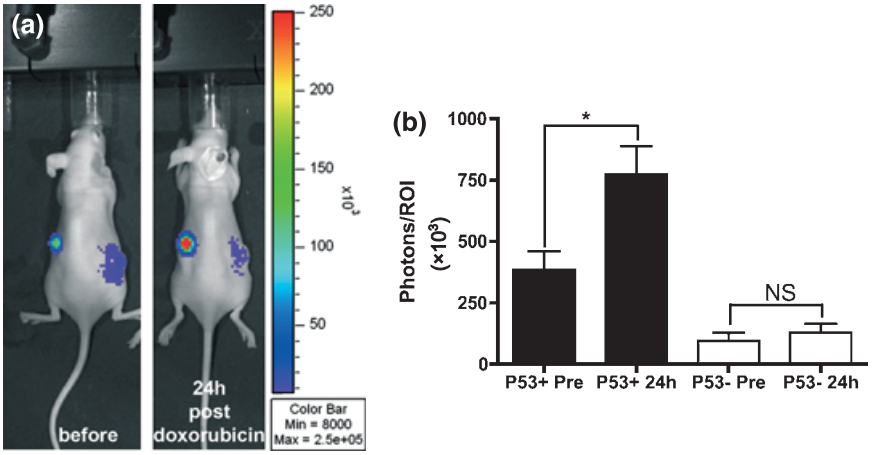

Fig. 2. Bioluminescence imaging of p53 induction in HCT116-p53RE-Luc tumor-bearing BALB/c nude mice. Mice bearing a $p53^{+/+}$ tumor on the left flank and a $p53^{-/-}$ tumor on the right flank were imaged before and 24 h after receiving an intraperitoneal injection of doxorubicin (1.5 mg/kg). (a) Gray-scale image of a representative mouse overlayed with a pseudo-color image of bioluminescence. All window and level settings depict a range between 8000 and 250 000 photons/s and are consistent for all images. (b) quantification of bioluminescence on tumor regions of interest (ROI) before and after treatment with doxorubicin. *Columns*, averaged bioluminescence ($n = 5$ mice) expressed as total photons flux in the ROI; *bars*, standard error of the mean. *$P = 0.0103$; NS: non-significant ($P = 0.4207$).

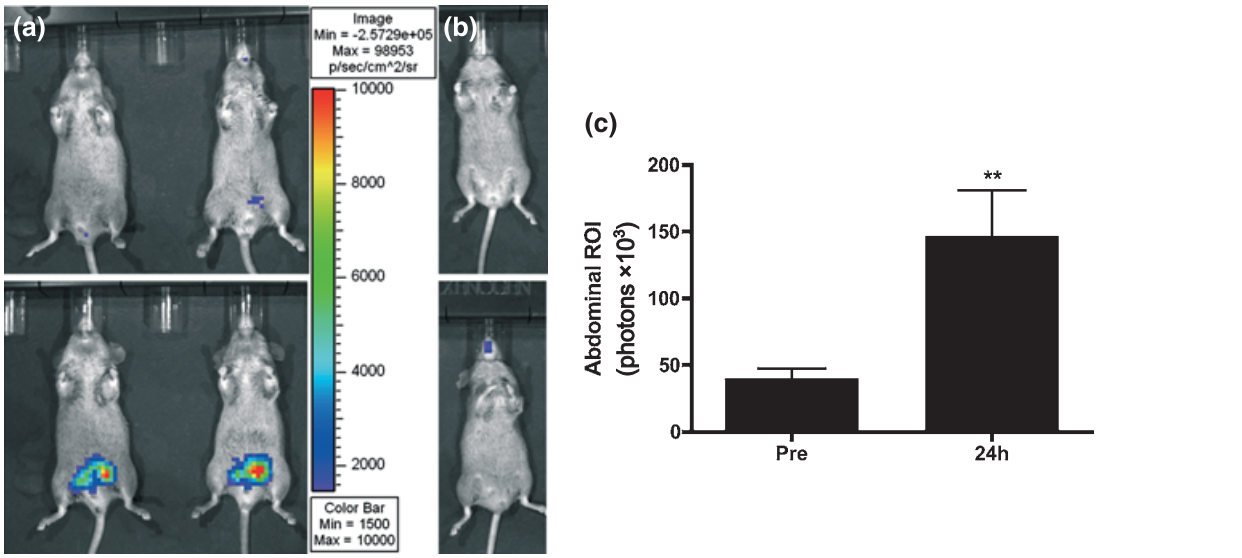

Fig. 3. Bioluminescence imaging of p53 induction in p53RE-Luciferase mice. Mice were imaged before (top) and 24 h after administration of a single intraperitoneal injection of doxorubicin (1.5 mg/kg) (bottom). Gray-scale image of the mice overlayed with a pseudo-color image of bioluminescence. All window and level settings depict a range between 1500 and 10 000 photon/s and are consistent for all images. (a) male transgenic mice ($n = 7$); (b) female transgenic mouse ($n = 3$); (c) quantification of bioluminescence on abdominal regions of interest (ROI) in males before and after treatment with doxorubicin ($P = 0.0096$). *Columns*, averaged bioluminescence expressed as total photons flux in the ROI; *bars*, standard error of the mean.

