ATP Hydrolysis by Mammalian RAD51 Has a Key Role during Homology-directed DNA Repair*

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Disruption of the gene encoding RAD51, the protein that catalyzes strand exchange during homologous recombination, leads to the accumulation of chromosome breaks and lethality in vertebrate cells. As RAD51 is implicated in BRCA1- and BRCA2-mediated tumor suppression as well as cellular viability, we have begun a functional analysis of a defined RAD51 mutation in mammalian cells. By using a dominant negative approach, we generated a mouse embryonic stem cell line that expresses an ATP hydrolysis-defective RAD51 protein, hRAD51-K133R, at comparable levels to the endogenous wild-type RAD51 protein, whose expression is retained in these cells. We found that these cells have increased sensitivity to the DNA-damaging agents mitomycin C and ionizing radiation and also exhibit a decreased rate of spontaneous sister-chromatid exchange. By using a reporter for the repair of a single chromosomal double-strand break, we also found that expression of the hRAD51-K133R protein specifically inhibits homology-directed double-strand break repair. Furthermore, expression of a BRC repeat from BRCA2, a peptide inhibitor of an early step necessary for strand exchange, exacerbates the inhibition of homology-directed repair in the hRAD51-K133R expressing cell line. Thus, ATP hydrolysis by RAD51 has a key role in various types of DNA repair in mammalian cells.

The accurate repair of DNA damage, such as a chromosomal double-strand break (DSB),[1] is essential to prevent chromosomal alterations and the loss of genetic information. DSBs can be formed by oxygen free radicals, topoisomerase failure, radiation treatment, and possibly most commonly, DNA replication. Repair of DSBs in mammalian cells, as in other organisms (1), occurs by multiple pathways (2, 3). One pathway of DSB repair involves homologous recombination in which a homologous sequence templates repair by gene conversion after strand invasion of a broken end, and thus can be termed homology-directed repair (HDR). The sister chromatid is by far the preferred template for HDR in mammalian cells (4). Two other pathways for repair are non-homologous end-joining (NHEJ), which results in deletions or insertions at a DSB, and single-stranded annealing (SSA), which involves the annealing of complementary strands at or near the DSB site. Various components of DSB repair pathways are known to be essential for cellular viability, embryonic development, and tumor suppression in mammalian cells (5). Therefore, characterizing the roles of such components in mammalian cells is essential for an understanding of how cells maintain genomic integrity.

Insight into mechanisms of HDR has come in large part from analysis of genes of the *RAD52* epistasis group in *Saccharomyces cerevisiae*, which includes genes important for meiotic and mitotic recombination and cellular resistance to DNA-damaging agents such as methylmethane sulfonate (MMS) and x-rays (1), and for which mammalian homologues have been identified (6, 7). One of these genes, *RAD51* (8, 9), encodes the homologue of the RecA protein which is pivotal for homologous recombination in *Escherichia coli* (10, 11). Like RecA, RAD51 forms helical nucleoprotein filaments on DNA in an ATP-dependent manner (12–14). These filaments catalyze DNA strand exchange, an early step in homologous recombination pathways (15).

Although RecA in *E. coli* and its counterpart Rad51 in *S. cerevisiae* are dispensable for viability of these organisms, disruption of the *RAD51* gene in vertebrate cells leads to increased numbers of chromosome aberrations in the absence of DNA-damaging agents, including chromosome breaks, and cellular and embryonic lethality (16–18). These phenotypes imply that RAD51 plays an important role in the repair of spontaneously arising DNA damage in vertebrate cells. As strand breaks can arise during DNA replication, vertebrate RAD51 may catalyze strand exchange-mediated replication fork restart, as has been proposed for RecA in *E. coli* (19, 20). Besides being essential for cell viability, RAD51 has also been implicated in the tumor suppression functions of BRCA1 and BRCA2 (21). RAD51 binds directly to regions of the BRCA2 protein and, together with BRCA1, interacts in nuclear foci. Importantly, cell lines containing mutations in *Brca1* or *Brca2* are defective in HDR (22–25). However, mammalian cell lines carrying defined mutations within *Rad51* have not as yet been examined for recombination phenotypes.

