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Rad51C deficiency destabilizes XRCC3, impairs recombination and radiosensitizes S/G_2-phase cells

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ABSTRACT

The highly conserved Rad51 protein plays an essential role in repairing DNA damage through homologous recombination. In vertebrates, five Rad51 paralogs (Rad51B, Rad51C, Rad51D, XRCC2, XRCC3) are expressed in mitotically growing cells, and are thought to play mediating roles in homologous recombination, though their precise functions remain unclear. Here we report the use of RNA interference to deplete expression of Rad51C protein in human HT1080 and HeLa cells. In HT1080 cells, depletion of Rad51C by small interfering RNA caused a significant reduction of frequency in homologous recombination. The level of XRCC3 protein was also sharply reduced in Rad51C-depleted HeLa cells, suggesting that XRCC3 is dependent for its stability upon heterodimerization with Rad51C. In addition, Rad51C-depleted HeLa cells showed hypersensitivity to the DNA cross-linking agent mitomycin C, and moderately increased sensitivity to ionizing radiation. Importantly, the radiosensitivity of Rad51C-deficient HeLa cells was evident in S and G_2/M phases of the cell cycle but not in G_1 phase. Together, these results provide direct cellular evidence for the importance of human Rad51C in homologous recombinational repair.

Keywords: homologous recombination/Rad51C/radiosensitivity/RNA interference/XRCC3

INTRODUCTION

In mammalian cells, DNA double-strand breaks (DSBs)[1] are repaired primarily by two distinct mechanisms (Khanna and Jackson, 2001; Pierce *et al*, 2001): non-homologous end joining (NHEJ), a non-templated, potentially error-prone process in which nucleotide alternations are tolerated at the site of rejoining, and homologous recombination (HR), a largely error-free process in which a sister chromatid or homologous chromosome is used as a template for repair. Homologous recombinational repair (HRR) provides high fidelity in repairing DNA damage, and is therefore essential and critical for the maintenance of genome stability and tumor avoidance (Thompson and Schild, 1999; 2001). The Rad51 protein plays a key role in HR, functioning to mediate homologous DNA pairing and strand exchange (Baumann *et al*, 1996; Baumann and West, 1997). Five vertebrate Rad51 paralogs are expressed in mitotically growing cells: Rad51B (Albala *et al*, 1997; Rice *et al*, 1997; Cartwright *et al*, 1998a), Rad51C (Dosanjh *et al*, 1998), Rad51D (Cartwright *et al*, 1998a; Pittman *et al*, 1998; Kawabata and Saeki, 1999), XRCC2 (Liu *et al*, 1998; Cartwright *et al*, 1998b; Johnson *et al*, 1999), and XRCC3 (Tebbs *et al*, 1995; Liu *et al*, 1998; Pierce *et al*, 1999). These proteins share 20-30% sequence identity with Rad51 and with each other. Only vertebrates appear to contain all five of these Rad51 paralogs. In human cells, Rad51C participates in various paralog complexes, including Rad51B-Rad51C, Rad51C-XRCC3, and Rad51B-Rad51C-Rad51D-XRCC2 (Masson *et al*, 2001; Sigurdsson *et al*, 2001; Wiese *et al*, 2002; Liu *et al*, 2002; Miller *et al*, 2002). In terms of protein-protein interactions, Rad51C apparently has a central role, interacting directly with Rad51B, Rad51D, XRCC3, and also weakly with Rad51 (Schild *et al*, 2000; Lio *et al*, 2003). However, the functional significance of these complexes is not yet clear.

Mutant studies provide a direct means for identifying the function of genes. A knockout mutation of Rad51C was previously generated in DT40 chicken B-lymphocyte cells (Takata *et al*, 2001). The mutant cells showed elevated spontaneous chromosomal aberrations, high sensitivity to killing by the cross-linking agent mitomycin C (MMC), mild sensitivity to γ-rays, and defective Rad51 nuclear focus formation after exposure to γ-rays. Similar phenotypes were also found in DT40 knockouts generated for the other four paralogs (Takata *et al*, 2000; 2001), suggesting that each of the paralogs functions in HRR, and that fully efficient repair may require all five. In addition, two hamster cell lines, irs3 and CL-V4B, have been identified as Rad51C mutants. Both were found to show reduced sister chromatid exchange and genomic instability (French *et al*, 2002; Godthelp *et al*, 2002), and HRR of a specifically induced chromosome break by gene conversion was reduced in irs3 cells (French *et al*, 2003). However, the relative importance of Rad51C in human cells has not been examined, because no Rad51C-mutant human cell line has been available.

RNA interference (RNAi) has rapidly emerged as a powerful technique for investigating gene function (Bass, 2000; Sharp, 2001; Zamore, 2001; Hannon, 2002), and a valuable complement to mutant studies. RNAi is a sequence-specific post-transcriptional gene silencing mechanism that uses double-stranded RNA as a signal to trigger the degradation of homologous mRNA (Fire *et al*, 1998; Montgomery *et al*, 1998). Chemically synthesized duplexes of 21-25 nucleotide small interfering RNA (siRNA) can induce specific gene silencing in a wide range of mammalian cell lines without causing apoptosis (Caplen *et al*, 2001; Elbashir *et al*, 2001; Harborth *et al*, 2001; Elbashir *et al*, 2002).

To determine the functions of Rad51C in human cells, we have previously used *in vitro* assays to demonstrate that the purified human Rad51C protein exhibits DNA binding, ATPase, and double-stranded DNA separation activities (Lio *et al*, 2003). These findings underscore the potential significance of the human Rad51C in the DNA strand exchange events of HR. Additionally, it was recently reported that Rad51C is required for Holliday junction processing in human extracts (Liu *et al*, 2004), implying a role for Rad51C in the resolution of HR intermediates. Here we report the depletion of Rad51C expression in two human cell lines, a fibrosarcoma line HT1080 and a cervical carcinoma line HeLa, using 21-nucleotide siRNA duplexes, and we directly examine the effect of Rad51C inhibition on HR, using an *in vivo* HR assay. The effect of Rad51C depletion on the endogenous level of other Rad51 paralogs in HeLa cells was also examined. In addition, the sensitivity of Rad51C-depleted HeLa cells to mitomycin C (MMC) and ionizing radiation (IR) was characterized, and the dependence of radiosensitivity in Rad51C-deficient HeLa cells upon cell cycle phase was investigated, using synchronized G_1 and $S+G_2/M$ populations.

RESULTS

Inhibition of Rad51C reduces the level of XRCC3 protein and other Rad51 paralogs

To determine optimal conditions for depleting the expression of the Rad51C protein, nine different transfection condition sets with siRNA #1 were examined in HeLa cells, varied by siRNA concentration, seeded cell density, and presence or absence of serum. Western analysis was used to monitor the expression level of Rad51C after siRNA treatment. Among the nine condition sets tested, one condition set achieved about 90% inhibition of the endogenous level of the Rad51C protein in HeLa cells (data not shown). This condition was therefore used as our standard protocol for Rad51C depletion, as described in **Materials and methods**, for both HeLa and HT1080 human cells.

