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14. **ABSTRACT**
Using an HR assay system, we found that individual expression of several small BRCA2 regions in human HT1080 cells causes a reduced frequency in homologous recombination. Our data provide the direct cellular evidence that the BRCA2-Rad51 interaction is crucial for HR repair and multiple regions of BRCA2 protein are involved in regulating HR repair. Using the baculovirus co-expression and Ni-NTA pull-down strategies, we demonstrated that BRCA2 forms a multiprotein complex with Rad51, Rad51B and Rad51C DNA repair proteins involving a strong interaction between BRCA2 and Rad51, and between Rad51B and Rad51C. A weak interaction between Rad51 and Rad51C was observed as well. We also found that the BRC repeats of BRCA2 do not directly interact with Rad51B or Rad51C. In addition, we have successfully expressed three BRC fragments using baculovirus expression system. These protein expressions were confirmed by Western analysis. The purification of these proteins was found to be difficult because these proteins were extremely unstable and tended to be degraded during the purification process. We have tested several conditions to stabilize the proteins, including use of different salts, different concentration of salts, different expression temperature, and co-expression of the proteins with Rad51. We have established three biochemical assays for Rad51 activities, including DNA binding, ATPase and DNA strand exchange. The investigation regarding whether the BRC1-4, BRC5-8 or BRC1-8 proteins affects the Rad51 activities are underway.

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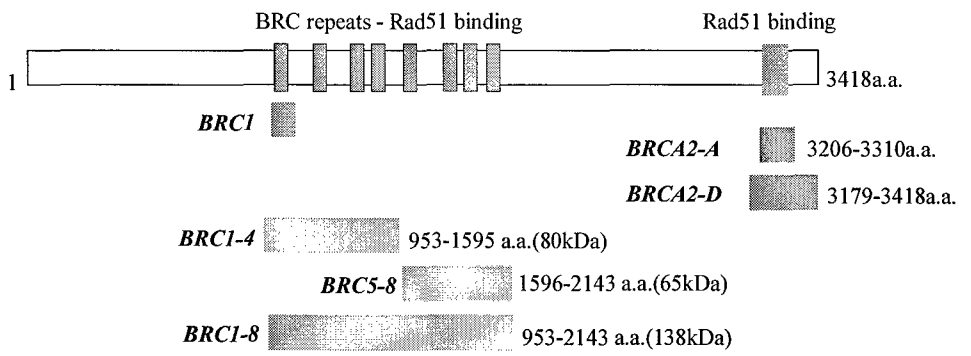
## INTRODUCTION

The BRCA2 gene is associated with hereditary tendency to breast cancer. Exactly how defects in BRCA2 causes predisposition to breast cancer is not yet understood. Recent evidence indicates that the BRCA2 protein has a critical function in DNA repair through homologous recombination (HR). It is very likely that defective HR repair causes the accumulation of unrepaired DNA in genome and results in cancers. We propose to investigate how BRCA2 functions in DNA HR repair using both cellular and biochemical approaches.

The BRCA2 gene encodes a large protein of 3418 amino acids with a molecular weight of 384-kDa. The BRCA2 protein physically interacts with Rad51, the key protein in DNA HR repair via two Rad51-binding domains, eight BRC repeats and a extreme C-terminal region (amino acids 3196-3232). These eight conserved BRC repeats (designated as BRC1 to BRC8), located in the central portion of the protein and cover nearly a third of the protein. These two Rad51-binding domains of BRCA2 have been shown to be essential for normal sensitivity to DNA-damaging agents, indicating they are functionally significant. Therefore, we focus our efforts on investigation of the effects of these Rad51-interacting regions of BRCA2 in HR repair. The domain constructs used in our study are diagrammed in Fig. 1. **Our specific aims and final research results are summarized as the following:**

**Fig. 1. Domain constructs of human BRCA2 protein**




**Specific aim 1. To test the hypothesis that disruption of the BRCA2-Rad51 interaction affects homologous recombination (HR) in human HT1080 cells.** We propose that the human BRCA2 participates directly to homologous recombination through the interaction with Rad51 and regulates this process in some critical way, such that disruption of the BRCA2-Rad51 interaction results in a reduction in the capacity of repairing chromosome breaks by HR. We established a cellular approach to assay HR frequency *in vivo*. To measure HR, an artificial reporter locus was installed into a chromosome of human HT1080 cells and clones of cells that integrated the reporter were isolated. A defined DNA double-strand break can be introduced at the integrated reporter locus, by transient expression of a highly site-specific endonuclease I-SceI of *S. cerevisiae*. This reporter locus can detect repair of a specifically I-SceI induced chromosomal double-strand break by HR. The HT1080 cells harboring the reporter were therefore used for the HR assay.

**Final results:**

Using this HR assay system, we investigated the effects of three BRCA2 regions, including a BRC1 motif and two C-terminal fragments (BRCA2-A and BRCA2-D). Fragment BRCA2-A consists of amino acids 3206-33310 and fragment BRCA2-D consists of amino acids 3179-3418. We found that transient expression of the BRC1 motif of BRCA2 during double-strand break induction in HT1080 cells caused a great suppression (~22-fold) as compared with the control (vector alone) in the frequency of HR (Fig. 2.). The result suggests that the BRCA2-Rad51 interaction through BRC1 is important for regulating HR repair, such that disruption of the normal protein-protein interaction between BRCA2 and Rad51 by overexpression of the small BRCA2 domain causes impaired HR. In addition, expression of fragment BRCA2-A or BRCA2-D in HT1080 cells resulted in a smaller but significant reduction in the HR frequency (Fig. 3.), indicating that the C-terminal region of BRCA2 also plays a role in the function of BRCA2 in HRR. Our results provide direct cellular evidence that the BRCA2-Rad51 interaction is crucial for HR repair and that multiple regions of BRCA2 protein are involved in regulating HR repair.

