

## Human cells deficient in p53 regulated p21<sup>waf1/cip1</sup> expression exhibit normal nucleotide excision repair of UV-induced DNA damage

Manzoor A.Wani<sup>1</sup>, Gulzar Wani<sup>1</sup>, Jihonag Yao<sup>1</sup>, Qianzheng Zhu<sup>1</sup> and Altaf A.Wani<sup>1,2,3,4</sup>

<sup>1</sup>Department of Radiology, <sup>2</sup>Department of Molecular and Cellular Biochemistry, and <sup>3</sup>James Cancer Hospital and Solove Research Institute, the Ohio State University, Columbus, OH 43210, USA

<sup>4</sup>To whom correspondence should be addressed at: Molecular Carcinogenesis Laboratory, 103 Wiseman Hall, 400 W. 12th Avenue, Columbus, OH 43210, USA  
Email:wani.2@osu.edu

Cancer development requires the accumulation of numerous genetic changes, which are believed to initiate through the presence of unrepaired lesions in the genome. In the absence of proficient repair, genotoxic agents can lead to crucial mutations of vital cellular genes via replication of damaged DNA. Many cell cycle regulatory proteins are known to modulate the repair capacity and consequently the fate of cells. We and others have recently shown that p53 tumor suppressor gene product is required for efficient global genomic repair (GGR) but not the transcription coupled repair (TCR) of the nucleotide excision repair (NER) sub-pathways. In order to discern the nature of the p53 modulation to be direct or indirect through a downstream mediator, we have investigated the processing of UV radiation induced lesions in human colon carcinoma, HCT116 cells expressing wild-type p53 but having different p21<sup>waf1/cip1</sup> (hereafter p21) genotypes (p21 +/+, p21 +/-, p21 -/-). Following 20 J/m<sup>2</sup> UV, all the three cell lines showed rapid increase in p53 protein but the accompanying increase in the expression of its downstream target protein p21 could only be seen in p21 +/+ and p21 +/- cells and not in p21 -/- cells. Nevertheless, an absence of detectable p21 protein in deficient cells had no demonstrable effect on DNA repair response to UV irradiation, as measured by an immunoassay to detect removal of UV photoproducts from genomic DNA (GGR) and by individual strand specific removal of endonuclease-sensitive CPD from a target gene fragment (TCR). Introduction of cytomegalovirus (CMV)-driven luciferase reporter plasmid, UV damaged *in vitro*, into the unirradiated cells of varying p21 background, revealed a relatively small but statistically significant decrease in the reporter expression in the host p21 -/- as compared with p21 +/+ and p21 +/- HCT116 cells. Super-expression of p21 protein upon reintroduction of p21 expression construct, showed an enhanced recovery of UV damaged reporter activity that was not greatly different from a similar enhancement observed with undamaged plasmid reporter DNA. Taken together, the results indicate that (i) the p21 protein does not have a significant role in the repair of genomic DNA at chromosomal level; (ii) the well-

established p53 dependent modulation of NER is distinct and independent of its cell cycle checkpoint function; and (iii) the reproducible enhancing effect of p21 expression observed through host cell reactivation (HCR) of extra-chromosomal DNA is mainly attributable to an effect exerted on transcription rather than repair.

### Introduction

Mammalian cells possess a remarkable repertoire of responses to diverse stresses, particularly those inflicting structural damage to the genome. Upon exposure to DNA damaging agents, mammalian cells initiate a series of complex biochemical reactions designed to ensure the integrity of the genetic material. Mechanisms, which have evolved to detect structural alterations of the genome and induce cell cycle checkpoints, function in parallel with biochemical machinery that repairs the DNA damage. Much progress has already been made in identifying the molecular components and delineating the underlying mechanism of various DNA repair pathways (1). Furthermore, it has become clear that UV and other types of DNA damaging agents activate cell cycle checkpoints that help prevent cells from undergoing division while unrepaired lesions are present in their genome (2). Thus, cell cycle checkpoints presumably function to allow adequate time for the repair and to prevent deleterious consequences of incurred damage. Moreover, proteins that have a direct function in DNA repair pathways are also known to be involved in transcription and DNA replication processes (3,4). Despite unraveling many coincident properties and connection between these fundamental cellular processes (5–8), an in-depth understanding of the nature of their interaction, extent of coordination, and temporal sequence of events remains an active area of research in molecular carcinogenesis.

In mammalian cells, DNA damage increases the levels of the nuclear tumor suppressor protein, p53. This protein is functional both in cultured cells (9,10) and intact animal tissues (11,12). In response to genotoxic insult, p53 can transcriptionally activate a range of target genes that affect cell cycle arrest and aids in the enhancement of DNA repair (2,13,14) and apoptosis (12,15). p53 not only engages a plethora of downstream pathways but itself seems to possess a biochemical flexibility that allows it to act beyond a mere transcription factor. Thus, p53 has recently been shown to play a direct role in NER pathway. Removal of a variety of DNA damage, including UV-induced photolesions and bulky adducts induced by chemical agents, are influenced by the presence or absence of p53 in the cells (16–20). In mammalian cells, removal of most of these bulky DNA lesions by NER is non-random across the genome as well as occurs in a strand-specific manner (21). Loss or disruption of wild-type p53 function has clearly been established to correlate with decreased GGR but not the TCR in various mammalian, including human, cells (16). Decreased repair of bulk of the DNA in p53-

**Abbreviations:** CMV, cytomegalovirus; CPD, cyclobutane pyrimidine dimers; GGR, global genomic repair; HCR, host cell reactivation; NER, nucleotide excision repair; NTS, non-transcribed strand; PCNA, proliferating cell nuclear antigen; TCR, transcription-coupled repair, TS, transcribed strand.

deficient cells is also associated with a concomitant increase in the cellular sensitivity and apoptosis (22).

While the role of p53 pathways in apoptosis, cell cycle checkpoints and now global genomic repair is reasonably well established, little has been resolved about the role of its downstream effectors in the maintenance of genomic stability, particularly DNA repair. Following DNA damage wild-type p53 protein functions as an effective and relatively stable sequence-specific transcriptional activator for the synthesis of a number of proteins that act as cell cycle regulators. p21 (also known as Waf1, Cip1 or Sdi1), is among one of these gene targets that acts as a downstream effector molecule for p53 action (14,23). However, p53 is not the only transcriptional inducer of p21 as it can also be regulated independent of p53 (24). p21 acts by binding to cyclin-dependent kinases, inhibiting their activity, and has been shown to be involved predominantly in cell cycle arrest at the G1 phase, presumably to allow time for the repair of damaged DNA (25,26). Studies have shown that p21 can also inhibit proliferating cell nuclear antigen (PCNA) activity thereby blocking DNA replication (27). NER of DNA damage induced by UV and other genotoxic agents is also shown to require active PCNA (28). Based on the p53 and PCNA inter-connection, the possibility that p21 might also be somehow involved in the modulation of GGR and TCR has continued to be a subject of intense investigation. But, the published reports, using diverse approaches, have so far presented conflicting results regarding a definitive role of p21 in DNA repair. Earlier studies found that p21 was able to inhibit PCNA-mediated DNA replication with no discernible effect on NER (29,30). Other attempts reported an inhibitory effect of p21 on NER (31). A recent study, using a specialized approach of CPD detection at nucleotide resolution and concluding that ablation of p21 expression actually enhances the repair capacity of p53-deficient human tumor cells, tends to support the inhibitory effect of p21 protein (32). On the contrary, another recent report, based upon the quantitative assessment of CPD removal from the bulk DNA or individual target gene strands in multiple cellular systems, was unable to observe any meaningful differences to assign a role for p21 in NER (33,34).