To begin to understand the role of RAD51 in mammalian cells, we sought to characterize the DNA repair functions of the mammalian RAD51 protein. In this report, we have examined two mutants of human RAD51, hRAD51-K133A and hRAD51-K133R, that are defective for ATP binding and ATP hydrolysis,

¹ The abbreviations used are: DSB, double-strand break; HDR, homology-directed repair; NHEJ, non-homologous end-joining; SSA, single-stranded annealing; MMS, methylmethane sulfonate; MMC, mitomycin C; SCE, sister-chromatid exchange; IR, ionizing radiation; GFP, green fluorescent protein; PBS, phosphate-buffered saline; ES, embryonic stem; Gy, grey.

respectively (26). Overexpression of hRAD51-K133R, but not hRAD51-K133A, has been shown previously (26) to complement a *RAD51* null allele in hyper-recombinagenic chicken DT40 cells, with the only reported phenotype being a decreased frequency of gene targeting. By using a dominant negative approach, we generated mouse ES cell lines that express hRAD51-K133R at comparable levels to endogenous RAD51, whose expression is retained in these cells, although we have not obtained cell lines that similarly express the hRAD51-K133A protein. We found that cells expressing hRAD51-K133R exhibit hypersensitivity both to the cross-linking agent mitomycin C (MMC) and to ionizing radiation (IR), a decrease in spontaneous sister-chromatid exchange (SCE), and a defect in HDR of an induced chromosomal DSB. Moreover, expression of a peptide inhibitor of RAD51 nucleoprotein filament formation, derived from BRCA2, exacerbates the HDR defect in this line. These results indicate that ATP hydrolysis by mammalian RAD51 has a key role during homologous recombination and DNA repair.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Lines—Generation of the E14-DRGFP cell line in which the DR-GFP reporter was targeted to the *hprt* locus has been described previously (27, 28). To generate cell lines expressing hRAD51-WT, hRAD51-K133R, and hRAD51-K133A, cDNAs for these human *RAD51* genes (26) were cut with *Bam*HI and *Sal*I and cloned into a modified form of pCAGGS (29), pCAGGS-BSKX-H3/Not, which expresses cDNAs from a human cytomegalovirus enhancer/chicken β-actin promoter. The vector pCAGGS-BSKX-H3/Not contains a polylinker (*Bgl*II-*Sma*I-*Kpn*I-*Xho*I) inserted between the *Xho*I and *Bgl*II sites of pCAGGS, such that the orientation of the *Bgl*II and *Xho*I sites is reversed, compared with pCAGGS, and a *Not*I linker inserted into the *Hin*dIII site just downstream. Expression cassettes were inserted as *Sal*I/*Not*I fragments into the *Pim1* locus gene-targeting vector p59.N/RV at unique *Sal*I/*Not*I sites, which are downstream of the hygromycin resistance gene coding sequence fused to *Pim1* exon 4 (30). The p59.N/RV vector is a version of p59 that has a *Not*I linker inserted at the unique *Sma*I site just downstream of the *Sal*I site and also contains *Eco*RV sites on both ends of the genomic fragment, allowing for linearization of the targeting vector with *Eco*RV. Each of these linearized vectors (70 μg) was electroporated into E14-DRGFP cells (5×10^6 ES cells suspended in 650 μl of phosphate-buffered saline (PBS) in a 0.4-cm cuvette) by pulsing the cells at 800 V, 3 microfarads. Hygromycin selection was applied 48 h later at 110 μg/ml. After 10 days, surviving colonies were isolated and analyzed for targeting to *Pim1* as described previously (30).

To construct the BRC3 expression vector, the coding sequence for amino acids 1415–1483 of BRCA2 was PCR-amplified from a human *BRCA2* cDNA using primers flanking this sequence. The PCR primers generated a stop codon at the 3′ end and restriction endonuclease sites at both ends of the PCR product (*Eco*RI and *Bbs*I sites at the 5′ end; *Bgl*II site at the 3′ end). A PCR product for mBRC3 was created the same way, except that the 5′ primer contained the Phe^{1428}–Lys^{1434} deletion mutation (31). PCR products were digested with *Eco*RI and *Bgl*II and cloned into these sites of the pCAGGS expression vector. Subsequently, an *Eco*RI/*Bbs*I fragment encoding a start methionine followed by three copies of a nuclear localization signal (32) was cloned in-frame, upstream of the BRC repeats. The BRC3 and mBRC3 sequences containing the nuclear localization signal were verified by the Sloan-Kettering Institute Core Facility.

