Suppression of DNA-PKcs and Ku80 individually and in combination: Different effects of radiobiology in HeLa cells

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Abstract. DNA-dependent protein kinase (DNA-PK), including Ku80, Ku70 and DNA-PK catalytic subunit (DNA-PKcs), is the key protein in non-homologous end-joining (NHEJ) after DNA double-strand breaks (DSBs) appear. In this study, small hairpin interfering RNAs (siRNAs) targeting Ku80 and DNA-PKcs were used both individually and in combination, to explore the effects of these DSB proteins on HeLa cell functional changes after X-ray irradiation. HeLa cells co-transfected with Ku80-siRNA and DNA-PKcs-siRNA were more radiosensitive than the ones transfected individually. HeLa in the absence of Ku80 and pretreated with LY294002, a chemically specific PI 3-kinase inhibitor, resulted in cells that were even more sensitive to X-rays than HeLa/Ku80-siRNA transfected with DNA-PKcs-siRNA. The cells inhibited by Ku80 either individually or in combination with DNA-PKcs showed cell accumulation in the G2/M phase 48 h post-irradiation, similarly to control cells. However, cells transfected with DNA-PKcs-siRNA or pretreated with LY294002 had a prolonged G2/M delay, suggesting the accumulation of significant un-repaired DNA damage following inhibition of DSB repair proteins. In conclusion, these data indicate that the role of Ku80 in DSB repair could be compensated by other DSB repair proteins; co-inhibition would be a suitable strategy to enhance the radio-sensitivity of cancer cells.

Introduction

DNA double-strand breaks (DSBs) are generally regarded as the most lethal of all DNA lesions after radiation and are repaired by two major repair pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ) (1-3). It is generally believed that NHEJ plays a more important role than HR in mitotically replicating cells, especially in human cells (4). The known NHEJ proteins include DNA-dependent protein kinase (DNA-PK), ligase IV, and its cofactor XRCC4, among others. DNA-PK is a serine threonine kinase that contains a Ku heterodimer (Ku70 and Ku80, Ku80 also named Ku86) and a DNA-PK catalytic subunit (DNA-PKcs), and is crucial to the NHEJ process and in the maintenance of telomere stability (3,5). Correspondingly, the ataxia-telangiectasia mutated (ATM) protein plays a main role in HR that is only a pre-emergency DSB repair mechanism in human cells during late S, G2 and M phase (6,7). In contrast, NHEJ is present at all times during the entire cell circle, especially in the G0/G1 and early S stages (8,9). When DSBs are produced by irradiation, the Ku hetero-dimer, which has a high affinity for DNA, can bind preferentially to the free DNA ends, possibly to prevent nuclease attack; subsequently, the conformation of Ku changes, allowing it to interact with the DNA-PKcs/Artemis complex (5). This complex acts as an endonuclease at both the 5' and 3' overhangs, and trims the 5' overhangs, resulting in a blunt end. In contrast, the 3' overhangs are clipped with a preference for leaving a 4- or 5-nuleotide single-stranded overhang (10). After autophos-phorylation, DNA-PKcs recruits XRCC4/ligase IV complex to process the DNA ends and to initiate re-ligation to form a single DNA molecule (5,11,12). Thus, targeted inhibition of DSB repair proteins is an attractive approach in the development of potent radiation therapy strategies (13,14).

Previous studies have investigated the consequences of the deficiencies of individual components of DNA-PK complex for DSB repair. However, only a few investigations have focused on co-inhibition. For prokaryotic or lower eukaryotic cells, the ability to obtain and study double or even triple mutants has been useful, but such mutants are not easily obtained for mammalian cells, as sometimes even single (let alone multiple) mutant phenotypes prove lethal (15). RNA interference (RNAi), which can highly specifically target gene silencing in mammalian cells using small interfering RNA (siRNA), has become a powerful tool in studying the cell function of any gene (16,17). In this study, siRNA targeting Ku80 and DNA-PKcs were used both individually and in combination, to explore the effects of these DSB proteins on HeLa cell functional changes after X-ray irradiation. Because DNA-PKcs and ATM are both members of the PI 3-kinase gene family (18), a chemically

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specific PI 3-kinase inhibitor named as LY294002, which can increase radiosensitivity by blocking the activity of both DNA-PKcs and ATM (19,20), was applied in our study.

Materials and methods

Cell line and cell culture. Human cervical adenocarcinoma cell line HeLa was obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen), 50 U/ml penicillin, and 50 μg/ml streptomycin. The HeLa cells were maintained in a humidified 37°C incubator with 5% CO_2, fed every two to three days with a complete medium (contain 10% FBS).

Plasmid construction. The pGenesil-1 (which was pEGFP-C1 inserted into a human U6 promoter by Genesil Biotechnology Co., Ltd, Wuhan, China) was used for the construction. The fragments, which transcribed the siRNA, are shown below (nucleotides and the sense and antisense sequences that will form a hairpin structure upon transcription inside cells are underlined). They were designed using a computer program available on the Internet (http://www.ambion.com/techlib/misc/siRNA_finder.html) and the principle reported by (17) Ku80-siRNA was the sense: 5'-GATCC<u>GACAGA CACCCTTGAAGAC</u>TTCAAGACG<u>GTCTTCAAGGGTGT CTGTC</u>TTTTTGTCGACA-3' and the antisense: 5'-AGCTT GTCGACAAAAAGACAGACACCCTTGAAGACCGTCTT GAAGTCTTCAAGGGTGTCTGTCG-3'. Negative-siRNA was the sense: 5'-GATCC<u>GACTTCATAAGGCGCATGC</u>TT CAAGACG<u>GCATGCGCCTTATGAAGTC</u>TTTTTGTCG ACA-3' and the antisense: 5'-AGCTTGTCGACAAAAAGACT TCATAAGGCGCATGCCGTCTTGAAGCATGCGCCTTA TGAAGTCG-3'. These above siRNA-encoding complementary single-stranded oligonucleotides, which were hybridized to give *BamH*I- and *Hind*III-compatible overhangs, then were ligated into pGenesil-1. Bacterial colonies were pooled and used for the plasmid preparation. The positive clones were confirmed by sequencing. The constructed plasmid, which transcripted DNA-PKcs-siRNA, was presented by Dr Xiao-Yu Tian, who applied RNAi-Ready pSIREN-DNR-DsRed-Express Donor Vector (Clontech, USA), which could express red fluorescent proteins for construction according to the manufacturer's protocol. The siRNA-encoding complementary single-stranded oligonucleotide fragment, which had *BamH*I and *EcoR*I overhang, had sense: 5'-GATCC<u>CTTTATGGTGG CCATGGAGTTCAAGAGA</u>CTCCATGGCCACCATAAAG TTTTTTGGAAG-3' and antisense: 5'-AATTCTTCCAAAA AACTTTATGGTGGCCATGGAGTCTCTTGAACTCCAT GGCCACCATAAAGG-3'.