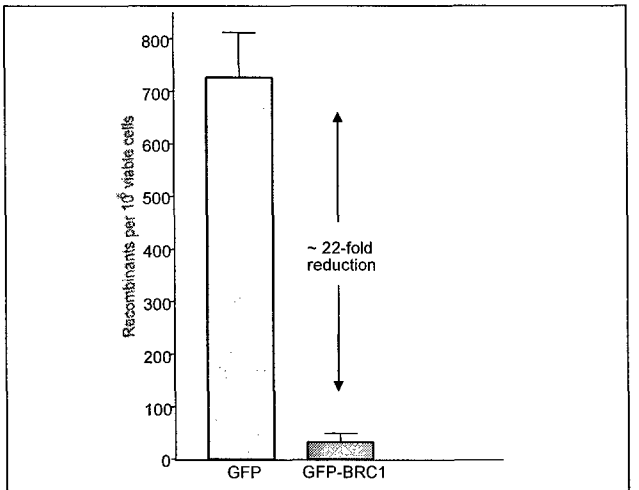



**Fig. 2. Expression of BRC1 motif of BRCA2 greatly inhibits repair of a chromosome break by homologous recombination**

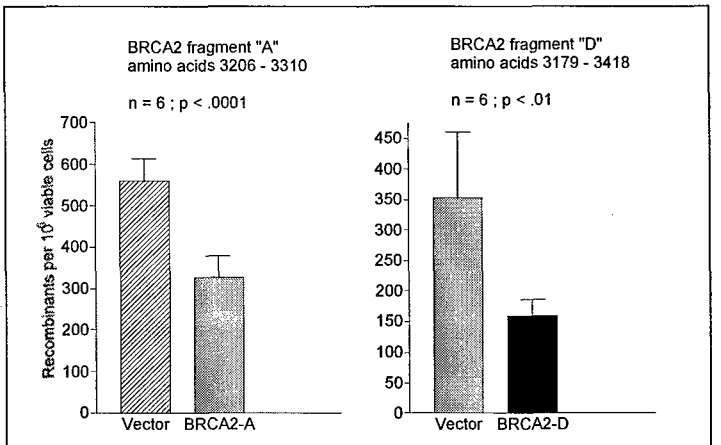



**Fig. 3. Expression of C-terminal fragments of BRCA2 causes smaller but significant reductions in HR frequency**

**Specific aim 2. To test the hypothesis that the BRCA2 protein functions to mediate the biochemical activities of Rad51.** It is not clear how the Rad51-BRCA2 interaction regulates DNA homologous recombinational repair. We hypothesize that BRCA2 directly mediates the biochemical activities of Rad51 and subsequently affects homologous recombination. To investigate the effects of BRCA2 protein on the Rad51 activities, we proposed to purify the BRCA2 protein and examine its effects on Rad51 using three biochemical assays, including DNA binding, ATPase and DNA strand exchange. Because the very large size of BRCA2 might hamper attempts to obtain the full-length protein, we decided to purify the functional domain of BRCA2, the BRC repeats. We proposed to express and purify three BRC fragments, including BRC1-4, BRC5-8 and BRC1-8. The purification of the human Rad51 protein is required for the assays as well.

**Results on protein purification:**

We have expressed the human Rad51 protein in insect cells Sf9 using the baculovirus system. The Rad51 protein was subsequently purified to

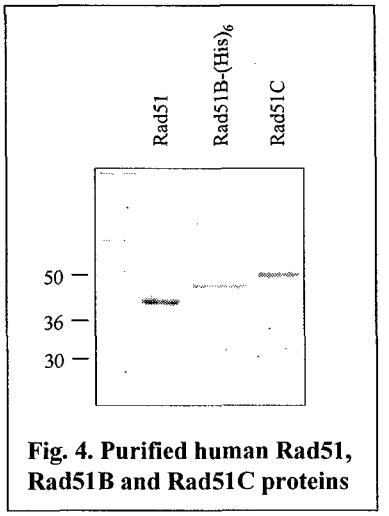



**Fig. 4. Purified human Rad51, Rad51B and Rad51C proteins**

homogeneity using spermidine precipitation and sequential column chromatography with hydroxyapatite, Q-Sepharose, heparin affinity and MonoQ column (Fig. 4.). We have been able to obtain about 10 mg of the purified Rad51 protein during our study and this amount of the protein is sufficient for the biochemical assays. To obtain the BRCA2 protein, we constructed the BRC1-4, BRC5-8 and BRC1-8 fragments of BRCA2 (as diagrammed in Fig. 1.) into a 6xHis-tagged baculoviral vector. Employing the baculovirus strategies, we successfully expressed the three BRC fragments of BRCA2 in Sf9 cells. The BRC1-4, BRC5-8, and BRC1-8 domains encode the proteins of 80-kDa, 65-kDa, and 138-kDa, respectively. The identity of these proteins was confirmed by Western Blotting using either α-BRC4 or α-BRC5 antibody as well as α-His antibody (Fig. 5.). When these proteins were subjected to purification, we discovered that these BRC proteins were extremely unstable and tended to be degraded during the purification process. We have been working on several approaches to solve the problem. We tested several conditions to stabilize the proteins, including use of different salts, different concentration of salts, different expression temperature, and co-expression of each protein with Rad51. Although some of the methods improved the stability of the protein, none of them can ultimately produce a protein, which is stable enough for biochemical assays.

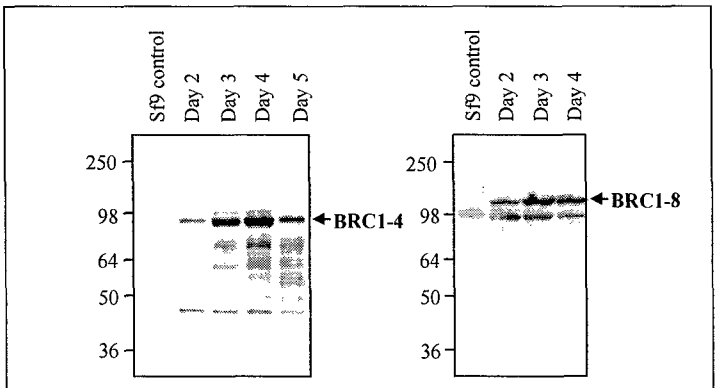



**Fig. 5. Baculovirus expression of BRC1-4 and BRC1-8 fragments.** Cells were harvested on days 2, 3, 4, 5 post-infection of baculoviruses

**Results on activity assays:**

We established three *in vitro* assays for Rad51 activity, including DNA binding, ATPase and DNA strand exchange. Using these biochemical assays, we examined the activity for Rad51 and Rad51 paralogs, Rad51B and Rad51C. We also examined the effects of small BRCA2 domains on the activity of Rad51.

**1) DNA binding assay.** We established a gel shift assay to determine the DNA binding activity using [$^{32}$P]-labeled oligonucleotides, including single-stranded DNA (ssDNA) 63mer, double-stranded DNA (dsDNA) 63/63mer, and 3'-tailed dsDNA 63/32mer. Using this assay, we have examined the DNA binding activity of Rad51 and two Rad51 paralogs, Rad51B and Rad51C. We demonstrated that Rad51B and Rad51C bind both ssDNA and dsDNA, and show preference for tailed dsDNA. We also compared the DNA binding capabilities of Rad51B and Rad51C with Rad51 using the same three DNA substrates: 63mer, 63/63mer and 63/32mer. We found that Rad51C possesses a higher affinity for all of three DNAs than does Rad51. The relative affinity of these three proteins for DNA was found to be: Rad51C > Rad51 > Rad51B.

**2) ATPase assay.** We established an *in vitro* ATPase assay using [γ-$^{32}$P]ATP as the substrates and have determined the ATPase activity of the Rad51, Rad51B and Rad51C proteins. We also examined whether the BRC1 domain of BRCA2 has effects on the ATPase activity of Rad51. As shown in Fig. 6., various ratios of

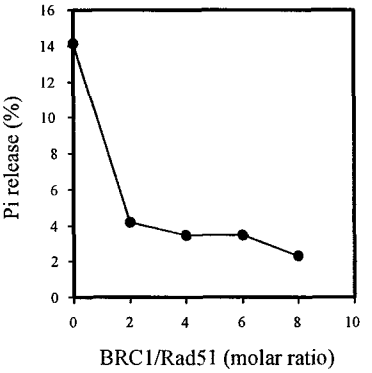



**Fig. 6. BRC1 motif of BRCA2 inhibits the ATPase activity of the human Rad51 protein**

BRC1 and Rad51 were tested in the assay and an inhibitory effect on the ATPase activity of Rad51 was observed. We found that the ATPase activity of Rad51 was inhibited at approximately 4.0 fold with the molar ratio of BRC1 and Rad51 is equal to 2 and that the inhibitory effect was slightly increased as the BRC1/Rad51 ratio increases. The inhibitory effect of the BRC1 domain on the ATPase activity of Rad51 indicates that the BRC1 domain of BRCA2 is important for regulating the ATP binding and/or hydrolysis of Rad51.

