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Silencing Expression of the Catalytic Subunit of DNA-dependent Protein Kinase by Small Interfering RNA Sensitizes Human Cells for Radiation-induced Chromosome Damage, Cell Killing, and Mutation

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Abstract

Targeted gene silencing in mammalian cells by RNA interference (RNAi) using small interfering RNAs (siRNAs) was recently described by Elbashir *et al.* (S. M. Elbashir *et al.*, Nature (Lond.), *411:* 494–498, 2001). We have used this methodology in several human cell strains to reduce expression of the *Prkdc* (*DNA-PKcs*) gene coding for the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) that is involved in the nonhomologous end joining of DNA double-strand breaks. We have also demonstrated a radiosensitization for several phenotypic endpoints of radiation damage. In low-passage normal human fibroblasts, siRNA knock-down of DNA-PKcs resulted in a reduced capacity for restitution of radiation-induced interphase chromosome breaks as measured by premature chromosome condensation, an increased yield of acentric chromosome fragments at the first postirradiation mitosis, and an increased radiosensitivity for cell killing. For three strains of related human lymphoblasts, DNA-PKcs-targeted siRNA transfection resulted in little or no increase in radiosensitivity with respect to cell killing, a 1.5-fold decrease in induced mutant yield in TK6- and p53-null NH32 cells, but about a 2-fold increase in induced mutant yield in p53-mutant WTK1 cells at both the hypoxanthine quanine phosphoribosyl transferase (hprt) and the thymidine kinase loci.

Introduction

Isolation and characterization of mammalian cell mutants hypersensitive to ionizing radiation has provided key evidence that a principal underlying defect involves faulty processing of DNA double-strand breaks (1–3). Many of the genes involved and their products are now known, including both the main system of NHEJ,[4] and homology directed repair (4). For prokaryotic or lower eukaryotic cells, the ability to obtain and study double or even triple mutants has been very useful, but such mutants are not easily obtained for mammalian cells, and in some cases even single (let alone multiple) mutant phenotypes are lethal. Last year, Elbashir *et al.* (5) reported a highly specific targeted gene silencing in mammalian cells by RNAi using siRNAs. To examine the potential general use of this approach for studying the genetic control of radiosensitivity in human cells and in what situations comparisons could be made easily with cells of identical genetic backgrounds, we targeted the mRNA transcript of the *Prkdc* (*DNA-PKcs*) gene coding for the DNA-PKcs. This protein is central to the NHEJ process as well as being involved in the maintenance of telomere stability (6, 7). The aim for the present experiments was to measure the effectiveness of this siRNA approach for "knocking down" DNA-PKcs levels using Western blot analysis and immunocytochemistry and, also, to measure functional (phenotypic) effects on radiosensitization. The latter included measurements to determine whether the knock-down resulted in (*a*) reduced interphase (G_1) chromosome break restitution as measured by PCC 4 h after irradiation; (*b*) increased frequencies of chromosome aberrations in the first postirradiation mitosis; and (*c*) increased cell killing in low-passage normal human fibroblasts. In addition, experiments were included to detect changes in radiation-induced mutant yields as well as cell killing in three strains of TK6 human lymphoblasts differing in p53 status.

Materials and Methods

Cells

Low-passage normal human fibroblast cultures GM08399 were obtained from the National Institute of General Medical Sciences Coriell Cell Repositories. These cells were routinely grown in αMEM containing 15% fetal bovine serum as described previously (8–10). Three strains of WIL2 lymphoblast-derived cells were used. The TK6, WTK1, and NH32 derivatives are heterozygous for the *thymidine kinase* gene ($TK^{+/-}$). WTK1 cells produce a mutant form of p53, and NH32 cells are p53 null. Growth and use of these cells for mutagenesis assays were identical to the procedures described on previous occasions (11–14).

siRNA Transfections

The siRNA sequence used for targeted silencing of *DNA-PKcs* was chosen as described by Tuschl (http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna_u.html) and recommended by the siRNA supplier (Dharmacon). We used two duplex siRNA sequences separately or together. One was targeted 352 bases downstream from the start codon. This double-stranded siRNA was

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GAUCGCACCUUACUCUGUUdTdT
dTdTCUAGCGUGGAAUGAGACAA.
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The other siRNA, targeted to the kinase domain, was