Transfection of cells. Plasmids were transfected into cells according to the manufacturer's protocol of Lipofectamine™ 2000 (Invitrogen). In short, one day prior to transfection, cells were seeded without antibiotics in a 6-well plate at $3x10^5$ cells/ well, corresponding to a density of 70-80% at the time of transfection. Plasmids (3 μg) and 6 μl Lipofectamine 2000 were mixed with DMEM without FBS and incubated for 5 min at room temperature. These reagents were combined

and incubated for 20 min before being added to the cells in DMEM without FBS, penicillin or streptomycin. Cells were incubated at 37°C for 4 h, then fresh DMEM with 10% FBS was added. While the cells that transfected Ku80-siRNA and negative-siRNA were planted into the 6-well plates again at $1x10^5$ cells/well, DMEM with 10% FBS and 800 μg/ml G418 (Gibco, USA) were added, resulting in a concentration of G418 that decreased to 400 μg/ml when the cell clones were formed after 8-12 days of growth. The clones expressing GFP were selected out to maintain cultivation. Finally, two stable transfected HeLa cell clones (named HeLa/Ku80-siRNA and HeLa/Neg-siRNA) were collected for the next study. In order to ascertain the transfection efficiencies of cells stably transfected with siRNA-encoding plasmids, the green fluorescence of these cells was quantified by fluorescence-activated cell sorting (FACS) analysis. Cells (including HeLa/Neg-siRNA and HeLa/Ku80-siRNA) transfected with DNA-PKcs-siRNA were harvested at 24, 48 and 72 h after transfection for Western blot analysis.

Western blot analysis. Cells were harvested after transfection and whole cell proteins were isolated via M-PER™ mammalian protein extraction reagent (Pierce, Rockford, IL). Protein concentrations were determined with a BCA protein assay kit (Pierce) according to the manufacturer's instructions. An equal amount of total protein (50 μg) from each lysate was loaded onto 6-10% SDS-polyacrylamide gel. After electrophoresis, the separated proteins were transferred to nitrocellulose membranes, which were then blocked with TBS-T (0.1 μmol/l Tris-HCl, pH 7.5; 0.9% sodium chloride; 0.05% Tween 20) containing 5% powdered non-fat milk for 1 h at 37°C. Then this was incubated with mouse anti-human monoclonal antibodies against Ku80 (NeoMarkers, 1:2,000 dilution), DNA-PKcs (NeoMarkers, 1:2,000 dilution), or β-actin (Santa Cruz, 1:2,000 dilution) at 4°C overnight. After washing with TBS-T for 4x15 min, nitrocellulose membranes were incubated with 1:5,000 dilution horseradish peroxidase (HRP)-labeled goat anti-mouse second antibody for 1 h at room temperature and washed with TBS-T for 4x15 min. Immunodetection was performed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and the density of the bands in the resulting film was quantified using the NIH image analysis.