It has been shown that Rad51C directly interacts with Rad51, Rad51B, Rad51D and XRCC3, and together they form multiprotein complexes (Schild *et al*, 2000; Masson *et al*, 2001; Sigurdsson *et al*, 2001; Wiese *et al*, 2002; Liu *et al*, 2002; Miller *et al*, 2002; Lio *et al*, 2003). We examined the endogenous levels of Rad51 and the other four Rad51 paralogs in Rad51C siRNA-transfected HeLa cells on days 2, 3 and 4 post-transfection. As shown in Fig. 1, the cellular level of Rad51C decreased on day 2 after siRNA treatment and remained very low through days 3 and 4. Both siRNA #1 and #2 caused a similar level and time course of inhibition, indicating that these two siRNA duplexes are almost equally effective for suppressing Rad51C expression. Concomitantly with Rad51C inhibition, XRCC3 protein level was also greatly reduced on days 2, 3 and 4 post-siRNA transfection. The result indicates that depletion of Rad51C destabilizes XRCC3, presumably due to decreased formation of the stabilizing Rad51C-XRCC3 heterodimer. When the expression levels of Rad51 and Rad51B was examined, it was found that Rad51 level was low on day 2 (quantified as 53% and 85% reduction for the siRNA #1- and siRNA #2-treated cells, respectively), but recovered by day 3. Similarly, Rad51B level was reduced on day 3 (quantified as 67% and 88% reduction for the siRNA #1- and siRNA #2-treated cells, respectively), but recovered by day 4. These observations suggest that the level of Rad51C present may dynamically affect the expression or stability of Rad51 and Rad51B as well as XRCC3. In contrast, the inhibition of Rad51C had minimal effect on the level of Rad51D and XRCC2, indicating that Rad51C is not required for stabilization of these two proteins. Mixed siRNA #1 and #2 was also used for transfection, and a similar pattern of Rad51 paralog levels was observed (data not shown). The entire experiment was repeated twice and consistent results were obtained. The marked dependence of XRCC3 upon Rad51C for its stability is important, and means that other results described below may reflect depletion of Rad51C, or XRCC3, or both (*i.e.*, the Rad51C-XRCC3 heterodimer).

Inhibition of Rad51C results in a reduced frequency of homologous recombination

Rodent cells mutated for Rad51C or XRCC3 have previously been shown defective for HRR (Pierce *et al*, 1999; Brenneman *et al*, 2000; Brenneman *et al*, 2002; French *et al*, 2003). To measure HR in human cells, an artificial reporter locus (as diagrammed in Fig. 2A) was installed into a chromosome of human HT1080 cells by electroporation with a vector, pPGKpacIR-BSD, that carries the complete reporter locus. Clones of cells that integrated the reporter were isolated by selecting for a blasticidin resistance gene included on the vector, and the presence of a single integrated copy was confirmed by Southern blotting (data not shown). The reporter locus comprises two defective Pac (puromycin acetyltransferase) genes configured as an inverted repeat. The left copy of the Pac gene has complete regulatory sequences (the murine phosphoglycerate kinase promoter/enhancer and the SV40 polyadenylation region), but is defective because of a mutation that deletes 80 base pairs of coding sequence, and creates a cleavage site for the highly site-specific endonuclease I-SceI of *S. cerevisiae* (Rouet *et al*, 1994; Choulika *et al*, 1995; Brenneman *et al*, 2000). The right copy of the Pac gene has an intact reading frame and a polyadenylation region, but is defective because it lacks a promoter. A defined DNA double-strand break can be introduced at the integrated reporter locus, by transient expression of I-SceI. Cleavage by I-SceI creates a double-strand break within the left Pac gene of the reporter locus. HR initiated from the break can convert the left Pac gene back to a wild-type sequence, by using the right copy as a template. Frequency of HR is scored by counting puromycin-resistant colonies of cells. HT1080-1885 is a clonal isolate of HT1080 cells stably carrying the reporter, and produces puromycin-resistant colonies at a frequency of ~$5x10^{-3}$ per viable cell upon transient transfection for I-SceI expression (data not shown).

Rad51C siRNA #1 and #2 were individually transfected into HT1080-1885 cells. Two days after siRNA transfection, the cells were transiently transfected for expression of I-SceI endonuclease. Twenty-four hours after I-SceI transfection, a portion of the cells was subjected to Western analysis for Rad51C expression and a portion was replated for HR assay. As shown in Fig. 2B, both siRNA #1 and #2 duplexes inhibited the expression of the Rad51C protein by about 70% in HT1080-1885 cells. Using the *in vivo* HR assay, we found that the frequency of HR was reduced about 2-fold in the Rad51C siRNA-treated cells by both siRNA duplexes, as compared with the mock-transfected controls (t-test; siRNA #1, $P = 0.0088$; siRNA #2, $P = 0.0049$) (Fig. 2C). Our data provide direct *in vivo* confirmation that human Rad51C and/or XRCC3 function in HRR, as their rodent homologs do.

Suppression of Rad51C causes increased sensitivity to mitomycin C and ionizing radiation

Cells defective in HR show hypersensitivity to a number of DNA-damaging agents, but are particularly sensitive to agents that form interstrand crosslinks, such as mitomycin C (MMC). We examined the sensitivity of Rad51C siRNA-transfected HeLa cells using acute exposure to MMC. We found that the siRNA-treated cells were ~2-fold more sensitive to MMC as compared with mock-transfected control based on the estimated D_{10} values (*i.e.*, the dose that reduces survival to 10%) (Fig. 3A). The MMC sensitivity profile of siRNA #1-treated cells was very similar to that of siRNA #2-treated cells, in accord with the similar depletion levels of Rad51C produced by the two siRNA duplexes. The plating efficiencies of mock-transfected control and siRNA-transfected HeLa cells were about 70% and 50%, respectively (data not shown). Mock-transfected and untransfected HeLa cells had similar sensitivity to MMC (data not shown), indicating that our transfection protocol does not alter the sensitivity of HeLa cells to MMC. The HeLa control cells were much more sensitive to MMC (D_{10} about 1.3 μM) as compared with V79 hamster cells (D_{10} about 30 μM) (Liu *et al*, 1998) under acute conditions.

To determine the effects of IR on Rad51C-depleted HeLa cells, we first examined asynchronous cultures. Mock- and siRNA-treated cells were irradiated with various doses (0, 4, 6, 8, 10 Gy) of γ-rays. Suppression of Rad51C with siRNA transfection in HeLa cells resulted in mildly increased sensitivity to γ-rays as compared with mock-transfected controls (Fig. 3B). Both siRNA #1- and siRNA #2-transfection caused a similar effect on HeLa cells to γ-ray sensitivity. Treatment with the transfection reagent (Oligofectamine) alone had no effect on γ-ray sensitivity in HeLa cells (data not shown).

Rad51C siRNA treatment alters cell cycle progression, and increases radiosensitivity in S and G_2/M phases, but not in G_1 phase

Because homologous recombination is an important pathway for repairing DNA DSBs in mammalian cells, particularly in S and G_2 phases of the cell cycle (Cheong *et al*, 1994; Takata *et al*, 1998; Rothkamm *et al*, 2003), we investigated whether the IR sensitivity of Rad51C-deficient HeLa cells is cell-cycle dependent. The plant amino acid mimosine inhibits cell cycle traverse in late G_1 phase, and can effectively synchronize mammalian cells (Lalande, 1990; Watson *et al*, 1991; Talwar *et al*, 1997). HeLa cells were treated with mimosine for 16 hours and then released to progress through the cell cycle. This technique produced an excellent synchrony for HeLa cells as described previously (Talwar *et al*, 1997). Using this approach, Rad51C siRNA #1-treated HeLa cells and mock-transfected controls were synchronized. Flow cytometry was used to determine the percentage of the cell population in each cycle phase, and the results were plotted (Fig. 4). The distribution of mock-transfected HeLa cells through the cell cycle (Fig. 4A) was very similar to that previously observed for untreated cells (Talwar *et al*, 1997), *i.e.*, the majority of cells were in G_1 phase immediately following release from mimosine, whereas S phase and G_2/M phases represent the majority at 6.5 and 11.5 h, respectively, after release from mimosine. This indicates that transfection with Oligofectamine did not affect the cell cycle progression. However, Rad51C siRNA treatment resulted in delayed progression through the cycle after release from mimosine, with S- and G_2/M-phase cells reaching maxima at 10 and 12.5 hours, respectively (Fig. 4B). Based on these results, we chose two specific time points to irradiate the synchronous cell cultures with γ-rays. In the mock-transfected control populations enriched for G_1- and S+G_2/M-phase are predominant at 0 and 7.5 h after release from mimosine, respectively, and the cells were accordingly irradiated at 0 or 7.5 h after release. For the Rad51C siRNA-treated cells, however, the populations enriched for G_1 and S+G_2/M phases are predominant at 0 and 10 h after release from mimosine, respectively, and these cells were therefore irradiated at those times.