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CUUUAUGGUGGCCAUGGAGdTdT
dTdTGAAAUACCACCGGUACCUC.
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Searches of the human genome database (BLAST) were carried out to ensure the sequences would not target other gene transcripts. The concentration of siRNAs was 0.15 μM during transfections, which were facilitated by Oligofectamine (In Vitrogen), also according to the protocol of Tuschl and the siRNA supplier. For controls, we used Lamin A/C or Ku80-targeted siRNAs, or, in more recent experiments, a *DNA-PKcs*-derived sequence in which 2 or 3 bp were changed. None of these control siRNAs affected DNA-PKcs protein levels or the radiosensitivity phenotypes studied. Because protein turnover rates will determine the optimal transfection and irradiation- or assay-timing protocol, we carried out preliminary experiments to establish that two successive transfections of log phase of cultures (~50% confluent) at 2-day intervals produced marked reductions in DNA-PKcs. Of course, other protocols we have not tried may also produce similar or superior DNA-PKcs knock-downs. In most experiments with the fibroblasts, 2 days after inoculation and 1 day after siRNA transfection, cells were subcultured to one-half of the cell density and transfected again 1 day later. Then, 3 days later, when irradiations were carried out and other assays performed, cells were still

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[4] The abbreviations used are: NHEJ, nonhomologous end joining; RNAi, RNA interference; siRNA, small interfering RNA; PCC, premature chromosome condensation; DNA-PKcs, catalytic subunit of DNA-dependent protein kinase; DAPI, 4′,6-diamidino-2-phenylindole.

growing and cultures were about 80–90% confluent. In one of the human normal fibroblast experiments, the cells were not subcultured after the first transfection; after the second transfection, cells were allowed to reach confluence, and the following day, the irradiations were carried out. Then, 1 day later, the cells were subcultured, and aberrations were scored after colcemid collection of cells in the first postirradiation mitosis, as reported in Fig. 2*C*.

During the 6-day siRNA transfection protocol for the fibroblasts, in preparation for irradiation or other assays, we estimate that the growth rate (doubling time) of the DNA-PKcs-targeted siRNA-transfected cell populations decreased by ~10% relative to control (altered sequence) siRNA transfection, but the growth was interrupted twice for ~20 h for each transfection in serum-free medium; and in most cases, cells were subcultured to one-half density between transfections. For the lymphoblast-derived cell lines, the siRNA transfections had no effect on the cell population growth.

Western Blot and Immunocytochemistry

For Western blot analysis, cells were prepared as described by Song *et al.* (15). Gels were loaded with 20 μg of protein and, after electrophoresis in 6% Tris-glycine polyacrylamide gels, proteins were transferred to nitrocellulose membranes. After blocking in Tris-buffered saline (TBS) containing 7% nonfat dry milk, membranes were incubated in TBS with 5% nonfat dry milk containing mouse antibodies against DNA-PKcs (AB-4) or Ku 80 (Ab2) obtained from Neomarkers. Goat antimouse secondary antibodies labeled with horseradish peroxidase were then used to bind the primary antibodies, and detection was by chemiluminescence (ECL kit; Amersham Pharmacia). X-ray films to detect the chemiluminescence were then exposed for appropriate times depending on luminescence intensity.

For immunocytochemistry, after the siRNA transfections and at the time selected for optimum knock-down, human fibroblast cultures of attached cells were fixed *in situ* in 4% paraformaldehyde in PBS and were then permeabilized in PBS containing 0.2% Triton X-100. In the case of lymphoblast-derived cells, which grow in suspension culture, cells were prepared for analysis after being centrifuged onto slides (Cytocentrifuge) before fixation in a manner similar to that described above. To detect DNA-PKcs, the same primary antibodies were used, but detection in this case was with rhodamine-labeled goat antimouse IgG and was viewed by fluorescence microscopy.

Chromosome Damage Assays

Interphase PCC. Four h after irradiation of confluent cultures, to allow for completion of much of the postirradiation damage processing, cells were resuspended and fused to mitotic HeLa cells to induce PCC, and the total number of PCCs and fragments was scored as described previously (8–10, 16). The frequency of "excess PCC fragments per cell" was then estimated by subtracting the total number per cell in unirradiated samples from that for irradiated samples. For unirradiated cells, the total number of PCCs per cell was tightly distributed around 45–46.