Clonogenic survival assay. Cells were plated in triplicate onto 60-mm dishes at the required concentration to get 50-100 colonies per dish and were allowed to attach for 24 h. HeLa, HeLa/Neg-siRNA, HeLa/Ku80-shNRA cells and cells pretreated with 50 μmol/l LY294002 (Promega) for 30 min or transient were transfected with DNA-PKcs-siRNA for 48 h and were exposed to 0, 1, 2, 3, 4, 6, and 8 Gy X-ray. Then the cells were cultured for 10 to 14 days in 5% CO_2 to get viable colonies. The colonies were stained with 0.5 ml of 0.01% crystal violet (Sigma-Aldridge) solution for 1 h and were counted by using a microscope (x40 magnification). A viable colony was defined as having at least 50 cells after 10 days of growth. Colonies were counted from each triplicate sample and presented as average values ± SD. The surviving fraction of the treated cells was normalized to the plating efficiency of control (no irradiation) cells. Cell survival was plotted as a function of dose and fitted using the linear quadratic model SF=exp (-αD-βD²),
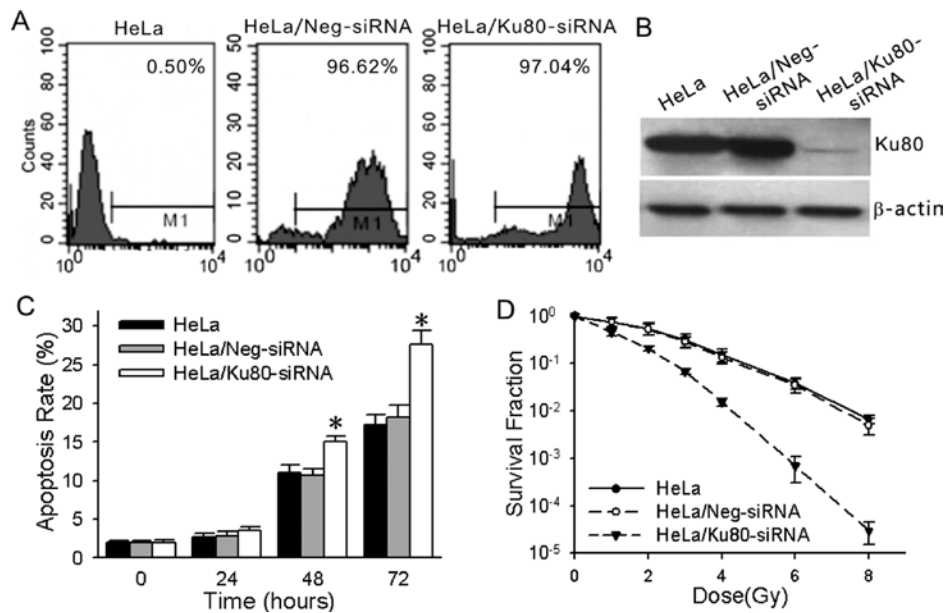
Figure 1. Effects of Ku80-siRNA on radiosensitivity of HeLa cells. (A) Transfection efficiencies of HeLa cells stably transfected with Ku80-siRNA and negative-siRNA-encoding plasmids that constitutively expressed green fluorescent protein (GFP). Cell clones stably transfected with siRNA-encoding plasmids were collected and green fluorescence was quantified by FACS analysis. (B) Western blot analysis of the Ku80 protein expression in HeLa cells transfected with Ku80-siRNA and Neg-siRNA. (C) HeLa cells were transfected with Ku80-siRNA and Neg-siRNA was treated with 6 Gy X-ray for 24, 48 and 72 h. The apoptosis rate was analyzed by FACS, *P<0.05. (D) HeLa cells were transfected with Ku80-siRNA and Neg-siRNA was exposed to 0 to 8 Gy X-ray prior to assessment of colony formation. The clonogenic survival assays were performed in triplicate. Standard errors are shown by error bars.

where SF is the cell survival, D is the dose of radiation, and α and β are constants. SF2 (surviving fraction of cells at 2 Gy) was calculated from the actual data. Dose-reduction factors (DRFs), the factor by which the dose of the radiation can be reduced in the presence of the sensitizing agent to achieve the same level of cell killing in the absence of the sensitizing agent, were calculated as the dose required to achieve 10% cell survival (90% clonogenic cell killing) from the fitted clonogenic survival curves for the transfected cells.

Irradiation and cell cycle analysis. Cells were plated and pretreated with LY294002 at 50 μmol/l for 30 min, and then were transported in a 37˚C insulated container in sealed flasks and exposed to 6 Gy 6MV X-ray (300 cGy/min). The cells were stably transfected with siRNA-encoding plasmids or transient transfected with DNA-PKcs-siRNA-encoding plasmid for 48 h. For the determination of cell cycle distribution, the cells were harvested after irradiation for 4 h (not for cells pretreated with LY294002), 24, 48 and 72 h, washed with PBS, and fixed in 75% ice-cold ethanol overnight at -20˚C. After being washed in PBS, the cells were treated with 50 μg/ml propidium iodide (PI) and 1 mg/ml RNase A for 30 min at 37˚C. Subsequent analyses of the apoptosis rate (Sub G1) and cell cycle distribution were performed using CellQuest software (BD Biosciences, Heidelberg, Germany).

Statistical analysis. SPSS v11.5 software was used for all statistical procedures. Data were presented as means ± SD of at least triplicate experiments and a two-tailed unpaired t-test was used to compare the statistical significance of the differences in the data between the two groups. Differences at P<0.05 were considered significant.

Results

Effects of Ku80-siRNA on radiosensitivity of HeLa cells. The plasmids that constitutively expressed green fluorescent protein (GFP) and could transcript Ku80-siRNA and negative-siRNA, were stably transfected into HeLa cells, named HeLa/Ku80-siRNA and HeLa/Neg-siRNA, respectively. The transfection efficiencies of HeLa/Ku80-siRNA and HeLa/Neg-siRNA were detected by FACS analysis and were all above 95%, with the efficiency of HeLa/Ku80-siRNA reaching 97.04% (Fig. 1A). More than 96% of the Ku80 protein was knocked down by Ku80-siRNA, while the Ku80 protein of HeLa/Neg-siRNA failed to decrease (Fig. 1B). These data indicate that the siRNA targeting of Ku80 was specific and the plasmid vector had no effect on Ku80 protein expression. HeLa/Neg-siRNA was used as control in the next study. HeLa cells were transfected with Ku80-siRNA and Neg-siRNA was treated with 6 Gy X-ray for 24, 48 and 72 h. The apoptosis rate of HeLa/Ku80-siRNA was significantly higher than that of HeLa and HeLa/Neg-siRNA (P<0.05) (Fig. 1C). A clonogenic survival assay showed that HeLa cell silencing of Ku80 was more sensitive to X-rays than were the control cells (Fig. 1D).