**3) DNA strand exchange assay.** The key biochemical activity of Rad51 in the recombinational DNA repair process is to promote strand pairing and exchange between two homologous DNA strands. It is important to investigate whether BRCA2 acts to mediate the DNA strand exchange activity of Rad51 and leads to effects on homologous recombination. We established a DNA strand transfer assay using ssDNA 63mers and [$^{32}$P]-labeled dsDNA 32/32mers as the substrates. The DNA strand transfer activity of Rad51 was determined as a control. The strand transfer products (3'-tailed 63/32mer) were observed and the amount of product formation is dependent on the concentration of Rad51. We also demonstrated that Rad51C displays apparent DNA strand transfer in an ATP-independent manner, while Rad51B shows no such activity (Fig. 7.).

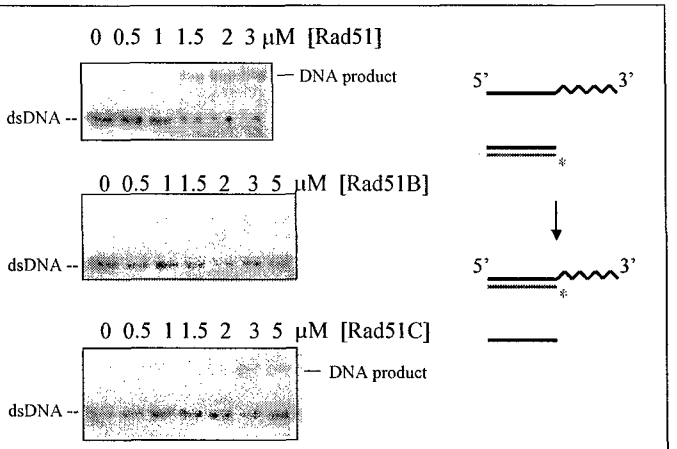



**Fig. 7. DNA strand transfer activity of Rad51.** The Rad51C protein displayed low activity compared to that of Rad51. The apparent DNA strand exchange ability of Rad51C is due to its DNA melting activity.

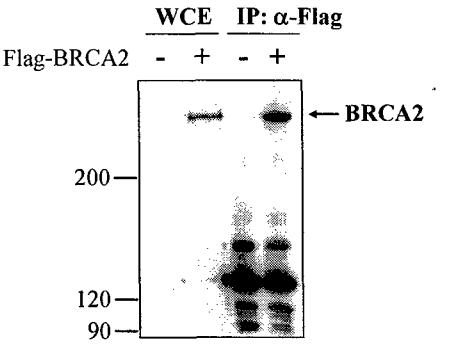



**Fig. 8. Expression and immunoprecipitation of BRCA2 in HeLa cells.** After transfection with or without the Flag-BRCA2 plasmid, whole cell extracts (WCE) and immunoprecipitated (IP) samples were subjected to western blotting with anti-Flag antibody.

**New finding regards radiation-induced *in vivo* phosphorylation of BRCA2:**

Using a Flag-tagged BRCA2-pME18S mammalian construct, we have demonstrated a successful transient expression of the full-length BRCA2 in HeLa cells and shown that the expressed BRCA2 can be immunoprecipitated by anti-Flag antibody (Fig. 8). Importantly, we found that the Flag-tagged BRCA2 can be phosphorylated with *in vivo* [$^{32}$P]-orthophosphate labeling and the phosphorylation is induced by 50 Gy of X-rays (Fig. 9). This preliminary result is the first evidence indicating that BRCA2 is phosphorylated *in vivo* in a DNA damage-dependent manner. We further analyzed BRCA2 in human, canine, chicken, and mouse using sequence alignments, and found that human BRCA2 contains eighteen potential (S/TQ) phosphorylation sites and that many of the sites are highly conserved among different species (Fig. 10). Because S/TQ has been shown to be a consensus sequence for the substrate of phophoinositide 3-kinase related kinases (PIKKs), our findings lead to a speculation that the IR-inducible in vivo phosphorylation of BRCA2 might be regulated by PIKKs. Although the overall sequence similarity of BRCA2 is low among different species, based on sequence alignments we found that BRCA2 has three highly conserved regions, an N-terminus end, a C-terminal region, and a small region containing the BRC1 and BRC2 domains.

Interestingly, the BRC1-2 region possesses three highly conserved S/TQ sites (Fig. 10). We therefore first examined whether this BRC1-2 region is phosphorylated in response to IR. We have constructed the BRC1-2 region (BRC-N) and this region with deletion of S1106 and S1123 (BRC-DN) in the Flag-tagged pME18S vector (Fig. 11). We have further demonstrated a successful transient expression of these two BRCA2 fragments in HeLa cells and shown that the expressed BRCA2 can be immunoprecipitated by anti-Flag antibody (Fig. 12). The cells will be treated with in vivo [$^{32}$P]-orthophosphate labeling, followed by X-ray irradiation. Whether either the BRC1-2 region is in vivo phosphorylated will be determined. We will also further characterize the nature of the IR-induced BRCA2 phosphorylation.

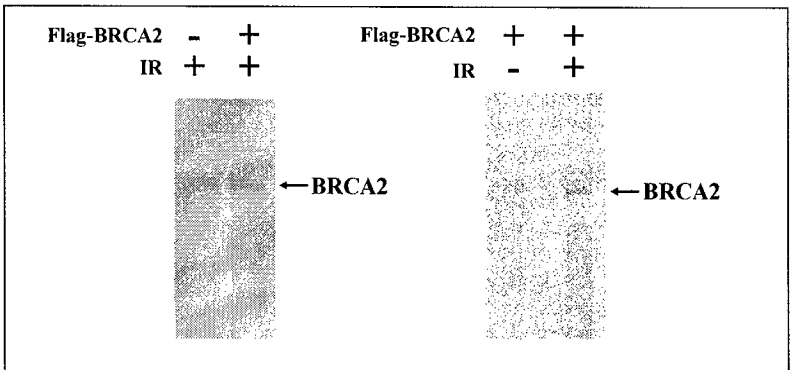



**Fig. 9. BRCA2 is phosphorylated in vivo in response to IR.** HeLa cells were transfected with the Flag-BRCA2 plasmid and *in vivo* labeled with [$^{32}$P], followed by 50 Gy of X-ray irradiation. Cells extracts were immunoprecipitated with anti-Flag antibody, and were resolved by SDS-PAGE and detected by autoradiography.

