# Gemcitabine Induces Radiosensitization Through Inhibition of RAD51-dependent Repair for DNA Double-strand Breaks

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**Abstract.** *Background/Aim: Gemcitabine (GEM) is used in clinical chemo-radiotherapy; however, the mechanism that contributes to enhanced radiosensitivity by GEM is not fully-understood. We evaluated the effect of GEM on radiosensitization in pancreatic cancer cell lines. Materials and Methods: Pancreatic cell lines PK-59 and PK-45p were used. A total of 5 μM GEM for 4 h were administered pre- or post-gamma irradiation. Results: Enhanced cell killing effects by GEM in radiotherapy were observed for pre-treatment but not post-treatment GEM. We focused on the dynamics of RAD51 and phospho-H2AX foci after irradiation. Significantly higher numbers of phospho-H2AX foci were observed in GEM pre-treated cells than in untreated cells after irradiation. We also found inhibition of the formation and degradation of RAD51 foci by GEM pre-treatment. The radiosensitizing effect of GEM was suppressed by knockdown of RAD51. Conclusion: RAD51-dependent homologous recombination is one of the key targets in the GEM-induced radiosensitizing effect.*

Gemcitabine (GEM) is a deoxycytidine analogue anti-tumor drug that can act as an inhibitor of, or a substrate for, replicative DNA polymerases (1-4). GEM is used as a standard therapy for patients with advanced pancreatic cancer. However, GEM treatment alone is not useful for pancreatic cancer treatment: it induces a partial response in a few people and can alleviate symptoms in some with advanced tumors (5-8). There is, thus, need for the use of another chemotherapy regime at the same time. GEM is known as a radiosensitizing agent both *in vitro* and *in vivo* (9-12). However, the mechanism that contributes to enhanced radiosensitivity by GEM treatment is not fully understood.

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DNA double-strand breaks (DSBs) are the major lethal damage caused by radiation. Cells have several DSB repair pathways, known as non-homologous end-joining (NHEJ) and homologous recombination (HR) (13-16). NHEJ ligates the two broken ends, whereas HR refers to the use of the sequence homologous to the DSB site, resulting in gene conversion. Different cell conditions can modify the choice between NHEJ and HR for DSB repair (17). NHEJ is prominent in the G1/early S phase and HR in the late S/G2 phase (18). Most somatic cells are in the quiescent cell cycle stage, thus targeting the HR pathway for radiotherapy is more specific to cancer cell death than targeting the NHEJ pathway. In the HR pathway, 3'-overhanging single-strand DNA is formed at the first stage. Next, RAD51 is recruited to the single-strand DNA and promotes DNA homologous pairing and strand exchange in association with other proteins of the gene conversion complex (19, 20). After homologous pairing, DNA synthesis starts from the 3'-end. The RAD51 protein is a pivotal component of the HR pathway (21-24).

We hypothesized that the radiosensitizing effect of GEM is caused by inhibition of DNA damage repair. In the present study, we examine how GEM affects radiation sensitivity. We demonstrated that pre-treatment with GEM delays the formation of RAD51 foci 4 h after gamma irradiation. In addition, GEM inhibits the decrement of both phospho-H2AX foci and RAD51 foci. Moreover, knockdown of RAD51 expression suppresses the radiosensitizing effect of GEM. These results suggest that RAD51-dependent homologous recombination is one of the key targets of the gemcitabine-induced radiosensitizing effect.