Knockdown of DNA-PKcs by siRNAs after Ku80 silencing. Reconstructed DNA-PKcs-siRNA-transcripted plasmid, which could express red fluorescence protein in the host cell, was transfected into HeLa/Neg-siRNA and HeLa/Ku80-siRNA cells (Fig. 2A). Protein extracts were obtained at 24, 48 and 72 h after transfection and a Western blot analysis was performed for the DNA-PKcs protein and normalized to β-actin expression. The results disclosed that 48 h post-transfection was the time point at which the greatest DNA-PKcs protein
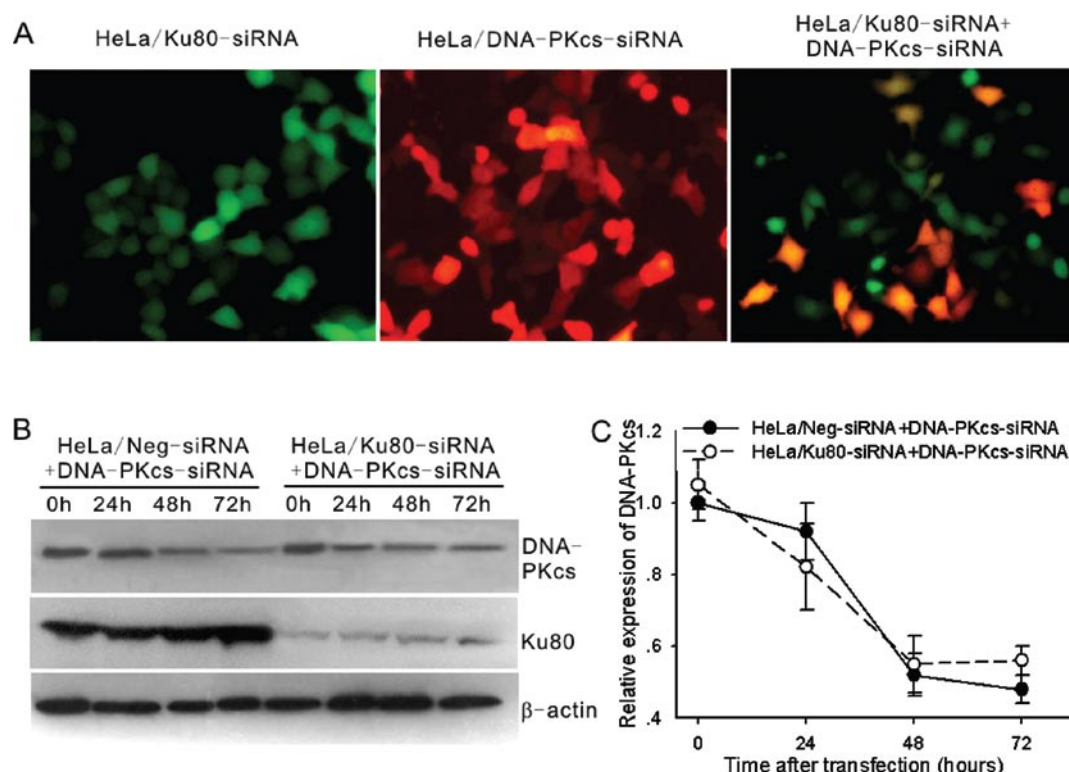
Figure 2. DNA-PKcs was down-regulated by DNA-PKcs targeting siRNA. (A) Reconstructed DNA-PKcs-siRNA-transcripted plasmid that expressed red fluorescence protein was transfected into HeLa/Ku80-siRNA cells (x200). (B) Cell extracts were obtained from 24 to 72 h post-transfection and target proteins were detected by Western blot analysis and normalized to β-actin expression. (C) The density of bands was quantified by NIH image analysis and the data presented as mean values of each data point ± standard error (n=3).

down-regulation occurred (about 50% lower) (Fig. 2B and C). No obvious changes in Ku80 protein were found at any time, either for the HeLa/Neg-siRNA or HeLa/Ku80-siRNA cells. Thus, the DNA-PKcs-targeting siRNA did not affect the expression of Ku80 protein and also suggested that Ku80 protein did not vary in HeLa/Ku80-siRNA after a long-time culture after the cell line was established. In the next experiment, cells transfected with DNA-PKcs-siRNA for 48 h were analyzed by clonogenic survival assay and apoptosis rate and cell cycle analysis after X-ray irradiation.

HeLa cells cotransfected with Ku80-siRNA and DNA-PKcs-siRNA in combination were more radiosensitive than either individually. The apoptosis analysis displayed that the apoptosis rate of HeLa cells cotransfected with Ku80-siRNA and DNA-PKcs-siRNA were higher than that of other cells (P<0.05, Fig. 3A). The clonogenic assay showed that HeLa and HeLa/Neg-siRNA had similar results in terms of SF2 and α values (Fig. 3B, Table I), confirming the notion that the plasmid vector had no effect on radiosensitivity either. In order to remove the effect of the plasmid vector, HeLa/Neg-siRNA was regarded as the control cells. As shown above, HeLa/Ku80-siRNA had 96% Ku80 protein knockdown, meaning that the function of NHEJ would be nearly completely inhibited in normal inference. But when HeLa/Ku80-siRNA cells were transfected with DNA-PKcs-siRNA, its SF2 value became significantly decreased (Fig. 3B, Table I) at only 0.08±0.01, which is far lower than HeLa/Ku80-siRNA (0.20±0.05) and HeLa transfected only with DNA-PKcs-siRNA (0.25±0.03). The DRF of 10% cell survival compared to HeLa/Neg-siRNA even reached 2.47. The α values

of these cell lines had opposite change patterns (as shown in Table I), again showing that HeLa cells co-transfected with Ku80-siRNA and DNA-PKcs-siRNA were more radiosensitive than the ones transfected individually.

The cell cycle distributions of HeLa cells cotransfected with Ku80-siRNA and DNA-PKcs-siRNA showed interesting changes after 6 Gy X-ray irradiation. Because HeLa and HeLa/Neg-siRNA had similar cell cycle distributions, HeLa/Neg-siRNA was used as the control cells in order to remove the effect of the plasmid vector (as before). It is known that on response to X-rays, a lower LET (linear energy transfer) radiation, mammalian cells normally are arrested (≤10 h) in G1, which is followed by DNA repair and/or apoptosis (21). At 4 h post-radiation in this study, the percentage in the G1 phase of HeLa/Ku80-siRNA was (55.45±3.49)%, which was lower compared to (63.59±2.64)% of the HeLa/Neg-siRNA (P<0.05). S arrest appeared in HeLa/Ku80-siRNA cells (36.41±3.05) % of the time compared to (25.90±2.16)% of the HeLa/Neg-siRNA cells (P<0.05). HeLa/Neg-siRNA or HeLa/Ku80-siRNA cells that were transiently transfected with DNA-PKcs-siRNA both showed decreases in G1 arrest and increases in S arrest, similar to changes seen in the HeLa/Ku80-siRNA cells (Fig. 3C).