In the present study, we have investigated the possible role of p21 in DNA repair by comparing the kinetics of repair of UV induced DNA damage in human colon carcinoma HCT116 cells expressing wild-type p53 but having a different status with regards to p21 expression. Our results demonstrate that, whereas, p21<sup>-/-</sup> cells undergo significant programmed cell death upon UV irradiation, the surviving fraction exhibited efficient repair of UV-induced DNA damage. Absence of p21 did not affect the removal of CPD from the TS or NTS of the p53 target gene sequence or the overall genome. Nevertheless, *in vivo* HCR of the *in vitro* UV-damaged CMV-driven luciferase reporter containing plasmid DNA revealed a measurable decrease in the reporter expression in p21<sup>-/-</sup> cells. Our previous findings have clearly shown that wild-type p53 is required for efficient NER repair. However, the combined results presented here show that p21 protein has little or no role in NER and the p53 based NER modulation is independent of its known regulation of p21 mediated checkpoint pathway.

## Materials and methods

### Cell cultures

Human colon adenocarcinoma HCT116 cells (p21<sup>+/+</sup>, p21<sup>+/-</sup> and p21<sup>-/-</sup>; kindly provided by Dr Bert Vogelstein, The John Hopkins Oncology Center,

Baltimore, MD) were generated by homologous recombination to create homozygous deletion of p21 (35). The cells were grown in a humidified atmosphere of 5% CO<sub>2</sub> in DMEM (GIBCO-BRL, Bethesda, MD) supplemented with 10% fetal calf serum (Atlanta Biological, Atlanta) and penicillin/streptomycin (GIBCO-BRL). For some experiments, to separate the newly synthesized from parental DNA, cells were pre-labeled with 25 nCi/ml [<sup>3</sup>H] thymidine (86 Ci/mmol) in the medium for 3–7 days. The medium was changed every 2–3 days.

### UV-irradiation and post-treatment incubation of cells

For the assessment of DNA damage and repair in the different cell lines, the confluent cells in 150 mm dishes were washed and replaced with a thin layer of prewarmed PBS. The monolayers were irradiated at 20 J/m<sup>2</sup> of 254 nm UV dose and either lysed immediately or after further incubation in the medium containing 10 μM bromo-deoxyuridine and 1 μM fluoro-deoxyuridine for different post-exposure times. The irradiation was carried from a germicidal lamp at a dose rate of 0.5 J/m<sup>2</sup> as measured by a Kettering model 65 radiometer (Yellow Springs Instruments).

### Western blot analysis

Exponentially growing cells were washed with PBS and exposed to 20 J/m<sup>2</sup> of UV dose and maintained in fresh medium for a 0 to 24 h period. At the indicated time periods, the cells were recovered by gentle trypsinization and immediately lysed by boiling for 10 min in sample buffer (2% SDS, 10% glycerol, 10 mM DTT in 62 mM Tris-HCl (pH 6.8), 10 μg/ml pepstatin, and 10 μg/ml leupeptin). Western blot analysis was performed as described earlier (36). Protein extracts from ~1.5 × 10<sup>5</sup> cells were separated in 8 or 12% SDS-polyacrylamide gels and transferred to PVDF membranes using a semidry electroblotter (Hoeffer, San Francisco, CA). Equal protein loading was confirmed as described earlier (20). For p53 protein detection, a mixture of anti-p53 antibodies (Ab-2 and Ab-6 from hybridoma clones 1801 and DO-1, Neomarkers, CA) was used at 1:200 dilution. Antibodies for the detection of p21 (clone DC60.2) were also used at a 1:200 dilution. Following incubation with the corresponding enzyme-conjugated secondary antibody (Boehringer Mannheim, Indianapolis, IN), the filter bound peroxidase enzymatic activity was detected using the enhanced chemiluminescence substrate reaction (Pierce, Rockford, IL) essentially according to the manufacturer's instructions using Kodak X-OMAT AR film. Rainbow protein size markers were run in parallel in all the experiments to localize the gel transfer regions for specific proteins and determine the transfer efficiency.

### Isolation, purification and separation of parental DNA

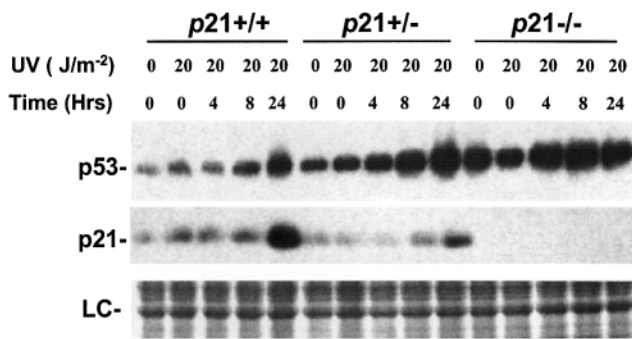
Cells were harvested at desired incubation periods by gentle trypsinization to maintain the high genomic integrity and immediately lysed for DNA isolation by a salt precipitation method (37) modified in our laboratory. Ethanol precipitated DNA was resuspended in 1 mM EDTA/10 mM Tris-HCl, pH 8.0 and extensively digested to completion with EcoRI (5 unit/μg of DNA) at 37°C for 6–8 h. Complete digestion was verified by electrophoresis of sample aliquots on agarose minigels. The digested DNA was purified by extraction with phenol/chloroform (3.5:6), precipitated with ethanol and quantitated. Unreplicated DNA was then separated and isolated using cesium chloride isopycnic density gradient centrifugation. Gradients were fractionated and radioactivity in samples was measured in a scintillation counter to determine the amounts and position of parental and hybrid density DNA. Fractions of parental DNA were pooled and dialyzed against TE. The dialyzed parental DNA was ethanol precipitated and quantitated by absorption measurement at 260 nm.

### Quantitation analysis of global genomic repair by ISB assay

The amounts of initial CPD formed and the damage remaining after genomic DNA repair from cells collected at various times following UV irradiation was determined using non-competitive immuno-slot assay, as previously described (38,39). Briefly, confluent cells were maintained overnight in serum free medium and exposed to 20 J/m<sup>2</sup> of UV dose from a germicidal UV lamp delivering at 254 nm. Cells were either immediately lysed for DNA isolation or incubated further in fresh medium for different repair times. For CPD estimation, several increasing concentrations of unirradiated, irradiated and repaired DNA samples were evaluated by standard immunoassay using dimer specific polyclonal antibody, as described earlier (40). The damage levels were calculated by comparing the band intensities of the samples with UV modified DNA standard samples run in parallel on each filter.