## Materials and Methods

*Cell culture and gamma irradiation*. Human pancreatic cancer cells (PK-59, PK-45p; RIKEN CELL BANK, Tsukuba, Japan) were cultured in alpha-MEM medium (alpha-minimum essential medium; Life Technologies Japan, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA) in a 5% $CO_2$ incubator at 37˚C. For GEM treatment, cells were treated with 5 μM GEM (Tokyo Kasei, Tokyo, Japan) pre-treatment (treatment of

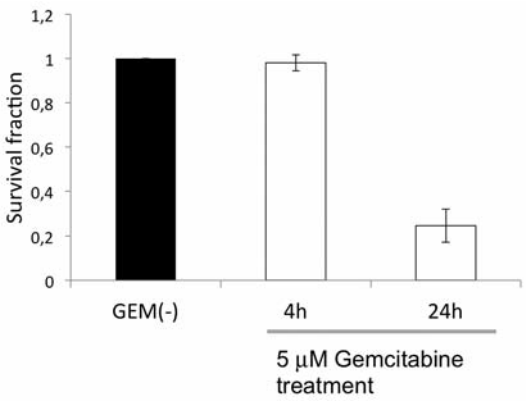


Figure 1. *Treatment with 5 μM GEM for 4 h is non-toxic in PK-59 pancreatic cancer cells. PK-59 cells were treated with medium with or without 5 μM GEM for 4 h or 24 h. After treatment, the cells were plated onto 6-well plates in order to form surviving colonies for 10 days. Colonies containing more than 30 cells were counted.*

GEM from 4 h before irradiation to just after irradiation) or post-treatment (treatment of GEM from immediately after irradiation for 4 h). Cells were irradiated with each dose of gamma rays from $^{137}Cs$.

*Survival assay.* Cells were irradiated with each dose of gamma rays. After irradiation, cells were plated onto a 6-well plate and cultured for 14 days. After incubation, colonies were fixed with 100% ethanol and stained using 5% Giemsa (Roche Tissue Diagnostics, Tokyo, Japan). Colonies containing more than 30 cells were counted.

*Immunofluorescence staining.* Cells grown on coverslips were incubated with or without 5 μM GEM for 4 h at 37˚C. After GEM treatment, cells were irradiated with 6 Gy of gamma rays. Cells were washed once with cytoskeleton (CSK) buffer and permeabilized with 0.5% Triton X-100 in CSK buffer for 2 min on ice at each time point. Cells were then fixed by treatment with 4% formaldehyde in $PBS^-$ solution for 20 min at room temperature. After fixation, cells were washed once with $PBS^-$ and treated with 0.5% NP-40 in $PBS^-$ for 5 min at room temperature. Primary antibodies for phosphorylated histone H2AX at Ser139 (mouse, clone 2F3; Biolegend, San Diego, CA, USA), RAD51 (rabbit, clone H-92; Santa Cruz, Dallas, TX, USA) dissolved in TBS-DT (20 mM Tris–HCl, 137 mM NaCl, 0.1% Tween 20, 125 g/ml ampicillin, 5% skim milk) were applied for 2 h in a 37˚C incubator. Cells were washed with $PBS^-$- three times. Secondary antibody conjugated with Alexa 488 or 594 (Molecular Probes®, Life Technologies Japan, Tokyo, Japan) was then applied for 1 h in an 37˚C incubator. After washing with $PBS^-$- five times, coverslips were mounted onto slide glasses with 10% glycerol in $PBS^-$. Digital images of the primary antibodies were acquired using fluorescence microscopy (DP72, Olympus, Tokyo, Japan).

*Transfection of small interfering RNA (siRNA).* A siRNA pool for *RAD51* was purchased from GE Dharmacon (Lafayette, CO, USA). Control siRNA was purchased from Qiagen (Tokyo, Japan). Lipofectamine 2000 and each siRNA were diluted in Opti-MEM as described in the manufacturer's protocol (Life Technologies Japan, Tokyo, Japan). This Lipofectamine2000 and siRNA complex solution was added to cells grown on a 35-mm dish. Cells were incubated for 1 day and then washed with medium. Two days after transfection, cells were treated with 5 μM GEM and irradiated at each dose. After irradiation, cells were plated onto 100-mm dishes in order to make 100 surviving colonies and grown for 14 days in a 5% $CO_2$ incubator at 37˚C.

*Western blot analysis.* The method for Western blot analysis was as described previously (25). RAD51 antibody (Santa Cruz, Dallas, TX, USA) and anti-tubulin (Cell Signaling, Danvers, MA, USA) were used.

