p53-independent DNA repair and cell cycle arrest in embryonic stem cells

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Received 21 February 1998

Abstract The role of p53 in DNA repair and cell cycle checkpoint after ultraviolet irradiation was investigated in an embryonic stem cell line homozygous for a targeted deletion of p53. Results indicate that loss of p53 does not alter the capacity of ES cells to respond to DNA damage. Wild-type and p53-deficient cells showed similar cessation of DNA synthesis after UV damage and similar ultimate capacity to repair a transiently transfected reporter plasmid. Interestingly, in the absence of DNA damaging treatment, the transit of p53-deficient cells through S phase was slower than wild-type cells. We suggest that this may result from the absence of a p53-dependent response to endogenous DNA damage: without p53 sensing endogenous damage leading to immediate repair, such damage may persist and thus delay DNA synthesis.

Key words: Embryonic stem cell; p53; Cell cycle; DNA repair; UV; DNA damage

1. Introduction

The cellular response to DNA damage includes activation of DNA repair, cell cycle arrest and, in some situations, death by apoptosis [1–3]. DNA damage can take many different forms and rather little is known of the factors that permit its recognition and coupling to these responses. There is however increasing evidence for involvement of a small set of common regulatory molecules that include the onco-suppressor protein p53. The precise responses regulated through p53 in cells sustaining DNA injury depend on the damage stimulus [4,5], the cell type [6], and the differentiation state but are frequently associated with cell cycle checkpoints or initiation of apoptosis. Whilst the consequences of p53 deficiency or dysfunction for growth arrest and apoptosis in different tissues are well described [5], the effects of p53 on DNA repair process are less certain. Early observations led to the hypothesis that growth arrest provided time for completion of repair prior to replication. More recent in vitro data have suggested that p53 can directly regulate proteins involved in nucleotide excision repair [7–9]. At present there remain conflicting data from different systems about the role of p53 deficiency in DNA repair [10–15].

The present study aimed to better define the consequences of p53 deficiency for DNA repair by investigating the relationship between p53 genotype, cell cycle activity and DNA repair in mouse embryonic stem (ES) cells stimulated by genotoxic injury (UV irradiation). Such cells are continuously proliferating but non-transformed and remain capable of contributing to embryogenesis. They thus represent a system in which maintenance of genetic integrity and hence efficient repair should be critical, and a sensitive model to evaluate a role for p53 in DNA repair. Moreover, as totipotential cells they are free from modifying influences imposed by differentiation and thus provide a general paradigm for p53 and repair in proliferating cells.

2. Materials and methods

2.1. Cell cycle analysis by flow cytometry

All analyses were performed on exponentially growing ES cells (passages 23 to 27 and 18 to 21 for wild-type and p53-null cells respectively). For single parameter flow cytometry analysis, cultures were trypsinised, pelleted and nuclei prepared and stained by propidium iodide as described by Vindelov [16]. The relative proportion of cells in different phases of the cell cycle (average ± S.E.M.) were determined from eight independent experiments.

For dual parameter flow cytometry cells were cultured with BrdU 10 μM for 20 min and then, depending on experimental protocol, either harvested immediately or washed 3 times and cultured for a further chase period in BrdU-free fresh medium. After trypsinisation, cells were fixed in ethanol and stored at −20°C until use. Nuclei were then prepared by pepsin digestion (20 min at 0.2 mg/ml in 2 M HCl) and incorporated BrdU was labelled by a standard indirect immunofluorescence technique. The primary antibody was monoclonal rat anti-bromodeoxyuridine (Sera Labs) and the secondary antibody FITC-conjugated Rabbit anti-Rat (Serotec) (both at 1/100 dilution in PBS containing 0.5% Tween 20 and 5% normal serum). After two final washes in PBS, nuclei were resuspended in 10 mg/ml of ice cold propidium iodide solution containing 0.04% RNAse and analysed for integral red and green fluorescence on a Coulter EPICS flow cytometer.

