Adenovirus-mediated wild-type p53 radiosensitizes human tumor cells by suppressing DNA repair capacity

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Abstract

Functional inactivation of the p53 gene and robust DNA repair capacity may be among the salient causes of radioresistance in tumor cells. We expressed the wild-type (wt) p53 gene in a p53-mutant human epidermoid carcinoma cell line, A431, using an adenoviral vector [adenovirus-p53 (Ad-p53), INGN 201], examined its radiosensitivity, and correlated p53 status and radiosensitivity with cellular repair functions. Using clonogenic survival assays and the terminal deoxynucleotidyl transferase-mediated nick end labeling assay for apoptosis, we demonstrated that preirradiation treatment with Ad-p53 significantly increased the radiosensitivity of A431 cells over controls. Induction of p53 expression using a construct where p53 expression was under the control of an inducible promoter also significantly increased radiosensitivity of H1299 lung tumor cells, which are otherwise null for p53. These results did not correlate with radiation-induced apoptosis but did correlate with functional impairment of DNA repair and suppressed expression of several repair-related genes, such as Ku70, DNA-dependent protein kinase, ataxia telangiectasia mutated, and X-ray-sensitive complementation group 4. Normal human fibroblast MRC-9 cells showed no impairment in the repair capability due to Ad-p53 despite the suppression of some repair genes. Expression of Ku70, which is known to mediate diverse cellular functions, correlated with the differential effects of p53 on radiosensitivity in the normal and tumor cells. (Mol Cancer Ther. 2003;2:1223–1231)

Introduction

Radiotherapy continues to be a frontline treatment for cancer despite the risk of normal tissue complications. Enhancing the antitumor effects by combining radiation with other agents often allows lower doses to be used, thereby minimizing side effects. Gene therapy in combination with radiation is one such promising strategy (1–6). It has been estimated that about 50% of all tumors have mutations in p53, and the p53 pathway may be nonfunctional for other reasons in many more. A number of investigators have shown that exogenous expression of wild-type (wt) p53 sensitizes human tumor cells to radiation *in vitro* and *in vivo* (7–16).

p53 is well known for its role in monitoring genomic stability. The mechanisms underlying this function of p53 are not fully understood (17). Nevertheless, it is known that genetic insults activate p53, which in turn induce downstream repair genes including GADD45, p48XPE, and XPC (17–19) that are involved in the nucleotide excision repair (20) and base excision repair (21, 22) processes. Genetic insults may also lead to DNA double-strand breaks (DSB) that are repaired by interchromosomal and intrachromosomal homologous recombination (HR) and nonhomologous end joining (NHEJ). The NHEJ pathway is especially important for repairing radiation-induced DSBs. Factors that are known to participate in NHEJ include Ku70, Ku80, DNA-dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKcs), Artemis, X-ray-sensitive complementation group 4 (XRCC4), and DNA ligase IV (23, 24). Deficiency of any of these factors also results in defects in V(D)J recombination, leading to severe combined immunodeficiency. There are conflicting reports in the literature regarding the role of wt-p53 in the regulation of NHEJ. Several reports show a positive involvement of wt-p53 in accentuating NHEJ (25–28), but others have demonstrated down-regulation of NHEJ-mediated DSB repair by wt-p53 (29). The multiple functions of wt-p53 are tightly regulated by several molecular processes including post-translation modification (30, 31), multiple-site phosphorylation (32–34), acetylation (35), multiple protein-DNA interactions (36), conformational changes (37), and sumoylation and glycosylation (36) depending on the cell type. We therefore hypothesized that p53 may play distinct roles in governing DNA repair capacity in normal and transformed cells.

To test whether radiosensitization of human tumor cells by the gene therapy vector adenovirus-p53 (Ad-p53) is due to suppression of NHEJ, we treated human p53-mutant epidermoid carcinoma A431 cells and examined the expression of proteins that participate in NHEJ. We found that Ad-p53 radiosensitized A431 cells and that this effect correlated with a down-modulation of proteins involved in NHEJ. Normal human fibroblasts were not radiosensitized by Ad-p53, which suggests that Ad-p53 has a differential effect on DNA repair in tumor cells *versus* normal cells.

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Materials and Methods

Cell Culture

The human epidermoid carcinoma cell line A431 was obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in DMEM-F12 growth medium supplemented with 10% FCS, 10-mM glutamine, and 100-unit/ml penicillin G + 100-μg/ml streptomycin. Cells were grown at 37°C in 95% air/5% CO_2. The H1299 non-small cell lung cancer cell line carrying a stably transfected ecdysone-inducible p53 construct (38) was maintained in RPMI 1640 with 10% FCS, 10-mM glutamine, 100-unit/ml penicillin G, 100-μg/ml streptomycin, 200-μg/ml G418 (Life Technologies, Inc., Rockville, MD), and 100-μg/ml zeocin (Invitrogen, Carlsbad, CA). The MRC-9 normal human fibroblast cells were cultured in α-MEM containing 10% FCS, 10-mM glutamine, 100-unit/ml penicillin G, and 100-μg/ml streptomycin supplemented with nonessential amino acids and vitamins.

Adenovirus

The E1-deleted adenovirus-β-gal (Ad-β-gal), Ad-p53, and adenovirus-luciferase (Ad-luc) vectors were obtained from Introgen Therapeutics (Houston, TX). Ad-luc was used as a control vector for all experiments.

Antibodies

Antibodies to p21 and Ku70 were obtained from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Antibody to p53 was purchased from DAKO Corp. (Carpinteria, CA). Antibodies to β-actin and Ku80 were obtained from Sigma (St. Louis, MO). Antibodies to DNA-PKcs, XRCC4, and ataxia telangiectasia mutated (ATM) were purchased from GeneTex (San Antonio, TX).

Gene Delivery

A431 cells (0.5×10^6) were plated in 100-mm culture dishes. Forty-eight hours after plating, cells were infected with vector for 1 h in 1 ml of serum-free medium. After infection with the appropriate vector, complete medium with 10% FBS was added to the cells. Cells were incubated for an additional 48 h. Infectivity of the cells was tested using Ad-β-gal followed by β-gal expression. For this, 25×10^3 cells/well were seeded in each well of a six-well plate, infected (10^3 viral particles [vp]/cell) with Ad-β-gal, and incubated for 24 h. X-gal (1 mg/ml) was used to stain cells for β-gal, and the cells were incubated overnight. The cells were then fixed in 10% formalin, washed in PBS, and kept in PBS at 4°C. Blue-stained cells were considered infected with Ad-β-gal.

Irradiation

Cells growing in log phase were exposed to ^{137}Cs at a dose rate of 3.5 Gy/min.