Mitotic Cells. In the same experiments described for PCC break measurements, other parallel samples were subcultured and allowed to progress to mitosis for scoring aberrations in metaphase cells. Samples were fixed after incubation in the presence of 0.1 μg/ml Colcemid during the intervals 18–22, 22–26, 26–30, and 30–34 h after subculture. We have shown that virtually no second-division cells are present during these fixation intervals after irradiation, incubation, and subculture of these contact-inhibited cells. Aberration scoring was carried out as described previously (10, 17–19).

Cell Survival

Cell survival responses were measured after irradiation of log phase cultures using colony formation as the criterion for cell survival. The siRNA transfection protocol for log phase cultures was similar to that for the treatment of the contact-inhibited plateau-phase cultures except, as mentioned earlier, cells were subcultured after the first siRNA transfection so that they would still be growing and cultures would be ~80% confluent 2 days after the second transfection.

Mutation Assays

Lymphoblast cells were transfected with siRNA as described above. The next day (day 1), they were treated with CHAT medium to eliminate preexisting hprt and tk mutants. A second siRNA treatment was performed on day 3, and cells were used for irradiation on day 5. Cells were untreated or irradiated with 1, 3, 5, or 7 Gy for cytotoxicity experiments, or 1.5 Gy for mutation experiments.

To determine surviving fractions, cells were seeded into 96-well microtiter dishes immediately after treatment, at concentrations ranging from 1 to 10,000 cells per well, depending on the expected surviving fractions.

To determine mutation frequencies, cells were grown in nonselective media for 3 or 6 days to allow phenotypic expression of newly induced mutants at tk or hprt, respectively. At those times, cells were seeded at high density in 96-well microtiter dishes in the presence of trifluorothymidine to measure mutation at tk, or 6-thioguanine to measure mutation at hprt. Cells were also seeded at low density to determine plating efficiency. Plates were scored 12–20 days later, and mutant fractions were calculated as described previously (11).

Results and Discussion

Studies Using Low-Passage Normal Human Fibroblasts. Fig. 1*A* shows a Western blot analysis for one experiment in which two different concentrations were used of the siRNA targeted to DNA-PKcs mRNA, as well as a Ku80-targeted siRNA or a mock transfection without siRNA. The blots were incubated simultaneously with mouse anti-DNA-PKcs and mouse anti-Ku80 antibody followed by detection with horseradish peroxidase-labeled goat antimouse IgG and a chemiluminescence assay. A more severe knock-down of DNA-PKcs was seen for the 0.15 μM concentration of siRNA targeted to DNA-PK_{cs} than for the 5-fold lower (0.03 μM) concentration. In several other experiments, the level of knock-down of DNA-PKcs varied somewhat depending on the protocol. No effect of DNA-PKcs-targeted siRNA transfection on Ku 80 was seen, nor did Ku 80-targeted siRNA affect DNA-PKcs levels. Similar results were obtained in other experiments showing a lack of cross-reactivity with Lamin A/C-targeted siRNAs (data not shown), although, in those cases, the Lamin A/C-targeted siRNA drastically reduced Lamin A/C protein. In the experimental result shown here, Ku80 protein was only partially reduced by Ku80-targeted siRNA. In this case, either the Ku80-targeted siRNA sequence was relatively ineffective or the turnover time of Ku80 was sufficiently different that the timing, or other aspects of the transfection protocol, were suboptimal for that protein.

Fig. 1*B*) shows two fluorescence microscope fields from an experiment in which DNA-PKcs was measured by immunocytochemistry. The siRNA transfection protocol was similar to that used for the result shown in Fig. 1*A* but was from an experiment carried out on another occasion. In Fig. 1*B*, *panels a* and *b* are the same field of cells from a mock-transfected culture showing the cells with a DAPI filter to identify DAPI-stained nuclei (Fig. 1*B*, *a*); and with a rhodamine filter to identify nuclei containing measurable DNA-PKcs (Fig. 1*B*, *b*). Of 156 cells examined, strong DNA-PKcs signals appeared in 85% of the cells, weaker signals in 12%, and no detectable signals in 3%. In cells transfected with DNA-PKcs-targeted siRNA (Fig. 1*B*, *c* and *d*), it appeared that one cell was virtually unaffected, (normal level of DNA-PKcs) another showed reduced DNA-PKcs, but the rest did not have obviously detectable levels. Scoring numerous other microscope fields from this experiment (322 cells total) indicated an average of ~22% unaffected cells (strong signals comparable with controls mentioned above); or with slightly reduced levels of DNA-PKcs, ~13% had appreciably reduced levels, and the remainder had no visibly detectable levels. Similar results were obtained in several replicate experiments, although the proportion unaffected differed. DNA-PKcs is a very abundant protein in human cells and the immunocytochemical detection efficiency range in our experiments from minimum to maximum is not known. For this reason and known nonlinearities in film densitometry (at both low- and high-exposure levels), the Western blot and immunocytochemical analyses in