### Quantitation of transcription coupled repair by Southern blot analysis

TCR was analyzed by the modification of procedure originally developed by Hanawalt and coworkers (41). Equal amounts (20 μg), of each parental DNA sample isolated from cells incubated for varying post-irradiation periods, were either treated with T4 endonuclease-V or enzyme buffer alone and incubated at 37°C for 1 h. A linearized plasmid (5 pg) DNA containing the p53 cDNA sequence was added into each sample as an internal standard. After incubation



**Fig. 1.** Time dependent UV-induction response of p53 and p21 proteins in HCT 116 cells. Exponentially growing cells were irradiated with 20 J/m<sup>2</sup> UV-irradiation and maintained further in fresh medium. At the indicated times, cells were harvested and cellular extracts (equivalents of 1 × 10<sup>5</sup> cells/lane) were separated by SDS-PAGE, transferred onto PVDF membranes, processed with monoclonal antibody specific for p53 and p21 proteins and visualized by enhanced chemiluminescence as described in Materials and methods. Fast green staining of the bottom portion of the protein blots was used as loading control (LC). The representative results were independently repeated, at least, three times.

the DNA was purified by extraction with phenol/chloroform and precipitated with ethanol. The samples were then dissolved in denaturation buffer (20) and incubated at 37°C for 30–45 min to denature the DNA followed by electrophoresis in a neutral agarose gel (0.7%) for 18–20 h. The DNA in the gel was partially depurinated, neutralized and transferred to a nylon membrane (42). Membranes were hybridized in 10 ml of solution containing 50% (v/v) formamide, 6× SSC, 0.5% SDS, 5% dextran sulfate, denatured salmon sperm DNA (100 µg/ml), and 1–2 × 10<sup>8</sup> c.p.m. <sup>32</sup>P-labeled single stranded exon specific probes generated by asymmetric PCR (43). After 20–22 h hybridization time at 42°C, the membranes were washed to a final stringency at 62°C in 1× SSC/1% SDS and exposed to a phosphorimager screen. The ratio of full-length restriction fragment in the endonuclease-treated and untreated sample was determined by quantifying the individual band intensities upon imaging and processing by Imagequant software (Molecular Dynamics). The average number of UV-lesions per fragment were calculated by Poisson distribution as described earlier (44).

#### Cell transfection and reporter assay

Cells were transfected as described earlier (6). Briefly, for each transfection experiment, exponentially growing cells (3 × 10<sup>5</sup>) were plated in triplicate in 35 mm dishes 18–20 h prior to plasmid transfection. Cells were transfected with 1 µg/dish of 1000 J/m<sup>2</sup> UV-damaged or undamaged control pCMV-Tag DNA. Transfection was performed using FUGENE 6 transfection reagent (Promega) according to manufacturer's instructions. Following 5 h of transfection, the transfection mix was removed and cultures were supplied with fresh medium for another 8 or 24 h. At the indicated times cells were harvested and lysed in 100 µl luciferase cell culture lysis reagent (Promega). Luciferase activity from 20 µl of cell lysates was assayed in a standard luciferase assay system (Promega). Reference standards and negative controls were run in each experiment, and the luminescence was recorded with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). For co-transfection experiments, 1.0 µg of reporter plasmid was premixed with increasing amounts (0.25–1.0 µg) of p21 expression vector and transfected into p21<sup>-/-</sup> cells. Empty vector DNA was used to keep the total amount of DNA at a constant 2 µg for each transfection. After 8 and 24 h, cells were harvested, lysed and luciferase activity determined as described above.

## Results

### UV-induction response of p53 and p21 proteins in HCT 116 cells

The effects of UV-induced DNA damage on the p53 protein level and p53 regulated p21 gene product were examined in human adenocarcinoma HCT 116 cell lines and corresponding somatic cell knockouts of different p21 status (Figure 1). All three cell lines express wild-type p53 protein and upon DNA damage following 20 J/m<sup>2</sup> UV-irradiation exhibited a characteristic time dependent increase in their p53 protein levels. Previously, we have reported an induction in the levels

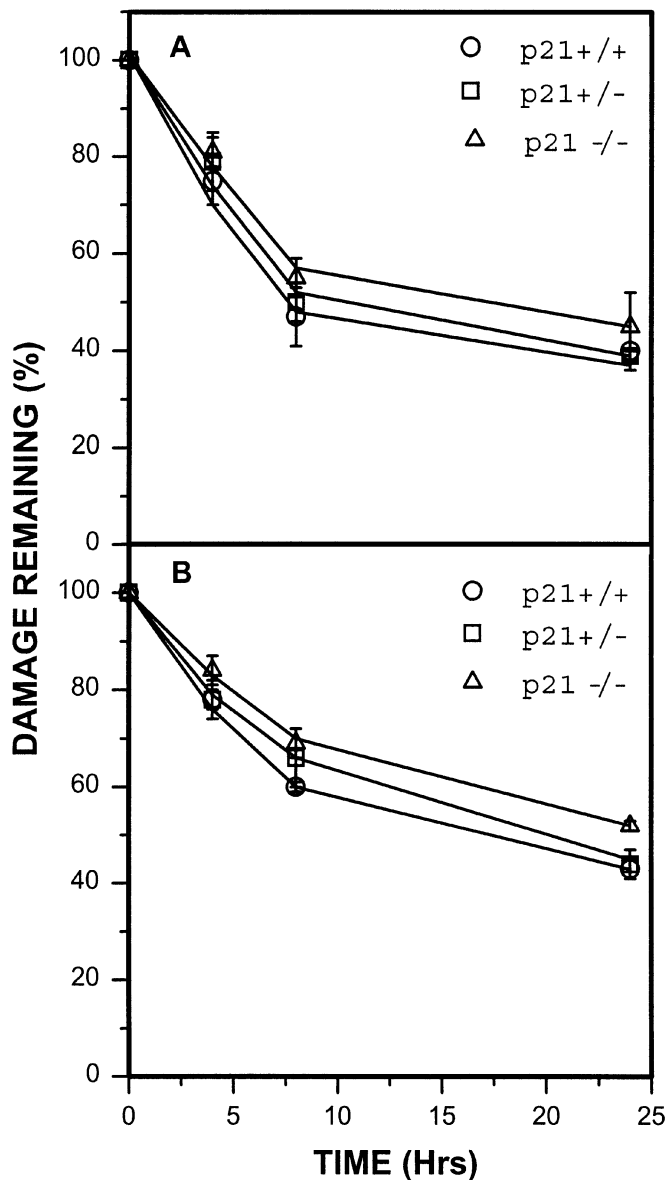
of p53 protein at 6–8 h following 20 J/m<sup>2</sup> UV-irradiation of human fibroblast cells and the induced levels were sustained up to 24 h (19). Current results are consistent with these findings as HCT116 cells also showed elevated levels of p53 protein with a several fold increase beginning 4–8 h and sustained higher levels up to 24 h following 20 J/m<sup>2</sup> UV-irradiation. In comparison to p21<sup>+/+</sup> cells, both the p21<sup>+/-</sup> and p21<sup>-/-</sup> cells showed relatively higher basal levels of p53 protein. Consistent with the wild-type p53 protein phenotype of these three cell lines, the p21 protein, a known transcriptional target of p53, responded to the induction of p53 by its elevated protein levels following UV-irradiation. However, no detectable levels of p21 protein were apparent in p21<sup>-/-</sup> cells, and a somewhat reduced amount of p21 protein was present in p21<sup>+/-</sup> cells compared with p21<sup>+/+</sup> cells.