DR-GFP Assays—To measure the repair of an I-*Sce*I-generated DSB, 50 μg of the I-*Sce*I expression vector pCBASce (33) was mixed with 5×10^6 ES cells suspended in 650 μl of PBS in a 0.4-cm cuvette, followed by pulsing the cells at 300 V, 950 microfarads. In experiments with the BRC repeat, 70 μg of the BRC3 or mBRC3 expression vectors were additionally added. To determine the amount of HDR, the percentage of cells that were GFP-positive was quantitated by flow cytometric analysis 7 days after electroporation on a Becton Dickinson FACScan, as described previously (28).

To determine the percentage I-*Sce*I site loss for each electroporation (27), genomic DNA was isolated 7 days after transfection. 2 μg of genomic DNA was used as the template for PCR with primers at 200 nM in a reaction volume of 50 μl. The primer sequences were DRGFP1 5′ AGGGCGGGGGTTCGGCTTCTGG and DRGFP2 5′ CCTTCGGGCATGGCGGACTTGA. PCRs were performed using the Roche Molecular Bio-

chemicals GC-rich PCR System in a PerkinElmer Life Sciences Gene-Amp PCR System 9600. Amplification was for a total of 35 cycles, the first 10 cycles using a 1-min elongation time and the final 25 cycles using a 2-min elongation time. Following amplification, PCR products were purified using an Amersham Biosciences GFX PCR DNA and Gel Band Purification Kit. PCR products were digested with I-*Sce*I for 20 h with 10 units of I-*Sce*I (Roche Molecular Biochemicals) and separated on a 1.5% agarose gel. The gel was stained with ethidium bromide, and the ethidium signals for the I-*Sce*I-resistant and I-*Sce*I-cleaved band were quantified using a Bio-Rad Chemidoc 2000 system with rolling disc background subtraction.

Western Blotting—Cells (5×10^6) suspended in 100 μl of PBS and mixed with an equal volume of protein extraction buffer (34) were heated at 100 °C for 10 min and subsequently spun for 10 min. 5 μl of this protein extract was separated on two 12% SDS-PAGE gels; one was stained with Coomassie Blue to confirm equal loading, and the other was transferred to Polyscreen polyvinylidene difluoride membrane (PerkinElmer Life Sciences). The membrane was probed with anti-RAD51 IgG goat antibodies (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated mouse anti-goat secondary antibodies (Pierce), and immunoblot signals were detected using ECL reagent (Amersham Biosciences).

Mitomycin C and Ionizing Radiation Survival Assays—MMC and IR survival assays were performed as described previously (22, 35). For MMC treatment, 2×10^5 cells for each line were exposed to various doses of MMC for 4 h and washed three times with PBS. Cells were trypsinized, counted, and then plated at various concentrations for 7–10 days of cell growth. For IR treatment, 2×10^5 cells, in solution, were exposed to various doses of IR by varying the duration of exposure to a ^{137}Cs source. Cells were diluted and plated at various concentrations for 7–10 days of cell growth. For both the MMC and IR experiments, clonogenic survival was determined for a given concentration of cells that were plated by dividing the number of colonies on each treated plate by the number of colonies on each untreated plate.

Sister-Chromatid Exchange Assays—Subconfluent ES cells cultured on 100-mm plates were incubated with bromodeoxyuridine (Sigma) at 10 μM for 48 h. Colcemid (Invitrogen) was then added at a final concentration of 0.15 μg/ml for 1 h after which time cells were harvested for metaphase preparation. Slides were aged for 3 days and stained with 10^{-4} M 33258 Hoechst (Sigma) for 10 min. After washing in water, slides were mounted and then placed under a 120-watt Plant Lite (General Electric, Inc.) at a distance of 10 cm for 2 h. Slides were then washed and stained in 2% Giemsa (LabChem, Inc.) in Gurr's buffer (Bio/Medical Specialties, Inc.) for 15 min and then washed again and examined under an Olympus microscope mounted with a Sony CCD camera. Metaphases that showed no more than 5 undifferentially stained chromosomes were selected for counting SCEs. Statistical analysis was performed using the Student's two-tailed *t* test.