## Results

*Radiosensitization was observed by pre-treatment with GEM but not by post-treatment.* First, we examined the toxic effect of 5 μM GEM by a colony formation assay in a human pancreatic cancer cell line, PK-59. As shown in Figure 1, there was no difference in survival between cells treated with 5 μM GEM for 4 h and untreated control cells. By contrast, 24 h of treatment with GEM significantly decreased survival. The data indicate that 4 h of treatment with 5 μM GEM had no effect on cell death. We chose 4 h treatment with 5 μM GEM to determine the radiosensitizing effect of GEM. Next, we applied GEM by two different methods, as depicted in Figure 2A. For pre-treatment cells, cells were treated with 5 μM GEM for 4 h before irradiation. Post-treatment cells were treated with 5 μM GEM for 4 h after irradiation. As shown in Figure 2B and C, pre-treatment with GEM had significant radiosensitive effects at doses of 4 Gy and 6 Gy in both PK-59 cells and PK-45p cells. On the other hand, radiosensitization was not observed by post-treatment with 5 μM GEM in PK-59 cells (Figure 2D). We hypothesized that the radiosensitizing effect of GEM was caused by inhibition of DNA damage repair; thus, post-treatment of GEM did not have a radiosensitizing effect.

*Inhibition of DNA damage repair by GEM treatment.* Next, we observed DNA repair kinetics by staining phosphorylated histone H2AX at Ser139 (phospho-H2AX) and RAD51 in PK-59 cells (Figure 3A). Since histone H2AX is phosphorylated at sites of DNA damage, we counted phospho-H2AX foci as the total number of sites of DNA damage. RAD51 foci were counted as the number of sites of homologous recombination repair (HR). As shown in Figure 3A, the number of phospho-H2AX foci peaked at 0.5 h after 6 Gy irradiation and gradually decreased until 24 h. The number of RAD51 foci peaked at 4 h after irradiation and decreased until 24 h. Thus, we examined the number of phospho-H2AX and RAD51 foci from 4 h to 24 h after irradiation with or without 5 μM GEM pre-treatment (Figure 3B and 3C). Interestingly, pre-treatment