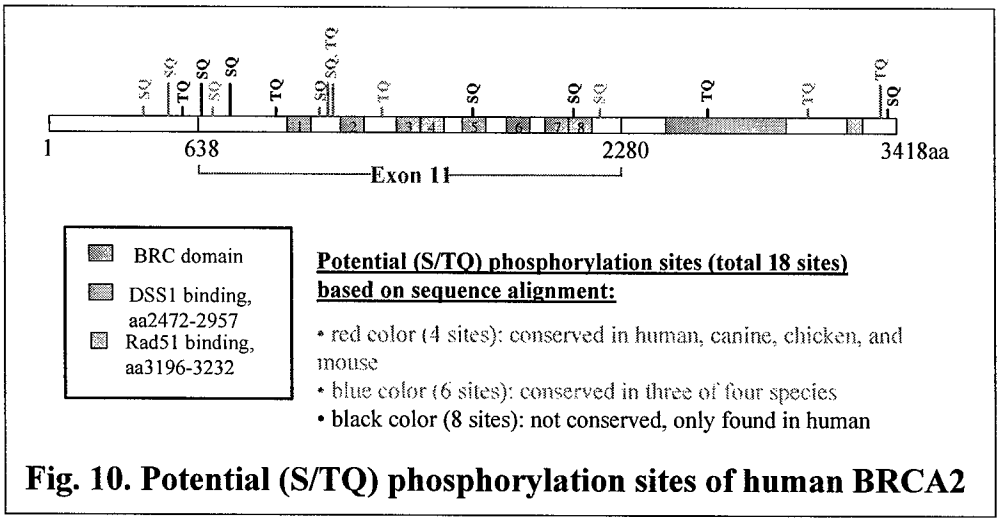



**Fig. 10. Potential (S/TQ) phosphorylation sites of human BRCA2**

**Specific aim 3. To test the hypothesis that the BRCA2 protein forms a stable complex with Rad51 and two Rad51 paralogs, Rad51B and Rad51C.** Using a baculovirus co-expression system and Ni-NTA pull-down strategies, we have demonstrated that Rad51 and two Rad51 paralogs (Rad51B and Rad51C) interact simultaneously and form a multiprotein complex. We have changed the initial aim to demonstrate that BRCA2 is an essential component in the Rad51-dependent recombinational complex, interacting with Rad51, Rad51B, and Rad51C to form a stable complex. The Ni-NTA pull-down strategies were used to test this hypothesis. In addition, we also

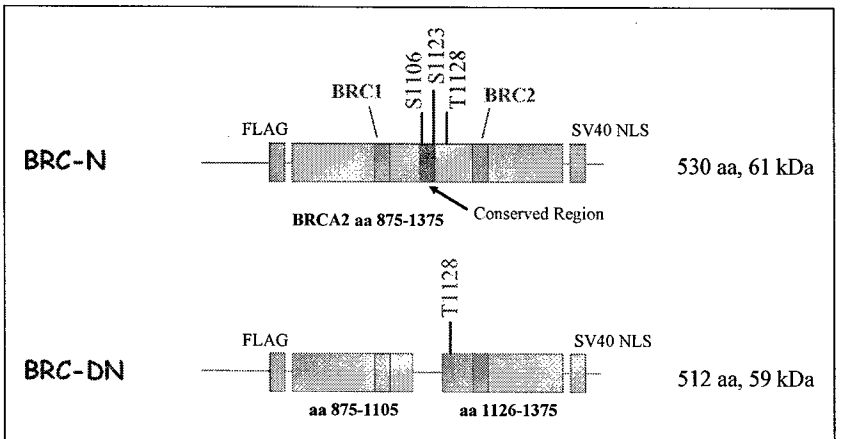



**Fig. 11. BRC1-2 Expression plasmids.** BRC-N and BRC-DN were cloned into pCD-F2 (without S40 NLS) and pFN (with S40 NLS) vectors.

investigated whether BRCA2 directly interacts with Rad51B or Rad51C.

**Final results:**

Using baculovirus system, we co-expressed the 6xHis-tagged BRC1-4 (or BRC1-8) domain of BRCA2 with untagged Rad51, Rad51B and Rad51C in Sf9 cells. The Ni-NTA magnetic beads were used to pull-down 6xHis-tagged BRC1-4 (or BRC1-8) protein and the associated proteins. We examined the pull-downed fractions using Western blotting analysis with α-BRC, α-Rad51, α-Rad51B and α-Rad51C antibodies. We found that a large amount of Rad51 was pull-downed by BRC fragments as well as a little amount of Rad51C and Rad51B. The result indicates that BRCA2 interacts simultaneously with Rad51, Rad51B and Rad51C, and forms a complex with these proteins *in vitro*. It also shows that the interaction between the BRC repeats of BRCA2 and Rad51 is strong and BRC1-4/Rad51 (or BRC1-8/Rad51) interacts weakly with Rad51C/Rad51B heterodimer through the interaction between Rad51 and Rad51C.

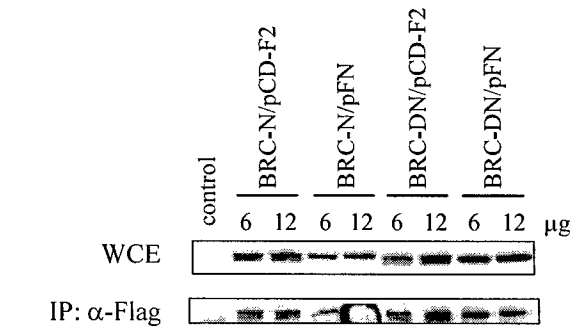



**Fig. 12. Expression and immunoprecipitation of BRC1-2 fragments in HeLa cells.** After transfection of BRC-N or BRC-DN fragment-containing expression plasmid, whole cell extracts (WCE) and immunoprecipitated (IP) samples were subjected to western blotting analysis with anti-Flag antibody.

In addition, to examine whether the BRC repeats of BRCA2 interacts directly with Rad51 paralog Rad51B (or Rad51C), we co-expressed the 6xHis-tagged BRC1-4 (or BRC1-8) fragment and untagged Rad51B (or Rad51C) proteins in Sf9 insect cells. We tested BRC1-4/Rad51B, BRC1-8/Rad51B, BRC1-4/Rad51C and BRC1-8/Rad51C. The protein expressions were confirmed using Western blotting analysis with α-BRC4, α-BRC5, α-Rad51B and α-Rad51C antibodies. The Ni-NTA magnetic beads were used to bind the 6xHis-tagged BRC domains and whether the untagged Rad51B (or Rad51C) can be pull-downed by the BRC domains was examined. We found that no detectable Rad51B or Rad51C was observed in the pull-downed fraction with either BRC1-4 or BRC1-8 fragment. The results suggest that there is no direct interaction between the BRC repeats of BRCA2 and Rad51B, or BRCA2 and Rad51C.

Together, our data demonstrated that: (1) BRCA2 forms a multiprotein complex with Rad51, Rad51B and Rad51C through a strong interaction between BRCA2 and Rad51 and a weak interaction between Rad51 and Rad51C. (2) There is no direct interaction between the BRC repeats of BRCA2 and Rad51B (or Rad51C).

## KEY RESEARCH ACCOMPLISHMENTS

1. Using an in vivo assay, the BRC1 motif and two C-terminal fragments (amino acids 3206-3310 and 3179-3418) of the BRCA2 protein was shown to function in DNA repair via homologous recombinational (HR). Our results provide direct cellular evidence that BRCA2-Rad51 interaction is crucial for HR repair and that the multiple regions of BRCA2 protein are involved in regulating HR repair.
2. We found that BRCA2 forms a multiprotein complex with Rad51, Rad51B and Rad51C through a strong interaction between BRCA2 and Rad51 and a weak interaction between Rad51 and Rad51C. No direct interaction between the BRC repeats of BRCA2 and Rad51B (or Rad51C) was found.