HeLa cells were synchronized using mimosine treatment and irradiated with various doses of γ-rays at the time points determined above. After irradiation, cells were replated at low density either immediately or after an 18-h delay, and assayed for colony-forming ability. For the population enriched for S+G_2/M phase, the Rad51C siRNA #1 and #2-treated cells were found to be ~1.2-fold more sensitive to IR as compared with the mock-transfected control (Fig. 5A) using the immediate plating protocol, as assessed by D_{10} values. This modestly increased radiosensitivity is similar to that observed for asynchronous cells (Fig. 3B). Interestingly, the siRNA-treated G_1-enriched cells did not show any additional sensitivity to γ-rays as compared with the mock-transfected G_1 cells (Fig. 5A). These important findings indicate that human

Rad51C functions in repair of IR-induced DNA damage specifically in S and G_2/M phases, but not in G_1 phase, and supports a role of Rad51C in HRR. The mock-transfected control cells displayed greater radiosensitivity in G_1 than in the S and G_2/M phases (Fig. 5A). This observation is in agreement with earlier studies in HeLa cells by Terasima and Tolmach (1963a; 1963b), who found that radioresistance is greatest in the latter stage of S phase, and that G_1-phase cells are comparatively more sensitive to IR. Although the radioresistance of siRNA-treated S+G_2/M-enriched cells was less than that of control S+G_2/M-enriched cells, it did not fall to the level of G_1 cells (Fig. 5A). This suggests that HRR in S and G_2 phases is impaired but not abolished in the siRNA-treated cells, possibly reflecting the incomplete inhibition of Rad51C.

In addition to the immediate plating after γ-irradiation, a parallel set of cells was returned to 37°C incubation and then replated after an 18-h delay. It has been observed that the fraction of cells surviving a given dose of IR increases if a time interval is allowed between irradiation and replating because, during this interval, potentially lethal damage (PLD) is repaired (Phillips and Tolmach, 1966; Belli and Shelton, 1969; Barendsen *et al*, 2001). As shown in Fig. 5B, the surviving fraction of Rad51C siRNA- and mock-transfected cells was increased, in both populations enriched for G_1 and S+G_2/M phases, with delayed plating compared to that with immediate plating (Fig. 5A). In the G_1 phase, no difference in IR sensitivity between Rad51C siRNA-treated cells and the control was observed (Fig. 5B). This result is consistent with the results obtained with immediate plating (Fig. 5A), suggesting that Rad51C inhibition does not affect the repair of PLD in G_1 phase. Importantly, we found that Rad51C siRNA-treated cells displayed a greater IR sensitivity than the control in the population enriched for S+G_2/M phase (Fig. 5B), and that this difference was somewhat larger (1.4-fold) than that observed with immediate plating (Fig. 5A). This result suggests that Rad51C depletion inhibits the repair of

PLD in S+G_2 phase and thus results in a higher radiosensitivity. Both the immediate and delayed plating experiments were carried out twice and consistent results were obtained. Our findings suggest a role for human Rad51C in repairing DSBs induced by γ-irradiation that is specific to the S+G_2 phase of the cell cycle.

DISCUSSION

Rad51C dynamically influences the protein levels of other Rad51 family members

The five mitotic Rad51 paralogs have been shown to form two distinct complexes in human cells, *i.e.*, a heterodimer of Rad51C-XRCC3, and a larger complex comprising Rad51B, Rad51C, Rad51D, and XRCC2 (Masson *et al*, 2001; Wiese *et al*, 2002; Liu *et al*, 2002; Miller *et al*, 2002). The transient existence of Rad51B-Rad51C and Rad51D-XRCC3 heterodimers has also been suggested (Miller *et al*, 2002; Liu *et al*, 2002; Kurumizaka *et al*, 2002). It seems probable that the various paralog complexes form dynamically during the process of HR (Liu *et al*, 2002). In these complexes, Rad51C interacts directly with XRCC3, Rad51B and Rad51D. Rad51C was also shown to weakly interact with Rad51 *in vitro* (Schild *et al*, 2000; Lio *et al*, 2003). The previous protein-protein interaction data have suggested a central role for Rad51C among the five paralogs. Our findings of dynamic correlations between the expression level of Rad51C and those of XRCC3, Rad51 and Rad51B in Rad51C-deficient HeLa cells support this concept. Particularly, our results indicate that Rad51C directly stabilizes XRCC3, most probably through participation in the Rad51C-XRCC3 heterodimer. In cells treated with siRNA against Rad51C, XRCC3 protein levels were reduced nearly as much as Rad51C, and remained low over three days. This dependence upon heterodimerization appears to be mutual: in complementary experiments, we have previously shown that overexpression of XRCC3 produces an elevated level of Rad51C as well (Weiss *et al*, 2002). Rad51C appears also to affect the expression or stability of Rad51 and Rad51B, though to a lesser extent and more transiently. It is interesting that the expression of Rad51D and XRCC2 seemed not to correlate with Rad51C expression, perhaps reflecting weaker or indirect associations between these proteins. The co-depletion of Rad51C and XRCC3 we observed in cells treated with siRNA against Rad51C has an important implication for the other results obtained in this study. The phenotypic changes we report in regard to frequency of HRR, MMC sensitivity, and IR sensitivity might, in principle, be attributable to deficiency of either Rad51C or XRCC3. However, we favor the possibility that these changes reflect a reduced level of functions carried out by the Rad51C-XRCC3 heterodimer.

Our results provide direct *in vivo* evidence for the function of human Rad51C in HRR

Studies with chicken and hamster cells have indicated a role for Rad51C in HRR (Takata *et al*, 2001; French *et al*, 2002; Godthelp *et al*, 2002; French *et al*, 2003). However, no direct evidence of the biological functions of Rad51C has been available from human cells. Our results demonstrate a reduced frequency of repair of a specific chromosomal DSB by recombination after Rad51C siRNA treatment, thereby providing direct confirmation that Rad51C functions in HRR in human cells. Although the observed reduction in HR frequency in Rad51C-depleted HT1080 cells is modest, the difference was reproducible and statistically significant. The incomplete inhibition of HR is likely due to partial depletion of Rad51C in siRNA-transfected HT1080 cells; approximately a 70% reduction as assessed by Western blot. It is possible that constitutive expression of siRNA would produce a more effective inhibition of Rad51C, and thus further decrease the frequency of HR. Further work remains to confirm this speculation.

Hypersensitivity to interstrand crosslinking agents such as MMC is a consistent feature of HRR-deficient mutants in vertebrate cell lines. It was previously reported that Rad51C-knockout chicken DT40 cells are ~3-fold more sensitive than wild-type to MMC after acute exposure (Takata *et al*, 2001), and that the Rad51C-mutant hamster irs3 and CL-V4B cell lines are ~20-fold and ~32-fold more sensitive to chronic treatment with MMC, respectively (French *et al*, 2002; Godthelp *et al*, 2002). Our results show that the Rad51C-deficient HeLa cells display ~2-fold greater sensitivity to MMC using acute treatment, demonstrating that these cells have a similar phenotype for MMC sensitivity to that of the chicken and hamster mutants. An *XRCC3* gene knockout in the human colon cancer cell line HCT116 has been recently reported, and the XRCC3-deficient cells showed ~2-fold excess sensitivity to MMC (Yoshihara *et al*, 2004). The MMC sensitivity level we observe in Rad51C-deficient HeLa cells is thus very close to that of the XRCC3-deficient human cells. Although the relative hypersensitivity of Rad51C-deficient human (and chicken) cells to MMC is not as remarkable as that observed in hamster cells, the results in aggregate indicate that a role of Rad51C in HRR of interstrand crosslinks is conserved across vertebrate species.