Clonogenic Assay

The effectiveness of the combination of Ad-p53 and ionizing radiation was assessed by clonogenic assays as detailed in a previous publication (13). Briefly, A431 cells growing in log phase were infected with Ad-p53 or Ad-luc vector as mentioned above and after 48 h exposed to radiation. Following irradiation, the cells were trypsinized and counted, and known numbers were seeded in 100-mm culture dishes in two sets of triplicates for each dose of radiation; sufficient numbers were seeded to ensure that about 50–100 macroscopic colonies would appear in each plate at the end of 8–10 days. Colonies were stained with 0.5% gentian violet solution and counted. The percent plating efficiency and surviving fraction were calculated for each radiation dose. The percent plating efficiency for each dish was calculated by dividing the number of colonies by the number of cells plated and multiplying by 100. The surviving fraction for each radiation dose was then calculated by dividing the plating efficiency determined for that treatment by the plating efficiency for the appropriate unirradiated control. The survival curves shown were normalized for the toxicity of the vectors by using the treatments with vector alone as the appropriate unirradiated controls for the vector + radiation survival curves. The width of the shoulder region of the survival curves, a classical indication of the relative repair capacity of the cells (39), was calculated by graphical analysis as the quasi-threshold dose or Dq (40).

Western Blot

Cells were harvested after treatment with Ad-p53 or Ad-luc and lysed by vortexing in a lysis buffer containing 50-mM HEPES (pH 7.0), 150-mM NaCl, 1.5-mM MgC2, 1-mM EGTA, 100-mM NaF, 10-mM sodium pyrophosphate, 10% glycerol, and 1% Triton X-100. The buffer was fortified with 1-mM sodium orthovanadate, 2-mM phenylmethylsulfonyl fluoride, 10-μg/ml aprotinin, and 10-μg/ml leupeptin at the time of use. The lysate was incubated on ice for 20 min and centrifuged at 12,000 rpm at 4°C for 15 min to remove any cellular debris. Protein concentration of the lysates was determined by the Bio-Rad protein assay method. Equal amounts of the protein were resolved on 7–12% polyacrylamide gels and transferred to Immobilon membrane (Millipore, Billerica, MA). The membrane was washed in Tris (20 mM)-buffered saline (150 mM; pH 7.4) + 0.05–0.1% Tween (TBS-T) and blocked in a blocking buffer as suggested by the manufacturer for 1 h at room temperature (RT) or at 4°C overnight. After being washed with TBS-T again, the membrane was incubated with primary antibody in a suitable buffer for 1 h at RT or overnight at 4°C. The membrane was then incubated with a compatible secondary antibody tagged with horseradish peroxidase for 1 h at RT and developed by enhanced chemiluminescence (Amersham, Piscataway, NJ) on a STORM 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay by Flow Cytometry

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was performed using the APO-BRDU kit (PharMingen, San Diego, CA) to quantify induction of apoptosis following manufacturer's instructions. Briefly, A431 cells were fixed in 1% (w/v) paraformaldehyde in PBS and incubated on ice for 15 min. Then, the cells were washed thrice with PBS and preserved in 70% (v/v) ethanol overnight. About 1×10^6 cells/treatment, in duplicate, along with positive and negative controls, were counted, pelletized, washed twice with wash

buffer, and subjected to labeling reaction using terminal deoxynucleotidyl transferase overnight at RT. At the end of the reaction, cells were rinsed twice before treatment with fluorescein-labeled anti-BrdU antibody solution in the dark for 30 min at RT. The cells were stained with propidium iodide/RNase solution for 30 min in the dark and analyzed by flow cytometry (Epics XL-MCL, Beckman Coulter Corp., Miami, FL).

RNase Protection Assay

Cells were harvested following treatment with the vectors, and RNA was extracted using TRIZOL (Life Technologies) reagent following the company's protocol. RNase protection assay (RPA) was performed using a kit (PharMingen) following manufacturer's instructions. Briefly, a radioactive probe using RPA template and [α^{32}P]UTP was synthesized and purified. The probe was then hybridized with 20-μg total RNA over a period of 15 h. The hybridized samples were RNase digested to get rid of the single-strand extensions. The hybridized, blunt-ended nucleic acid samples were resolved on a 5% polyacrylamide gel as per manufacturer's instructions. The gel was blotted onto a piece of Whatman paper (3 mm), dried for 1 h at 80°C under vacuum, and analyzed on a STORM 860 PhosphorImager (Molecular Dynamics). The intensity of the bands in different samples was quantified using a densitometer and the Image Quant program (Molecular Dynamics) and normalized to the intensity of the housekeeping gene transcript (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). The differences were represented as percent of control.

Host Cell Reactivation Assay

Ten thousand cells were plated in each well of six-well plates. The cells were infected with Ad-p53 or Ad-luc at 500–1000 vp/cell followed by a 48-h incubation. The cells were then infected with Ad-β-gal (1×10^3 vp/cell) that had been irradiated with 0–4000 Gy of γ-radiation (source, ^{137}Cs) and incubated for an additional 24 h. This dose of 4000 Gy is necessitated by the small genome size of the adenoviral vector compared with a mammalian cell. Calculations indicate that this dose will produce about 1–2 DSBs/vector particle. The cells were then stained with X-gal following the procedure described above and fixed in 10% formalin. β-gal-positive (blue) cells were scored under high power (40×) of a light microscope. The data are presented as percent of control.

Results

Adenovirus-Mediated p53 Gene Expression Enhances Radiosensitivity of A431 Cells in a Dose-Dependent Manner

Having confirmed by β-gal assay that human epidermoid A431 carcinoma cells are significantly infectable by adenoviral vectors (data not shown), we treated them with the gene therapy vector Ad-p53 and examined p53 protein expression by Western blot analysis. Expression of p53 increased with the increasing dose of vector (Fig. 1C). We observed that A431 cells were significantly sensitized by Ad-p53 to γ-radiation in a dose-dependent manner (Fig. 1A). A431 exhibited a typical clonogenic survival curve with a shoulder signifying cellular repair capacity (39). Doses of 2, 4, and 6 Gy of γ-radiation alone killed