3. The human Rad51 protein was expressed in insect cells and purified to homogeneity using spermidine precipitation and sequential column chromatography. The BRC1-4 and BRC1-8 fragments of BRCA2 were expressed. We discovered that these BRC proteins were extremely unstable and tended to be degraded during the purification process. We have been working on several approaches to solve the problem.
4. We found that the BRC1 domain of BRCA2 suppresses the ATPase activity of human Rad51. The result indicates that the BRC1 domain of BRCA2 functions to modulate the ATP binding/hydrolysis of Rad51.
5. Our preliminary data demonstrate the first evidence indicating that BRCA2 is phosphorylated in vivo in a DNA damage-dependent manner.

## REPORTABLE OUTCOMES

1. An abstract "Lio, Y-C, Schild, D, Brenneman, MA, Redpath, JL, and Chen, DJ. siRNA depletion of Rad51C suppresses homologous recombination frequency and causes hypersensitivity to mitomycin C in human cells." was presented in American Association for Cancer Research Special Conference at Dana Point, California in February 2004.
2. A manuscript related to this project entitled "Human Rad51C deficiency destabilizes XRCC3, impairs recombination and radiosensitizes S/$G_2$-phase cells." has been accepted for publishing in the Journal of Biological Chemistry.

## CONCLUSIONS

Using an HR assay system, we found that individual expression of several small BRCA2 regions in human HT1080 cells causes a reduced frequency in homologous recombination. These results indicate that disruption of the normal BRCA2-Rad51 interaction by introducing the small BRCA2 fragments impairs homologous recombination. Our data provide the direct cellular evidence that the BRCA2-Rad51 interaction is crucial for HR repair and multiple regions of BRCA2 protein are involved in regulating HR repair.

Using the baculovirus co-expression and Ni-NTA pull-down strategies, we demonstrated that BRCA2 forms a multiprotein complex with Rad51, Rad51B and Rad51C DNA repair proteins involving a strong interaction between BRCA2 and Rad51, and between Rad51B and Rad51C. A weak interaction between Rad51 and Rad51C was observed as well. We also found that the BRC repeats of BRCA2 do not directly interact with Rad51B or Rad51C.

In addition, we aim to purify the Rad51-binding domain (BRC repeats) of BRCA2 and investigate its effects on Rad51 activities. We have successfully expressed three BRC fragments using baculovirus expression system, including BRC1-4, BRC5-8 and BRC1-8. These protein expressions were confirmed by Western analysis using specific antibodies. The purification of these proteins was found to be difficult because these proteins were extremely unstable and tended to be degraded during the purification process. We have tested several conditions to stabilize the proteins, including use of different salts, different concentration of salts, different expression temperature, and co-expression of the proteins with Rad51. We have established three biochemical assays for Rad51 activities, including DNA binding, ATPase and DNA strand exchange. We found that the BRC1 domain of BRCA2 inhibits the ATPase activity of Rad51, indicating a role for the BRC1 domain in modulating the ATP binding and/or hydrolysis activity of Rad51. The investigation regarding whether the BRC1-4, BRC5-8 or BRC1-8 proteins affects the Rad51 activities are underway.

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APPENDICES


1. Lio, Y-C, Schild, D, Brenneman, MA, Redpath, JL, and Chen, DJ (2004) Human Rad51C deficiency destabilizes XRCC3, impairs recombination and radiosensitizes S/$G_2$-phase cells. *J. Biol. Chem.* **279**, 42313-42320.

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# Human Rad51C Deficiency Destabilizes XRCC3, Impairs Recombination, and Radiosensitizes S/$G_2$-phase Cells*

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**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, and XRCC3) are expressed in mitotically growing cells and are thought to play mediating roles in homologous recombination, although their precise functions remain unclear. Among the five paralogs, Rad51C was found to be a central component present in two complexes, Rad51C-XRCC3 and Rad51B-Rad51C-Rad51D-XRCC2. We have shown previously that the human Rad51C protein exhibits three biochemical activities, including DNA binding, ATPase, and DNA duplex separation. 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 function of human Rad51C in homologous recombinational repair.**

In mammalian cells, DNA double strand breaks (DSBs)[1] are repaired primarily by two distinct mechanisms, 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 (for review, see Refs. 1 and 2). 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 (for review, see Refs. 3 and 4). The Rad51 protein plays a key role in HR, functioning to mediate homologous DNA pairing and strand exchange (5, 6). Five vertebrate Rad51 paralogs are expressed in mitotically growing cells: Rad51B (7–9), Rad51C (10), Rad51D (9, 11, 12), XRCC2 (13–15), and XRCC3 (13, 16, 17). 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 (18–22). In terms of protein-protein interactions, Rad51C apparently has a central role, interacting directly with Rad51B, Rad51D, and XRCC3 and also weakly with Rad51 (23, 24). However, the functional significance of these complexes is not yet clear.

Mutant studies provide a direct means for identifying the function of genes. A knock-out mutation of Rad51C was generated previously in DT40 chicken B-lymphocyte cells (25). 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 (25, 26), 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, and both were found to show reduced sister chromatid exchange and genomic instability (27, 28). However, how Rad51C functions in human cells has not yet been understood, and no Rad51C-mutant human cell line has been available.

RNA interference (RNAi) has rapidly emerged as a powerful technique for investigating gene function (29–32) and a valuable complement to mutant studies. RNAi is a sequence-specific posttranscriptional gene-silencing mechanism that uses double-stranded RNA as a signal to trigger the degradation of homologous mRNA (33, 34). 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 (35–38).

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[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; α-MEM, minimal essential medium; Gy, gray; Pac, puromycin acetyltransferase; $D_{10}$, dose that reduces survival to 10%.

To determine the functions of Rad51C in human cells, we have used previously *in vitro* assays to demonstrate that the purified human Rad51C protein exhibits DNA binding, ATPase, and double-stranded DNA separation activities (24). 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 (39), 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 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.

## EXPERIMENTAL PROCEDURES

*Rad51C siRNA Design*—The siRNA duplexes were designed according to published procedures (36, 38) by selecting sense and antisense oligoribonucleotides homologous to the mRNA sequence. siRNA 1 is ~80 bases from the initiating AUG codon, and siRNA 2 is about 120 bases before the carboxyl-terminal encoding sequence. Both siRNA 1 and siRNA 2 are complementary 21-mers with a 2-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 (38). The siRNA sequences of Rad51C used in the study are siRNA 1, CUCCUAGAGGUGAAACCCUtt, and siRNA 2, GUCACCCAGCCAGAAGGAAtt.

*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 (Invitrogen). The cells were maintained in a humidified 4.3% $CO_2$ incubator at 37 °C. Twenty-four h prior to transfection, cells were seeded in a 6-well plate at 400,000 cells/well. For each well, 15 μl of siRNA stock oligonucleotide (20 μM) was diluted into 0.26 ml of Opti-MEM (Invitrogen). 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 dropwise to the cells and incubated at 37 °C. After 6 h, 0.75 ml of α-MEM 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 posttransfection.