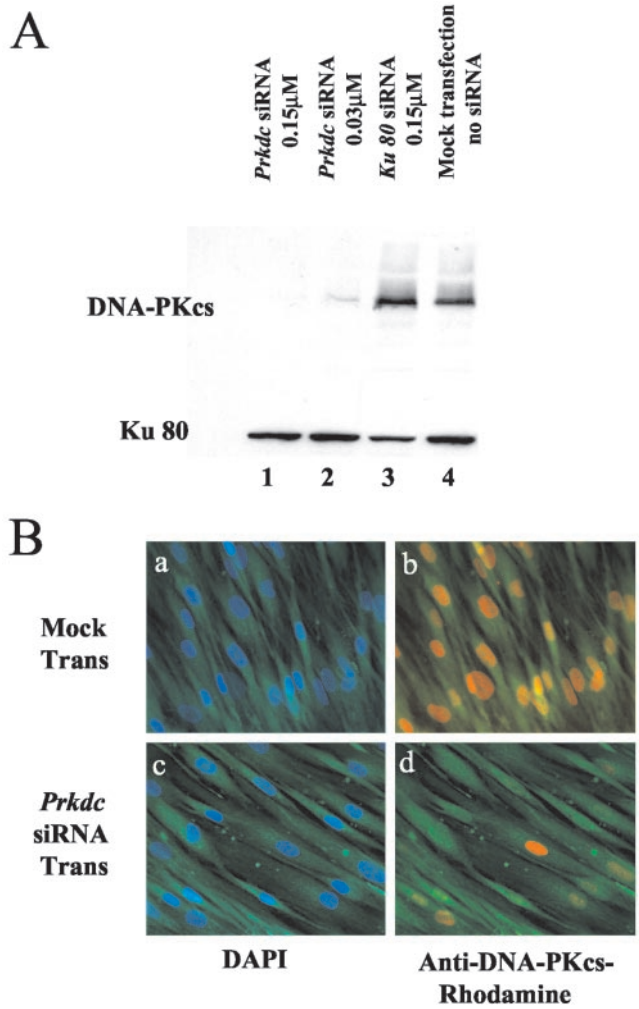

Fig. 1. Expression of DNA-PKcs in low-passage normal human fibroblasts (GM08399) after transfection of siRNA targeted to DNA-PKcs mRNA. *A*, a Western blot from cells after two Oligofectamine-mediated transfections (see "Materials and Methods"); the measurement of both DNA-PKcs and Ku80 protein from cells using siRNA targeted to DNA-PKcs at a concentration of 0.15 μM (*Lane 1*) and at a concentration of 0.03 μM (*Lane 2*). *Lanes 3* and *4*, respectively, cells that were transfected with Ku80-targeted siRNA or mock transfected (two Oligofectamine treatments but without RNA). *B*, fluorescence microscope images of cells either mock transfected (*Mock Trans*, *a* and *b*, same cells) or transfected twice with siRNA targeted to DNA-PKcs (*c* and *d*, same cells). Cells were stained with DAPI (*blue nuclei*) and Vybrant CFDA SE (*green cytoplasm*) and with rhodamine-labeled mouse anti-DNA-PKcs; photographs were taken with DAPI and FITC filters and images merged (*a* and *c*) or with a rhodamine filter and an FITC filter and merged (*b* and *d*).

the present study are not quantitatively comparable. The immunocytochemistry was carried out principally for the purpose of indicating heterogeneity among cells, which was not reflected in Western blots. Another unknown for these studies is the quantitative level of DNA-PKcs reduction necessary for radiosensitization.