a dose dependent increase in luciferase activity on HCT116-p53RE-Luc $p53^{+/+}$, while doxorubicin treatment does not result in increased luciferase expression in HCT116-p53RE-Luc $p53^{-/-}$. In addition, infection of HCT116-p53RE-Luc $p53^{-/-}$ with a recombinant adenovirus encoding p53 restored the p53 response (Fig. 1b). Altogether, these results demonstrate that the luciferase activity is strictly dependent on p53 presence and activation *in vitro*.

Imaging of p53 transcriptional activity in HCT116-p53RE-Luc tumor xenografts. To determine the p53-selectivity of the p53RE-Luc construct *in vivo*, HCT116-p53RE-Luc $p53^{+/+}$ and $p53^{-/-}$ were injected subcutaneously in nude mice. Before treatment, both HCT116-p53RE-Luc $p53^{+/+}$ and $p53^{-/-}$ tumors were producing luciferase (Fig. 2a). HCT116-p53RE-Luc $p53^{+/+}$ tumors produced more than five times more luciferase activity than $p53^{-/-}$ control tumors (Fig. 2b). Imaging of the same cohort of mice following an intraperitoneal injection of doxorubicin (1.5 mg/mL) led to a two-fold (2.136 ± 0.378) increase in luciferase activity in HCT116-p53RE-Luc $p53^{+/+}$ tumors (773 800 ± 115 700 vs 387 200 ± 72 300, $P = 0.0103$), without significantly affecting the luciferase activity in $p53^{-/-}$ tumors (128 460 ± 35 760 vs 94 900 ± 33 100, $P = 0.4207$) (Fig. 2).

***In vivo* localization of p53 activation by doxorubicin in p53RE-Luc transgenic mice.** The p53RE-Luc construct was used to generate transgenic animals. P53RE-Luc male mice from the resulting line were imaged by BLI before treatment with doxorubicin and showed only a weak and diffuse signal in the lower peritoneal cavity in some animals of the cohort (Fig. 3a). The same animals were imaged 24 h after receiving an intraperitoneal injection of 1.5 mg/kg doxorubicin. The drug induced a strong increase of luciferase activity in the peritoneal region (Fig. 3a). Quantitative analysis showed a 4-fold increase in luciferase activity in a regions of interest placed over the abdominal region after doxorubicin injection in males (146 900 ± 33 900 vs 40 220 ± 7300, $P = 0.0096$) (Fig. 3c). By contrast, injection of doxorubicin in females resulted in a non-statistically significant increase (11 950 ± 4210 vs 6388 ± 1048, $P = 0.3284$) of luciferase activity in the oral cavity region only (Fig. 3b). These results were