*Antibodies*—Rad51B and Rad51C antibodies were generated as described previously (24). 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/well. The next day, each well was transfected with 1 μg of I-SceI expression vector pCMV(3×NLS)I-SceI and 4 μl of Superfect transfection reagent (Qiagen) or transfected with Superfect alone in serum-free α-MEM at a final volume of 1 ml. After 5 h of incubation at 37 °C, the transfection mixture was removed; 3 ml of complete α-MEM was added into each well, and the cells were returned to incubation at 37 °C. Twenty-four h after I-SceI transfection, cells were replated in duplicate for puromycin selection at 120,000 cells/100-mm culture dish. Parallel platings for measurement of plating efficiency were made in duplicate at 250 cells/100-mm dish. On day 2 after transfection with I-SceI, selection cultures were refed with fresh medium 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 11 days without refeeding and then were fixed and stained. Colonies with 50 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/100-mm dish for each MMC dose to be tested. After a 4-h 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, and the cells were returned to incubation at 37 °C for colony formation. After 11 days, cell colonies were fixed and stained for counting. For γ-irradiation treatment, cells were seeded at 200–6,000 cells/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 and then returned to incubation at 37 °C for colony formation. After 11 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 (40–42). 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 were washed three times with fresh medium. 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 (43). Measurements of cell cycle distribution were performed using a FACScan flow cytometer (BD Biosciences), 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/T25 flask for each radiation dose to be tested. A parallel set of cells was returned to incubation at 37 °C and replated in triplicate for colony formation after an 18-h delay. After 11 days of incubation, cell colonies were fixed and stained for counting.

## 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 under "Experimental Procedures," 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 (18–24). 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 posttransfection. As shown in Fig. 1*A*, 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

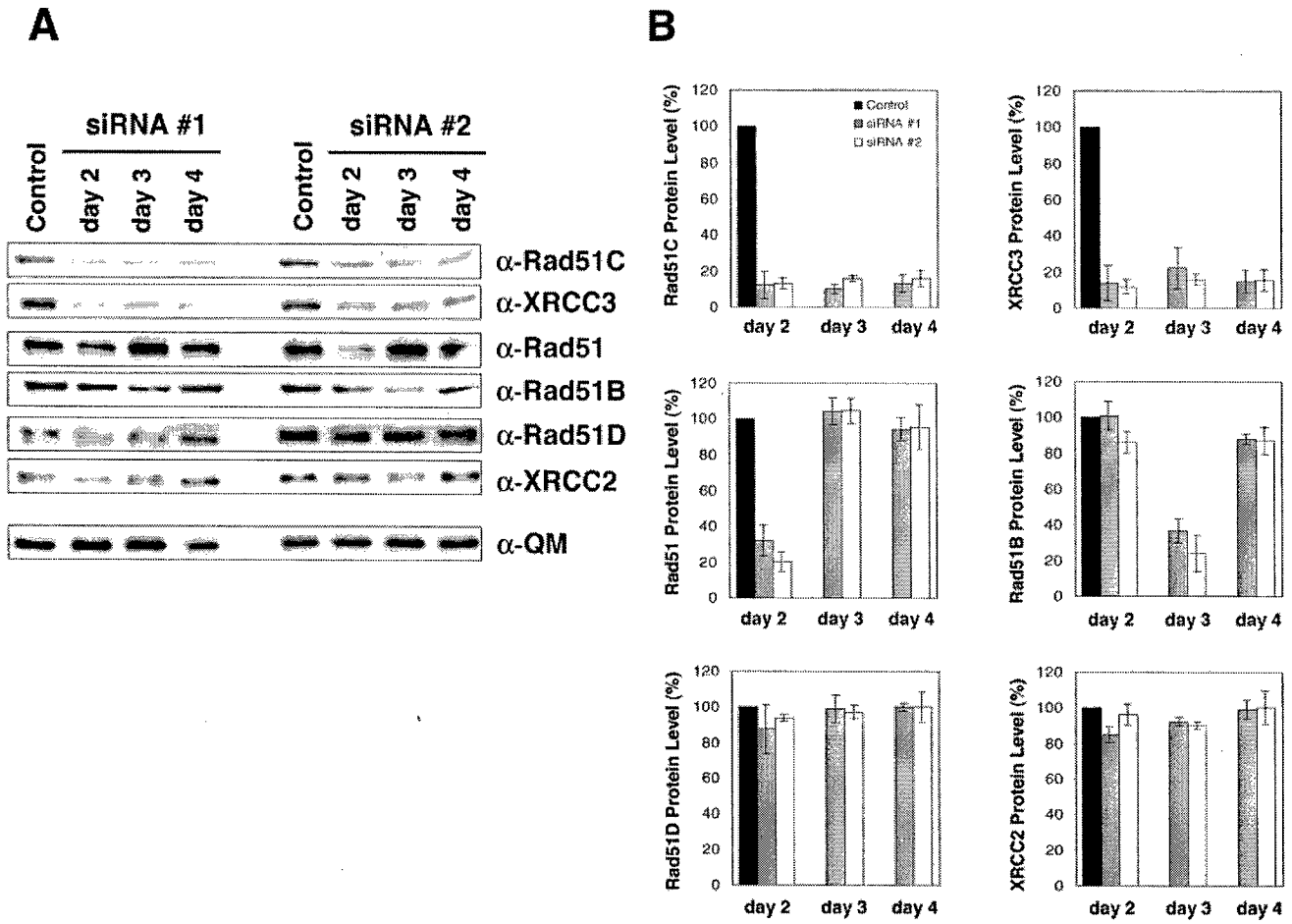


FIG. 1. **Inhibition of Rad51C suppresses XRCC3 expression and other Rad51 family proteins.** *A*, Western analysis of the endogenous levels of Rad51 and Rad51 paralogs. HeLa cells were individually transfected with Rad51C siRNA 1 and 2 duplexes or mock-transfected (control). On days 2, 3, and 4 posttransfection, 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. *B*, quantification and statistical analysis of the protein levels of Rad51 and Rad51 paralogs. The means and standard deviations for three independent experiments are shown.

of Rad51C destabilizes XRCC3, presumably because of decreased formation of the stabilizing Rad51C-XRCC3 heterodimer. When the expression levels of Rad51 and Rad51B were examined, it was found that the Rad51 level was low on day 2 but recovered by day 3. Similarly, the Rad51B level was reduced on day 3 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 a minimal effect on the level of Rad51D and XRCC2, indicating that Rad51C is not required for the stabilization of these two proteins. The entire experiment was carried out three times, and similar results were obtained. The Western data were quantified by densitometry, and statistical analysis for three independent experiments is shown in Fig. 1*B*. Mixed siRNA 1 and 2 were also used for transfection, and a similar pattern of Rad51 paralog levels was observed (data not shown). 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*—To measure HR, an artificial reporter locus (as diagrammed in Fig. 2*A*) 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 *Saccharomyces cerevisiae* (44–47). 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 $\sim 5 \times 10^{-3}$/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 en-