The cell cycle-dependence of radiosensitivity in Rad51C/XRCC3-depleted cells suggests that HRR operates mainly in the S and G_2/M phases of higher eukaryotes

Studies undertaken with various DSB repair-defective mutants have produced evidence for the contribution of HRR to IR resistance in the S and G_2/M phases of the cell cycle. Disruption of the HRR-related gene *Rad54* causes a modest increase in radiosensitivity (Bezzubova *et al*, 1997; Essers *et al*, 1997) that is associated primarily with the late-S/G_2 phase (Takata *et al*, 1998). By contrast, the NHEJ-defective CHO mutants for *XRCC4*, *Ku86* and *DNA-PKcs* (the XR-1, xrs5/6, and V3 cell lines, respectively) are highly sensitive to IR in G_1 and early S phases, compared to the wild-type, but are more IR-resistant in late S/G_2 (Stamato *et al*, 1983; Giaccia *et al*, 1985; Stamato *et al*, 1988; Whitmore *et al*, 1989; Jeggo 1990). A similar pattern was reported for murine pre-B cells carrying the *scid* mutation (Lee *et al*, 1997). These results suggest that DSBs occurring in replicated DNA are repaired efficiently by HRR. HRR is likely favored in the S-phase cells due to the presence of sister chromatids as proximal repair templates (Johnson and Jasin, 2000). During the normal cell cycle, Rad51 transcription is induced in the S and G_2 phases (Flygare *et al*, 1996), and Rad51 protein expression is found to be lowest in G_1, increasing in S, and reaching a maximum in G_2/M (Chen *et al*, 1997). Mammalian Rad51 forms discrete nuclear foci during the S phase (Tashiro *et al*, 1996). All of this evidence argues that the critical function of HRR takes place in the S/G_2 phase of the cell cycle.

Our results with HeLa cells co-depleted for Rad51C and XRCC3 further support this view. We examined the radiosensitivity of Rad51C siRNA-depleted HeLa cells using asynchronous cultures, and synchronized G_1 and S+G_2 populations. The asynchronous cells displayed moderately increased sensitivity to IR, which is in consistent with the phenotype shown by other HR-defective mutants, *i.e.* Rad54-deficient chicken DT40 (Bezzubova *et al*, 1997) and mouse embryonic stem cells (Essers *et al*, 1997); Rad51C, XRCC2 or XRCC3-mutant hamster cells (French *et al*, 2002; Godthelp *et al*, 2002; Liu *et al*, 1998); and Rad51 paralog-knockout DT40 cells (Takata *et al*, 2000; 2001). When the radiosensitivities of G_1- and S+G_2-phase cells were investigated separately, however, distinct IR responses were observed for these two populations. In G_1 phase, Rad51C/XRCC3-deficient cells are no more sensitive to IR than controls. In S+G_2

phase, however, the Rad51C/XRCC3-deficient cells show increased IR sensitivity relative to controls. In a related manner, it has been previously reported that hamster V79 parental cells were sensitive to IR at the G_1/S border whereas they are IR-resistant in late S phase; however, the irs-1 (XRCC2-mutant) cells lost the S-phase-dependent resistance to IR (Cheong *et al*, 1994). Current models propose that, in mammalian cells, NHEJ dominates DSB repair in G_1/early S, but that HRR and NHEJ both contribute substantially during late S/G_2 (Lee *et al*, 1997; Rothkamm *et al*, 2003). The cell cycle-dependent radiosensitivity we observe in Rad51C siRNA-treated HeLa cells strongly supports a role for human Rad51C and/or XRCC3 in S/G_2 phase-specific HRR.

In conclusion, our studies demonstrated that: (i.) RNA interference effectively depletes Rad51C in human cells; (ii.) Depletion of Rad51C destabilizes the XRCC3 protein; (iii.) Inhibition of Rad51C impairs HRR of chromosomal DSBs; (iv.) Rad51C-deficient HeLa cells are sensitive to MMC and IR and; (v.) Radiosensitivity of Rad51C-deficient HeLa cells is associated with the S/G_2 phase of the cell cycle. These findings are the first *in vivo* evidence for the functions of human Rad51C in repairing DNA DSBs through homologous recombination.

MATERIALS AND MATHODS

Rad51C siRNA design

The siRNA duplexes were designed according to published procedures (Elbashir *et al*, 2001; 2002) by selecting sense and anti-sense oligoribonucleotides homologous to the mRNA sequence. siRNA #1 is approximately 80 bases from the initiating AUG codon and siRNA #2 is about 120 bases before the carboxyl-terminal encoding sequence. Both siRNA #1 and #2 are complementary 21-mers with a two-base overhang, and start at an AA dinucleotide in the mRNA. Selected sequences were subjected to BLAST analysis to rule out homology to other human mRNA sequences. The siRNAs were synthesized by Dharmacon Research and provided as purified and annealed duplexes (Elbashir *et al*, 2002). The siRNA sequences of Rad51C used in the study are: siRNA #1: CUCCUAGAGGUGAAACCCUtt; siRNA #2: GUCACCCAGCCA GAAGGAAtt

Cell Culture and siRNA transfection

The human HT1080-1885 and HeLa cell lines were cultured as monolayers in minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (Hyclone) and penicillin-streptomycin (Gibco BRL). The cells were maintained in a humidified 4.3% CO_2 incubator at 37°C. Twenty-four hours prior to transfection, cells were seeded in a 6-well plate at 400,000 cells per well. For each well, 15 μl of siRNA stock oligonucleotide (20 μM) was diluted into 0.26 ml of Opti-MEM (Gibco BRL). In a separate tube, 6 μl of Oligofectamine transfection reagent (Invitrogen) was diluted into 17 μl of Opti-MEM and incubated at room temperature for 10 min. The Oligofectamine dilution was added into the diluted siRNA duplex and incubated at room temperature for 20 min. For control, cells were mock-transfected with Oligofectamine alone. Cells were washed twice with Opti-MEM, and 1.2 ml of Opti-MEM was subsequently added to each well. The Oligofectamine-siRNA complex was added drop-wise to the cells and incubated at 37°C. After 6 h, 0.75 ml of α-MEM media supplemented with 30% fetal bovine serum was added to each well without removing the transfection mixture, and the cells were returned to incubation at 37°C. Cells were harvested for assay of Rad51C expression by Western blotting analysis at 48, 72, and 96 h post-transfection.

Antibodies

Rad51B and Rad51C antibodies were generated as described previously (Lio *et al*, 2003). Polyclonal antiserum against human Rad51D was raised in rabbits using a synthetic peptide (CGTWGTSEQSATLQGDQT, Zymed Lab) as immunogen. The Rad51D antibody was affinity-purified from the antiserum. Rad51 antibody was kindly provided by Dr. Akira Shinohara. Both XRCC2 and XRCC3 antibodies were kind gifts of Dr. Nan Liu. QM antibody was purchased from Santa Cruz Biotechnology, Inc.