### Repair of CPD in global genomic DNA following UV-irradiation

The extent of induction and removal of CPD following DNA damage by UV-irradiation was immunologically determined using a sensitive immuno-slot blot assay with polyclonal antibodies specific for UV-induced CPD (39,40). HCT116 cells having different p21 (+/+, +/-, and -/-) genotypes were exposed to 20 J/m<sup>2</sup> UV-irradiation and the levels of CPD measured immediately or at different repair times following UV-irradiation. The three cell lines exhibited an identical response with regards to the loss of antibody binding sites, a measure of CPD removal from the DNA. p21<sup>+/+</sup> and p21<sup>+/-</sup> cells demonstrated about 60% and 61% removal of CPD, respectively, from their genome within 24 h following UV-irradiation. Similarly, p21<sup>-/-</sup> cells were equally proficient and removed 55% of induced CPD within the same period. Neither the total extent nor the rate of elimination of CPD was significantly different between the cells of varying p21 status (Figure 2A). To ensure that the loss of CPD was actually due to repair and not due to dilution of damage through DNA replication in actively dividing HCT116 cells, the GGR was measured in parental DNA after its separation from the replicated DNA by isopycnic cesium chloride density gradient sedimentation. As shown in Figure 2B, the pattern of repair with the parental DNA, both in terms of the extent and the rate, was again identical in the three cell lines. The same magnitude of DNA repair observed with the total DNA or upon separation of parental DNA is consistent with the growth arrest of HCT116 cells upon exposure to UV radiation.

### Strand specific repair of CPD

Since p53 protein has been shown to participate in the GGR and no clearly demonstrable influence on the TCR, it is conceivable that this modulation is mediated through a downstream effector like p21. To determine such a differential strand-specific repair response the analysis of CPD was performed within the individual strands of target human p53 gene sequence of cells of varying p21 status. The rate of removal of CPD from the TS and NTS of the 16 kb EcoRI fragment of p53 gene was measured using T4-endonuclease site sensitivity of CPD and probing of individual DNA strands by Southern blotting. The reappearance of a full-length p53 target fragment was used as an index of disappearance of enzyme sensitive CPD due to *in vivo* cellular repair and the measurement of band intensities gave a direct comparison of the extent of damage processed in HCT116 cells under varying genetic backgrounds. Representative autoradiograms of these repair experiments are presented in Figure 3A. The three cell lines



**Fig. 2.** Global NER of CPD in HCT116 cells. (A) Confluent cultures of HCT116 cells were exposed to 20 J/m<sup>2</sup> UV-irradiation and further incubated in fresh medium for indicated time periods before harvesting for DNA isolation. Immunoblot analysis of constant amounts of isolated DNA samples was performed and the DNA repair determined as the loss of antibody binding sites from DNA obtained from indicated cell types at different time periods following UV-irradiation. The extent of DNA repair is expressed as the percent of initial damage remaining at the indicated times. (B) DNA repair determined by immunoblot analysis with the unreplicated (parental) DNA after separation of hybrid (daughter) DNA by CsCl density gradient sedimentation as described in Materials and methods.

did not exhibit significant differences in the removal of CPD from either strand of the *p53* gene. For example, 45% T4 endonuclease sensitive sites were removed from the TS of p21+/+ cells compared with 32% and 40% for p21+/- and p21-/- cells, respectively, at 4 h after irradiation. The repair was almost identical at 8 h and completed at 24 h in all three cell lines (Figure 3B). A similar pattern of repair was observed within the NTS of the three cell lines, although the overall repair was slightly lower compared with that of TS.

#### Host cell reactivation of exogenously damaged DNA

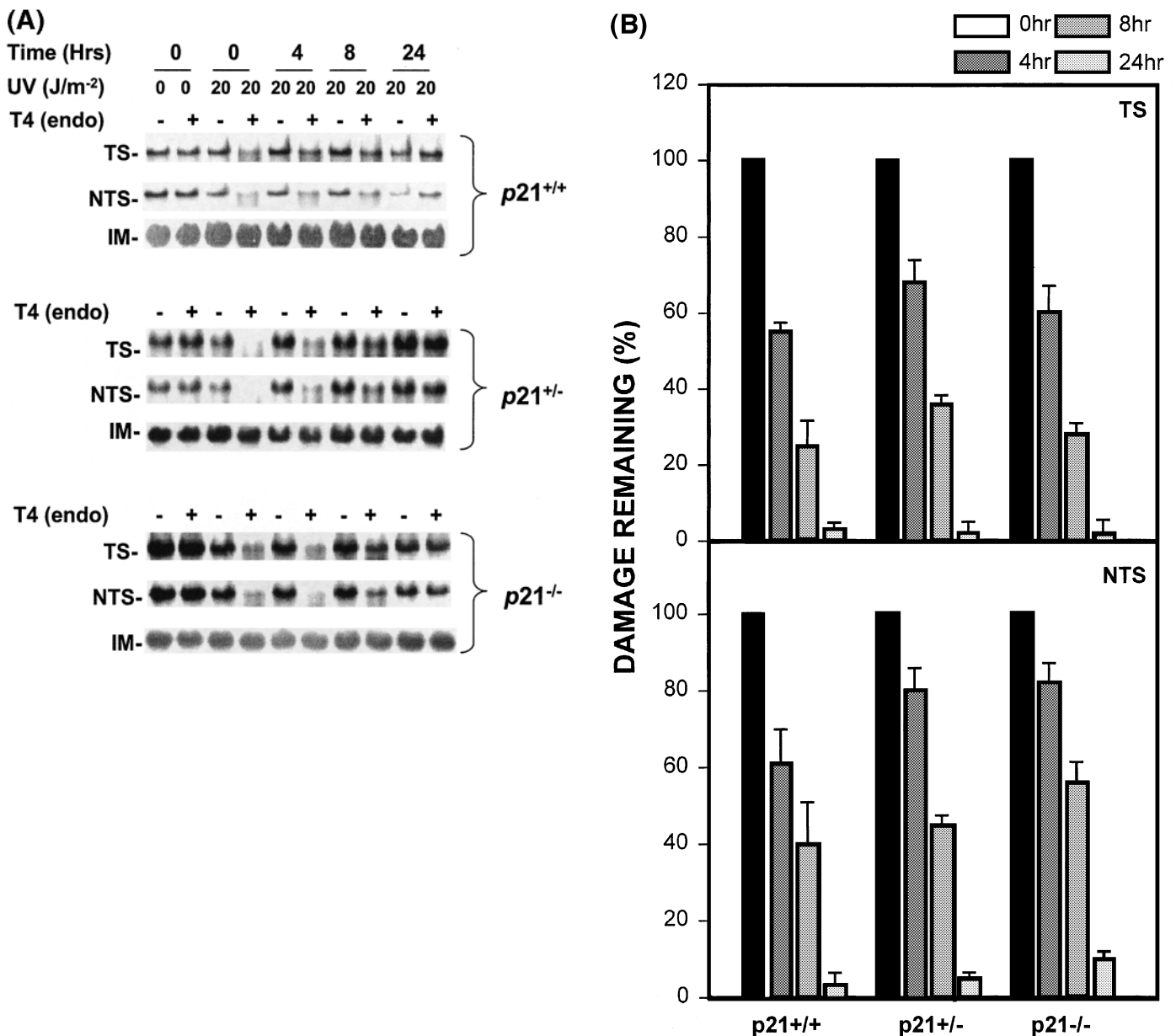
To further investigate the relationship of p21 function in DNA repair processes involving UV type DNA damage, HCR