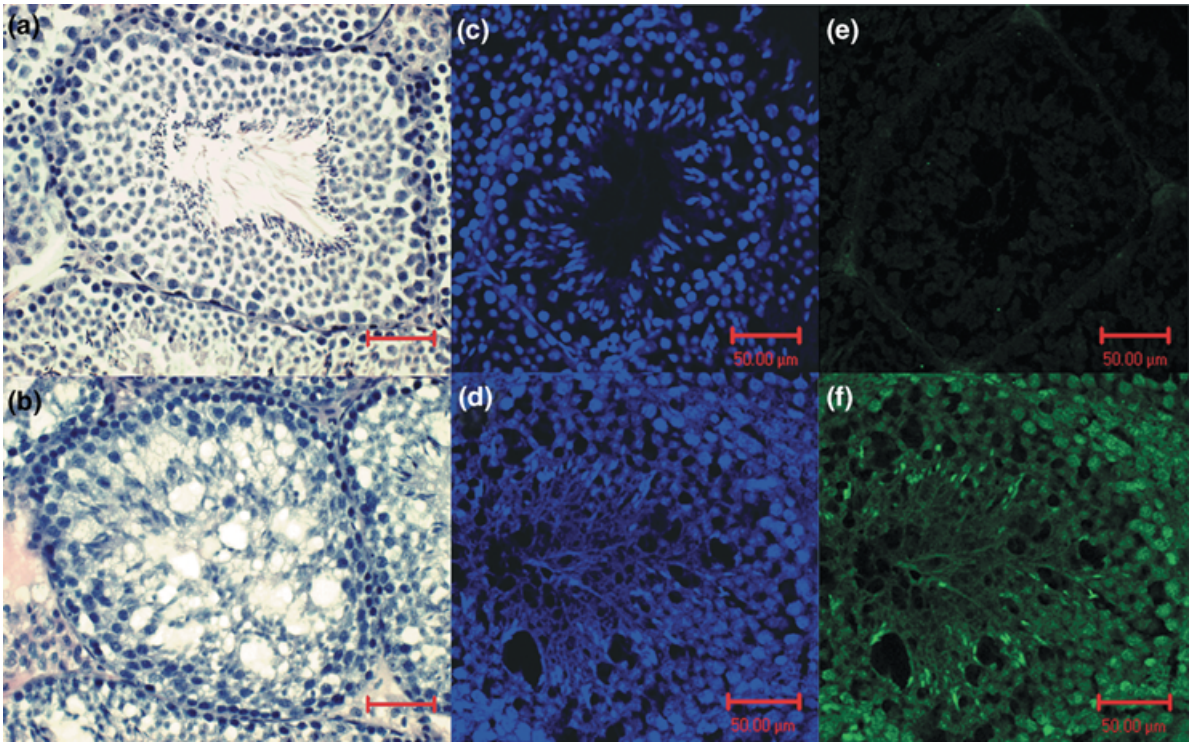

Fig. 4. Immunohistological analysis of seminiferous tubules sections from a control (a, c, e) or a p53RE-Luc mouse following a single intraperitoneal injection of Doxorubicin (b, d, f). (a, b) Haematoxylin and eosin staining (magnification ×20). (c–f) confocal microscopy: fluorescence visualization of 4′-6-Diamidino-2-phenylindole (c, d), immunofluorescence staining for luciferase (e, f). Bar: 50 micrometers.

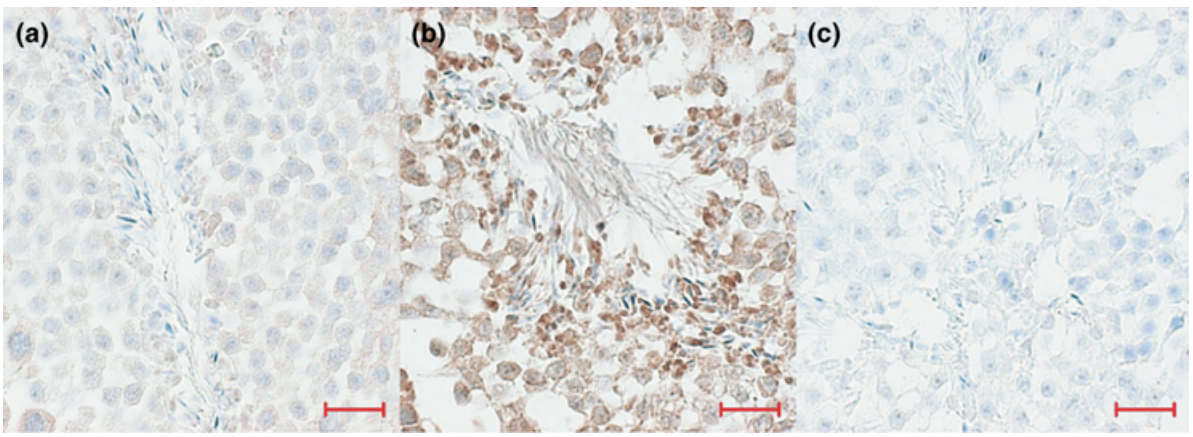

Fig. 5. Immunohistological analysis of seminiferous tubules sections: immunoperoxidase staining of p53. (a) p53RE-Luc mouse before doxorubicin treatment; (b) p53RE-Luc mouse after treatment and (c) control section with no primary antibody (magnification ×40). Bar: 100 micrometers.

obtained on seven males and three female transgenic mice. Qualitatively, the results were identical in all animals.

Anatomical and histological localization of doxorubicin-induced p53 activation. To identify the organs responsible for the doxorubicin-induced increase in luciferase activity, p53RE-Luc mice were culled and quickly dissected 10 min after receiving an intraperitoneal injection of D-luciferin. Various organs were positioned in the field of the camera and images were taken. The quantitative analysis of the luciferase activity associated with these organs clearly established that the bioluminescence was mainly generated from the testes, suggesting that doxorubicin induces p53 activation in this organ. To a lower extent, luminescence was also generated from the liver and the colon. Histological analysis of testis sections revealed that doxorubicin treatment lead to a dramatic disorganization in the structure of the seminiferous tube (Fig. 4a,b). Immunohistofluorescence for luciferase demonstrated that the cell population of the seminiferous tubules was luciferase-positive (Fig. 4c–f). Cells with the highest activity were the spermatids and the spermatogonia. Immunohistochemistry confirmed that p53 was expressed at a high level in these tubular cells following doxorubicin treatment (Fig. 5).