Fig. 2, *A* and *B*, shows the result of an experiment to test the effect of DNA-PKcs-targeted knock-down on the rejoining of broken chromosomes during interphase as measured by inducing PCC 4 h after irradiation. Because the immunocytochemical studies showed that the cells in the population irradiated were not likely to be uniformly reduced in DNA-PKcs, we might expect differences among cells in levels below which radiosensitization occurs and, therefore, a nonuniform radiosensitization. Cytogenetic assays such as those carried out to obtain the results shown in Fig. 2, *A* and *B*, are capable of detecting such heterogeneity because damage (excess PCC fragments) is measured in each individual cell. Fig. 2 shows for control (*A*) and siRNA-transfected cells (*B*) the distribution of cells with various numbers of excess fragments 4 h after irradiation with 5 Gy of ^{137}Cs γ rays. Each chromosome break results in an excess fragment. After some time elapses for rejoining, remaining excess fragments can result from either unrejoined breaks or, as is often the case, for longer times in normal human cells, from mis-rejoined breaks that result in acentric or centric rings, *i.e.*, asymmetrical intra-arm intrachanges (8, 10). At the shortest time measurement possible after irradiation with the PCC system (~20 min), a dose of 5 Gy results in an average of ~25–30 excess PCC fragments per cell for several other human fibroblast cell strains, and the excess fragment frequency decreases because of rejoining with a half-time of about 1.5 to 1.7 h (8–10). If these fibroblasts were similar in response, we would expect some five or six excess fragments per cell at the 4-h sample time after a dose of 5 Gy. Fig. 2*A* shows that an average of about eight excess fragments per cell remained, a value not far from the expectation. For cells transfected with DNA-PKcs-targeted siRNA (Fig. 2*B*), the distribution of excess fragments appeared to be bimodal with one peak around 8 and another around 18 excess fragments per cell. These results suggest a sensitization factor of about 2-fold (for the subpopulation sensitized) assuming an approximately linear dose response in the 5-Gy dose range for excess fragments 4 h after irradiation.

The result of an experiment to measure radiosensitivity for induction of chromosomal aberrations measured in the first post-irradiation mitosis, is shown in Fig. 2*C*. We would expect fewer excess acentric fragments than in the interphase PCC experiments because cells were incubated 24 h after irradiation and are required to progress to mitosis for the assay. For nontransfected cells, there were about 0.8 induced dicentrics (a dicentric is scored as one dicentric plus its associated acentric fragment) and 1.0 deletions per cell (acentric fragments not associated with dicentrics or centric rings). For DNA-PKcs-targeted siRNA-transfected cells, we observed about 1.0 induced dicentric and 1.9 deletions per cell. This represents a nearly 2-fold increase in excess fragments or deletions for the DNA-PKcs-targeted siRNA-transfected cells. In this experiment, we did not observe the greatly