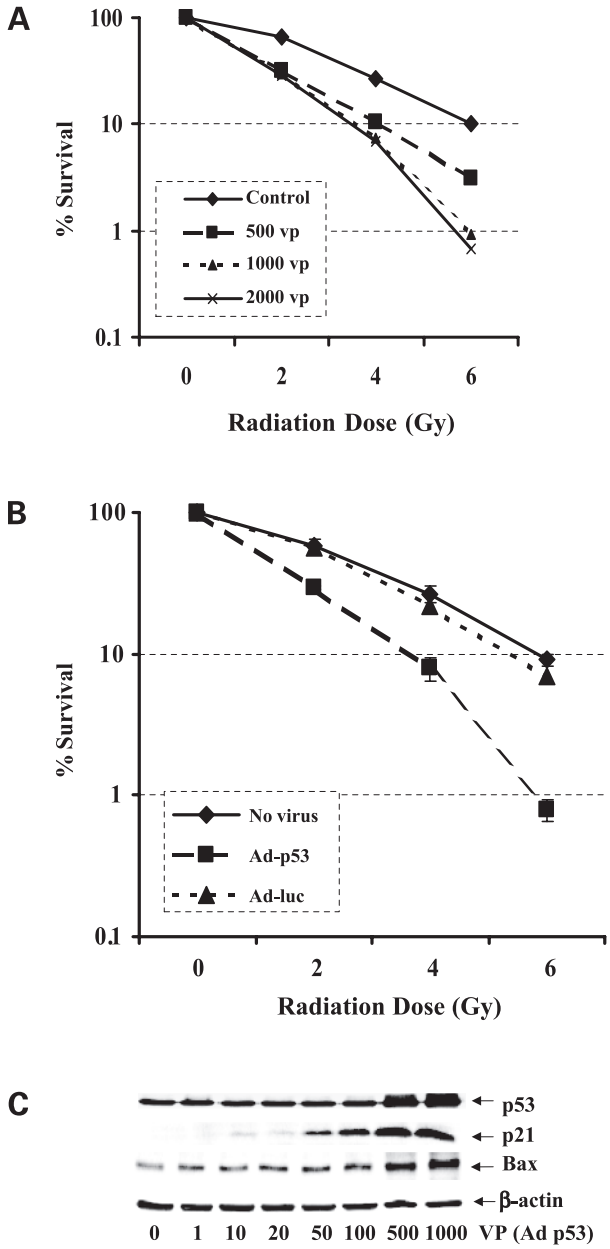

Figure 1. Ad-p53 expression enhances radiosensitivity of A431 cells as a function of vector dose (vp/cell) and radiation dose (Gy) in A431 cells. **A,** cells growing in log phase were infected with 500–2000 vp/cell as described in ''Materials and Methods'' and incubated for 48 h followed by exposure to 0–6 Gy of γ-radiation. The cells were harvested, counted, and plated in triplicate for clonogenic assay. **B,** equal doses of control vector (Ad-luc) and Ad-p53 (1000 vp/cell) were used for infecting A431 cells as described and the cells were exposed to γ-radiation and subjected to clonogenic assay. *Points,* average of two independent experiments; *bars,* SE. **C,** Western blot results showing expression of p53, Bax, and p21 as a function of vp/cell. *Blots,* representative of at least two independent experiments. β-actin is shown as a loading control.

about 35%, 63%, and 90% of cells, respectively. Ad-p53 (500 vp/cell) enhanced the cell killing to 68%, 90%, and 97% at 2, 4, and 6 Gy of γ-radiation, respectively. The shoulder of the survival curve was lost at this level of expression of p53 as indicated by a reduction in Dq from 1.25 Gy for the control curve to 0.2 Gy for the Ad-p53 (500 vp/cell) curve. A further increase in p53 expression, produced by 1000 vp/cell, increased the radiosensitizing effect somewhat, but 2000 vp/cell did not increase it further. These results led us to conclude that p53 expression equivalent to that produced by 1000 vp/cell in A431 cells produces maximum radiosensitivity. As shown in Fig. 1B, Ad-luc (1000 vp/cell) as a control vector did not significantly change radiosensitivity compared with that in untreated cells. As expected, the Ad-p53 vector produced a toxic effect when used as a single agent reducing plating efficiency of A431 cells from about 70% in the controls to about 16% following treatment of 1000 vp/cell. This effect appeared to be a result of the expression of wt-p53 as the Ad-luc vector was not toxic in these cells. These survival curves described above have been normalized for vector toxicity as described in ''Materials and Methods.''

Ad-p53 Enhances Radiation-Induced Apoptosis

The TUNEL assay was used to determine if Ad-p53 enhances radiation-induced apoptosis in A431 cells. Data presented in Fig. 2 are representative of three independent observations. Radiation alone was very ineffectual in inducing apoptosis in A431 cells. However, Ad-p53 potently induced apoptosis and substantially sensitized A431 cells to radiation-induced apoptosis. This degree of sensitization appeared to be greater than additive. For example, 6 Gy alone induced about 2% apoptosis over the background level and Ad-p53 induced about 13% apoptosis over background. The combination, however, induced about 26% over the background level. This sensitizing effect was not observed after Ad-luc infection.

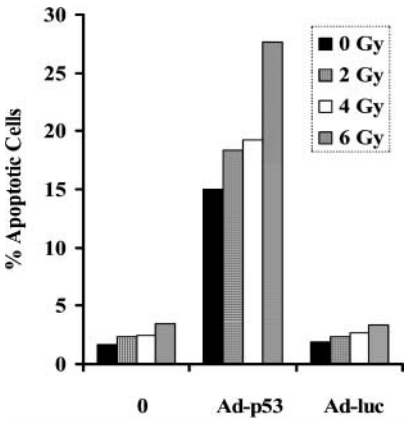

Figure 2. Ad-p53 induces apoptosis in A431 cells, which is further accentuated by radiation. Cells were either mock infected or infected with Ad-luc or Ad-p53 at 1000 vp/cell, incubated for 48 h, and irradiated (0–6 Gy). The cells were harvested 48 h after irradiation, fixed in 1% paraformaldehyde, and processed following manufacturer's instructions for apoptosis assay using the APO-BRDU kit.

Ad-p53 Suppresses the DNA Repair Capacity of A431 Cells

Based on the large reduction in Dq seen in the survival curves (Fig. 1), we performed host cell reactivation (HCR) assay to determine if the radiosensitizing effect of Ad-p53 could be explained as a suppression of the DNA repair capacity of the A431 cells. For this, A431 cells, pretreated with either Ad-p53 or Ad-luc, were subsequently infected with Ad-β-gal that had been irradiated with 0–4000 Gy of γ-radiation. The ability of the A431 cells to reactivate the irradiated Ad-β-gal based on β-gal expression was assessed 24 h later. The results of these experiments, presented as percent of controls using unirradiated Ad-β-gal (Fig. 3), show that Ad-p53-pretreated A431 cells had a significantly ($P \geq 0.01$) lower capacity to reactivate irradiated Ad-β-gal compared with A431 cells that received either no treatment or Ad-luc pretreatment.

Ad-p53 Treatment Suppresses the Expression of Proteins Involved in NHEJ

As shown in Fig. 4, Ad-p53 infection led to overexpression of p53 as compared with control and Ad-luc-treated cells. Expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and proapoptotic protein Bax followed a pattern of enhanced expression similar to that of p53 in Ad-p53-treated cells. Irradiation of untreated or Ad-luc-treated cells did not enhance p21 or Bax expression consistent with the mutant status of p53 in this cell line. It appears that radiation-induced expression of these genes was masked by their overexpression in the Ad-p53-treated cells. The expressions of several proteins involved in NHEJ (*i.e.*, DNA-PKcs, Ku70, and XRCC4) were significantly suppressed by Ad-p53. This effect was not produced to the same degree in the Ad-luc controls. The most dramatic effect of Ad-p53 was observed for XRCC4, the expression of which appeared to be completely inhibited. Expression of Ku80, on the other hand, was unaffected by Ad-p53.