experiments were carried out in HCT116 cells expressing wild-type p53 but having different p21 (+/+, +/-, and -/-) genotypes. The HCR assay utilized in this study consisted of a UV damaged or undamaged reporter plasmid encoding the CMV driven luciferase gene. The assay is based on the principle that UV induced DNA lesions strongly block transcription and these lesions must be repaired in order for the reporter gene to be expressed (45,46). Thus the luciferase activity can be apparent only to the extent of damage being repaired upon transfection and recovery of functional plasmid in a given cell. We treated CMV-driven luciferase reporter plasmid with 1000 J/m<sup>2</sup> UV radiation and then transiently transfected undamaged and damaged plasmid into p21+/+, p21+/- and p21-/- cells. Control luciferase activity with unirradiated plasmid was in the order of 10<sup>6</sup> and 10<sup>7</sup> RLU at 4 and 8 h, respectively, in all the three cell lines. Data show that the reactivation of CPD compromised luciferase reporter activity during 8 h post-transfection period. Nevertheless, at 24 h post-transfection, a small but statistically significant (~1.5-fold) decrease in the luciferase reporter activity could be seen in p21-/- cells (Figure 4). p21-/- cells revealed 50% of control reporter activity compared with 65% and 78% respectively in p21+/- and p21+/+ cells at 24 h post-transfection. Moreover, when a wild-type p21 expression plasmid was co-transfected together with a UV-damaged or undamaged plasmid into p21-/- cells, we observed a dosage-dependent increase in percentage of the reporter activity at 24 h following transfection (Figure 5). On the other hand, stimulation of transcription from both UV-damaged and undamaged plasmid was also observed (Figure 5B), suggesting that enhanced DNA repair by p21 may be due to an effect on transcription rather than repair.

#### Discussion

Normal cells, challenged with stressor genetic insults, trigger a variety of intra-cellular programs that lead to cell growth arrest, senescence, or apoptosis. Eukaryotic cells have developed a network of highly conserved surveillance mechanisms (checkpoints), which ensure that damaged chromosomes are repaired before they are replicated during mitosis or segregated during meiosis. These mechanisms are essential for maintaining genomic integrity and cell and tissue viability. Cells respond to genotoxic stress by the induction of genes whose products can have protective function. A variety of DNA damaging agents, both physical and chemical, have been shown to activate p53 protein (47,48), which is one of the critical mediators of genotoxin induced cellular responses, acting at different levels of control during the cell cycle (11). p53 is known to exert its multipotent biological function by inducing downstream mediator genes, e.g. *p21*, *Bax* and *Gadd45* or through its interactions with other regulatory proteins which are involved either in cell cycle control, apoptosis or DNA repair (49). Thus p53 centered signal transduction represents a very important mechanism in the cellular response to a variety of DNA damaging agents.

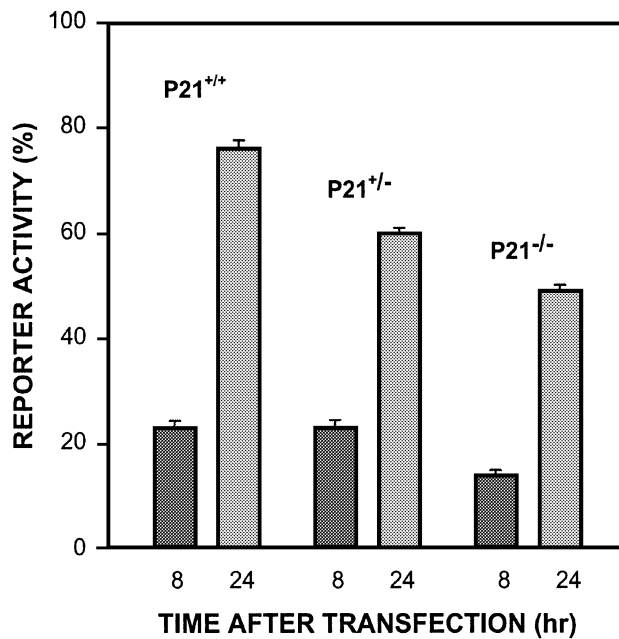
UV-induced DNA damage is predominantly repaired via the NER subpathways that require functionally active PCNA (28). Ever since the reports that p21 interacts physically with PCNA (27), and that p53 stimulates NER, there has been a high degree of interest in exploring the possibility whether downstream effectors of p53 are in some fashion involved in NER. In logical continuation of the previous work, identifying a



**Fig. 3.** Strand-specific DNA repair of CPD from human p53 gene in HCT116 cells after 20 J/m<sup>2</sup> UV-irradiation. **(A)** Autoradiograms representing the extent of removal of CPD in the TS and NTS of the human p53 gene. DNA isolated from untreated confluent cells (first two lanes of each panel) or from cells incubated for indicated time periods after UV-irradiation was digested with EcoRI and the unreplicated (parental) DNA separated from hybrid (daughter) DNA by CsCl density gradient sedimentation as described in Materials and Methods. DNA fragments containing the sequences to be probed were included in each DNA sample as internal marker. Samples of 20 µg EcoRI digested DNA were then treated (+) or mock-treated (-) with T4-endonuclease, electrophoresed under formamide denaturing conditions, Southern transferred to nylon membrane and hybridized with p53 strand-specific <sup>32</sup>P-labeled DNA probes. Bands marked as TS and NTS correspond to the 16 kb fragment of the human p53 gene, and the bands marked as IM correspond to the DNA fragments serving as internal markers. **(B)** Repair profile for the rate of removal of CPD within the TS and NTS of the human p53 gene. The frequency of induction of CPD and their rates of removal were determined as the re-appearance of the full-length restriction fragments in the T4 endonuclease-V treated and untreated sample upon quantitation by phosphorimager analysis. The average number of lesions (endonuclease-sensitive sites) per fragment was calculated using the Poisson equation. The bars represent SD of three independent experiments.

modulating role of p53 in GGR of physical and chemical agent-induced lesions (16–20,33,50), we have now investigated whether p21 is directly or indirectly involved in the regulation of NER by p53. Approaches, analogous to our use of human cells expressing wild-type p53 but having different p21 (+/+, +/-, and -/-) genotypes, have proven quite useful in understanding the nature and extent of repair modulation by cellular regulatory proteins. Thus, as expected, the data clearly show a UV-induced response of p53 as well as p21 proteins

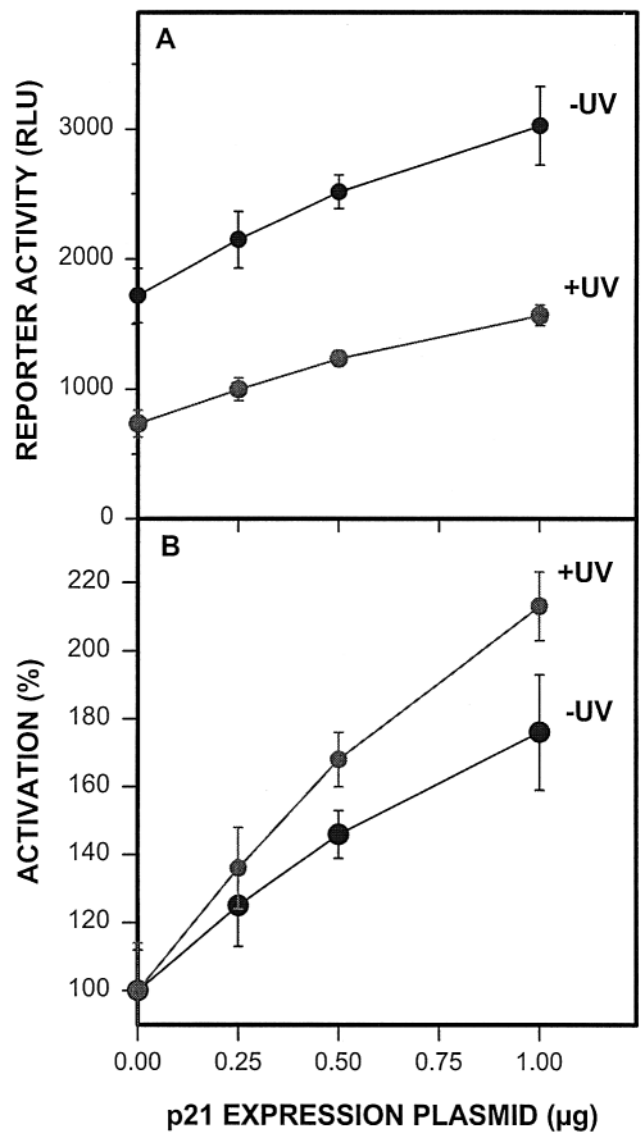
that is consistent with their genotypes. Several fold increase in the p53 protein level suggested a normal response of cells to DNA damaging agents with respect to the induction of p53. Consistent with the augmented p53 expression, the p21 +/+ and p21 +/- cells also responded with an increase in their p21 protein levels. Notably, a complete absence of p21 protein in p21 -/- and a relatively reduced amount of p21 protein present in the p21 +/- compared with p21 +/+ cells provided a suitable background for repair assessment under inherently different