2.2. p53 reporter plasmid

Cells cultured for 24 h in 24 well plates were transfected independently with three different plasmids: pRGCΔFosLacZ is a p53 reporter plasmid containing two copies of the RCG p53-specific binding site upstream of a non-functional fos promoter and a lacZ gene; the negative control plasmid pΔFosLacZ is identical except lacking the RGC p53 binding site [17]; the positive plasmid pCMVβ (Clontech) carries the LacZ gene under the control of a constitutive promoter.

Transfections were performed using Lipofectin (Gibco). Briefly, plasmid DNA (1 μg) and Lipofectin (4 μg), each diluted in serum-free medium (200 and 100 μl respectively), were incubated for 30 min at room temperature. Plasmid and Lipofectin were then mixed and further incubated for 45 min before laying the complex over the cells. After 6 h of incubation at 37°C in a CO_2 incubator, the DNA-containing medium was replaced by fresh medium containing 10% serum. Forty-eight hours after transfection, cells were UV-C irradiated (10 J/m^2), cultured for further 3 to 24 h and then lysed in 100 μl of Reporter Lysis Buffer (Promega). β-galactosidase activity was determined using ONPG substrate (Promega β-galactosidase enzyme assay), and expressed relative to the amount of protein (Biorad protein assay) recovered from each well.

2.3. Reactivation of a UV-C irradiated reporter plasmid

The pOP13 CAT reporter plasmid (Stratagene) was treated with UV-C at various doses and then cotransfected with unirradiated pCMVβ (1:1 ratio) into embryonic stem cell cultures (using Lipofectin as described above). The cells were lysed in Reporter Lysis Buffer (Promega) either 24 or 48 h after transfection.

The CAT activity in transfected cells was measured with Quan-T-

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PII S0014-5793(98)00296-8

CAT assay system (Amersham) used according to the manufacturer's instructions. This assay is based upon acetylation of biotylated chloramphenicol with radiolabelled acetylCoA. This binds to streptavidin coated polystyrene beads that are pelleted, washed and resuspended in scintillation liquid.

The results for each lysate were corrected for the transfection efficiency, as given by the β-galactosidase activity resulting from the undamaged pCMVβ expression (Promega assay) per μg of protein (Biorad protein assay). Reactivation of CAT activity from the irradiated plasmid was calculated relative to the activity for an undamaged plasmid.

3. Results

3.1. UV irradiation induces accumulation and increased transcriptional activity of p53

The kinetics of p53 protein accumulation in wild-type ES cells following UV-C irradiation were studied by immunocytochemistry using the monoclonal antibody pAb 421 (Oncogene Science; 1/1000 dilution). Non-irradiated cultures showed a low prevalence of weak nuclear p53 immunopositivity (less than 5% of cells). However, within 1 h after UV treatment (10 J/m^2, 254 nm) over 80% of cells had become strongly immunopositive for p53 and the intensity of positive staining continued to increase for 6 h. This response was sustained until at least 10 h after irradiation (data not shown). Changes in the transcriptional activity of p53 were also studied, using a p53-specific reporter plasmid (pRGCΔ-FosLacZ) that was introduced into wild-type ES cells by lipofection. UV-C irradiation of transfected cultures was followed by an increase in reporter β-galactosidase activity (1.8-fold) that peaked 9–12 h post treatment (Fig. 1A), whilst control plasmid (pΔFosLacZ) transfectants showed no significant activity throughout (Fig. 1B and data not shown). Interestingly, βGal activity of unirradiated cells transfected with the p53-specific reporter plasmid was still approximately 3-fold greater than cells transfected with the control p53 unresponsive plasmid (0.39 mU/μg of protein versus 0.14), indicating a significant baseline p53 transactivation activity in these cells. This is in keeping with the significant level of p53 immunopositivity observed in the non-transfected cultures and in agreement with the work from Sabapathy et al. [18] reporting that proliferating undifferentiated ES cells express a high level of transcriptionally active p53. Taken together these results suggest physiological p53 activity in these actively dividing cultures.