Transcription of DNA Repair Genes Is Significantly Suppressed by Ad-p53 Treatment of A431 Cells

We performed a RPA to determine if the lowered expression of proteins involved in DNA repair by Ad-p53 could be explained by an inhibition of transcription of the genes that encode these proteins. Fig. 5 indicates that, after normalizing for expression of the loading control (GAPDH), Ad-p53 suppressed the transcription of ATM, DNA-PKcs, XRCC4, Ku70, and Ku80. Transcription of ATM appeared to be the most affected (95%). Ad-luc appeared to exert a slight inhibitory effect on ATM and XRCC4 gene transcription but significantly less so than that seen in the Ad-p53-treated cells.

Expression of p53 Radiosensitizes Cells and Impairs NHEJ in H1299 Cells

As a further test of the ability of overexpressed p53 to down-regulate DNA repair proteins and radiosensitize tumor cells, we examined H1299 cells that had been stably transfected with p53 under the control of the ponesterone A (Pon A)-inducible promoter (38). Exposure of the cells with induced p53 to γ-radiation significantly reduced clonogenic survival compared with cells lacking induced p53 (Fig. 6).

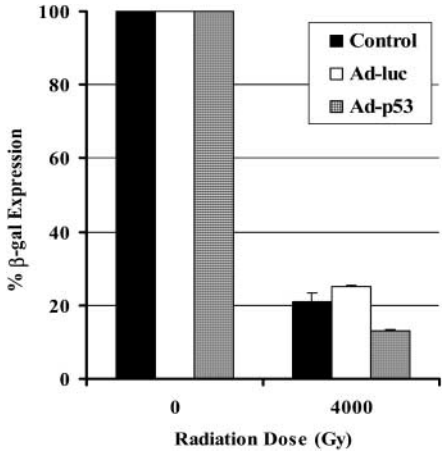

Figure 3. Ad-p53 suppresses HCR in A431 cells. Cells (2×10^4) were seeded in each well of six-well plates, infected with Ad-p53 or Ad-luc at 1000 vp/cell or mock infected, and incubated for 48 h. They were then reinfected with irradiated (0–4000 Gy) Ad-β-gal at 1000 vp/cell for another 24 h. The cells were then stained for β-gal. The β-gal-positive cells were counted and recorded. The percent positive cells were normalized to controls for comparison. *Points,* average of at least four independent experiments; *bars,* SE.

Data presented in Fig. 7A show that induction of p53 by Pon A significantly ($P \geq 0.01$) suppressed HCR in the H1299 cell system. Western blot analysis revealed that a robust expression of p53 and its downstream cyclin-dependent kinase inhibitor, p21, was induced in these cells by Pon A (Fig. 7B). Under these conditions, there was a modest suppression of DNA-PKcs, ATM, Ku70, and XRCC4 protein levels. Expression of Bax and Ku80, however, remained unaffected. The negligible effect of p53 on the expression of Bax in Pon A-treated cells was somewhat surprising. However, this was consistent with the results of TUNEL assay in which Pon A-induced p53 showed no tangible effect on radiation-induced apoptosis in these cells; total TUNEL-positive cells measured 48 h after 6 Gy was 5.4% in the Pon A-treated cells *versus* 5.1% in the control. Taken together, these results largely confirmed our observations obtained using Ad-p53 in A431 cells.

Ad-p53 Does Not Suppress NHEJ Repair of DSB in Normal MRC-9 Cells

We previously reported (13) that Ad-p53 does not affect the radiosensitivity of the normal human fibroblast MRC-9 cell line. Therefore, we examined the effect of Ad-p53 expression on the ability of these cells to carry out HCR. Treatment of MRC-9 cells with Ad-p53 does not suppress their capacity to carry out HCR as compared with untreated controls or treatment with Ad-luc (Fig. 8A). Ad-p53 had a suppressive effect on DNA-PKcs, ATM, and XRCC4 protein levels (Fig. 8B). Ku70 and Ku80 levels, however, remained unaffected. Interestingly, there was no enhanced expression of p21 or Bax by Ad-p53 in MRC-9 cells, correlating with the lack of any restoration of radiation-induced apoptosis seen in these cells following this treatment (13).

Discussion

The p53 tumor suppressor gene plays a critical role in preventing cancer development by regulating a number of cellular processes that are initiated following genotoxic stress (36). In addition to its ability to initiate the demise of damaged cells by apoptosis, it is now understood that p53 is involved in other signaling pathways leading to growth arrest in cells with damaged DNA (41). It has also been hypothesized that p53 may participate in the mechanisms of DNA repair. Thus, loss of p53 function may contribute to tumor cell progression through enhanced genomic instability (23). Based on this concept, strategies have been developed for manipulating the p53 pathway in the treatment of human cancer (42). One such approach involves replacement of wt-p53 into tumor cells by gene therapy. A phase I clinical trial of this strategy

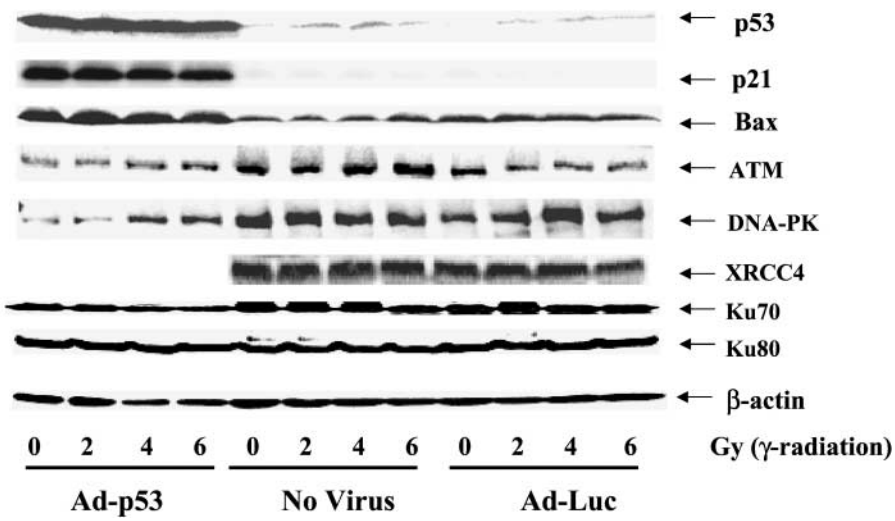

Figure 4. Ad-p53 suppresses DSB repair proteins in A431 cells. Cells were mock infected or treated with Ad-p53 or Ad-luc at 1000 vp/cell and incubated for 48 h followed by exposure to 0–6 Gy. The cells were harvested and lysed, and 30-μg protein was electrophoresed and subjected to Western blot analysis as described in ''Materials and Methods.''