Homologous recombination assays

Human HT1080-1885 cells were treated with siRNA transfection (or mock-transfection) as described above. Two days after transfection, both sets of cells were trypsinized and individually seeded in a 6-well plate at 400,000 cells per well. The next day, each well was transfected with 1 μg of I-SceI expression vector pCMV(3xNLS)I-SceI and 4 μl of Superfect transfection reagent (Qiagen), or transfected with Superfect alone in serum-free α-MEM media at a final volume of 1 ml. After 5-h incubation at 37°C, the transfection mixture was removed, and 3 ml of complete α-MEM was added into each well, and the cells were returned to incubation at 37°C. Twenty-four hours after I-SceI transfection, cells were replated in duplicate for puromycin selection, at 120,000 cells per 100-mm culture dish. Parallel platings for measurement of plating efficiency were made in duplicate at 250 cells per 100-mm dish. On day 2 after transfection with I-SceI, selection cultures were refed with fresh media containing 1 μg/ml puromycin. Cells were refed again with puromycin on days 6 and 10. Thirteen days after plating, cells were fixed and stained. The cultures for plating efficiency assay were incubated at

37°C for eleven days without refeeding, then fixed and stained. Colonies with fifty cells or more were counted.

Mitomycin C and γ-ray survival using asynchronous cells

Two days after siRNA transfection, HeLa cells were trypsinized and seeded at 1,000 cells per 100-mm dish for each mitomycin C (MMC) dose to be tested. After a four-hour incubation at 37°C, cells were treated with graded concentrations (0-2 μM) of MMC for 1 h at 37°C. The MMC-containing medium was then removed and replaced with 10 ml of complete α-MEM medium, and the cells were returned to incubation at 37°C for colony formation. After eleven days, cell colonies were fixed and stained for counting. For γ-irradiation treatment, cells were seeded at 200-6,000 cells per T25 flask for each radiation dose to be tested. Cells were irradiated with a ^{137}Cs γ-ray source at a dose rate of 1.50 Gy/min for various doses (0-10 Gy) at room temperature, then returned to incubation at 37°C for colony formation. After eleven days, cell colonies were fixed and stained for counting.

Cell synchronization and flow cytometry

HeLa cells were synchronized in late G_1 phase using the plant amino acid mimosine (Lalande 1990; Watson *et al*, 1991; Talwar *et al*, 1997). Cells were treated with 200 μM mimosine (Calbiochem) for 16 h. At the end of this period, the mimosine-containing medium was removed and the cultures was washed three times with fresh media. Cells were thus released from mimosine blockade and allowed to proceed through the cell cycle. The distribution of cells in the phases of the cell cycle was assessed by flow cytometric analysis. At intervals after release from mimosine, cells were prepared by standard methods using propidium iodide staining of

DNA (Taylor, 1980). Measurements of cell cycle distribution were performed using a FACScan flow cytometer (Becton-Dickinson) and data were analyzed using MODFIT software.

γ-ray survival using synchronous cells

HeLa cells were transfected with Rad51C siRNA (or mock-transfection) as described above. Two days after transfection, both sets of cells were individually synchronized using mimosine treatment. The mock-transfected cultures were irradiated with various doses of γ-rays (0-10 Gy) at time point 0 or 7.5 h after release from mimosine, and the siRNA-transfected cultures were irradiated at 0 or 10 h after release from mimosine. After irradiation, cells were immediately trypsinized and replated in triplicate at 200-10,000 cells per T25 flask for each radiation dose to be tested. A parallel set of cells was returned to incubation at 37°C and replated for colony formation after an 18-h delay. After eleven days of incubation, cell colonies were fixed and stained for counting.

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FOOTNOTES

[1] The abbreviations used are: DSB, double-strand break; NHEJ, non-homologous end joining; HR, homologous recombination; HRR, homologous recombinational repair; MMC, mitomycin

C; RNAi, RNA interference; siRNA, small interfering RNA; IR, ionizing radiation; PLD, potentially lethal damage.

FIGURE LEGENDS

Fig. 1. Inhibition of Rad51C suppresses XRCC3 expression and other Rad51 paralogs. HeLa cells were individually transfected with Rad51C siRNA #1 and #2 duplexes. On days 2, 3 and 4 post-transfection, cells were harvested and subjected to Western blotting analysis with α-Rad51C, α-XRCC3, α-Rad51, α-Rad51B, α-Rad51D, α-XRCC2, and α-QM antibodies. QM is a transcription factor and was used as a loading control.

Fig. 2. (**A**) Homologous recombinational repair of a site-specific chromosomal break in the reporter locus. PGK: mouse phosphoglycerate kinase enhancer/promoter. Pac: puromycin acetyltransferase gene (confers puromycin resistance). Deletion mutation/I-SceI cleavage site: inactivates the pac gene and creates a site for chromosomal cleavage by I-SceI endonuclease. pA: SV40 polyadenylation region. Ori/Amp: bacterial origin of replication and ampicillin resistance gene (needed for propagation of the vector as a plasmid). CMV-BSD: blasticidin deaminase (blasticidin resistance) gene, with Cytomegalovirus promoter/enhancer and bovine growth hormone polyA signal. Heavy line indicates flanking chromosomal sequence. (**B**) Transfection of siRNA duplexes inhibits the expression of Rad51C in HT1080-1885 cells. HT1080-1885 cells were transfected with Rad51C siRNA #1 or siRNA #2, or mock-transfected with Oligofectamine alone (control). On day 2 post-siRNA transfection, cells were harvested and subjected to Western blotting analysis with α-Rad51C and α-QM antibodies. (**C**) siRNA

depletion of Rad51C causes a reduction in HR frequency. On day 2 post-siRNA transfection, HT1080-1885 cells were transfected for expression of I-SceI endonuclease. Each transfection was replated in duplicate for HR frequency measurements. The HR frequency was calculated as the average number of colonies per dishes divided by the plating efficiency for that transfection, divided by 120,000 (the total number of cells plated). The means and standard deviations for six experiments are shown.

Fig. 3. Inhibition of Rad51C causes increased sensitivity to mitomycin C and γ-rays in asynchronous HeLa cells. (**A**) MMC sensitivity: HeLa cells were transfected with Rad51C siRNA #1 or siRNA #2, or mock-transfected with Oligofectamine alone (control). On day 2 post-siRNA transfection, cells were seeded and treated with various concentrations of MMC (0, 0.2, 0.4, 0.8, 1, 2 μM) for 1 h. (**B**) γ-ray sensitivity: HeLa cells were transfected with Rad51C siRNA #1 or siRNA #2, or mock-transfected with Oligofectamine alone (control). On day 2 post-siRNA transfection, cells were seeded and treated with various doses of γ-rays (0, 4, 6, 8, 10 Gy). The surviving fractions were calculated and normalized for plating efficiency. The data points graphed are the means of four independent experiments; the error bars represent standard deviations.

Fig. 4. Distribution of HeLa cells in the phases of the cell cycle as a function of time after release from mimosine treatment. On day 2 post-transfection, the mock-transfected (**A**) and Rad51C siRNA #1-treated (**B**) cells were exposed to 200 μM mimosine for 16 h, at which time the cultures were rinsed three times with fresh media to remove mimosine. Subsequently, cells were collected and fixed at the indicated time points (0, 6.5, 10, 12, 13.7 h) and analyzed by flow

cytometry. The time points chosen for irradiation of the G_1- and S+G_2-enriched populations are indicated by arrows.

Fig. 5. Rad51C-deficient HeLa cells show increased sensitivity to γ-rays in S+G_2/M phase, but not in G_1 phase. After irradiation with various doses (0, 4, 6, 8, 10 Gy) of γ-rays, mock- and Rad51C siRNA-transfected cells were either (**A**) immediately replated in triplicate for colony formation assay, or (**B**) returned to incubation at 37°C for 18 h, and then replated for colony formation assay. The surviving fractions were calculated and normalized for plating efficiency. The means and standard deviations for six replicate experiments are shown.