**Fig. 4.** HCR of damaged reporter plasmid DNA. p21<sup>+/+</sup>, p21<sup>+/-</sup>, and p21<sup>-/-</sup> cells were transfected with CMV-driven luciferase reporter plasmid following *in vitro* exposure of DNA to 1000 J/m<sup>2</sup> UV-irradiation. After 5 h transfection time, the DNA containing medium was replaced with fresh medium and cell incubation continued for another 8 and 24 h. At the indicated time, cells were harvested, lysed and assayed for luciferase reporter activity. The data are expressed as percentage of undamaged reporter activity at the corresponding times and is an average of three individual experiments each run with triplicate or duplicate plates.

*in vivo* concentrations of p21 protein. In this regard, our cellular system is similar to that of DLD1 tumor cells in which the ablation of p21 has been reported to enhance the NER (32). It should be noted, however, that DLD1 tumor cells are mutant for p53 and considered functionally p21-deficient in cellular response to genotoxic damage. No p21 protein was detectable before or after UV irradiation in either the p21<sup>+/+</sup> or p21<sup>-/-</sup> DLD1 strains (32), making any conclusions regarding the effect of p21 in these cells difficult. Moreover, the difference in NER results of these two analogous studies could perhaps be attributed to the DLD1 study's application of the more rigid LMPCR for monitoring the fate of CPD at individual damage sites that are known to have heterogeneity vis-à-vis excision under varying sequence contexts and presumably chromatin assembly related factors (16,51,52). It may be noted that the LMPCR approach has also identified a role for retinoblastoma protein (51) while several other studies, using diverse systems, have failed to indicate any such function for this protein (16,18,50,53).

All the studies, reported so far, are unequivocal regarding the participation of p53 in NER (16–20,34,50,51,53,54). However, the question as to what extent the p53 protein participates directly in DNA repair (55,56) versus indirect modulation through p53 regulated gene products that may interact with NER components remains to be fully established. Evidence has just begun to appear for the potential participation of some of the p53 regulated gene products in the NER process (9,54). But, the evidence related to p21 protein has been controversial perhaps because of the diverse systems and approaches applied to discern the p21 dependent effects. Some times application of even the similar schemes has produced divergent results. For example, using *in vitro* cell free systems, two reports



**Fig. 5.** Stimulation of reporter activity upon restoration of wild-type p21 expression in p21<sup>-/-</sup> cells. Increasing concentrations of p21 expression plasmid together with either undamaged or 1000 J/m<sup>2</sup> UV-irradiated CMV-driven luciferase reporter were co-transfected in p21<sup>-/-</sup> cells. At 24 h following transfection, cells were assayed for reporter activity as described in Materials and methods. (A) Actual luciferase reporter activity observed from undamaged and UV-damaged plasmid DNAs as a function of increasing p21 expression. (B) The activity of the undamaged and damaged reporters was normalized to the initial activity observed in p21<sup>-/-</sup> cells in the absence of p21 expression vector. The results are an average of three independent experiments with each point run in triplicate.

indicated that p21 was able to inhibit PCNA mediated DNA replication without affecting NER (29,30). Whereas, the similar *in vitro* approach reported an inhibitory effect of p21 on NER (31). Using a direct CPD measurement by antibodies or dimer sensitivity to T4-endonuclease, we found that p21 deficiency did not affect the removal of CPD to a significant degree. Our data clearly show that, although the repair being slightly lesser in p21<sup>-/-</sup> cells at 4 h compared with p21<sup>+/+</sup> and p21<sup>+/-</sup> cells, all the three cell lines efficiently remove CPD from both the strands of the p53 gene at later time points. The three cell lines also maintained preferential repair within the TS of the p53 gene. Identical results were also obtained by direct quantitation of GGR of CPD, using the dimer specific antibody-

ies. These experiments also showed that the three cell lines were proficient in the removal of CPD from the genome overall with equal efficiency, however, a slight difference in repair (less than 10%) in p21<sup>-/-</sup> cells was observed at 24 h compared with p21<sup>+/+</sup> and p21<sup>+/-</sup> cells. These results are fully consistent with recent reports applying same approaches for the CPD measurement (34,57). These later studies have assessed the kinetics of dimer removal from the overall genome as well as CPD from either strand of the mouse *dhfr* gene and concluded that p21 is not required for GGR or TCR. Similar conclusions regarding repair modulation were obtained with studies of human cells (34). Therefore, based upon these latest studies that were aimed at analyzing the primary recognition and/or incision step of NER process, the effect of p21 appears to be minimal, if any, particularly in the context of DNA damage processing involving the complexities and constraints operational at the whole chromosomal level.

To investigate the possibility of p21 acting at the level of post-incision repair synthesis, the ability to restore the activity of a damaged reporter target was analyzed in p21-proficient and -deficient cell lines. It may be noted that in assessing the HCR, the test cells are not subjected to damaging effects of the agents. So, unless the UV induced DNA lesions, that present a strong block for transcription, are fully repaired by the *in vivo* removal of lesion as well as repair replication at the excision site, the reporter gene cannot be expressed. Thus, the levels exhibited by the damaged compared with undamaged reporter gene expression reflect the extent of repair restoration of target gene during its transient existence and the repair proficiency of a given cell line (58,59). Earlier HCR studies observed a reduced repair of UV or *cis*-platinum damaged plasmid DNA in p21<sup>-/-</sup> HCT116 compared with their parental p21<sup>+/+</sup> cells and assigned a clear role for p21 in NER. Moreover, the absence of p21 was also indicated to coincide with the reduced sensitivity and increased mutagenesis in p21<sup>-/-</sup> cells (59). Our reporter expression experiments were able to confirm the decreased HCR in p21<sup>-/-</sup> cells as we observed a statistically significant difference in the reporter activity at 24 but not at 8 h after transfection. Our data also confirmed the restoration of enhanced HCR ability of p21<sup>-/-</sup> cells upon expression of exogenously incorporated p21 function. However, our data indicate that p21 expression had a greater effect on transcription than on DNA repair. The net effect, attributable to possible modulation of DNA repair, was quite marginal and makes it difficult to comment on potential implications or mechanistically linking p21 to discrete events of damage processing pathway either before or after lesion incision. Close examination of the results of the earlier study also reveal that the differences in the reporter activities within p21<sup>+/+</sup> versus p21<sup>-/-</sup> cells were typically in the modest range of ~10% (59). Nevertheless, this enhancement of reporter activity has been envisioned as the stimulation of repair capacity due to the interaction of p21 with PCNA and presumed modulation of processive DNA synthesis.