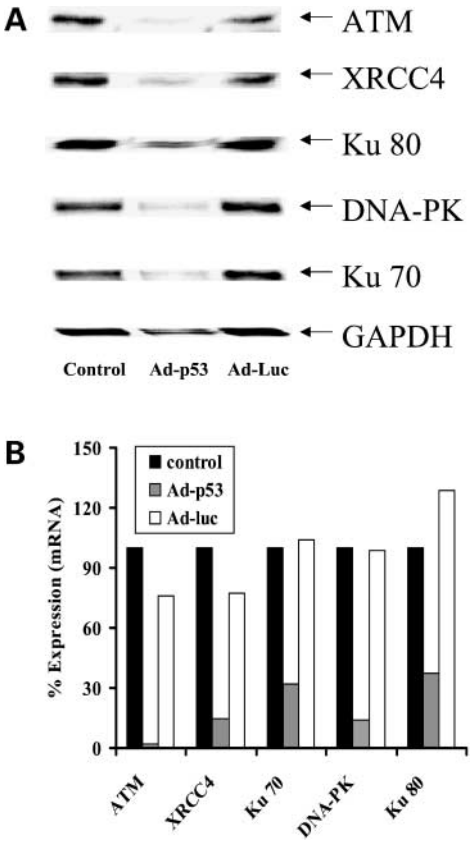

Figure 5. Ad-p53 significantly inhibits transcription of repair genes in A431 cells. **A,** cells were mock infected or treated with Ad-p53 or Ad-luc at 1000 vp/cell and incubated for 48 h. Total RNA was isolated from these samples, and 20 μg of RNA for each sample were used for analyzing the effect of Ad-p53 on transcription of repair genes by RPA as described in ''Materials and Methods.'' **B,** densitometric analysis of the RPA gel was carried out to quantitate the changes in DNA repair genes.

using Ad-p53 illustrated the safety and modest efficacy of this vector when used as a single agent. However, as it would be expected that Ad-p53 might also sensitize human tumors to DNA damaging agents, clinical trials have also been conducted where Ad-p53 was combined with either cisplatin or radiation therapy (43). Results of the first trial where patients with non-small cell lung cancer were treated with the combination of Ad-p53 and radiation therapy have recently been published (44). These investigators concluded that intratumoral injection of Ad-p53 followed by radiation therapy was well tolerated and demonstrated evidence of tumor regression. As additional trials of this strategy are now being planned, it becomes imperative to understand the mechanism by which restoration of wt-p53 radiosensitizes human tumor cells. The present study examined whether p53 radiosensitized cells by suppressing the repair of radiation-induced DNA damage.

As shown in Fig. 1, Ad-p53 significantly enhanced the radiosensitivity of human epidermoid A431 carcinoma cells as determined based on clonogenic survival. The control Ad-luc vector produced a negligible effect on A431 radiosensitization, indicating that the radiosensitizing

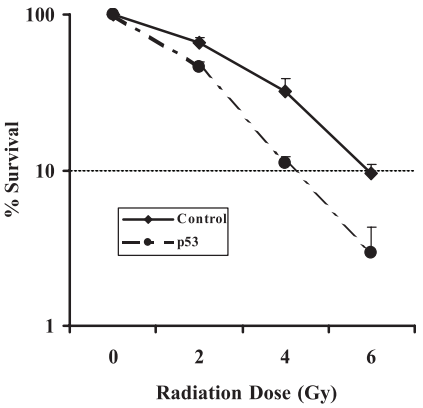

Figure 6. Induction of p53 also enhances radiosensitivity. H1299 cells, stably transfected with a construct carrying the p53 gene under control of a promoter that is inducible by Pon A, were treated with 5-μM Pon A for 24 h. The rest of the procedure was the same as in Fig. 1. *Points,* average of at least three independent experiments; *bars,* SE.

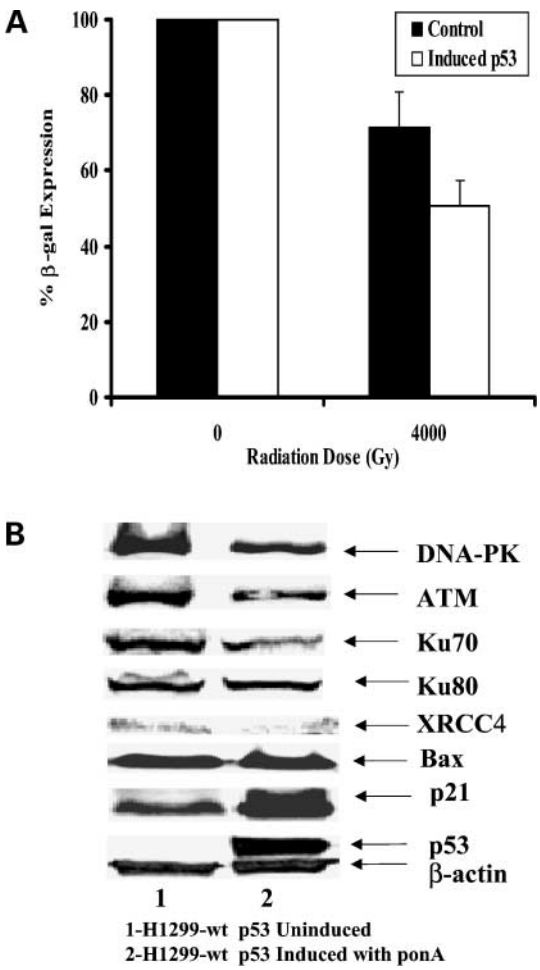

Figure 7. Induced p53 also suppresses HCR and related proteins in H1299 cells. The experimental details are described in Fig. 3. **A,** HCR assay results. *Points,* average of two independent experiments; *bars,* SE, **B,** Western blot for repair proteins. *Blots,* representative of at least two independent experiments. Actin was used as a loading control.