RESULTS

Stable Expression of the ATP Hydrolysis-defective hRAD51-K133R Mutant at Moderate Levels Is Not Lethal to Mouse ES Cells—To begin to identify the activities of mammalian RAD51 that are involved in DNA repair, we attempted to generate cell lines that express mutant forms of the protein. Considering that mutant RAD51 may not be able to support cellular viability in the absence of the wild-type protein, we took a dominant negative approach, expressing mutant forms of the protein exogenously, while maintaining expression of the endogenous wild-type protein. In this manner, we could take advantage of homotypic interactions of RAD51, such that mutant forms of the protein would have the potential to form mixed nucleoprotein filaments with the endogenous protein and thereby interfere with the function of the wild-type RAD51 without completely abolishing its activity (36–38). In these experiments, we attempted to generate mouse cell lines that exogenously express two mutant forms of human RAD51, which is 99% identical to mouse RAD51 (9): hRAD51-K133A, which is defective for ATP binding, and hRAD51-K133R which is competent for ATP binding but is defective for ATP hydrolysis (26). In parallel, we developed cell lines that exogenously express hRAD51-WT for comparison.

To generate these cell lines, we gene-targeted the RAD51 expression cassettes to a specific chromosomal locus in the
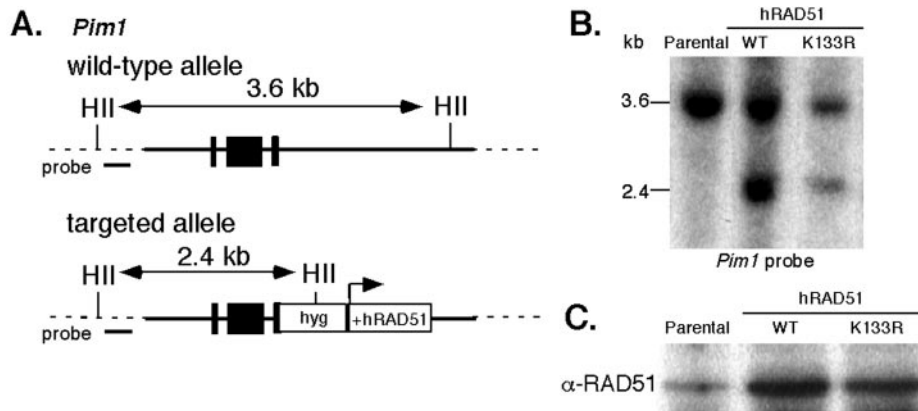
FIG. 1. **Expression of human *RAD51* at the *Pim1* locus in mouse ES cells**. *A*, expression cassettes for hRAD51 were cloned into a genomic fragment of the *Pim1* gene containing a linked promoterless hygromycin resistance gene (*hyg*) to allow for efficient targeting. Shown are diagrams of wild-type and targeted *Pim1* loci and the fragment of *Pim1* used as the probe for the Southern blot. Targeting vectors were transfected into the mouse ES cell line E14-DRGFP (parental), and hygromycin-resistant clones were isolated. *B*, Southern blot analysis of gene-targeting of the *Pim1* locus. Genomic DNA from hygromycin-resistant clones was digested with *Hinc*II and probed with a DNA fragment of the *Pim1* locus. Shown are *Pim1* Southern blot signals for the parental cell line and hRAD51-WT and hRAD51-K133R expressing cell lines. *C*, Western blot analysis of RAD51 expression in the targeted cell lines. Protein from equivalent numbers of cells of the parental cell line and the hRAD51-WT and hRAD51-K133R expressing lines was separated by SDS-PAGE and probed for RAD51 by immunoblotting. Shown is the immunoblot signal for RAD51 for each cell line from the same film exposure. Loading of equivalent amounts of protein was confirmed by Coomassie staining of a protein gel run in parallel (data not shown).

wild-type mouse ES cell line E14-DRGFP (parental), which contains a substrate for the quantification of HDR of a chromosomal DSB (27, 28). To this end, each RAD51 expression cassette was cloned into a *Pim1* genomic fragment containing a linked promoterless hygromycin gene (Fig. 1*A*), which allows efficient selection of gene-targeted clones (30, 33). The RAD51-targeting vectors, hRAD51-WT, hRAD51-K133R, and hRAD51-K133A, were introduced into the parental cell line by electroporation, and stably integrated clones were selected using hygromycin. In these experiments, the frequency of hygromycin-resistant clones was ~8-fold higher for the hRAD51-WT vector (2×10^{-6}) than for the hRAD51-K133R vector (1.5×10^{-7}), whereas no clones were found with the hRAD51-K133A vector ($<1 \times 10^{-8}$). The inability to generate viable clones with the hRAD51-K133A expression vector is consistent with similar findings in chicken DT40 cells (26) and suggests that its expression is lethal.