HeLa/Ku80-siRNA and HeLa/Neg-siRNA cell lines were both found to accumulate in the G2/M phase between 24 to 72 h after 6 Gy X-ray irradiation, and at 48 h, the percentages were at highest levels for both, reaching above 80%. The cells released from the accumulation of the G2/M phase and reentering the cell cycle resulted in apoptosis at 72 h. However, there were no obvious differences (P>0.05) in the cell proportions observed
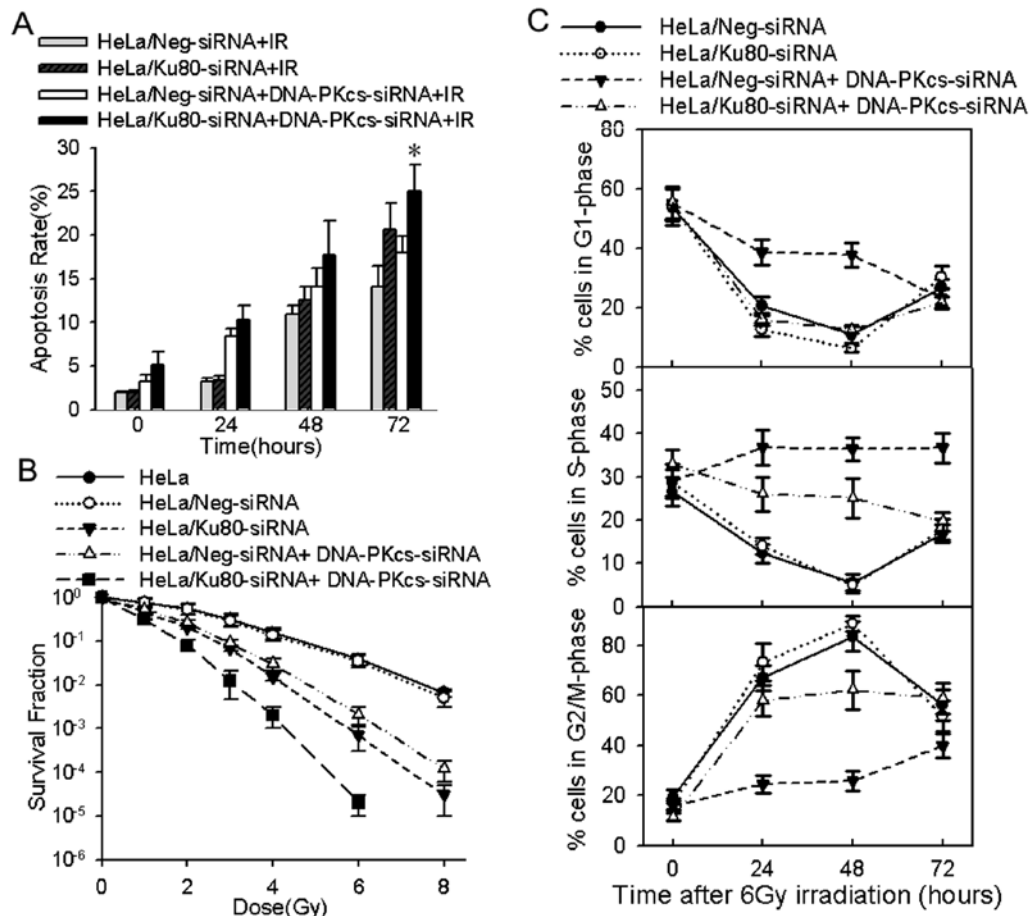
Figure 3. Radiobiology variability of HeLa after suppression of DNA-PKcs and Ku80 both singly and in combination with siRNA. (A) HeLa cells were transfected with Ku80-siRNA and/or DNA-PKcs-siRNA was irradiated for 6 Gy X-ray and was harvested at 24, 48 and 72 h after irradiation. Apoptosis rates (subG1) were analyzed by FACS. *P<0.05. (B) HeLa, HeLa/Neg-siRNA, HeLa/Ku80-siRNA, and cells cotransfected with DNA-PKcs-siRNA for 48 h were irradiated at the indicated dose and were assessed for clonogenic survival assay as described in 'Materials and methods'. (C) HeLa cells were transfected with Ku80-siRNA and/or DNA-PKcs-siRNA was irradiated with 6 Gy X-rays. All cells were harvested at 24, 48 and 72 h after irradiation, and were analyzed by FACS as described in 'Materials and methods'. The cell percents in G1, S and G2/M phase are shown, respectively. All illustrated data are presented as mean based on three separate experiments.

Table I. Response of irradiated cell lines.