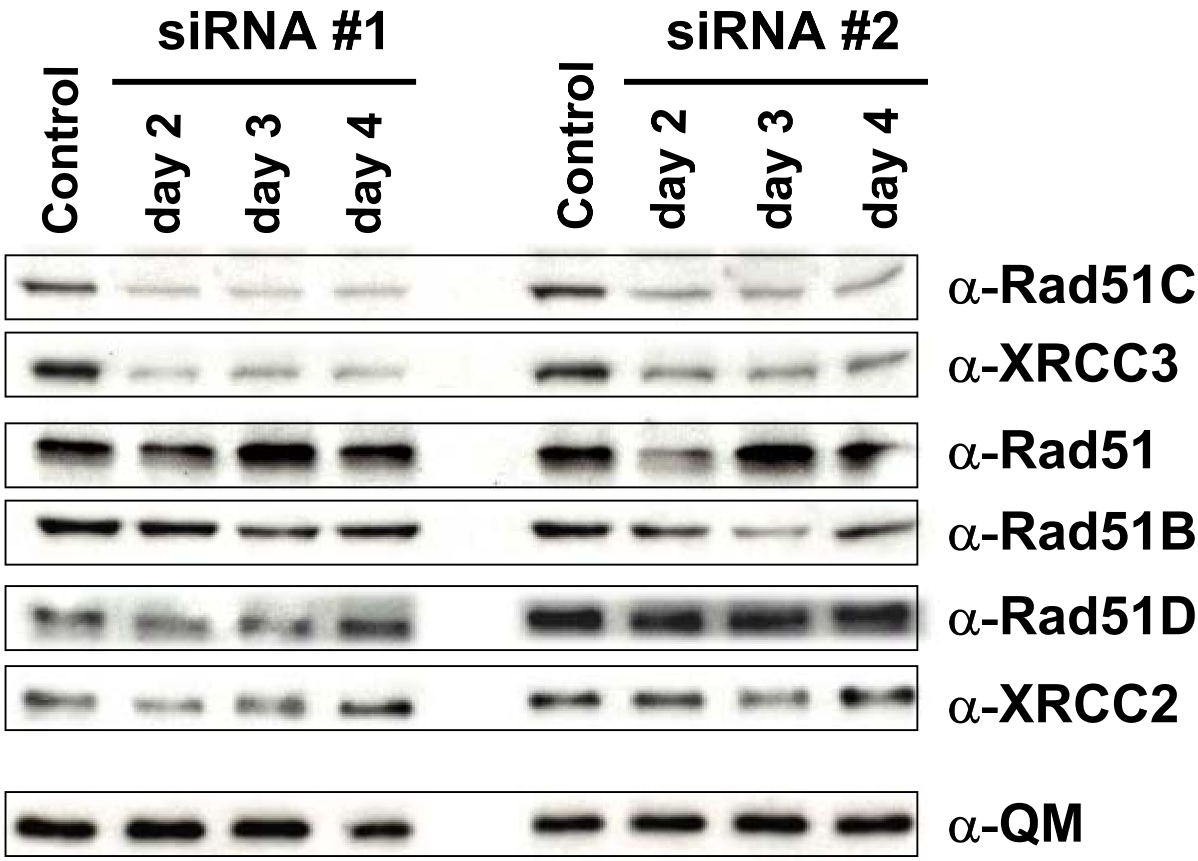
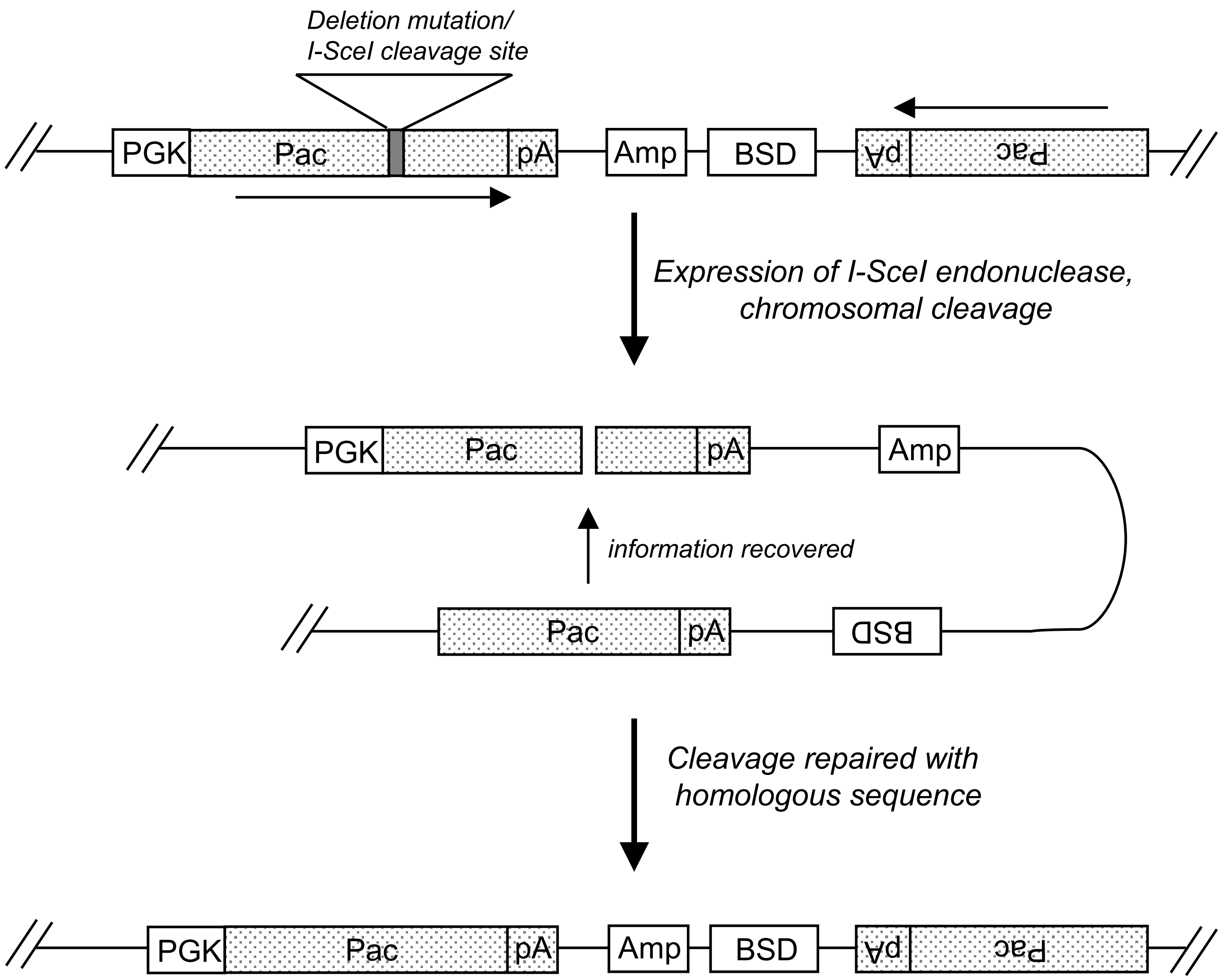

siRNA #1
siRNA #2
Control
day 2
day 3
day 4
Control
day 2
day 3
day 4
α-Rad51C
α-XRCC3
α-Rad51
α-Rad51B
α-Rad51D
α-XRCC2
α-QM

Fig. 1

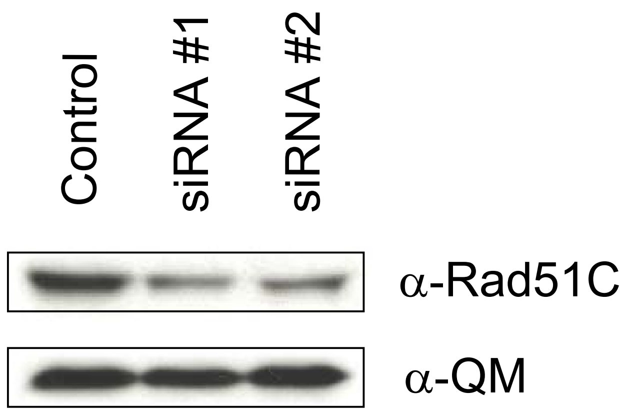
A



Deletion mutation/
I-SceI cleavage site
PGK
Pac
pA
Amp
BSD
pA
Pac
Expression of I-SceI endonuclease,
chromosomal cleavage
PGK
Pac
pA
Amp
information recovered
Pac
pA
BSD
Cleavage repaired with
homologous sequence
PGK
Pac
pA
Amp
BSD
pA
Pac