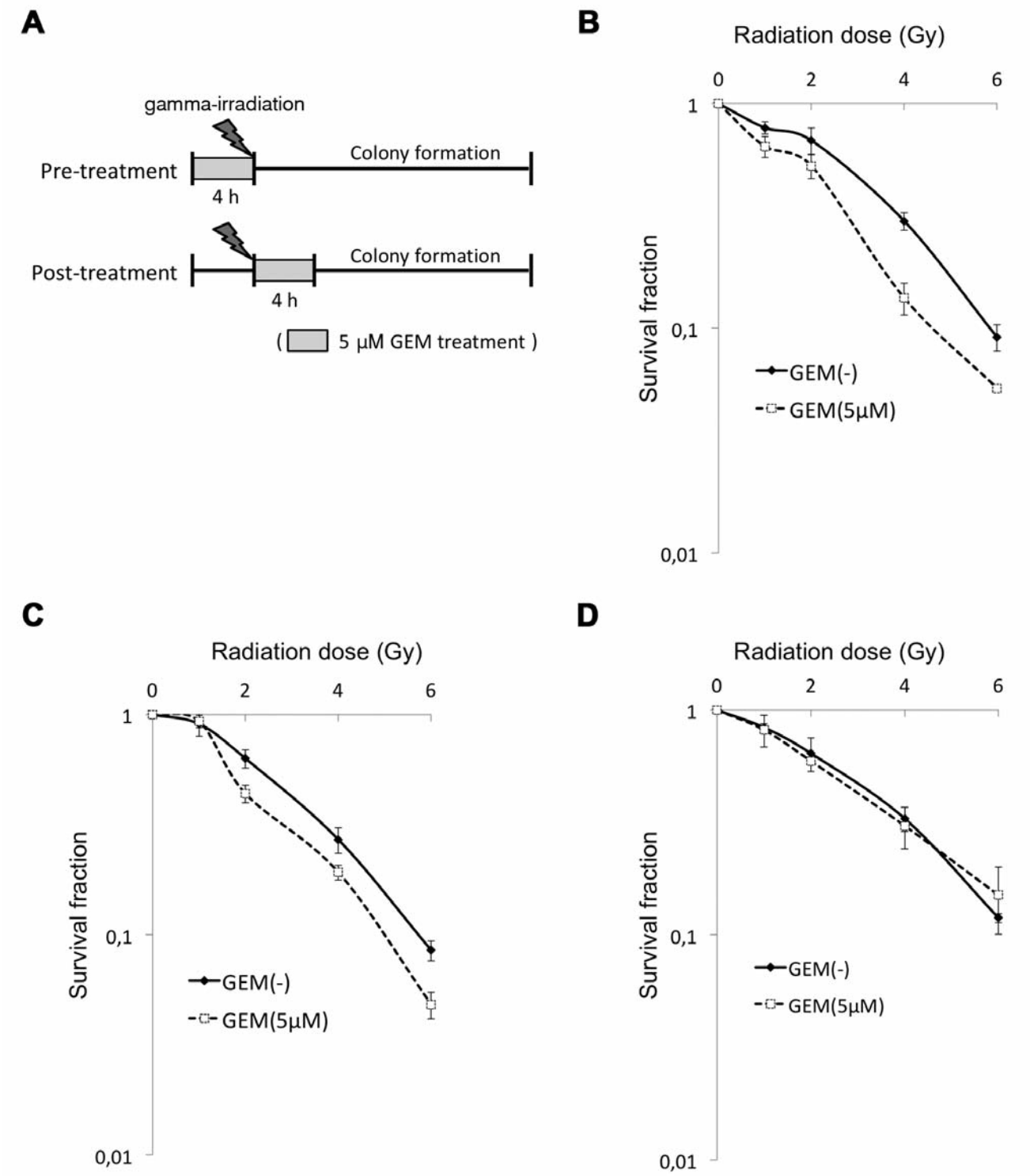


Figure 2. *A radiosensitizing effect was observed upon pre-treatment with 5 μM GEM. (A) Treatment with 5 μM GEM. GEM was administered at 5 μM, as described in the Materials and Methods section. Pre-treatment involved cells being treated with GEM from 4 h before irradiation to immediately after irradiation. Post-treatment involved cells being treated with GEM from immediately after irradiation to 4 h after irradiation. (B-D) Radiation survival curves for PK-59 cells and PK-45p cells irradiated with 0-6 Gy of gamma rays. Cells were plated onto 6-well plates in order to make surviving colonies and grown for 10 days after irradiation. Colonies containing more than 30 cells were counted and plotted as the log of the survival fraction of cells versus radiation dose. (B) Survival curve of PK-59 cells with or without 5 μM GEM pre-treatment. (C) Survival curve of PK-45p cells with or without 5 μM GEM pre-treatment. (D) Survival curve of PK-59 cells with or without 5 μM GEM post-treatment.*