Subsequently, these hygromycin-resistant clones were analyzed by Southern blotting to identify clones that had correctly integrated the vector at the *Pim1* locus. From this analysis, 6 of the 8 clones derived from the hRAD51-WT vector and 7 of 13 clones derived from the hRAD51-K133R vector were found to have undergone targeted integration (Fig. 1*B*, data not shown). These targeted clones were used for subsequent experiments to analyze the effects of wild-type and mutant RAD51 expression. Initially, multiple independently targeted clones were examined and found to behave similarly in the assays described below, such that one clone each for hRAD51-WT and hRAD51-K133R was chosen for detailed analysis. We refer to these cell lines as hRAD51-WT or hRAD51-K133R expressing cell lines, to indicate that they are exogenously expressing these RAD51 proteins in conjunction with the endogenous wild-type protein.

Expression of RAD51 protein in the targeted clones was analyzed by Western blotting of total protein using an antibody that recognizes both human and mouse RAD51. Targeted clones from both the hRAD51-WT and hRAD51-K133R expression vectors exhibit elevated levels of total RAD51 compared with the parental line, by ~4- and 2-fold, respectively (Fig. 1*C*). The 2-fold increase in total RAD51 in the hRAD51-K133R expressing ES cell line compared with the parental line suggests that the mutant protein accounts for approximately half of the RAD51 in these cells. It is unclear what accounts for the

somewhat higher amount of RAD51 in the hRAD51-WT expressing line relative to the hRAD51-K133R expressing line, although it is possible, for example, that the wild-type protein is somewhat more stable than the mutant protein. In summary, therefore, viable cell lines were generated that exogenously express moderate levels of both the hRAD51-WT and hRAD51-K133R proteins, together with the endogenous wild-type protein, whereas no viable cell lines were identified that express the hRAD51-K133A protein.

Cells Expressing hRAD51-K133R Exhibit an Increased Sensitivity to DNA-damaging Agents—Because moderate expression of the hRAD51-K133R protein, in otherwise wild-type cells, is not lethal, we could examine the impact of RAD51 with impaired ATP hydrolysis on the repair of different DNA lesions. As a first step, we compared the effects of the DNA-damaging agents MMC and IR on the relative viability of the parental and RAD51 expressing lines. We tested these agents because vertebrate cell lines deficient for homologous recombination have been found to be extremely hypersensitive to MMC, which produces interstrand cross-links, while having relatively less hypersensitivity to IR, which produces strand breaks and other types of lesions (22, 39, 40). To test whether mammalian cells expressing hRAD51-K133R are hypersensitive to these agents, the parental cell line and the hRAD51-WT- and hRAD51-K133R expressing lines were tested for clonogenic survival following brief exposure to varying concentrations of MMC or doses of IR. In both cases, clonogenic survival of untreated cells was established as 100% survival for each cell line.

In the MMC experiments, the survival of the parental line and hRAD51-WT expressing line was not compromised by exposure to 0.1 μM MMC, whereas the hRAD51-K133R expressing line showed a mild sensitivity at this concentration (52% survival) (Fig. 2*A*). At a higher concentration of 0.6 μM MMC, the parental line and the hRAD51-WT expressing line exhibited mild sensitivity (46 and 38% survival, respectively), whereas the hRAD51-K133R expressing line showed a marked sensitivity at this concentration (10.5% survival).