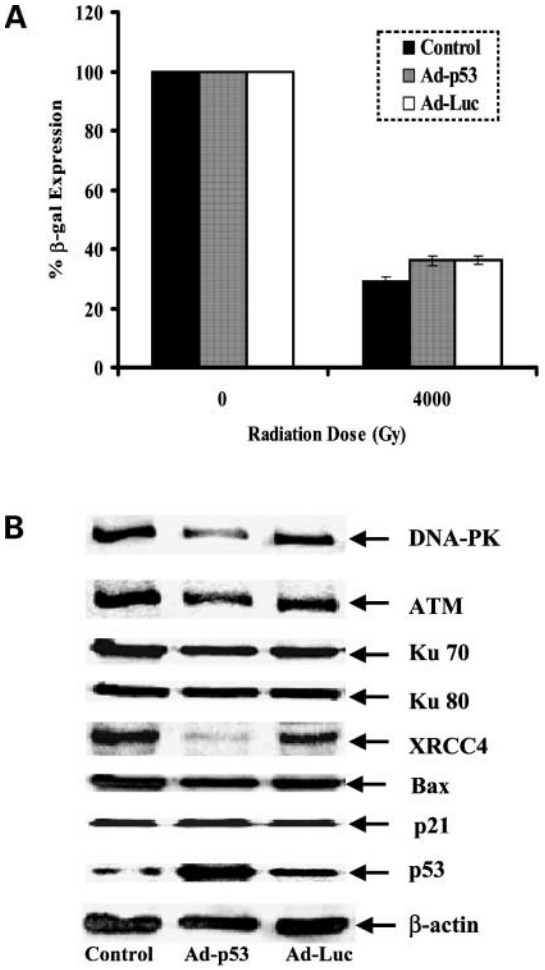

Figure 8. Effect of Ad-p53 on HCR and related proteins in normal human fibroblast MRC-9 cells. **A,** HCR assay results. *Columns,* representative of four independent experiments; *bars,* SE. **B,** Western blot for repair proteins.

effect of Ad-p53 is mediated by expression of p53 and not due to a nonspecific effect of the vector. These results are consistent with several previous reports (7–16) where Ad-p53 was shown to radiosensitize other types of human tumor cells. In one of these reports, we used isobologram analysis to demonstrate that the combination of Ad-p53 and radiation inhibited the growth of human lung cancer cells in a synergistic manner (12). Ad-p53 also restored radiation-induced apoptosis in A431 cells as shown in Fig. 2. However, this effect could not be reconciled with enhanced loss of clonogenic survival because radiation-induced apoptosis was not enhanced in the H1299 cells when wt-p53 was overexpressed although loss of clonogenic survival was enhanced. Thus, although restoration of radiation-induced apoptosis may partly explain enhanced radiosensitivity in some settings, this cannot be a universal explanation for the ability of p53 to radiosensitize human tumor cells. Especially in the case of ionizing radiation, mammalian cells may die by modes other than or in addition to apoptosis including senescence and mitotic catastrophe (45). Both of these modes may result from unrepaired or misrepaired DNA damage; therefore, we assessed whether cellular DNA repair processes were affected in the context of overexpression of wt-p53.

Although the repair of radiation-induced DSBs can be detected using pulsed-field gel electrophoresis techniques, this approach is insensitive to some types of misrepaired lesions. Thus, we chose to assess DNA repair in this investigation using HCR. This relatively old approach (46) was updated to incorporate expression of a reporter gene as the readout; radiation-induced DNA lesions in the reporter gene must be repaired by the host cell with complete fidelity in order for functional gene expression to be restored. The results of our HCR assays (Fig. 3) indicate that pretreating the A431 cells with Ad-p53 suppresses their ability to restore reporter gene expression using irradiated Ad-β-gal vector by nearly 50% when compared with controls. This degree of suppression has biological significance based on the fact that as part of another study, we have shown that a DNA-PK-deficient cell line, MO59J, displays a similar degree of suppression in this same HCR assay when compared with its DNA-PK-proficient control cell line, MO59K.[1] There, the β-gal expression following irradiation of the Ad-β-gal vector with 4000 Gy was reduced from 41.2 ± 7.9% in the MO59K cells to 22.9 ± 2.0% in the MO59J cells. The ability of overexpressed wt-p53 to radiosensitize human cells as determined based on clonogenic survival correlated with suppressed capacity for HCR for all three cell systems examined in this study [*i.e.*, A431 cells treated with Ad-p53 (Fig. 3), H1299 cells where wt-p53 expression was induced with an expression vector (Fig. 7), and MRC-9 cells treated with Ad-p53 (Fig. 8)]. We hypothesized based on these data that overexpression of wt-p53 in this context radiosensitized cells by suppressing their capacity for repairing radiation-induced DNA lesions.

The HCR assays detect changes in the total DNA repair capacity of the cell but do not reveal which DNA repair pathways are being affected because restoration of reporter gene expression would be expected to require the repair of all radiation-induced DNA lesions (*i.e.*, single-strand breaks, DSBs, damaged bases, etc.), whereas clonogenic survival is mainly affected by the NHEJ repair pathway. Thus, based on the correlation established above, we tested whether overexpression of wt-p53 could specifically suppress critical elements of the NHEJ pathway. As shown in Fig. 4, treatment of A431 cells, as expected, resulted in the enhanced expression of p21 and Bax protein. The genes for both of these products are known to be up-regulated by wt-p53. However, Ad-p53 treatment resulted in the suppressed expression of several proteins that are known to be critically important in

[1] T. Nishikawa, A. Munshi, M. D. Story, S. Chada, and R. E Meyn. Adenoviral-mediated MDA-7 expression suppresses DNA repair capacity and radiosensitizes a non-small cell lung cancer cells, manuscript in preparation.

NHEJ. Expression of DNA-PK, XRCC4, ATM, and Ku70 was lower in the cells following Ad-p53 compared with controls. The expression of Ku80 was not affected. A similar pattern was observed in the H1299 cells where wt-p53 was induced with an expression vector. In the H1299 cells, however, Bax expression was not enhanced, suggesting that p53-mediated Bax expression may be responsible for the restoration of radiation-induced apoptosis in the A431 cells that is absent in the H1299 cells. This lack of apoptosis in the H1299 cells also suggests that the suppressed expression of these proteins in the A431 cells following Ad-p53 treatment is not due to a general proteolytic degradation associated with the apoptosis seen in those cells. The normal human fibroblast cell line, MRC-9, displayed a somewhat different pattern following treatment with Ad-p53 (Fig. 8). In this case, although the expression of DNA-PK, ATM, and XRCC4 was lower in the Ad-p53-treated cells compared with controls, the degree of suppression appeared to be less than that seen in the A431 and H1299 cells. Moreover, there was essentially no suppression of Ku70 expression in the MRC-9 cells. These data when taken together suggest that one mechanism by which overexpressed wt-p53 radiosensitizes tumor cells is by suppressing the expression of certain proteins critical to the repair of radiation-induced DSBs by the NHEJ pathway. This suppression appears to occur at the level of gene transcription at least for the A431 cells treated with Ad-p53 based on the RNase protection data shown in Fig. 5.