Fig. 2

B


Control
siRNA #1
siRNA #2
α-Rad51C
α-QM

C

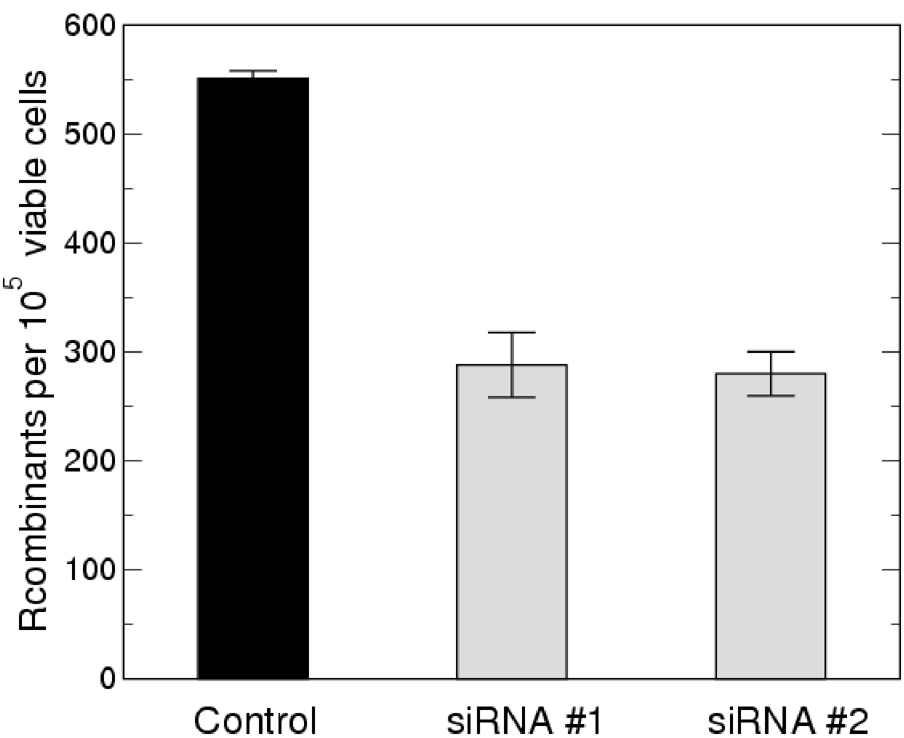

600
500
400
300
200
100
0
Rcombinants per 10^5 viable cells
Control
siRNA #1
siRNA #2

Fig. 2

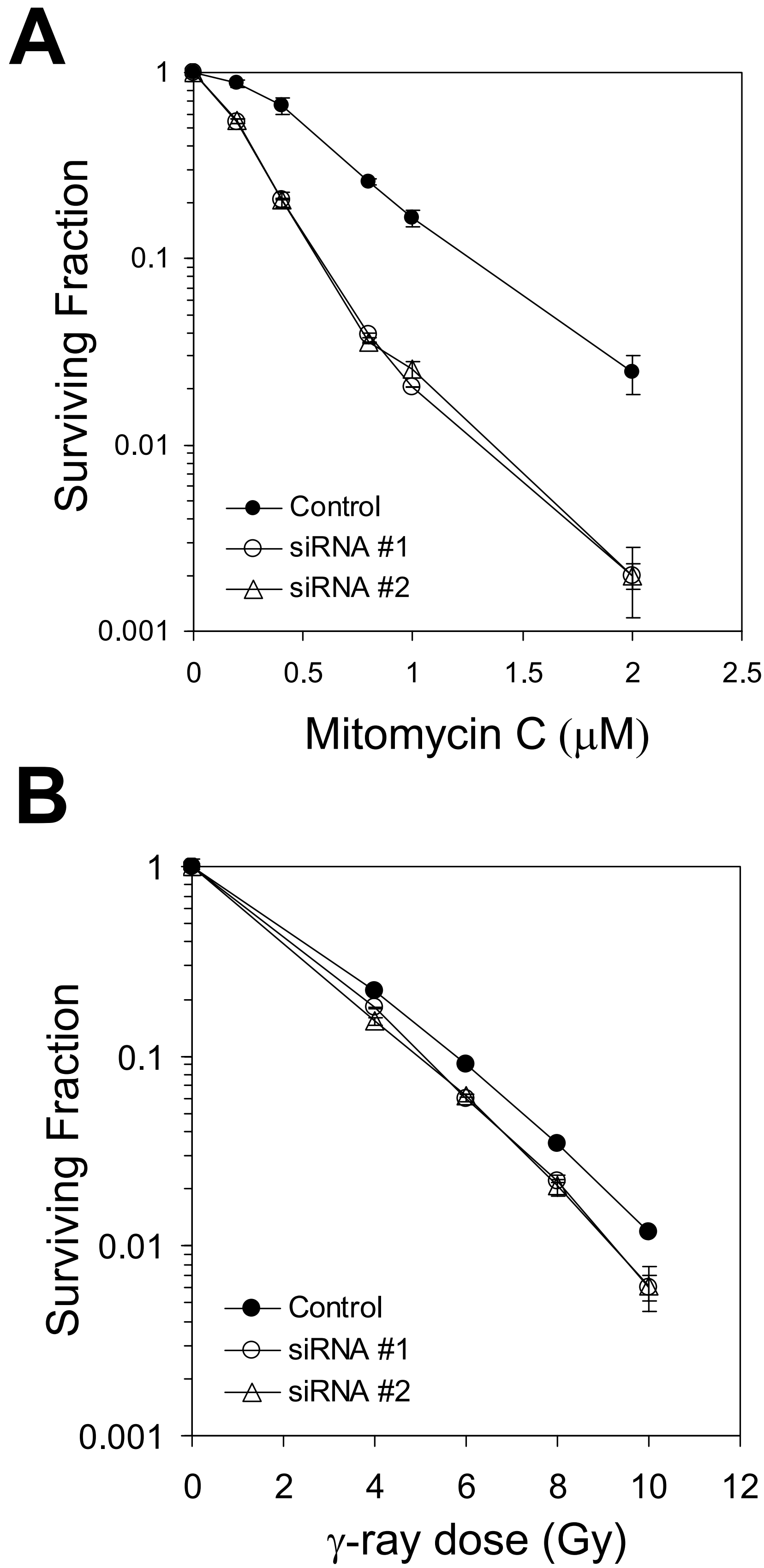

A
Surviving Fraction
1
0.1
0.01
0.001
0
0.5
1
1.5
2
2.5
Mitomycin C (μM)
Control
siRNA #1
siRNA #2
B
Surviving Fraction
1
0.1
0.01
0.001
0
2
4
6
8
10
12
γ-ray dose (Gy)
Control
siRNA #1
siRNA #2