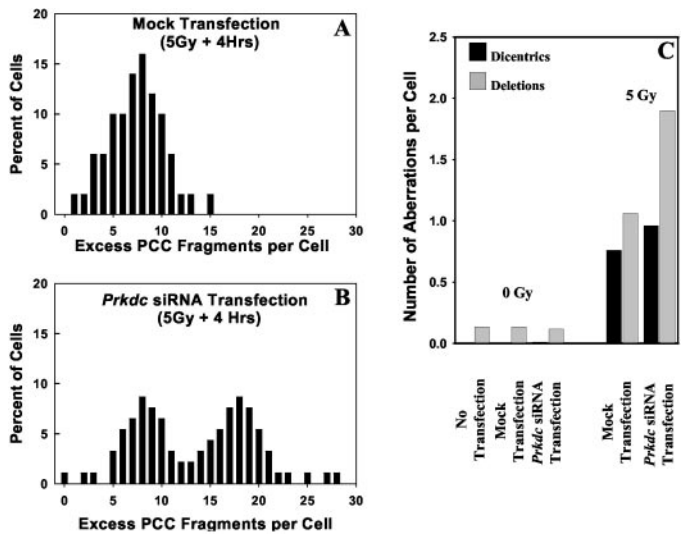

Fig. 2. Changes in chromosomal radiosensitivity of GM08399 human fibroblasts after two transfections with siRNA targeted to DNA-PKcs mRNA. Excess PCC fragments in interphase G_1 cells 4 h after 5-Gy Cs-137 γ rays were measured in mock-transfected cells (*A*) or cells transfected with siRNA targeted to DNA-PKcs mRNA (*B*). Approximately 50–60% of the cells apparently suffered sufficient knock-down of DNA-PKcs to impair their ability to rejoin broken chromosomes or to result in excessive incorrect rejoining to yield other excess fragments. *C*, the result of an experiment in which chromosome-type aberrations were scored after cells entered their first postirradiation mitosis. Dicentrics (*solid bars*) and deletions (*shaded bars*) were not produced by either mock or siRNA transfections alone. Five Gy of γ rays increased the dicentric yield, although not significantly more in the siRNA- than in the mock-transfected cells. In contrast, deletions from either unrejoined chromosome breaks or interstitial deletions (asymmetric intra-arm intrachanges) were increased about 2-fold by transfection with siRNA targeted to DNA-PKcs mRNA.

increased frequency of induced chromatid-type aberrations after irradiation of G_1 or G_0 cells that is usually seen for X-ray-sensitive cells with defects in NHEJ (3, 20–22).

The result of an experiment to examine DNA-PKcs-targeted siRNA radiosensitization with respect to cell killing is shown in Fig. 3. In addition to the heterogeneity in radiosensitivity always present for log phase cultures because of cell cycle-dependent variation in radiosensitivity, and despite some heterogeneity in the proportion of cells with severe or intermediate levels of DNA-PKcs knock-down, there was still a marked radiosensitization of DNA-PKcs-targeted siRNA-transfected cells relative to the two control cell populations. As expected, this was most pronounced in the low-dose region (0–1 Gy), in which survival in mixed heterogeneous cell populations is caused by the killing of the most sensitive subpopulations.

Also shown in Fig. 3 is a curve that passes through the survival estimates for the DNA-PKcs-targeted siRNA-transfected cells that was not fitted to the data but represents a curve for a mixed population of cells consisting of a fraction (20%) of unaffected cells with a radiosensitivity corresponding to curve *R*, and a fraction (80%) of sensitized cells whose response, curve *S*, was derived from the slope of the curve-sensitized cells in the low-dose region between 0 and 0.5 Gy. Thus, despite what appears as a greater knock-down of DNA-PKcs from the immunochemical results illustrated in Fig. 1, we suggest that this result indicates that DNA-PKcs is present in fairly large excess, far above that needed to confer normal radioresistance.

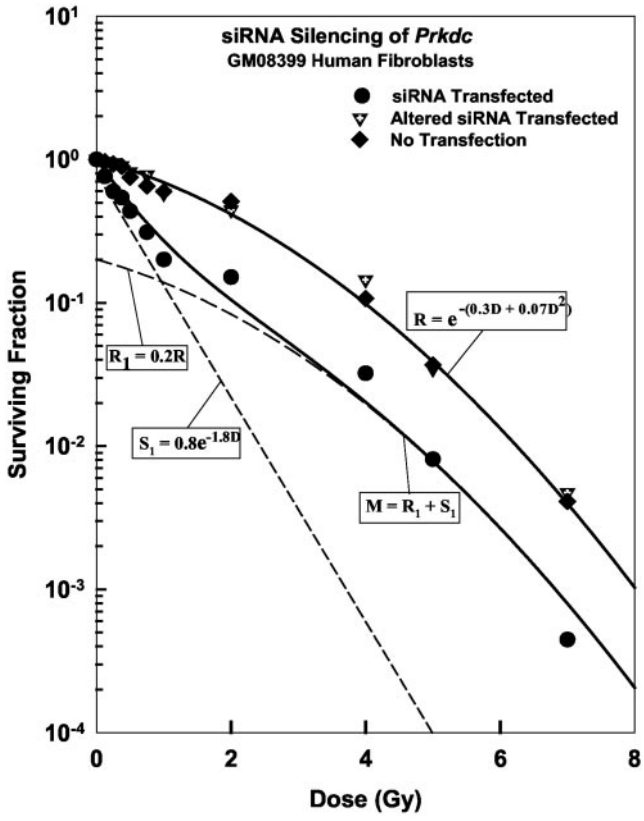

Fig. 3. Sensitization of GM08399 human fibroblasts with respect to cell killing by transfection with siRNA targeted to DNA-PKcs mRNA. Cell survival was measured by colony formation over a range of doses (0–7 Gy) of Cs-137 γ rays after transfection with siRNA targeted to DNA-PKcs mRNA (●, *lower solid curve*), a control siRNA in which the DNA-PKcs siRNA target sequence was altered slightly, as described in the "Results and Discussion" (*inverted triangles, upper solid curve*), or not transfected (◆, *upper solid curve*). The *lower solid curve* (*M*), which reasonably fits the data, is a composite curve that would be expected from a mixed population of cells consisting of 20% with the sensitivity of the cells that did not experience a DNA-PKcs knock-down (*curve labeled* R_1) and 80% with a sensitivity illustrated by *curve* S_1 estimated from the survival estimates for the sensitized cells in the low-dose (0–0.5 Gy) range. R and M represent surviving fraction, and D represents the doses.