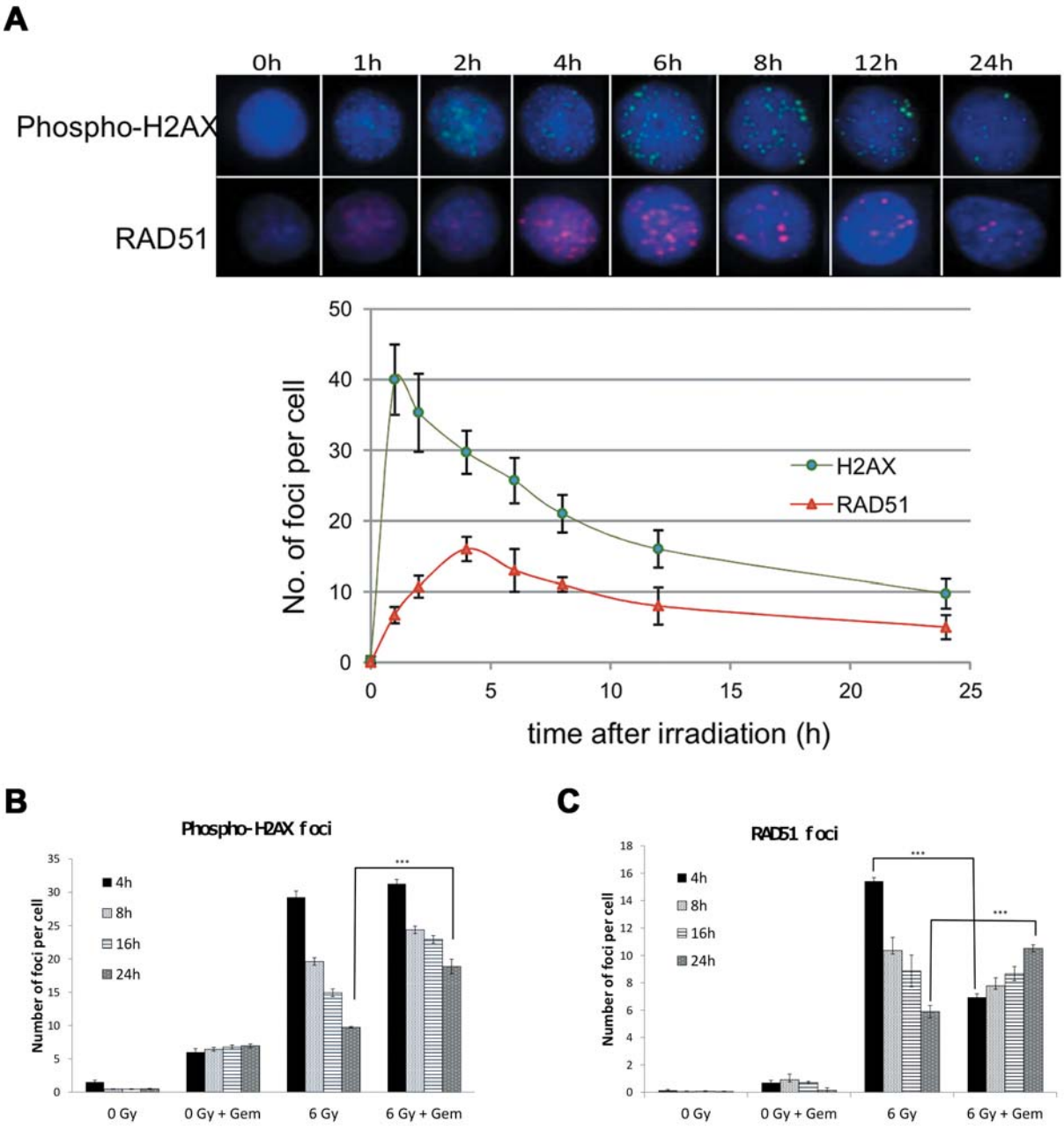


Figure 3. *Formation and disappearance of phospho-H2AX foci and RAD51 foci after 6 Gy irradiation in PK-59 cells. (A) Upper panel: representative picture of phospho-H2AX and RAD51 immunofluorescence staining after irradiation. PK-59 cells cultured on cover slips were irradiated with 6 Gy of gamma rays and then fixed at the indicated time points, followed by immunofluorescence staining for phosphor-H2AX and RAD51. Green, phospho-H2AX; Red, RAD51; Blue, 4',6-diamidino-2-phenylindole (DAPI) fluorescent stain. Lower panel: The average number of foci for phospho-H2AX and RAD51 after 6 Gy irradiation. (B) The average number of foci for phospho-H2AX after 6 Gy irradiation. (C) The average number of foci for RAD51 after 6 Gy irradiation. (A~C) More than 100 cells were analyzed for each case. The average number of foci per nucleus was calculated and is indicated above the bars in the graph. Data represent mean±SE of three independent experiments.* $^{***}p<0.001$.

with GEM suppressed the decrement of the number of phospho-H2AX foci. The remaining number of phospho-H2AX foci was significantly higher in cells pre-treated with GEM than in untreated cells at 16 h and 24 h after irradiation (Figure 3B). In addition, pre-treatment with GEM significantly suppressed the formation of foci of RAD51 at 4 h after irradiation. Furthermore, treatment with GEM suppressed the decrement of Rad 51 foci (Figure 3C).