Fig. 3

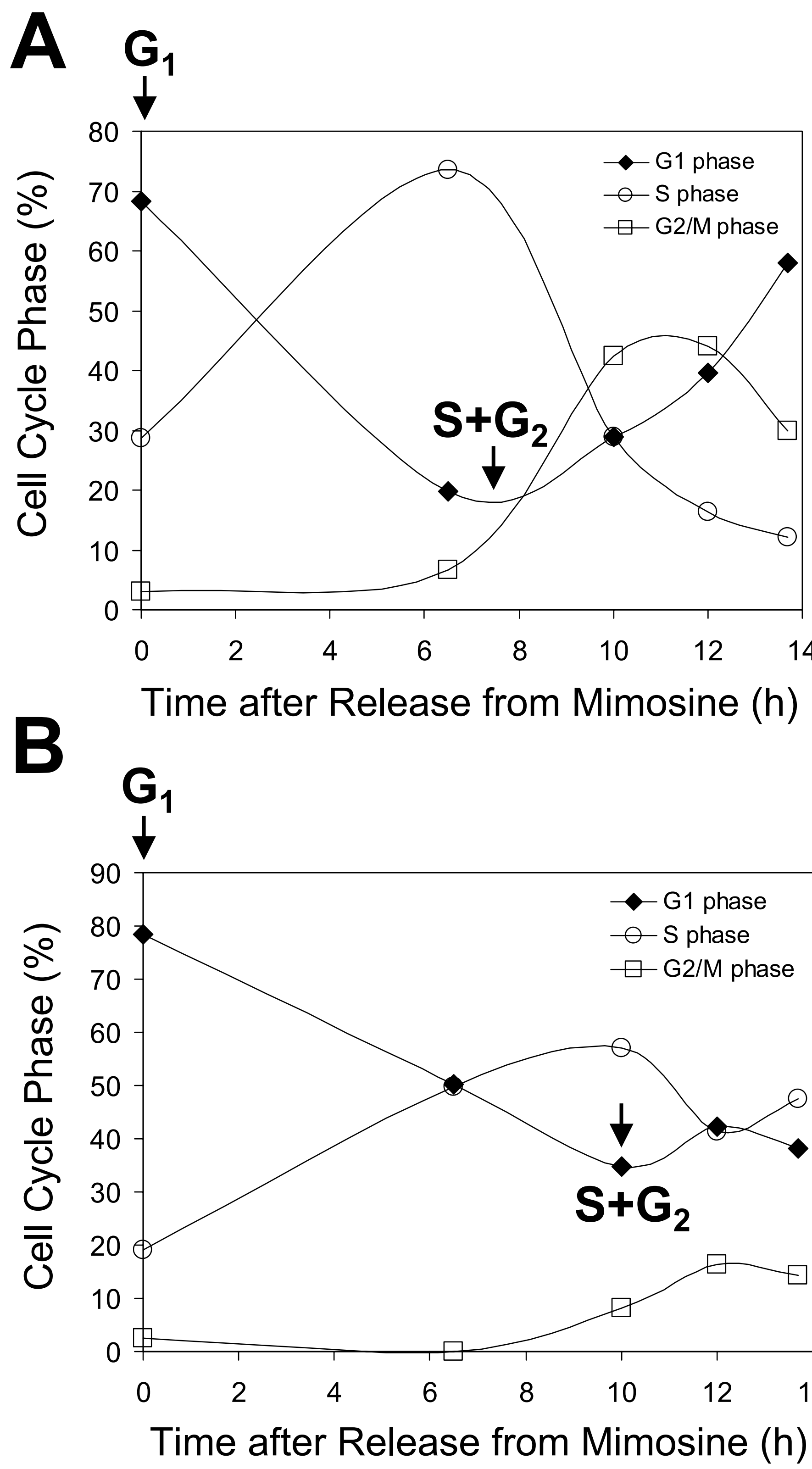

A
G1
S+G2
G1 phase
S phase
G2/M phase
Cell Cycle Phase (%)
Time after Release from Mimosine (h)
B
G1
S+G2
G1 phase
S phase
G2/M phase
Cell Cycle Phase (%)
Time after Release from Mimosine (h)

Fig. 4

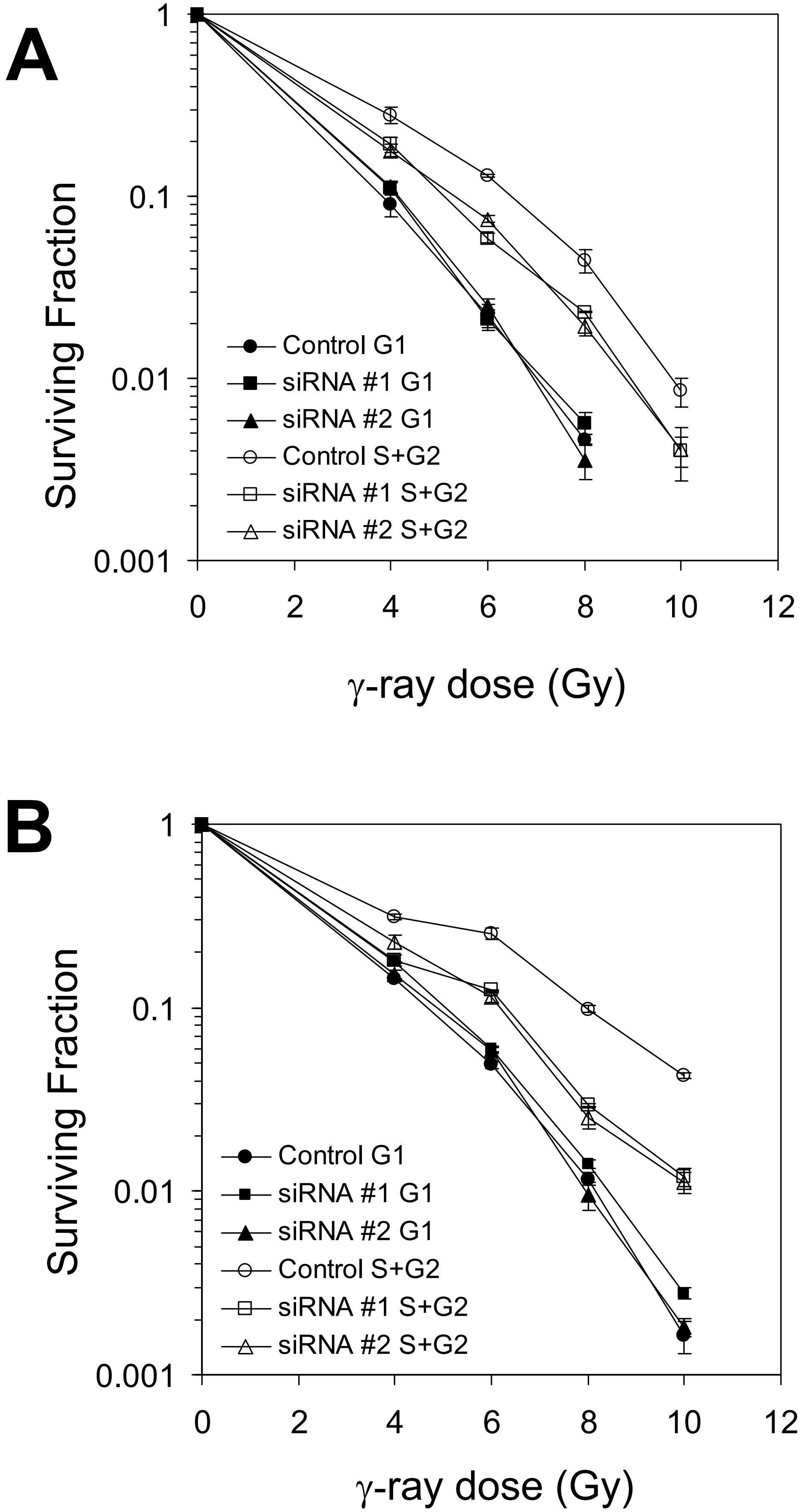

A
B
Surviving Fraction
γ-ray dose (Gy)
1
0.1
0.01
0.001
0
2
4
6
8
10
12
Control G1
siRNA #1 G1
siRNA #2 G1
Control S+G2
siRNA #1 S+G2
siRNA #2 S+G2

Fig. 5