We considered the possibility that sensitivity of the reporter plasmid assay might be reduced by general suppression of transcription following UV treatment [19]. Therefore, parallel cultures were transfected with plasmid constitutively expressing LacZ (pCMVβ). A 25% decrease in constitutive βGal activity was observed after UV treatment at the timepoint when pRGCΔFosLacZ activity was at peak (Fig. 1B), making even more significant the observed increase in βGal activity in pRGCΔFosLacZ transfectants.

3.2. Repair of a UV-damaged plasmid is faster in p53-deficient cells

DNA repair efficiency in p53-null and wild-type ES cells was evaluated using a host cell reactivation assay. A CAT reporter plasmid, damaged in vitro, and a control LacZ-expressing plasmid, were transiently cotransfected in ES cells. The CAT activity, 24 and 48 h after transfection reflects the DNA repair ability of the cells. Comparisons were made with a control, undamaged CAT reporter plasmid and appropriate corrections were made to take account of the transfection efficiency using the cotransfected undamaged LacZ plasmid. In addition to functional assessment of effective repair, these

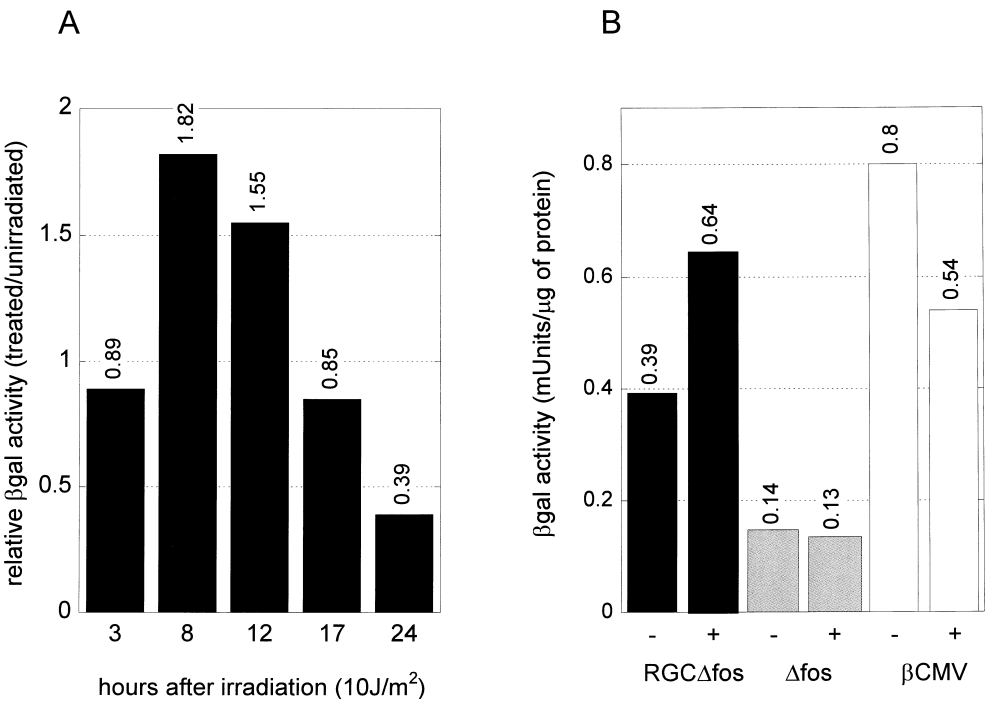

Fig. 1. p53 transactivation function after UV treatment (10 J/m^2) as determined with a reporter plasmid. A: Time course after 10 J/m^2 UV-C. β-galactosidase activity is expressed relative to untreated transfected cells. B: β-galactosidase activity 9 h after 10 J/m^2 UV-C (indicated by +) and without UV (indicated by −), for the p53 reporter plasmid (RGCΔFosLacZ), the negative control plasmid (ΔFosLacZ) and the constitutive pCMVβ plasmid. Note that A and B are results of independent experiments.