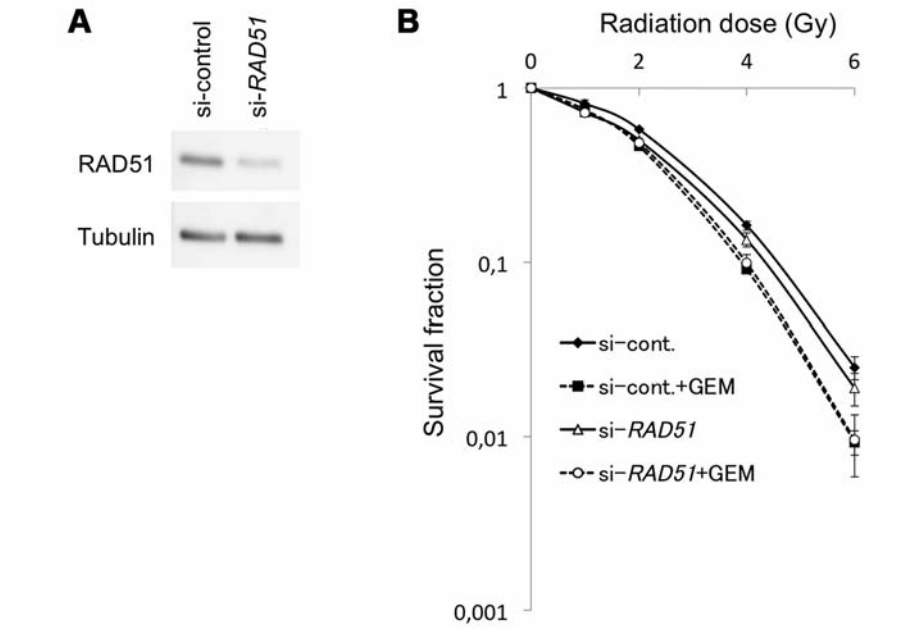


Figure 4. *Radiosensitization by gemcitabine was diminished by knockdown of RAD51 in PK-59 cells. (A) Western blot analysis of RAD51 expression in si-control cells and si-RAD51 cells. (B) Radiation survival curves for si-control cells and si-RAD51 cells with or without pre-treatment with 5 μM GEM. Cells were plated onto 6-well plates in order to make surviving colonies and grown for 10 days after irradiation. Colonies containing more than 30 cells were counted and plotted as the log of the survival fraction of cells versus radiation dose.*

*Radiosensitizing effect of GEM was RAD51-dependent*. We next down-regulated RAD51 expression to verify that the radiosensitizing effect of GEM was caused by inhibition of the RAD51-dependent DNA repair pathway. RAD51 protein expression was knocked down using specific siRNA (Figure 4A). In cells in which RAD51 expression was knocked down (*siRAD51* cells), a level of radiosensitivity comparable to that of control siRNA (siCont) cells was identified (Figure 4B). Importantly, in *siRAD51* cells with pre-treatment with GEM, there was no additive effect compared to *siRAD51* cells and cells pre-treated with GEM (Figure 4b). Knockdown of RAD51 expression resulted in a slightly decreased radiosensitizing effect of GEM.