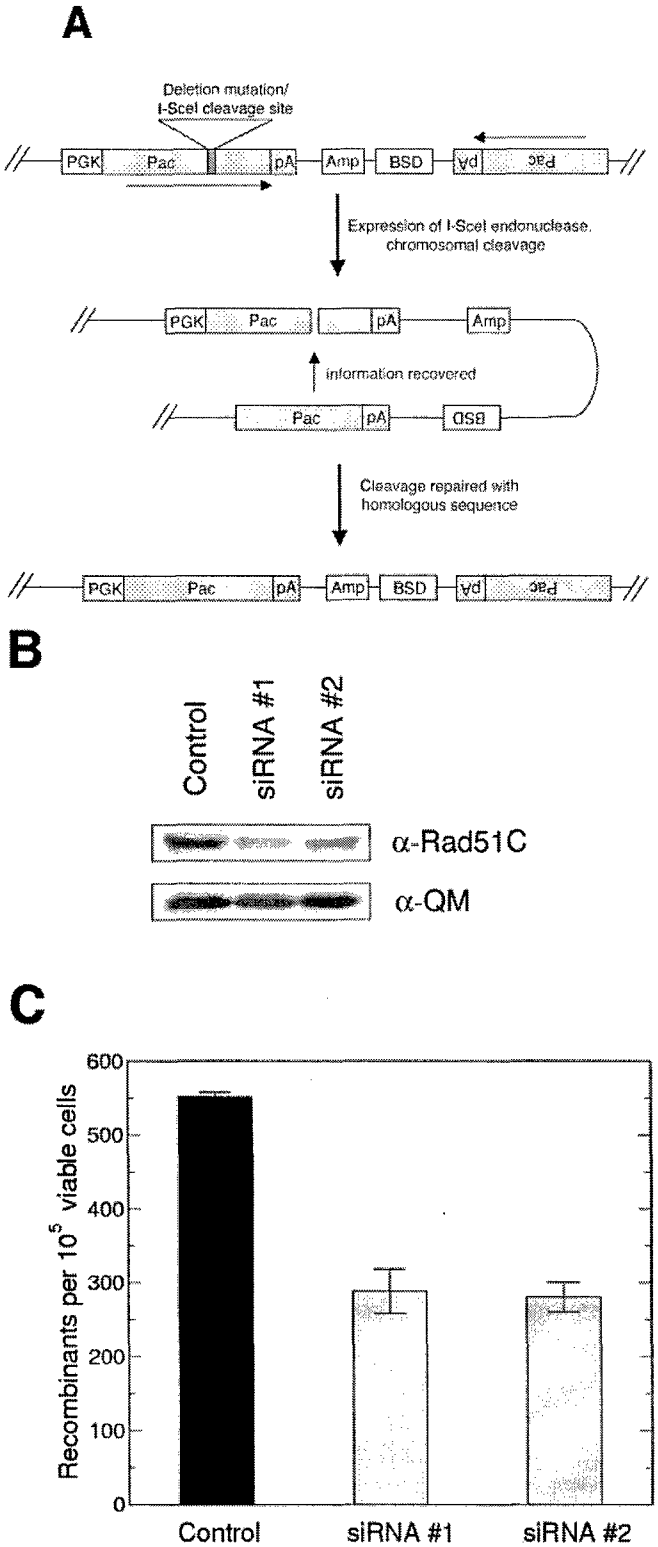


FIG. 2. **Rad51C depletion results in a reduced frequency in homologous recombination in human HT1080 cells.** *A*, homologous recombinational repair of a site-specific chromosomal break in the reporter locus. *PGK*, mouse phosphoglycerate kinase enhancer/promoter. *Pac*, puromycin acetyltransferase 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. *Amp*, bacterial origin of replication and ampicillin resistance gene (needed for propagation of the vector as a plasmid). *BSD*, blasticidin deaminase (blasticidin resistance) gene with cytomegalovirus promoter/enhancer and bovine growth hormone poly(A) signal. The pairs of *diagonal parallel lines* indicate flanking chromosomal sequences. *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/dish divided by the plating efficiency for that transfection and divided by 120,000 (the total number of cells plated). The means and standard deviations for six experiments are shown.

donuclease. Twenty-four h 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. 2*B*, 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, $p = 0.0088$, and siRNA 2, $p = 0.0049$) (Fig. 2*C*). Our data provide direct *in vivo* evidence that human Rad51C functions in HRR.

*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 cross-links, such as mitomycin C. 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. 3*A*). 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) (13) 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, and 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. 3*B*). 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 (48–50), 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 (40–42). HeLa cells were treated with mimosine for 16 h and then released to progress through the cell cycle. This technique produced an excellent synchrony for HeLa cells as described previously (42). Using this approach, Rad51C siRNA 1-treated HeLa cells and mock-trans-

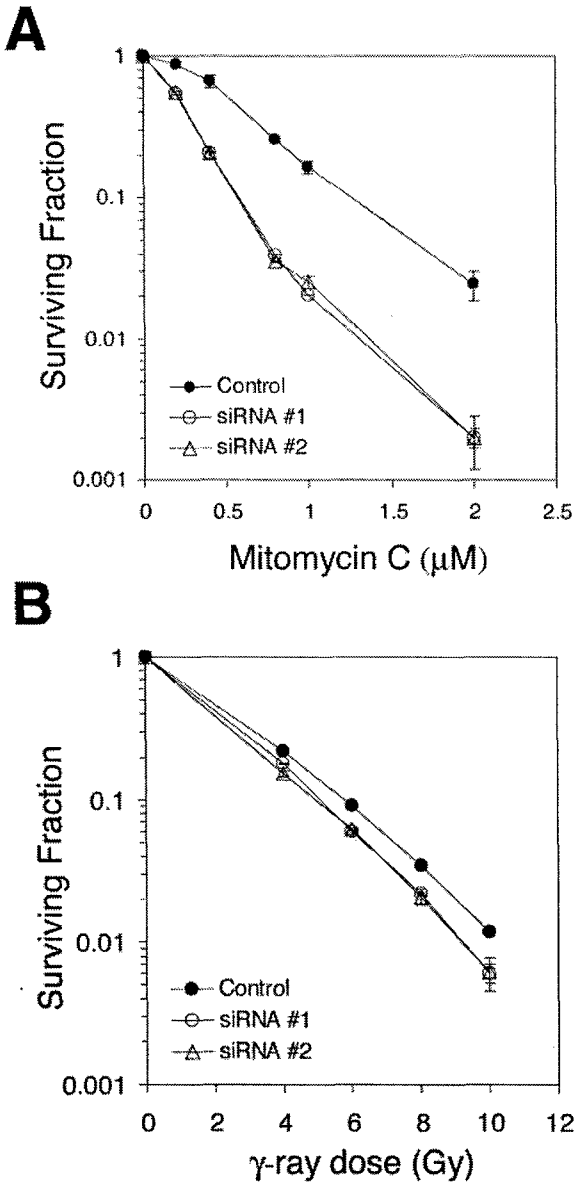


FIG. 3. **Inhibition of Rad51C causes increased sensitivity to mitomycin C and γ-rays in asynchronous HeLa cells.** *A*, MMC sensitivity. HeLa cells were transfected with Rad51C siRNA 1 or siRNA 2 or mock-transfected with Oligofectamine alone (control). On day 2 post-siRNA transfection, cells were seeded and treated with various concentrations of MMC (0, 0.2, 0.4, 0.8, 1, and 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, and 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.