It is also worth noting that one of the "control" cell populations in this experiment was not a "mock" transfection but, instead, cells that were transfected with a siRNA control duplex ($siRNA_c$) in which an adenine-uracil bp was substituted for a uracil-adenine bp in position 3 from the 5′ end of the strand of the duplex siRNA equivalent to the mRNA target, and also in which an AC sequence was replaced with a CA sequence in positions 12 and 13 from the 5′ end in the same strand. Transfection with this $siRNA_c$ duplex did not radiosensitize the cells, nor did it knock-down DNA-PKcs as measured in Western blots.

Toxicity and Mutagenesis Studies in Lymphoblast Cells. In our original report of the isolation of NH32 cells, we found that the background MF and the X-ray-induced MF were very similar to TK6, with both lines being much lower than WTK1 (14). However, in this study, we incubated the dishes for longer times (20 instead of 17 days), and observed a substantial increase in the MF for NH32. (The additional incubation time does not affect the MF at TK6 or WTK1.) Therefore, we now conclude that background and induced MF in these cell lines are: WTK1 (ile237 p53) > NH32 (p53 null) > TK6 (p53 wild-type).

As can be seen in Fig. 4*A*, knock-down of DNA-PKcs was achieved in all three of the lymphoblast lines. This result was mirrored in Western blot studies (data not shown). As expected, the two lines with mutant or null p53 were more resistant to radiation-induced cell killing than was the TK6 p53 wild-type. However, in all three lines, there was little or no effect of knocking down DNA-PKcs on cell killing, (data not shown). Surviving fractions were somewhat lower in cells transfected with siRNAs, with values ranging from 49 to 76% of those for the untransfected or mock-transfected cells. However the slopes of the survival curves for each particular cell line were not significantly different, with or without knock-down by siRNA.

Fig. 4*B* shows the results obtained for mutagenesis at the hprt and tk loci. At X-linked hprt, spontaneous mutations consist of one-half large deletions in both TK6 and WTK1 (23). The spectrum for NH32 cells is not known. In the present study, we saw no evidence that DNA-PKcs knock-down affected the quantitative levels of spontaneous mutations at either the hprt or the tk loci. After irradiation, however, there were clear, statistically significant differences for the mutation frequencies observed at the tk locus. The MF after knock-down was decreased in TK6 ($P = 0.002$ for the comparison of mock treated with siRNA), and in NH32 ($P < 0.001$), but, in contrast, the MF increased in WTK1 ($P = 0.004$). Although none of the MFs were significantly different at the hprt locus, nevertheless, there was a similar trend, in that TK6 ($P = 0.10$) and NH32 ($P = 0.10$) were lower and WTK1 ($P = 0.06$) was higher.

It is not straightforward to predict what effect the perturbation of the NHEJ system will have on the mutagenic impact of double-strand breaks. When damage is sustained on the X chromosome, for a cell that has not yet replicated the region (*i.e.*, in G_1 or early S phase), we would imagine that either it will die or it will require some kind of alternative end-joining reaction. In the former case, we would expect the observed MF to be reduced. In the latter case, it could be either increased or decreased, depending on the "error-proneness" of the mechanism, relative to NHEJ. We would have similar expectations for damage on chromosome 17, where tk is located, although it is more complicated because cells in G_1 or early S phase might be able to make use of the homologous chromosome as a template for repair. The fact that mutation was increased in WTK1 is intriguing. It is tempting to speculate that disruption of NHEJ, with the presence of large amounts of mutant p53, led to the operation of a more mutagenic mechanism.