Discussion

The lac-Z gene controlled by p53 responsive promoters has been used in transgenic mice to detect p53 activation *in vivo*.[14,22]

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However, transgene expression could only be detected using invasive methods, limiting the potential of these models. In addition, induction of endogenous β-galactosidase activity has been reported to complicate the analysis.[23] More recently, rodents bearing tumor xenografts encoding a p53-responsive promoter driving the expression of a relevant reporter gene were used to demonstrate the potential of molecular imaging technologies in the detection of p53 transcriptional activation *in vivo*.[20,24] A luciferase-based transgenic animal is an attractive strategy as it allows the whole body monitoring of gene expression in a non-invasive way. As we were developing the present p53RE-Luciferase mouse model, Hamstra *et al*. reported a transgenic line in which the MDM2 P2 minimal promoter was driving the expression of the reporter gene.[25] They showed that such a model could be used to monitor p53 activation following ionizing radiation. The transgenic model described in the present study is based on a promoter derived from the PUMA (p53 up-regulated modulator of apoptosis) gene. PUMA protein is involved in p53-dependant apoptosis and has been shown to be tightly regulated by p53.[17] In addition, Yu *et al*. showed that PUMA mRNA level was greatly increased *in vitro* following chemically induced DNA damage. p53 binding sites were extracted from the PUMA promoter and cloned in front of a minimal promoter driving the expression of the firefly luciferase gene.[17] We used this p53RE-Luc construct to generate HCT116 cell lines. Doxorubicin was able to induce luciferase expression in the $p53^{+/+}$ cells in a dose-dependant manner, but not in the $p53^{-/-}$ cells, thus confirming the p53 specificity of the reporter gene expression. HCT116-p53RE-Luc $p53^{-/-}$ were also infected with an adenovirus encoding the p53 protein to successfully induce luciferase expression. This was consistent with previous findings showing that endogenous expression of PUMA was induced following the infection of various $p53^{-/-}$ cell lines with Ad-p53.[17] *In vivo*, when these cells were implanted in BALB/c nu/nμ mice, a very weak signal was detected in $p53^{-/-}$ tumors. This signal was very significantly increased in $p53^{+/+}$ tumors. This increased luciferase activity is likely to be the result of p53 activation *in vivo* in response to the stressful conditions present in the tumor (e.g. hypoxia, infiltrating macrophages and other leukocytes, necrotic or apoptotic cells). A single, intraperitoneal injection of doxorubicin led the induction of luciferase expression in the wild-type tumors, with no significant effect in the $p53^{-/-}$ tumors, thus validating our approach *in vivo*.

In p53RE-Luc transgenic mice, BLI showed a very low background luciferase activity in the lower peritoneal cavity in males and no signal in females. Doxorubicin induced in a dramatic increase in luciferase activity in the lower peritoneal cavity in males only, suggesting p53 activation in the male reproductive tract. In females, we observed an increased in the signal in the oral cavity but quantitatively, this signal was not significantly affected by the treatment with the drug. This signal is weak and is likely to be due to a very weak 'leakiness' of the promoter driving the reporter gene in this anatomical site. This demonstrates that transgene expression is not limited to a single tissue and that it can be detected in both males and females. Dissection of the culled transgenic male mice and imaging of the tissues collected confirmed that the major source of luciferase bioluminescence was the testes. Immunohistology revealed that doxorubicin induced severe damages to the cells in the seminiferous tubules. Cells with the highest luciferase activity were the spermatids, secondary spermatocytes and spermatogonia. Immunohistochemistry confirmed a marked p53 expression in these cells. Moderate doses of doxorubicin have been reported to exert long-term testicular toxicity in rodents,[26] and to activate p53 and induce apoptosis in male germ line cells.[27] Altogether, these observations demonstrate that the luciferase activity detected *in vivo* is correlated to p53 activation. This toxicity in rodents is paralleled by a largely reversible sterility observed in male patients treated with doxorubicin.[28]

The current study is the first to demonstrate that chemically induced p53 activation can be visualized non-invasively *in vivo*. Therefore, we advocate that the p53RE-Luc mice could be a powerful tool to predict, map and characterize the toxicity of compounds in humans, and to help in the design of new therapeutic agents.

Acknowledgments

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