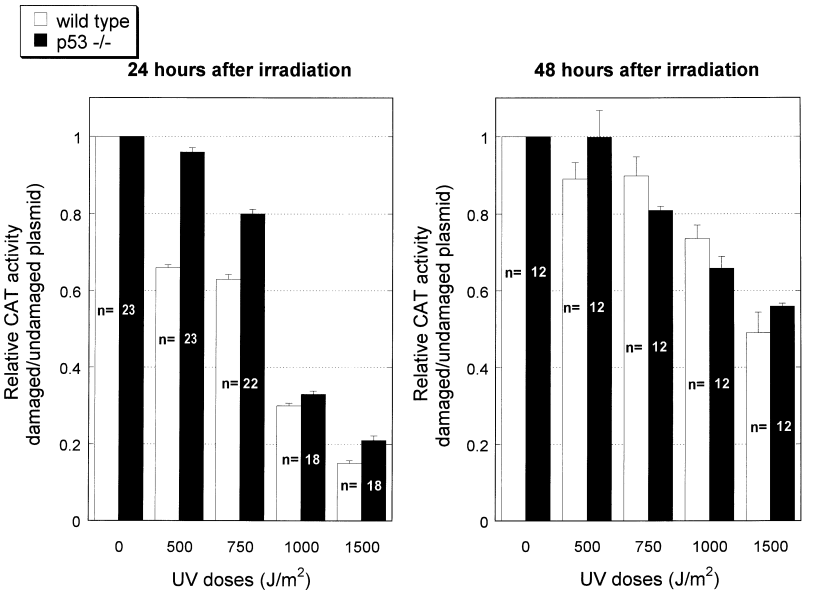

Fig. 2. Reactivation of a CAT reporter plasmid. The CAT activities 48 h after transfection, corrected according to the transfection efficiency, is given relative to the activity of an untreated plasmid transfected under the same conditions. The figure shows the average CAT activity from *n* independent transfections ± S.E.M. from four independent experiments.

experiments avoid the effects of whole cell UV irradiation, and a more physiological assessment of cellular repair capacity can be made.

As expected, there was a UV dose-related impairment of reporter function (Fig. 2). Twenty-four hours after transfection, p53-deficient cultures showed a significantly greater recovery of CAT reporter activity from plasmids damaged with the lower doses of UV, relative to controls, than did wild-type transfectants (Mann Whitney $P = 0.0001$ for 500 J/m^2, $P = 0.0012$ for 750 J/m^2). Moreover, for these lower doses of UV, there was no significant improvement in plasmid CAT activity between 24 and 48 h, indicating maximal repair by 24 h. By contrast, wild-type transfectants at 24 h had recovered only about 70% of the activity at 48 h. Taken together these findings indicate that p53-deficient cells repair weakly damaged plasmid more rapidly than wild-type, and this repair is completed as far as possible within 24 h, although the ultimate capacity to repair damage is similar between genotypes.

3.3. Cell cycle analysis of wild-type and p53-null ES cells

3.3.1. Wild-type and p53-deficient cells have different cell cycle distributions. DNA content and cell cycle analyses were performed by flow cytometry on exponentially growing wild-type and p53-null ES cells at various passage numbers. Despite similar doubling times for cultures of the two genotypes (19 and 21 h for p53-null cells and wild-type, respectively), cell cycle analysis on propidium iodide-stained cells showed a smaller proportion of p53-null cells in G_0/G_1 phase compared with wild-type cells (22.8 ± 1.7 and 35.5 ± 0.5% respectively). Proportions of both S and G_2/M phases in p53-null cells were increased commensurately (50.3 ± 4.3 versus 43.6 ± 2.9 for S phase and 26.9 ± 2.9 versus 20.9 ± 3.3 for G_2).

To better characterise these differences BrdU pulse-chase experiments were performed: unsynchronised cultures were pulse-labelled with BrdU for 20 min and, after removal of BrdU, were allowed to continue cycling for a chase period of up to 8 h before harvesting for analysis by dual parameter flow cytometry (Fig. 3). One major finding emerged: p53-de-