## Discussion

In the present study, we demonstrated that pre-treatment with GEM has radiosensitizing effects but post-treatment does not (Figure 2). It is noteworthy that GEM was applied under non-toxic conditions (Figure 1). In addition, pre-treatment with GEM causes suppression of the decrement of phospho-H2AX foci (Figure 3B). These results indicate that the radiosensitizing effect of GEM was caused by inhibition of DNA damage repair. In addition, we showed that the decrement of RAD51 foci was inhibited by pre-treatment with GEM (Figure 3C). Furthermore, knockdown of RAD51 protein expression attenuated the radiosensitizing effect of GEM (Figure 4). GEM is an inhibitor of DNA polymerase; therefore, these results suggest that GEM inhibits DSB repair through suppression of DNA synthesis in the HR pathway. Wachters *et al*. and Yong *et al*. previously reported that the radiosensitizing effect of GEM is related to suppression of the HR pathway in rodent cell lines (26, 27).

Surprisingly, our results showed that pre-treatment with GEM also inhibited the formation of RAD51 foci. In the HR pathway, 3'-overhanging single-strand DNA is formed at the first stage. Next, RAD51 is recruited to the single-strand DNA and promotes DNA homologous pairing and strand exchange in association with other proteins of the gene conversion complex. After homologous pairing, DNA synthesis is started from the 3'-end. Thus, inhibition of the formation of RAD51 foci occurs earlier than inhibition of DNA synthesis of the HR pathway (Figure 5). It is possible that incorporation of GEM to the DNA strand may inhibit RAD51 attachment to the DSB site or RAD51-mediated DNA homologous pairing.

It has been reported that over-expression of RAD51 was found in 66% of human pancreatic adenocarcinoma tissue specimens (28). Thus, targeting RAD51 for therapy appears as potential effective treatment. Furthermore, pre-treatment with GEM is expected to induce sensitization not only to radiation but also to other DNA-damage-inducing anti-cancer drugs, such as mitomycin C and cisplatin, because of the

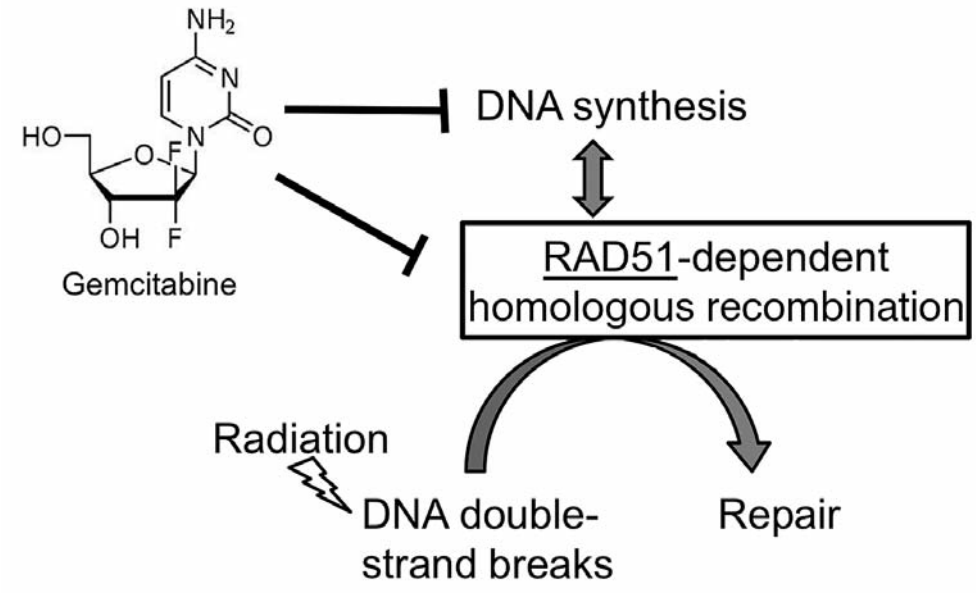


Figure 5. *A schematic model of inhibition of DNA double-strand break repair by GEM. GEM inhibits the HR pathway through both DNA synthesis and formation of RAD51 foci.*

inhibition of the HR pathway and DNA polymerase. Therefore, pre-treatment with GEM can be expected to be a useful tool for pancreatic cancer therapy.

## Conflicts of Interest

The Authors have no conflicts of interest.

## Acknowledgements

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