As shown in our previous report, normal human fibroblasts are not radiosensitized by Ad-p53 (13). Here, we extend this observation to the level of DNA repair as assessed using HCR. This may be explained based on a differential effect of overexpressed wt-p53 on suppression of the expression of proteins critical for repair. The degree of suppression of these proteins appeared to be different for the normal cells compared with the tumor cells, especially for Ku70, a protein required for NHEJ. It is possible that levels of Ku70 are rate limiting for the NHEJ pathway. wt-p53 is a transcription factor, the activity of which is governed by a number of post-translational modifications including phosphorylation at several sites in the protein (30). Thus, it is conceivable that replacing wt-p53 in a null or mutant p53 background produces different effects compared with overexpressing it in a normal background due to the different patterns of regulation of its activity that preexist in these different contexts. Indeed, it appears that some p53-defective tumor cells have increased NHEJ activity. Gaymes *et al.* have recently shown that HL60 cells (defective p53) have a 5–6-fold higher level of Ku70/Ku80-mediated ligation of DSBs compared with normal cells including normal fibroblasts (47). Thus, wt-p53 may normally act to suppress error-prone DNA repair pathways such as NHEJ as part of its overall maintenance of genomic stability. In the absence of this function, tumor cells may have enhanced NHEJ activity leading to radioresistance.

Other explanations for the ability of overexpressed wt-p53 to radiosensitize tumor cells but not normal cells include the possibilities that DNA repair pathways in addition to NHEJ are also differentially regulated in these cell types and that p53 may modulate DNA repair through mechanisms in addition to its activity as a transcription factor. Our observations reported here include the fact that levels of ATM were affected by p53. In addition to its role in NHEJ, ATM is also required for normal HR (48). Our HCR assay would be expected to include a component of HR. Thus, aspects of HR could also be differentially affected by wt-p53 in normal *versus* tumor cells. A direct participation of p53 protein in DNA repair has also been proposed. A recent report demonstrates that wt-p53 protein can suppress both HR and NHEJ through a mechanism that does not require its transactivation domain (29). Thus, wt-p53 may radiosensitize normal and tumor cells differentially through this mechanism as well.

In summary, we have shown that strategies for restoring wt-p53 function in p53-defective tumor cells such as Ad-p53 may radiosensitize the cells by suppressing the error-prone NHEJ DNA repair pathway. Although radiation-induced apoptosis is also restored in this context, this may be a less important factor. Inhibition of DNA repair by overexpressed wt-p53 may be due to a general suppression of the levels of proteins required for this process. DNA repair may be differentially governed in normal cells *versus* tumor cells through the complex pathways that regulate the activity of p53 itself. A complete understanding of these effects must await future studies. However, our observations reported here underscore the need for continued development of strategies for sensitizing human tumor cells to cancer therapies that kill cells by inducing DNA damage.

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References

1. Roth, J. A. Targeting lung cancer genes: a novel approach to cancer prevention and therapy. Semin. Thorac. Cardiovasc. Surg., *5:* 178–183, 1993.

2. Chmura, S. J., Advani, S. J., Kufe, D. W., and Weichselbaum, R. R. Strategies for enhancing viral-based gene therapy using ionizing radiation. Radiat. Oncol. Investig., *7:* 261–269, 1999.

3. Crystal, R. G. Transfer of genes to humans: early lessons and obstacles to success. Science, *270:* 404–410, 1995.

4. Nielsen, L. L. and Maneval, D. C. p53 tumor suppressor gene therapy for cancer. Cancer Gene Ther., *5:* 52–63, 1998.

5. Wadhwa, P. D., Zielske, S. P., Roth, J. C., Ballas, C. B., Bowman, J. E., and Gerson, S. L. Cancer gene therapy: scientific basis. Annu. Rev. Med., *53:* 437–452, 2002.

6. Sidransky, D. and Hollstein, M. Clinical implications of the p53 gene. Annu. Rev. Med., *47:* 285–301, 1996.

7. Spitz, F. R., Nguyen, D., Skibber, J. M., Meyn, R. E., Cristiano, R. J., and Roth, J. A. Adenoviral-mediated wild-type p53 gene expression sensitizes colorectal cancer cells to ionizing radiation. Clin. Cancer Res., *2:* 1665–1671, 1996.

8. Jasty, R., Lu, J., Irwin, T., Suchard, S., Clarke, M. F., and Castle, V. P. Role of p53 in the regulation of irradiation-induced apoptosis in neuroblastoma cells. Mol. Genet. Metab., *65:* 155–164, 1998.

9. Li, J. H., Lax, S. A., Kim, J., Klamut, H., and Liu, F. F. The effects of combining ionizing radiation and adenoviral p53 therapy in nasopharyngeal carcinoma. Int. J. Radiat. Oncol. Biol. Phys., *43:* 607–616, 1999.

10. Li, J. H., Huang, D., Sun, B. F., Zhang, X., Middeldorp, J., Klamut, H., and Liu, F. F. Efficacy of ionizing radiation combined with adenoviral p53 therapy in EBV-positive nasopharyngeal carcinoma. Int. J. Cancer, *87:* 606 – 610, 2000.

11. Cowen, D., Salem, N., Ashoori, F., Meyn, R., Meistrich, M. L., Roth, J. A., and Pollack, A. Prostate cancer radiosensitization *in vivo* with adenovirus-mediated p53 gene therapy. Clin. Cancer Res., *6:* 4402 – 4408, 2000.

12. Nishizaki, M., Meyn, R. E., Levy, L. B., Atkinson, E. N., White, R. A., Roth, J. A., and Ji, L. Synergistic inhibition of human lung cancer cell growth by adenovirus-mediated wild-type p53 gene transfer in combination with docetaxel and radiation therapeutics *in vitro* and *in vivo*. Clin. Cancer Res., *7:* 2887 – 2897, 2001.

13. Kawabe, S., Munshi, A., Zumstein, L. A., Wilson, D. R., Roth, J. A., and Meyn, R. E. Adenovirus-mediated wild-type p53 gene expression radiosensitizes non-small cell lung cancer cells but not normal lung fibroblasts. Int. J. Radiat. Biol., *77:* 185 – 194, 2001.

14. Gallardo, D., Drazan, K. E., and McBride, W. H. Adenovirus-based transfer of wild-type p53 gene increases ovarian tumor radiosensitivity. Cancer Res., *56:* 4891 – 4893, 1996.

15. Pirollo, K. F., Hao, Z., Rait, A., Jang, Y. J., Fee, W. E., Jr., Ryan, P., Chiang, Y., and Chang, E. H. p53 mediated sensitization of squamous cell carcinoma of the head and neck to radiotherapy. Oncogene, *14:* 1735 – 1746, 1997.

16. Lang, F. F., Yung, W. K., Raju, U., Libunao, F., Terry, N. H., and Tofilon, P. J. Enhancement of radiosensitivity of wild-type p53 human glioma cells by adenovirus-mediated delivery of the p53 gene. J. Neurosurg., *89:* 125 – 132, 1998.

17. Smith, M. L. and Seo, Y. R. p53 regulation of DNA excision repair pathways. Mutagenesis, *17:* 149 – 156, 2002.

18. Hwang, B. J., Ford, J. M., Hanawalt, P. C., and Chu, G. 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, 1999.

19. Amundson, S. A., Do, K. T., Shahab, S., Bittner, M., Meltzer, P., Trent, J., and Fornace, A. J., Jr. Identification of potential mRNA biomarkers in peripheral blood lymphocytes for human exposure to ionizing radiation. Radiat. Res., *154:* 342 – 346, 2000.