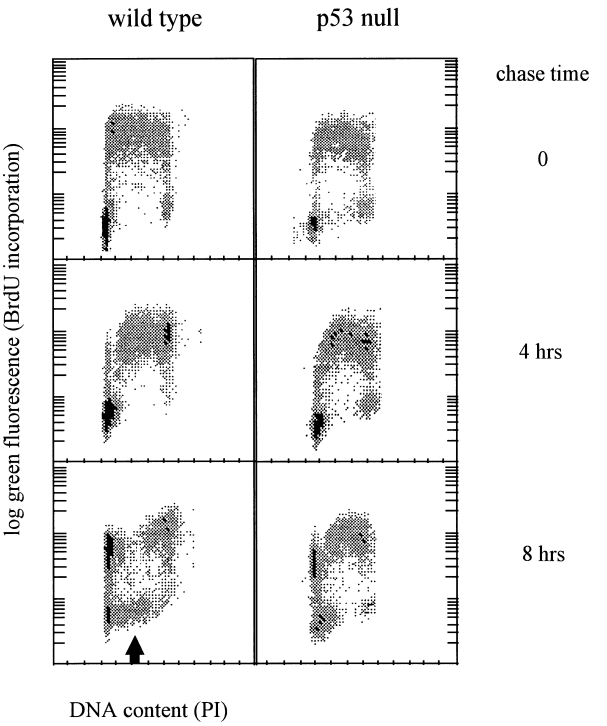

Fig. 3. Cell cycle progression in wild-type and p53-null ES cells by dual parameter flow cytometry. Unlabelled and BrdU pulse-labelled cell populations were followed for up to 8 h after a 20 min BrdU pulse exposure. The figure shows progression of the BrdU pulse-labelled cohort through S phase, increasing their DNA content with time, and the entry of unlabelled cells into S phase during this chase period (arrow). Note that progression of the labelled cohort through S phase (from 2*n* to 4*n*) is more advanced for wild-type cells compared with p53-null cells at the same timepoint, representing a faster rate of replicative DNA synthesis.