Cell lines and treatment	Surviving fraction at 2 Gy (SF2)	α	β	DRF[a] of 10% cell survival
HeLa	0.53±0.08	0.19±0.02	0.06±0.01	
HeLa/Neg-siRNA	0.51±0.07	0.19±0.02	0.07±0.01	1
HeLa/Ku80-siRNA	0.20±0.05	0.70±0.03	0.06±0.02	1.68
HeLa/Neg-siRNA + DNA-PKcs-siRNA	0.25±0.03	0.55±0.03	0.07±0.02	1.50
HeLa/Ku80-siRNA + DNA-PKcs-siRNA	0.08±0.01	1.09±0.04	0.09±0.02	2.47
HeLa/Neg-siRNA + LY294002	0.21±0.02	0.63±0.04	0.08±0.03	1.63
HeLa/Ku80-siRNA + LY294002	0.03±0.01	1.51±0.06	0.16±0.03	3.36

[a]DRF, dose-reduction factor, the factor by which the dose of radiation can be reduced in the presence of sensitizing agent to achieve the same level of cell killing in the absence of the sensitizing agent, were calculated as the dose required to give 10% cell survival (90% clonogenic cell killing) from fitted clonogenic survival curves for transfected or treated cells; in order to remove the effect of the plasmid vector, HeLa/Neg-siRNA was regarded as control cells. Results are shown as mean values of each data point ± standard error (n=3).

at 24, 48 and 72 h after irradiation (Fig. 3C). HeLa/Ku80-siRNA transfected with DNA-PKcs-siRNA showed similar cell cycle trends as the HeLa/Ku80-siRNA cells, accumulating at the

G2/M phase until 48 h post-irradiation, though the degree of change was not as pronounced (Fig 3C). However, HeLa/Neg-siRNA transfected with DNA-PKcs-siRNA displayed an
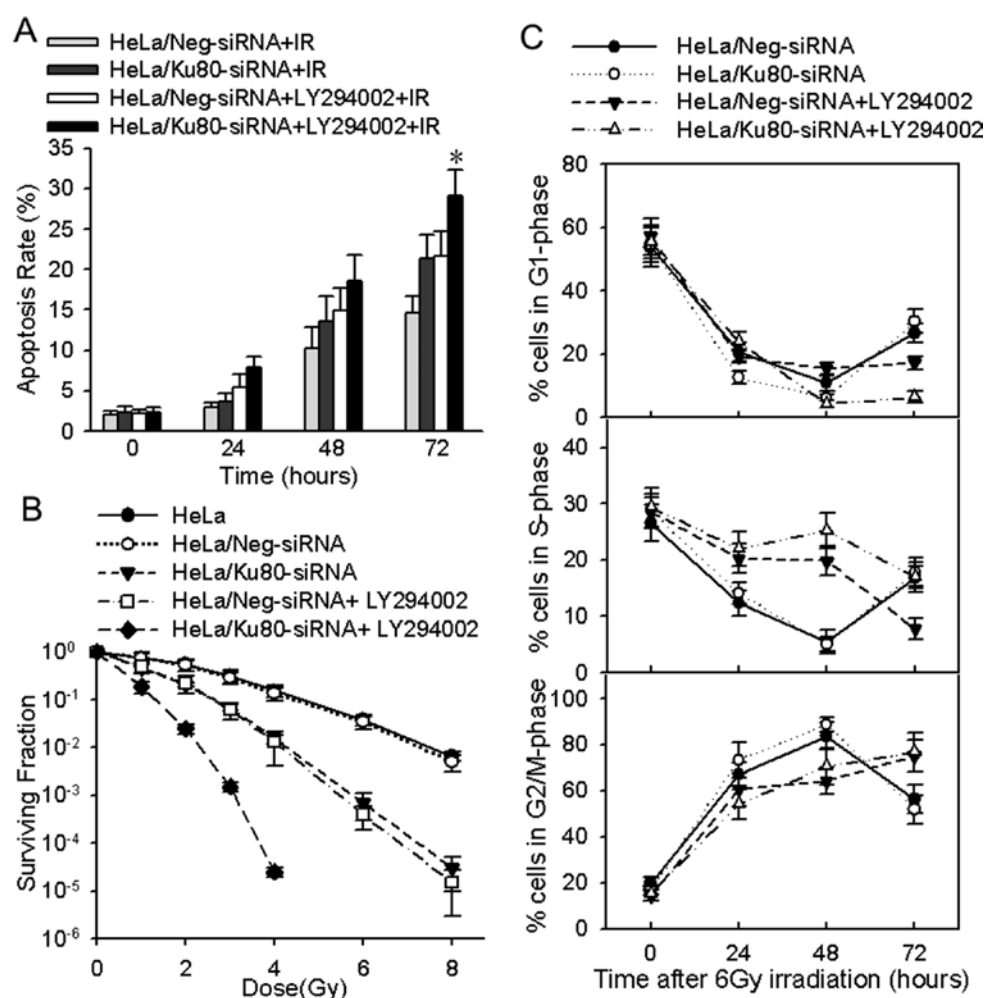
Figure 4. Radiobiology variability of HeLa after suppression of DNA-PKcs with LY294002 and/or Ku80 with siRNA both singly and in combination. (A) Cells pretreated with 50 μM LY294002 for 30 min were irradiated for 6 Gy X-ray and were harvested at 24, 48 and 72 h after irradiation. Apoptosis rates (subG1) were analyzed by FACS. *P<0.05. (B) HeLa, HeLa/Neg-siRNA, and HeLa/Ku80-siRNA cells pretreated with 50 μM LY294002 for 30 min were irradiated at the indicated dose and were assessed for clonogenic survival assay as described in 'Materials and methods'. (C) Cells pretreated with 50 μM LY294002 for 30 min were irradiated with 6 Gy X-rays. All cells were harvested at 24, 48 and 72 h after irradiation, and were analyzed by FACS as described in 'Materials and methods'. The cell percents in G1, S, and G2/M phase are shown, respectively. All illustrated data are presented as mean based on three separate experiments.

interesting phenomenon; in contrast to the other cell lines, the cell cycle initially accumulated in the G1 phase for a long time, then entered the S phase and G2/M phase slowly, in a step by step manner. The percentage of cells in the G2/M phase still did not get to a vertex even at 72 h post-irradiation. The percentage of cells in the G1 phase decreased gradually, though it did not reach the lowest point even at 72 h post-irradiation. In contrast, the other cell lines had reached the inflection point at 48 h post-irradiation, similar to G2/M phase accumulation.