Other approaches, involving stable expression of interfering RNAs for selective knock-down of gene products by introducing vectors that stably integrate and express interfering double-strand RNA hairpins

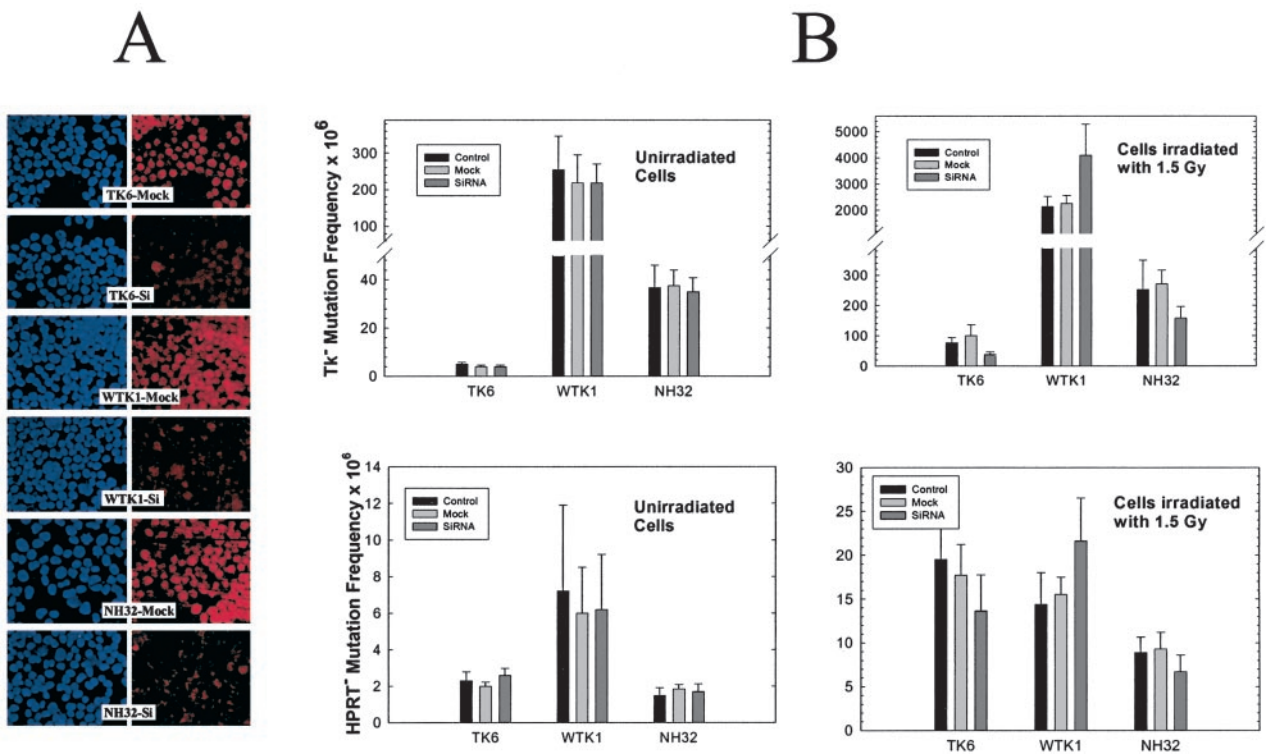

Fig. 4. Mutagenesis studies in human lymphoblast cell lines with altered DNA-PKcs expression. *A*, cells were mock transfected or transfected with siRNA as described in "Materials and Methods." The *left panel* in all cases is a DAPI stain; the *right panel* is immunofluorescence with antibody to DNA-PKcs. Cells were examined 3 days after transfection. *B*, spontaneous and induced mutation frequencies in lymphoblast cells. Experiments were performed 5 days after the initial transfection. Irradiation was with 1.5 Gy γ-irradiation. *Error bars*, SDs.

(24, 25), may offer some advantages over the transient transfection methods used in the present studies, but there may also be some disadvantages. With a stable expression approach, for example, if sufficient expression is achieved to produce a phenotypic change, and the cellular life span is not limiting, it should be possible to obtain clonal populations in which there is much less heterogeneity in levels of knock-down among cells than is possible with the transient expression knock-down. The transient approach, on the other hand may be more appropriate if the long-term knock-down is lethal, or for studies involving multiple gene silencing with little or no restriction on the choice of cells for study; therefore, it should be possible, for example, to study effects on cells with the same or different genetic backgrounds. Clearly the new approaches for RNAi in mammalian cells has many obvious applications as well as its intrinsic interest regarding regulation of gene expression in general.

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