To date, extensive investigations on the possible role of p21 on NER and the divergent nature of observations of most of the studies have led to interesting conjectures ranging from acquisition of tumorigenesis initiating mutations to the potential development of anti-cancer drugs based upon disrupting interactions of p21 with its target NER related proteins, e.g. PCNA. While these efforts are worthwhile and many suggestions appear quite logical in the context of differing but actual observations, the current state of the important and interesting

area of p21-dependent modulation of NER research warrants a more critical appraisal prior to embarking on any major scientific undertakings based upon loose or even faulty interpretation of the available information. Clearly, data presented here fail to support a role for p21 in DNA repair. But, this need not be the last word. However, future investigators should be able to rest their p21 related NER work on a firmer foundation that carefully considers the bulk of evidence for and against p21-NER connection.

### Acknowledgements

We are highly thankful to Dr Bert Vogelstein (The John Hopkins Oncology Center) for providing HCT116 cells, Dr Wafik El-Deiry (Howard Hughes Medical Institute) for pCEP-WAF1 expression vector and Dr Maqsood Wani (University of Cincinnati) for critical reading of the manuscript. This work was supported by NIEHS grant ES6074 to AAW.

### References

- Petit,C. andancar,A. (1999) Nucleotide excision repair: From *E.coli* to man. *Biochimie*, **81**, 15–25.
- Sanchez,Y. and Elledge,S.J. (1995) Stopped for repairs. *BioEssays*, **17**, 545–548.
- Shivji,M.K.K., Kenny,M.K. and Wood,R.D. (1992) Proliferating cell nuclear antigen is required for DNA excision repair. *Cell*, **69**, 367–374.
- Walker,L.J., Robson,C.N., Black,E., Gillespie,D. and Hickson,I.D. (1993) Identification of residues in the human DNA repair enzyme HAP1 (Ref-1) that are essential for redox regulation of Jun DNA binding. *Mol. Cell. Biol.*, **13**, 5370–5376.
- Zhu,Q.Z., Wani,M.A., El-mahdy,M., Wani,G. and Wani,A.A. (2000) Modulation of transcriptional activity of p53 by ultraviolet radiation: linkage between p53 pathway and DNA repair through damage recognition. *Mol. Carcinog.*, **28**, 215–224.
- Zhu,Q., Wani,G., Wani,M.A. and Wani,A.A. (2001) Human homologue of yeast Rad23 protein A interacts with p300/cyclic AMP-Responsive element binding (CREB)-binding protein to down-regulate transcriptional activity of p53. *Cancer Res.*, **61**, 64–70.
- Zhu,Q., Yao,J., Wani,G., Wani,M.A. and Wani,A.A. (2001) Mdm2 mutant defective in binding p300 promotes ubiquitination but not degradation of p53: evidence for the role of p300 in integrating ubiquitination and proteolysis. *J. Biol. Chem.*, **276**, 29695–29701.
- Wood,R.D., Mitchell,M., Sgouros,J. and Lindahl,T. (2001) Human DNA repair genes. *Science*, **291**, 1284–1289.
- 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. (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*, **71**, 587–597.
- Lu,X. and Lane,D.P. (1993) Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes. *Cell*, **75**, 765–778.
- Hall,P.A., McKee,P.H., Menage,H.P., Dover,R. and Lane,D.P. (1993) High levels of p53 protein in UV-irradiated normal human skin. *Oncogene*, **8**, 203–207.
- Midgley,C.A., Owens,B., Briscoe,C.V., Thomas,D.B., Lane,D.P. and Hall,P.A. (1995) Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type *in vivo*. *J. Cell Sci.*, **108**, 1843–1848.
- Smith,M.L. and Fornace,A.J., Jr. (1997) p53-mediated protective responses to UV irradiation. *Proc. Natl Acad. Sci. USA*, **94**, 12255–12257.
- El-Deiry,W.S., Tokino,T., Velculescu,V.E. *et al.* (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.
- Merritt,A.J., Potten,C.S., Kemp,C.J., Hickman,J.A., Balmain,A., Lane,D.P. and Hall,P.A. (1994) The role of p53 in spontaneous and radiation-induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. *Cancer Res.*, **54**, 614–617.
- Ford,J.M. and Hanawalt,P.C. (1997) Expression of wild-type p53 is required for efficient global genomic nucleotide excision repair in UV-irradiated human fibroblasts. *J. Biol. Chem.*, **272**, 28073–28080.
- Ford,J.M. and Hanawalt,P.C. (1995) Li-Fraumeni syndrome fibroblasts homozygous for p53 mutations are deficient in global DNA repair but exhibit normal transcription-coupled repair and enhanced UV resistance. *Proc. Natl Acad. Sci. USA*, **92**, 8876–8880.
- El-Mahdy,M.A., Hamada,F.M., Wani,M.A., Zhu,Q.Z. and Wani,A.A. (2000) p53-degradation by HPV-16 E6 preferentially affects the removal of