The effect of LY294002 on radiobiology of HeLa in the absence of Ku80. LY294002 inhibited the activity of two DSB repair proteins: DNA-PKcs and ATM. The apoptosis analysis displayed that the apoptotic rate of the HeLa cells treated with both Ku80-siRNA and LY294002 were higher than that of other cells (P<0.05, Fig. 4A). Even HeLa/Neg-siRNA showed increased radiosensitivity after pretreatment with LY294002, and the SF2 and DRF at 10% cell survival compared to the control cells were 0.21±0.02 and 1.63, respectively. HeLa in the absence of Ku80 and pretreated with LY294002 resulted

in cells that were even more sensitive to X-rays than HeLa/Ku80-siRNA transfected with DNA-PKcs-siRNA. The SF2 and DRF at 10% cell survival compared to the control cells were 0.03±0.01 and 3.36, respectively (Fig. 4B and Table I).

The irradiation of LY294002-treated cells led to a different cell cycle distribution, with HeLa/Neg-siRNA and HeLa/Ku80-siRNA showing similar accumulation in the G2/M phase (Fig. 4C). Similar to HeLa/Neg-siRNA transfected with DNA-PKcs-siRNA (Fig. 3C), the cells continued to slowly accumulate in the G2/M phase and did not reach the culminating point even at 72 h post-irradiation.

Discussion

It is known that the NHEJ pathway plays a crucial role in repairing DSBs during radiation in mammalian cells (4). In order to investigate the role of DNA-PK, reconstructed plasmids that could transcribe siRNA in cells were applied to inhibit the expression of Ku80. Human cervical adeno-carcinoma cell line HeLa, also a mammalian cell line, was
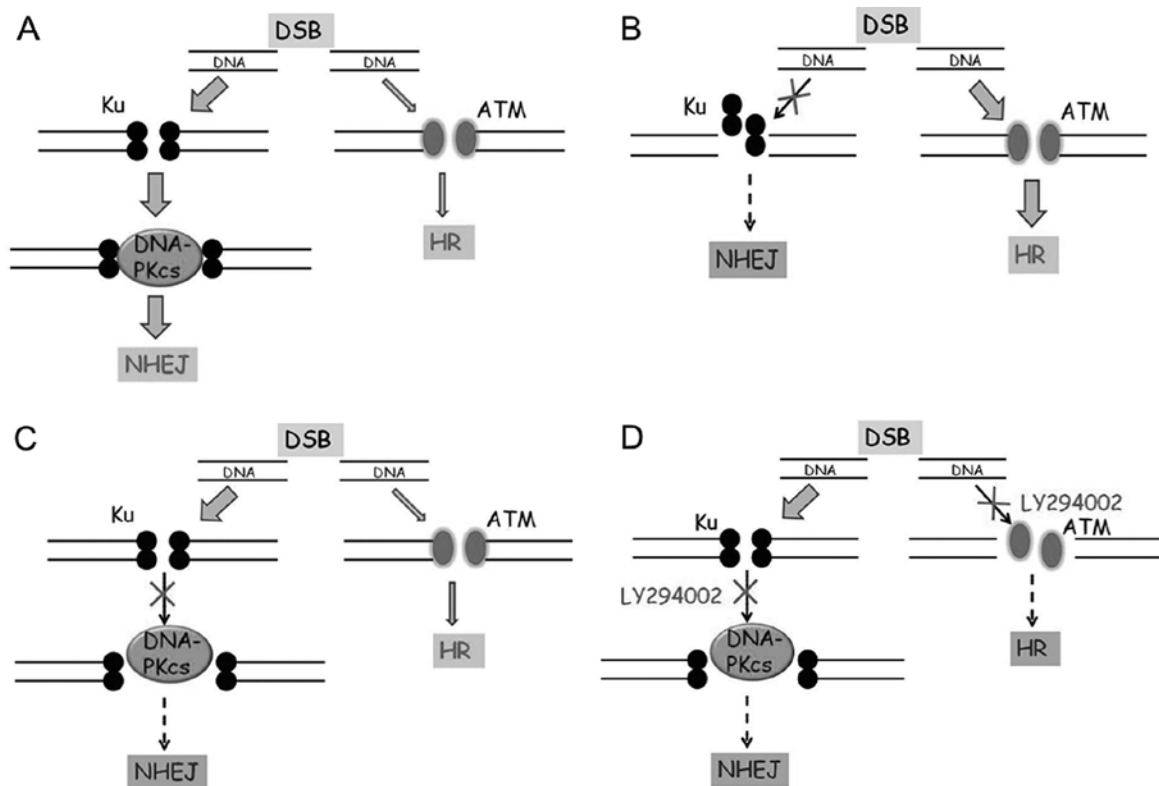
Figure 5. Schematic of two repair pathways of DSB. (A) Normal. (B) Ku80-defective. (C) DNA-PKcs-defective. (D) LY294002-treating cells. Ku means Ku heterodimer, including Ku70 and Ku80.

applied as a guide. We selected a stable transfection cell clone where >96% of the Ku80 protein was silenced. While X-ray sensitivity of this clone was not obviously increased, the DRF was only 1.68 compared to the control cells. However, re-transfection with siRNA targeting DNA-PKcs noticeably enhanced the radiosensitivity of HeLa/Ku80-siRNA (DRF at 10% cell survival reached 2.47, in comparison to the control cells). We know that Ku80 and DNA-PKcs are both components of DNA-PK and play same roles in the NHEJ pathway. Investigating if the two proteins play the same role upstream and downstream in the same pathway, the defecting of one protein and the knocking down of another nevertheless did not change the role of the pathway. This conclusion can be confirmed by the study that no effect was observed in M059J, which is a DNA-PKcs-deficient malignant glioma cell line after Ku80 antisense treatment (22). In order to explain the phenomenon that co-transfection with siRNAs targeting DNA-PKcs and Ku80 made the cells much more radiosensitive than transfection only with Ku80-targeting siRNA, we considered the following possible reasons. First, there are enormous differences in DSB repair protein expression between all kinds of eukaryotes, with human cells typically having more Ku80 and DNA-PKcs than rodents cells (23,24), and the expressive differences are even displayed between different human cells (25,26). Second, Ku80 proteins are abundant in human cells, so even a residual less than 4% of Ku80 protein could act to rejoin many DSBs. Third, DNA-PKcs can also bind to, and is activated by, free DNA ends in the absence of Ku (27,28). Fourth, there is competition or a sequence between the NHEJ and HR pathways (reviewed in refs. 3 and 11), with

several studies suggesting that when NHEJ is impaired, HR seems to increase and *vice versa* (29-31). Other studies have raised the possibility that NHEJ is the initial pathway that is attempted; when it fails, HR might take over (32,33).