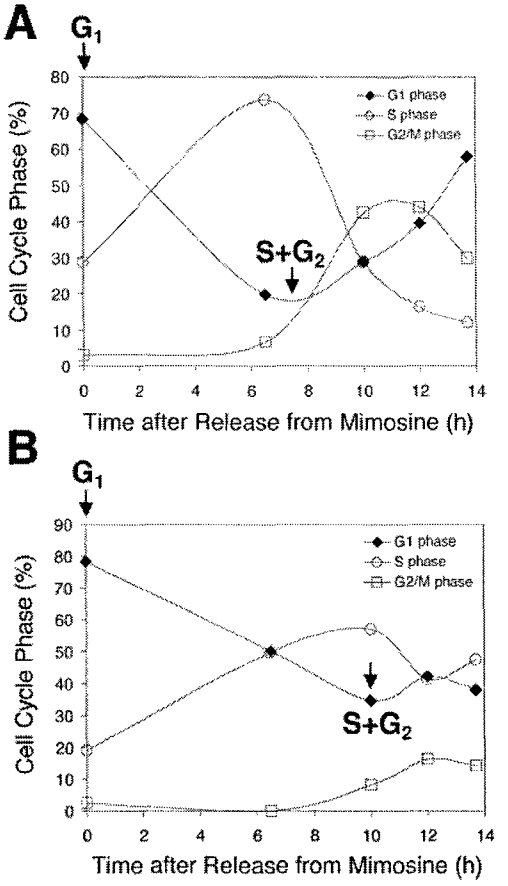


FIG. 4. **Distribution of HeLa cells in the phases of the cell cycle as a function of time after release from mimosine treatment.** On day 2 posttransfection, the mock-transfected (*A*) and Rad51C siRNA 1-treated (*B*) cells were exposed to 200 μM mimosine for 16 h, at which time the cultures were rinsed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

fected 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. 4*A*) was very similar to that observed previously for untreated cells (42); *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 h, respectively (Fig. 4*B*). 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. 5*A*) using the immediate plating protocol, as assessed by $D_{10}$ values. This modestly increased radiosensitivity is similar to that observed for asynchronous cells (Fig. 3*B*). 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. 5*A*). These important findings indicate that human Rad51C functions in the repair of IR-induced DNA damage specifically in S and $G_2$/M

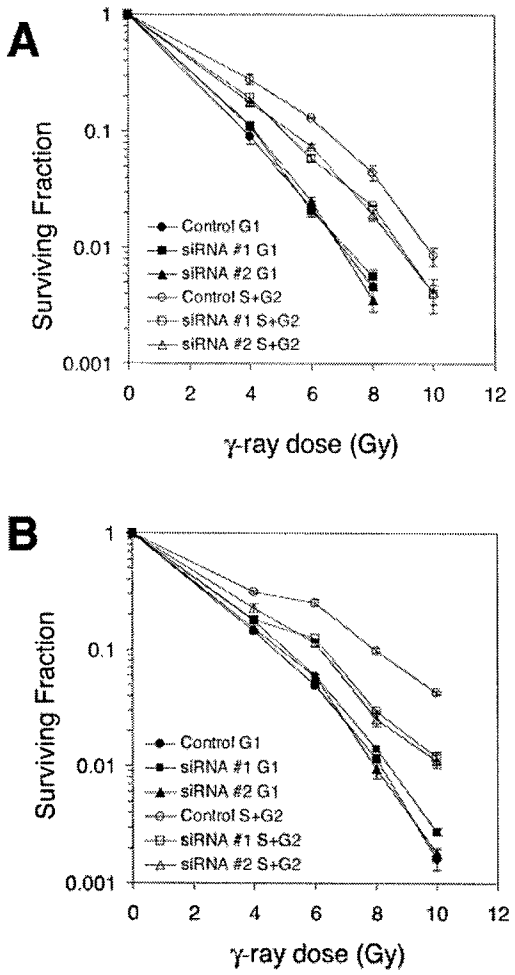


FIG. 5. **Rad51C-deficient HeLa cells show increased sensitivity to γ-rays in S + $G_2$/M phase but not in $G_1$ phase.** After irradiation with various doses (0, 4, 6, 8, and 10 Gy) of γ-rays, mock- (control) and Rad51C siRNA-transfected cells were either (*A*) immediately replated in triplicate for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

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. 5*A*). This observation is in agreement with earlier studies in HeLa cells by Terasima and Tolmach (51, 52), 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. 5*A*). 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 is repaired (53–55). As shown in Fig. 5*B*, 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 with cells with immediate plating (Fig. 5*A*). In the $G_1$ phase, no difference in IR sensitivity between Rad51C siRNA-treated cells and the control was observed (Fig. 5*B*). This result is consistent with the results obtained with immediate plating (Fig. 5*A*), suggesting that Rad51C inhibition does not affect the repair of potentially lethal damage 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. 5*B*) and that this difference was somewhat larger (1.4-fold) than that observed with immediate plating (Fig. 5*A*). This result suggests that Rad51C depletion inhibits the repair of potentially lethal damage 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 and XRCC3 and a larger complex comprising Rad51B, Rad51C, Rad51D, and XRCC2 (18, 20–22). The transient existence of Rad51B-Rad51C and Rad51D-XRCC3 heterodimers also has been suggested (21, 22, 56). It seems probable that the various paralog complexes form dynamically during the process of HR (21). In these complexes, Rad51C interacts directly with XRCC3, Rad51B, and Rad51D. Rad51C was also shown to weakly interact with Rad51 *in vitro* (23, 24). 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 3 days. This dependence upon heterodimerization appears to be mutual; in complementary experiments, it was shown previously that overexpression of XRCC3 produces an elevated level of Rad51C as well (20). Rad51C appears also to affect the expression or stability of Rad51 and Rad51B, although 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 that 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*—Mutant studies with chicken and hamster cells have implied a role for Rad51C in HRR (25, 27, 28). Very recently, it was reported that the hamster cell line irs3 has reduced homology-directed repair of a DNA DSB by gene conversion (57). However, no 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 evidence 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 because of 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 cross-linking agents such as MMC is a consistent feature of HRR-deficient mutants in vertebrate cell lines. It was reported previously that Rad51C-knock-out chicken DT40 cells are ~3-fold more sensitive than wild-type cells to MMC after acute exposure (25) and that the Rad51C-mutant hamster irs3 and CL-V4B cell lines are ~20- and ~32-fold more sensitive to chronic treatment with MMC, respectively (27, 28). 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 reported recently, and the XRCC3-deficient cells showed ~2-fold excess sensitivity to MMC (58). 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 cross-links 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 (59, 60) that is associated primarily with the late S/$G_2$ phase (49). By contrast, the NHEJ-defective Chinese hamster ovary 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 with the wild type but are more IR-resistant in late S/$G_2$ (61–64). A similar pattern was reported for murine pre-B cells carrying the *scid* mutation (65). These results suggest that DSBs occurring in replicated DNA are repaired efficiently by HRR. HRR is likely favored in the S-phase cells because of the presence of sister chromatids as proximal repair templates (66). During the normal cell cycle, Rad51 transcription is induced in the S and $G_2$ phases (67), and Rad51 protein expression is found to be lowest in $G_1$, increasing in S, and reaching a maximum in $G_2$/M (68). Mammalian Rad51 forms discrete nuclear foci during the S phase (69). 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 consistent with the phenotype shown by other HR-defective mutants, *i.e.* Rad54-deficient chicken DT40 (59) and mouse embryonic stem cells (60); Rad51C-, XRCC2-, or XRCC3-mutant hamster cells (13, 27, 28); and Rad51 paralog-knock-out DT40 cells (25, 26). 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 reported previously 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 (48). 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/$G_2$ (50, 65). 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 the following: (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.

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