- cyclobutane pyrimidine dimers from non-transcribed strand and sensitizes mammary epithelial cells to UV-irradiation. *Mutat. Res. DNA Repair*, **459**, 135–145.
19. Wani, M.A., Zhu, Q.Z., El-mahdy, M. and Wani, A.A. (1999) Influence of p53 tumor suppressor protein on bias of DNA repair and apoptotic response in human cells. *Carcinogenesis*, **20**, 765–772.
  20. Wani, M.A., Zhu, Q.Z., El-mahdy, M., Venkatachalam, S. and Wani, A.A. (2000) Enhanced sensitivity to anti-benzo[a]pyrene-diol-epoxide DNA damage correlates with decreased genomic repair attributable to abrogated p53 function in human cells. *Cancer Res.*, **60**, 2275–2280.
  21. Hanawalt, P.C. (1998) Genomic instability: environmental invasion and the enemies within. *Mutat. Res. Fundam. Mol. Mech. Mutagen.*, **400**, 117–125.
  22. Fan, S., Smith, M.L., Rivet, I.D.J., Duba, D., Zhan, Q., Kohn, K.W., Fornace Jr., A.J. and O'Connor, P.M. (1995) Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. *Cancer Res.*, **55**, 1649–1654.
  23. Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. (1993) p21 is a universal inhibitor of cyclin kinases. *Nature*, **366**, 701–704.
  24. Michieli, P., Chetid, M., Lin, D., Pierce, J.H., Mercer, W.E. and Givol, D. (1994) Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res.*, **54**, 3391–3395.
  25. Nakanishi, M., Adami, G.R., Robetorye, R.S., Noda, A., Venable, S.F., Dimitrov, D., Pereira-Smith, O.M. and Smith, J.R. (1995) Exit from G<sub>0</sub> and entry into the cell cycle of cells expressing p21<sup>Sdi1</sup> antisense RNA. *Proc. Natl Acad. Sci. USA*, **92**, 4352–4356.
  26. Gu, Y., Turck, C.W. and Morgan, D.O. (1993) Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit. *Nature*, **366**, 707–710.
  27. Sherr, C.J. and Roberts, J.M. (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.*, **9**, 1149–1163.
  28. Sancar, A. (1994) Mechanisms of DNA excision repair. *Science*, **266**, 1954–1956.
  29. Li, R., Waga, S., Hannon, G.J., Beach, D. and Stillman, B. (1996) Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature*, **371**, 534–537.
  30. Shivji, M.K.K., Grey, S.J., Strausfeld, U.P., Wood, R.D. and Blow, J.J. (1994) Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision-repair. *Curr. Biol.*, **4**, 1062–1068.
  31. Pan, Z.Q., Reardon, J.T., Li, L., Flores-Rozas, H., Legerski, R., Sancar, A. and Hurwitz, J. (1995) Inhibition of nucleotide excision repair by the cyclin-dependent kinase inhibitor p21. *J. Biol. Chem.*, **270**, 22008–22016.
  32. Therrien, J.P., Loignon, M., Drouin, R. and Drobetsky, E.A. (2001) Ablation of p21<sup>waf1/cip1</sup> expression enhances the capacity of p53-deficient human tumor cells to repair UVB-induced DNA damage. *Cancer Res.*, **61**, 3781–3786.
  33. Smith, M.L., Ford, J.M., Hollander, M.C., Bortnick, R.A., Amundson, S.A., Seo, Y.R., Deng, C.X., Hanawalt, P.C. and Fornace, A.J., Jr. (2000) p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21 and/or gadd45 genes. *Mol. Cell Biol.*, **20**, 3705–3714.
  34. Adimoolan, S., Lin, C.X. and Ford, J.M. (2001) The p53-regulated cyclin-dependent proteins kinases inhibitor, p21 (cip1, waf1, sid1), is not required for global genomic and transcription-coupled nucleotide excision repair of UV-induced DNA photoproducts. *J. Biol. Chem.*, **276**, 25813–25822.
  35. Waldman, T., Kinzler, K.W. and Vogelstein, B. (1995) p21 is necessary for the p53-mediated G<sub>1</sub> arrest in human cancer cells. *Cancer Res.*, **55**, 5187–5190.
  36. Venkatachalam, S., Denissenko, M. and Wani, A.A. (1997) Modulation of (+/-)-anti-BPDE mediated p53 accumulation by inhibitors of protein kinase C and poly(ADP-ribose) polymerase. *Oncogene*, **14**, 801–809.
  37. Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.*, **16**, 12–15.
  38. Venkatachalam, S. and Wani, A.A. (1994) Differential recognition of stereochemically defined base adducts by antibodies against anti-benzo[a]pyrene diol epoxide modified DNA. *Carcinogenesis*, **15**, 565–572.
  39. Wani, A.A., D'Ambrosio, S.M. and Alvi, N.K. (1987) Quantitation of pyrimidine dimers by immunoslot blot following sublethal UV-irradiation of human cells. *Photochem. Photobiol.*, **46**, 477–482.
  40. Wani, A.A., Gibson-D'Ambrosio, R.E. and D'Ambrosio, S.M. (1984) Antibodies to UV irradiated DNA: The monitoring of DNA damage by ELISA and indirect immunofluorescence. *Photochem. Photobiol.*, **40**, 465–471.
  41. Mellon, I., Spivak, G. and Hanawalt, P.C. (1991) Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. *Cell*, **51**, 241–249.
  42. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
  43. Ruven, H.J.T., Seelen, C.M.J., Lohman, P.H.M., Mullenders, L.H.F. and Van Zeeland, A.A. (1994) Efficient synthesis of <sup>32</sup>P-labeled single-stranded DNA probes using linear PCR; Application of the method for analysis of strand-specific DNA repair. *Mutat. Res.*, **315**, 189–195.
  44. Denissenko, M.F., Venkatachalam, S., Yamasaki, E.F. and Wani, A.A. (1994) Assessment of DNA damage and repair in specific genomic regions by quantitative immuno-coupled PCR. *Nucleic Acids Res.*, **22**, 2351–2359.
  45. Protic, M., Roilides, E., Levine, A.S. and Dixon, K. (1988) Enhancement of DNA repair capacity of mammalian cells by carcinogen treatment. *Somatic Cell Mol. Genet.*, **14**, 351–357.
  46. Smith, M.L., Kontny, U.U., Zhan, Q., Sreenath, A., O'Conner, P.J. and Fornace, A.J., Jr. (1996) Antisense GADD45 expression results in decreased DNA repair and sensitizes cells to u.v.-irradiation or cisplatin. *Oncogene*, **13**, 2255–2263.
  47. Oren, M. and Rotter, V. (1999) Introduction: p53 – the first twenty years. *Cell. Mol. Life Sci.*, **55**, 9–11.
  48. Levine, A.J. (1993) The tumor suppressor genes. *Ann. Rev. Biochem.*, **62**, 623–651.
  49. Gottlieb, T.M. and Oren, M. (1996) p53 in growth control and neoplasia. *Biochim. Biophys. Acta*, **1287**, 77–102.
  50. Zhu, Q.Z., Wani, M.A., El-mahdy, M. and Wani, A.A. (2000) Decreased DNA repair efficiency by loss or disruption of p53 function preferentially affects removal of cyclobutane pyrimidine dimers from non-transcribed strand and slow repair sites in transcribed strand. *J. Biol. Chem.*, **275**, 11492–11497.
  51. Therrien, J.P., Drouin, R., Baril, C. and Drobetsky, E.A. (1999) Human cells compromised for p53 function exhibit defective global and transcription-coupled nucleotide excision repair, whereas cells compromised for pRb function are defective only in global repair. *Proc. Natl Acad. Sci. USA*, **96**, 15038–15043.
  52. Tornaletti, S. and Pfeifer, G.P. (1994) Slow repair of pyrimidine dimers at p53 mutation hotspots in skin cancer. *Science*, **263**, 1436–1438.
  53. Ford, J.M., Baron, E.L. and Hanawalt, P.C. (1998) Human fibroblasts expressing the human papillomavirus E6 gene are deficient in global genomic nucleotide excision repair and sensitive to ultraviolet irradiation. *Cancer Res.*, **58**, 599–603.
  54. Hwang, B.J., Ford, J.M., Hanawalt, P.C. and Chu, G. (1999) Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. *Proc. Natl Acad. Sci. USA*, **96**, 424–428.
  55. Leveillard, T., Andrea, L., Bissonnette, N., Schaeffer, L., Bracco, L., Egly, J.-M. and Wasyluk, B. (1996) Functional interaction between p53 and TFIIH complex are affected by tumour-associated mutations. *EMBO J.*, **15**, 1615–1624.
  56. Wang, X.W., Yeh, H., Schaeffer, L. et al. (1995) p53 modulation of TFIIH-associated nucleotide excision repair activity. *Nature Genet.*, **10**, 188–195.
  57. Smith, M.L., Ford, J.M., Hollander, M.C., Bortnick, R.A., Amundson, S.A., Seo, Y.R., Deng, C.X., Hanawalt, P.C. and Fornace, A.J., Jr. (2000) p53-mediated DNA repair responses to UV radiation: Studies of mouse cells lacking p53, p21 and/or gadd45 genes. *Mol. Cell Biol.*, **20**, 3705–3714.
  58. Smith, M.L., Chen, I.-T., Zhan, Q., O'Connor, P.M. and Fornace, A.J., Jr. (1995) Involvement of the p53 tumor suppressor in repair of u.v.-type DNA damage. *Oncogene*, **10**, 1053–1059.
  59. McDonald, E., III, Wu, G.S., Waldman, T. and El-Deiry, W.S. (1996) Repair defect in p21<sup>WAF1/CIP1</sup> -/- human cancer cells. *Cancer Res.*, **56**, 2250–2255.

Received November 20, 2001; revised November 20, 2001;  
accepted November 21, 2001