20. Ford, J. M. and Hanawalt, P. C. 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, 1997.

21. Offer, H., Zurer, I., Banfalvi, G., Reha'k, M., Falcovitz, A., Milyavsky, M., Goldfinger, N., and Rotter, V. p53 modulates base excision repair activity in a cell cycle-specific manner after genotoxic stress. Cancer Res., *61:* 88 – 96, 2001.

22. Zhou, J., Ahn, J., Wilson, S. H., and Prives, C. A role for p53 in base excision repair. EMBO J., *20:* 914 – 923, 2001.

23. Ferguson, D. O. and Alt, F. W. DNA double strand break repair and chromosomal translocation: lessons from animal models. Oncogene, *20:* 5572 – 5579, 2001.

24. Bassing, C. H., Swat, W., and Alt, F. W. The mechanism and regulation of chromosomal V(D)J recombination. Cell, *109* (Suppl.)*:* S45 – S55, 2002.

25. Bill, C. A., Yu, Y., Miselis, N. R., Little, J. B., and Nickoloff, J. A. A role for p53 in DNA end rejoining by human cell extracts. Mutat. Res., *385:* 21 – 29, 1997.

26. Lee, H., Sun, D., Larner, J. M., and Wu, F. S. The tumor suppressor p53 can reduce stable transfection in the presence of irradiation. J. Biomed. Sci., *6:* 285 – 292, 1999.

27. Tang, W., Willers, H., and Powell, S. N. p53 directly enhances rejoining of DNA double-strand breaks with cohesive ends in γ-irradiated mouse fibroblasts. Cancer Res., *59:* 2562 – 2565, 1999.

28. Yang, T., Namba, H., Hara, T., Takmura, N., Nagayama, Y., Fukata, S., Ishikawa, N., Kuma, K., Ito, K., and Yamashita, S. p53 induced by ionizing radiation mediates DNA end-jointing activity, but not apoptosis of thyroid cells. Oncogene, *14:* 1511 – 1519, 1997.

29. Akyuz, N., Boehden, G. S., Susse, S., Rimek, A., Preuss, U., Scheidtmann, K. H., and Wiesmuller, L. DNA substrate dependence of p53-mediated regulation of double-strand break repair. Mol. Cell. Biol., *22:* 6306 – 6317, 2002.

30. Giaccia, A. J. and Kastan, M. B. The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev., *12:* 2973 – 2983, 1998.

31. D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., Gostissa, M., Coen, S., Marchetti, A., Del Sal, G., Piaggio, G., Fanciulli, M., Appella, E., and Soddu, S. Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser46 and mediates apoptosis. Nat. Cell Biol., *4:* 11 – 19, 2002.

32. Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell, *91:* 325 – 334, 1997.

33. Unger, T., Juven-Gershon, T., Moallem, E., Berger, M., Vogt Sionov, R., Lozano, G., Oren, M., and Haupt, Y. Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. EMBO J., *18:* 1805 – 1814, 1999.

34. Hofmann, T. G., Moller, A., Sirma, H., Zentgraf, H., Taya, Y., Droge, W., Will, H., and Schmitz, M. L. Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. Nat. Cell Biol., *4:* 1 – 10, 2002.

35. Prives, C. and Manley, J. L. Why is p53 acetylated? Cell, *107:* 815 – 818, 2001.

36. Vousden, K. H. p53: death star. Cell, *103:* 691 – 694, 2000.

37. Zacchi, P., Gostissa, M., Uchida, T., Salvagno, C., Avolio, F., Volinia, S., Ronai, Z., Blandino, G., Schneider, C., and Del Sal, G. The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. Nature, *419:* 853 – 857, 2002.

38. Nakamura, S., Roth, J. A., and Mukhopadhyay, T. Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. Mol. Cell. Biol., *20:* 9391 – 9398, 2000.

39. Hall, E. J. Radiobiology for the Radiologist, 5th ed., p. 588. New York: Lippincott Williams & Wilkins, 2000.

40. Joiner, M. C. Models of radiation cell killing. *In:* G. G. Steel (ed.), Basic Clinical Radiobiology, pp. 40 – 46. Boston: Edward Arnold Publishers, 1993.

41. Sharpless, N. E. and DePinho, R. A. p53: good cop/bad cop. Cell, *110:* 9 – 12, 2002.

42. Hupp, T. R., Lane, D. P., and Ball, K. L. Strategies for manipulating the p53 pathway in the treatment of human cancer. Biochem. J., *352* (Pt. 1)*:* 1 – 17, 2000.

43. Roth, J. A., Grammer, S. F., Swisher, S. G., Komaki, R., Nemunaitis, J., Merritt, J., Fujiwara, T., and Meyn, R. E., Jr. Gene therapy approaches for the management of non-small cell lung cancer. Semin. Oncol., *28:* 50 – 56, 2001.

44. Swisher, S. G., Roth, J. A., Komaki, R., Gu, J., Lee, J. J., Hicks, M., Ro, J. Y., Hong, W. K., Merritt, J. A., Ahrar, K., Atkinson, N. E., Correa, A. M., Dolormente, M., Dreiling, L., El-Naggar, A. K., Fossella, F., Francisco, R., Glisson, B., Grammer, S., Herbst, R., Huaringa, A., Kemp, B., Khuri, F. R., Kurie, J. M., Liao, Z., McDonnell, T. J., Morice, R., Morello, F., Munden, R., Papadimitrakopoulou, V., Pisters, K. M., Putnam, J. B., Jr., Sarabia, A. J., Shelton, T., Stevens, C., Shin, D. M., Smythe, W. R., Vaporciyan, A. A., Walsh, G. L., and Yin, M. Induction of p53-regulated genes and tumor regression in lung cancer patients after intratumoral delivery of adenoviral p53 (INGN 201) and radiation therapy. Clin. Cancer Res., *9:* 93 – 101, 2003.

45. Dewey, W. C., Ling, C. C., and Meyn, R. E. Radiation-induced apoptosis: relevance to radiotherapy. Int. J. Radiat. Oncol. Biol. Phys., *33:* 781 – 796, 1995.

46. Rainbow, A. J. Repair of radiation-induced DNA breaks in human adenovirus. Radiat. Res., *60:* 155 – 164, 1974.

47. Gaymes, T. J., Mufti, G. J., and Rassool, F. V. Myeloid leukemias have increased activity of the nonhomologous end-joining pathway and concomitant DNA misrepair that is dependent on the Ku70/86 heterodimer. Cancer Res., *62:* 2791 – 2797, 2002.

48. Morrison, C., Sonoda, E., Takao, N., Shinohara, A., Yamamoto, K., and Takeda, S. The controlling role of ATM in homologous recombinational repair of DNA damage. EMBO J., *19:* 463 – 471, 2000.

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