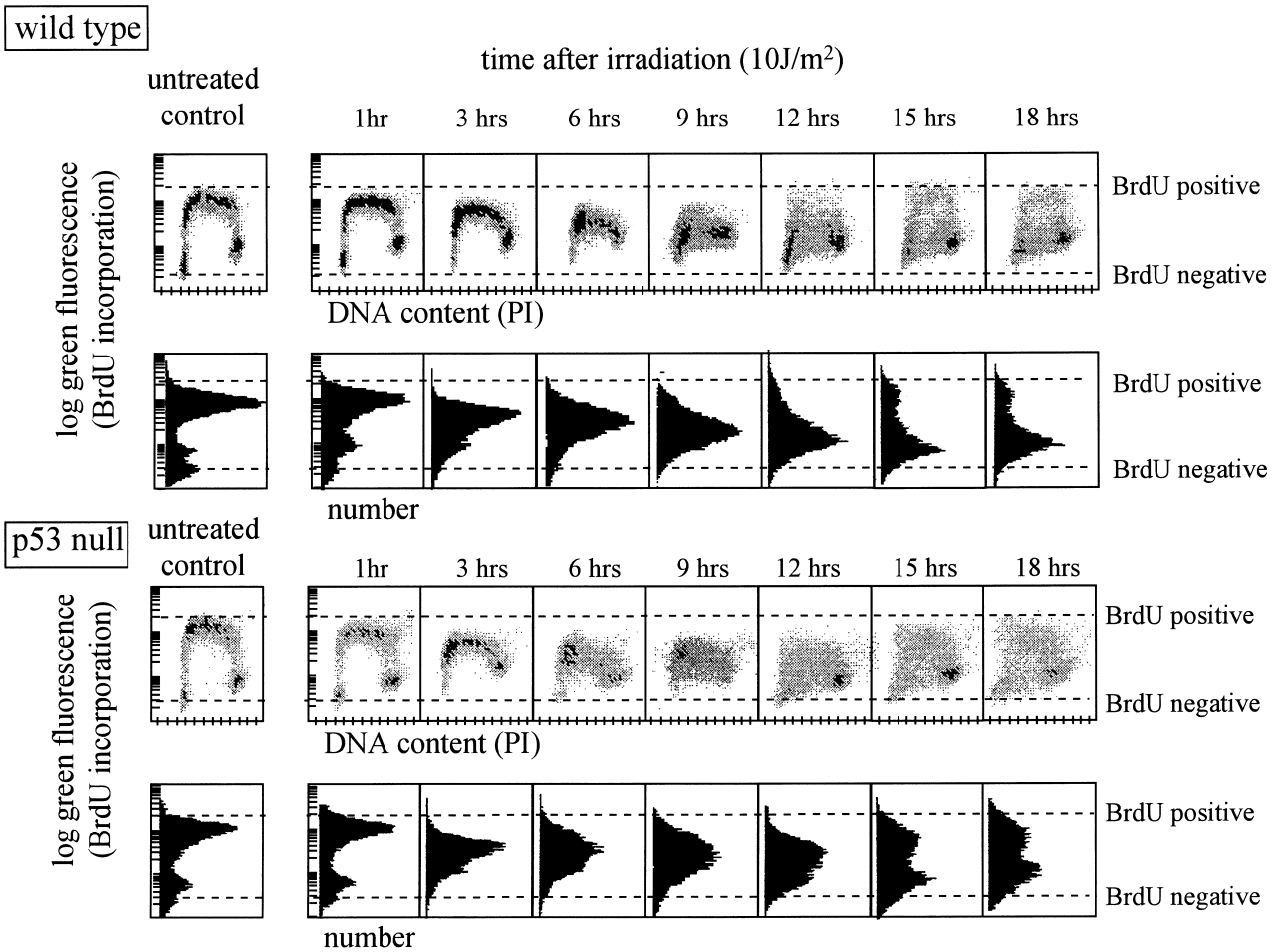

Fig. 4. Effect of UV irradiation on replicative DNA synthesis in wild-type and p53-deficient ES cells. Cells irradiated or not (controls) with UV-C (10 J/m^2) were then incubated for 20 min with BrdU at various timepoints, then harvested immediately and analysed by dual parameter flow cytometry. BrdU uptake is depicted as two-parameter (upper) and single parameter (lower) histograms for both genotypes.

ficient cells showed slower transit from 2*n* to 4*n* DNA content, indicating that S phase is longer than in wild-type (Fig. 3).

3.4. ES cells show p53-independent S phase arrest after DNA damage

Following the demonstration of differences in cell cycle characteristics between p53 genotypes it was of interest to study the effects of DNA damage (UV irradiation) on the cell cycle of wild-type and p53-deficient ES cells.

First, the effect of UV on replicative DNA synthesis was assessed by BrdU pulse labelling of cultures at various times after UV irradiation (10 J/m^2), and harvesting of cells at the end of the 20 min pulse (Fig. 4). By 1 h after irradiation, BrdU incorporation was reduced (decreased amplitude of log green fluorescence of S phase cells) and by 6 h was nearly completely abolished in both wild-type and p53-deficient cells already in S phase, indicating cessation of replicative DNA synthesis. By 9–12 h, the BrdU uptake began to recover towards a normal level in both genotypes. These results therefore demonstrate a delayed, transient arrest in S phase after UV irradiation that is independent of p53, and sustained for 3–6 h.

These experiments show a reduction of DNA synthesis after UV irradiation, but do not inform about checkpoints in G_1 or G_2/M. Therefore, a series of pulse-chase experiments were performed on UV irradiated cells. When compared with unirradiated controls, UV-treated cultures of both genotypes showed delay in the increase in DNA content of a BrdU pulse-labelled population, labelled immediately after UV treatment and followed for various chase intervals (Fig. 5). This is consistent with the transient S phase arrest after UV described above. Moreover, when DNA replication recommenced (9 h post irradiation), positive cells were also observed with 2*n* DNA content (G_1 phase), showing that at this time, cells had traversed G_2 and undergone mitosis. Furthermore, the entry from G_1 into S phase of unlabelled (9 h) and subsequently the BrdU-positive cells (12 h) showed also that there was no significant G_1 arrest at that time (Fig. 5).

4. Discussion

p53 function has been investigated in numerous cell systems, but comparison of results is confounded by variability of cell type, differentiation and transformation status (reviewed in [5]). Embryonic stem cells are pluripotent, permanent cells, capable of contributing to normal embryogenesis, and were therefore thought to be a good model to study the consequences of p53 deficiency for cell cycle and DNA repair, independently of ill-defined transformation or differentiation parameters.