Ionizing irradiation (IR) induces arrests in the G1, S and G2/M phases of cell cycle in <10 h (34); the work presented here demonstrated that the control cells were arrested in the G1 phase at 4 h after 6 Gy irradiation, just like other mammalian cells with wide-type p53. While HeLa cells inhibited Ku80 and DNA-PKcs both individually or in combination, they were arrested in the S phase at 4 h after 6 Gy irradiation, confirmed results reported previously (35). Similar to reasons explained above, the DSB repair was compensated by the HR repair pathway when the NHEJ proteins were inhibited, so the cells were arrested in the S phase in order to allow the DSBs to be repaired by the HR protein that plays a main role in the late S and G2/M phases (6,7,35).

As reported by other investigators (36,37), the increased number of G2/M cells that were detected several hours (≥24 h) after irradiation in this study was likely due to an accumulation of cells that had been in earlier phases of the cell cycle because of radiation exposure. Our results suggested that regardless of HeLa cell transfection (only with negative-siRNA or with Ku80-siRNA), they all accumulated at the G2/M phase, while no obvious difference was discovered. But when we transfected siRNA targeting DNA-PKcs into the control HeLa cells and exposed them with 6 Gy X-ray irradiation, the result was different; the cell cycle initially accumulated in the G1 phase for a long time, then entered the S and G2/M phases step by step at a rate far slower than the other cells. Interestingly, when

the HeLa/Ku80-siRNA cells were transfected with the DNA-PKcs-siRNA and exposed to 6 Gy X-ray irradiation, the distribution of the cell cycle was like that of the HeLa/Ku80-siRNA cells. All of the above results meet at a single conclusion, that the roles of Ku80 and DNA-PKcs in the cell cycle response are not the same, with DNA-PKcs perhaps acting in G2/M accumulation and being associated with Ku80, just like their roles in DSB repair. As shown in Fig. 5A, when DSB is induced, NHEJ proteins, Ku80 and Ku70, first bind to the free DNA end, and later recruit DNA-PKcs to complete the rest of the work (3,5). When the Ku protein is defective (shown in Fig. 5B), the HR proteins RAD51, RAD52 and ATM can bind to the DNA end and HR will repair the DSB. If DNA-PKcs is defective instead of the Ku protein (shown in Fig. 5C), considering that the DNA ends were occupied by the Ku protein, the HR protein ATM can not bind to these ends and DSBs cannot be rejoined (31). We discovered a similar situation in the cell cycle response after X-ray irradiation. It is presumed that NHEJ and HR proteins both take part in the cell cycle response, with Ku80 acting first and later inducing the participation of the DNA-PKcs. When the Ku80 protein is inhibited, the HR protein can compensate for it completely because the role of these HR proteins in the cell cycle response was more powerful than that of the NHEJ proteins. As such, HeLa cell inhibition of Ku80 showed the same level of G2/M accumulation compared to the control cells. When DNA-PKcs was inhibited, regarding the 'occupying' of the Ku protein, HR proteins could not join or compensate normally, resulting in a delay in the cell cycle. In cells cotransfected with the both kinds of siRNA and receiving radiation (Ku80 protein having been silenced 96%), the HR protein could compensate for major portion of the function, and the residual 4% of the Ku80 protein still 'occupied' a small part of the 'sites' that could be applied by neither the DNA- PKcs nor the HR protein. This maybe is the most possible explanation as to why the G2/M accumulation of the cotrans-fected cell line was slightly lower than that of the control cells and HeLa/Ku80-siRNA.

LY294002, a chemically specific PI 3-kinase inhibitor capable of blocking the activity of both NHEJ protein DNA-PK and HR protein ATM (shown in Fig. 5D) (19), was used to treat the cells before 6 Gy X-ray irradiation. The cells silenced by Ku80 were more sensitive to X-rays than cells inhibited by DNA-PKcs-targeted siRNA, which supports our presumption that the HR protein ATM could compensate major portions of the DSB repair function even in the absence of Ku80. Moreover, even when cells had 96% the Ku80 proteins knocked down by siRNA or when control cells had no Ku80 suppression, both scenarios displayed the same cell cycle distribution after LY294002 treatment and irradiation. Regarding the control cells, where previous results showed that irradiation of the LY294002-treated cells caused an increase in the length of G2/M arrest compared to cells exposed only to radiation (38), the inhibition of DNA-PKcs and ATM may block the recognition of DNA damage and prevent the recruitment of repair capacity in damaged sites. As for HeLa/Ku80-siRNA, although the HR protein ATM may compensate for DSB repair function in the absence of Ku80 and though ATM maybe the main target of LY294002 (38), LY294002-treated cells displayed that large DNA damage could not be repaired, displaying the same prolonged G2/M accumulation.

In conclusion, Ku80 and DNA-PKcs are both DSB repair proteins, though there still are many unknown mechanisms regarding their DSB repair function, cell cycle response and other radiobiology characteristics. In addition, the co-inhibition of both NHEJ and HR proteins would be a suitable strategy to enhance the radiosensibility of cancer cells.

Acknowledgements

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