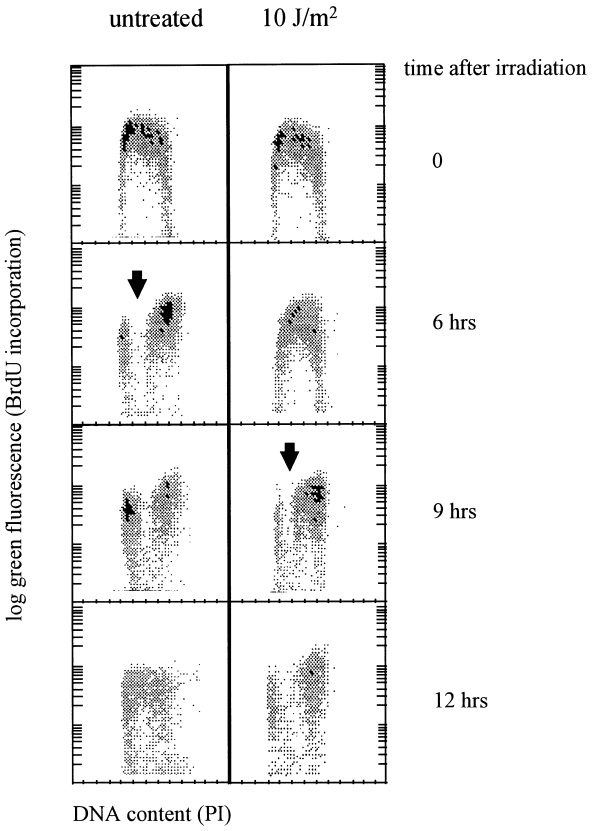

Fig. 5. Dual parameter flow cytometric cell cycle analysis in wild-type ES cells after UV irradiation. Unsynchronised wild-type cultures were pulse exposed to BrdU for 20 min just after UV irradiation, to label cells in S phase. BrdU was removed and cultures were harvested after various chase intervals, of which four are depicted. Unirradiated controls show the BrdU-positive cohort increasing its DNA content with time, and the appearance of BrdU-positive cells in G_1 ($2n$ DNA content). By contrast UV-treated cultures show similar but delayed changes (compare arrows), indicating a delayed transient S phase arrest, terminating by 9 h post treatment.

Surprisingly, UV treatment sufficient to stabilise and activate p53 did not produce any difference between genotypes in either cell cycle or repair responses. The only detectable effect on cell cycle in UV-treated ES cells was a p53-independent inhibition of DNA replication. The failure to detect a p53-dependent G_1 cycle arrest after DNA damage in these cells is perhaps not surprising since ES cells were found to have a low proportion of cells in G_1. p53 acts in G_1 by blocking the entry to S phase [20], but once the cells are in S phase p53 has no more effect on their cell cycle regulation [20]. The high proportion of ES cells in S phase and their short G_1 phase [21] therefore render them more likely to be hit in S phase. Moreover, it was recently reported that ES cells that express high level of functionally active p53 retain the capacity to proliferate rapidly and have a very short G_1 phase [18]. The authors suggested that the cell cycle regulation in embryonic stem cells could have evolved to tolerate high level of p53 expression [18].

The observation that cessation of replicative DNA synthesis after UV is p53-independent is in agreement with previous findings in bleomycin-treated fibroblasts [22]. The arrest is only apparent from 3 h after UV irradiation, suggesting that inhibition of DNA synthesis is not simply due to mechanical blockade of the replication fork by UV-induced pyrimidine dimers, but more probably from a second event that could be the appearance of DNA breaks during nucleotide excision repair. This inhibition of DNA synthesis (reviewed in [3]) has a clear checkpoint quality since it occurs at low doses, is maximal during the time of active DNA repair and resolves as repair subsides leading to the recovery of the normal level of DNA synthesis. There is common perception that the S phase checkpoint is equivalent to the G_1 checkpoint because both responses delay or inhibit the initiation of DNA synthesis. However, the inhibition of replicon initiation occurs in S phase and must utilise biochemical signals that are different from those that cause G_1 arrest. Results from Orren et al. [23] demonstrated that persistent UV-produced DNA damage, and not activation of signal transduction pathways resulting from general cell stress, is the cause of prolonged delays in the S phase progression. They also demonstrated that the DNA repair ability of the cells is directly related to the alteration of the S phase progression and extended cell cycle arrest. In agreement with this finding, we found that wild-type and p53-null ES cells exhibited both similar delays in S phase progression and ultimate DNA repair capacity. As expected by these results, mutation frequency after UV irradiation was increased to a similar level in wild-type and p53-null ES cells (Corbet et al., personal communication). To that extent therefore, repair in DNA-damaged ES cells can be considered p53-independent, in accord with results of Ishizaki [14] for photoproduct removal in UV irradiated wild-type and p53-null mouse embryo fibroblasts. In contrast to our finding, others have suggested that p53 does regulate NER of UV-induced DNA damage [10–13,24]. However, those studies differed from the present in design, utilising mutant p53 [10,11,13] or E6 overexpression [12], which in themselves present confounding factors to comparison with the present results and those of Ishizaki [14] for p53 deficiency: for example, possible gain of function of mutant p53 [25–27], interaction of the mutated p53 with the wild-type allele [11] and interaction of E6 with other proteins [28,29].

The findings that untreated p53-null ES cells exhibit a slower S phase than wild-type and that the repair of the damaged plasmid is faster for the lower doses of damage is rather interesting. It is possible that this reduced DNA synthesis reflects a response to endogenous DNA damages. DNA strand breaks occur during normal replication and may be induced by spontaneously produced free radicals. If p53 is normally involved in sensing [30–34] and signalling these types of injury then the absence of p53 might lead to persistence of endogenous DNA damage with compensatory S phase delay. The increased basal level of DNA repair could be an artifact reflecting the differences in the proportion of cells in G_1 and S phase of the cell cycle. This would explain the faster recovery of CAT activity when the reporter plasmid carries only a low level of damage but an ultimate DNA repair capacity similar to that of wild-type ES cells. Slower replicative DNA synthesis has not previously been reported as a feature of p53-deficient cells, however Ishizaki et al. described an increased S phase fraction in p53-deficient cells [14], which although ascribed to the loss of a p53-dependent G_1 checkpoint, could have been due to a prolonged S phase as observed here. In their study, the cells themselves were UV irradiated preventing the observation of a possible increase in basal DNA repair.

In summary, in normal undamaged proliferating ES cells, p53 is expressed and transcriptionally active (this study, [18]).

After DNA damage, p53 has no effect on the regulation of cell cycle checkpoints and no effect on the ultimate DNA repair capacity of ES cells. However, after high levels of DNA damage, these cells do undergo p53-dependent apoptosis (data not shown and Corbet et al., personal communication). More interestingly, the present work demonstrates that p53 may have a role in sensing basal levels of endogenous DNA damage, and that in the absence of p53, a compensatory increase in DNA repair can occur. There is an important prediction arising from this model: p53-deficient cells will repair DNA damage without acquiring potentially damaging mutations. This is consistent with results showing no effect of p53 deficiency on the incidence of point mutations [27,35–37]. A further prediction of the model is that the compensatory mechanisms might be effective on most occasions, but mutations occurring are likely to be qualitatively different to those of wild-type cells. Intriguingly, Ishizaki et al. have recently shown that p53-deficient cells do indeed accumulate different types of mutation to wild-type [35]. Taken together, these data indicate that p53 is important to provide the most efficient balance between DNA repair and replication under normal conditions. They are additional evidence for an active coupling between these fundamental cellular processes.

Acknowledgements: This work was supported by a European Communities Fellowship (Human Capital and Mobility ERB4001GT940112 and ERB4001GT954810). C.O.C.B. was a Cancer Research Campaign Gordon Hamilton Fairley Clinical Research Fellow. S.P. is recipient of a fellowship from the Scottish Hospital Endowments Research Trust.

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