In the IR experiments, the survival of the parental line was not compromised by exposure to 2 Gy, whereas the hRAD51-K133R expressing line showed a mild sensitivity at this dose (53% survival) (Fig. 2*B*). Exposure of cells to 4 Gy resulted in
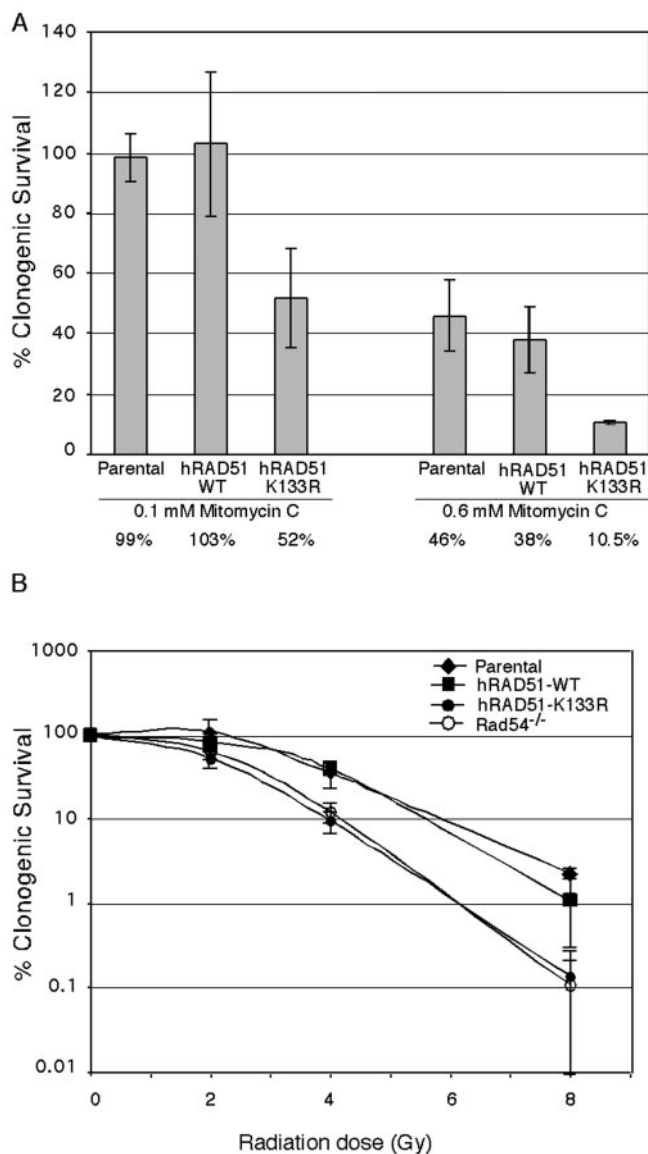
FIG. 2. **Cell lines expressing hRAD51-K133R exhibit an increased sensitivity to both the interstrand cross-linking agent MMC and IR.** The percent clonogenic survival of cells treated with these DNA-damaging agents was computed relative to that of untreated cells, which was set to 100% for each line. *A*, MMC sensitivity. Clonogenic survival of the parental cell line and the hRAD51-K133R and hRAD51-WT expressing lines was assessed after treatment for 4 h without MMC, with 0.1 μM MMC, and with 0.6 μM MMC. *B*, IR sensitivity. Clonogenic survival of the parental cell line, hRAD51-K133R and hRAD51-WT expressing lines, and a $Rad54^{-/-}$ cell line was assessed after treatment with 0, 2, 4, and 8 Gy of IR. Each percentage shown is the mean of three experiments, and *error bars* represent the S.D.

mild sensitivity of the parental line and hRAD51-WT expressing line (35 and 40% survival, respectively), whereas the hRAD51-K133R expressing line showed a marked sensitivity at this dose (10% survival) (Fig. 2*B*). In addition, a $Rad54^{-/-}$ mouse ES cell line was included in these experiments to compare the IR sensitivity of the hRAD51-K133R expressing ES cell line (35). Interestingly, these two cell lines showed similar degrees of sensitivity at these and higher doses (Fig. 2*B*). Thus, cells expressing the hRAD51-K133R protein at levels similar to the endogenous wild-type protein exhibit an increased sensitivity to both MMC and IR and thus are defective for the repair of some types of DNA damage.

Cells Expressing hRAD51-K133R Have Reduced Spontaneous Sister-Chromatid Exchange—Although damage induced by

MMC or IR may be repaired by homologous recombination, sensitivity to these agents does not provide a direct measure of recombination. To begin to analyze the efficiency of homologous recombination in the hRAD51-K133R expressing cell line, we investigated the spontaneous rate of SCE, an apparent indicator of homologous recombination (41), in this line and the parental line. Spontaneous SCEs were visualized cytologically in metaphase spreads following differential labeling of newly replicated chromatids with 5-bromodeoxyuridine. By using this method, the number of spontaneous SCEs per metaphase, normalized to the total number of chromosomes observed in each metaphase, was determined for metaphases from the two cell lines. In these experiments, the hRAD51-K133R expressing cell line exhibited a lower average number of SCEs per metaphase (5.25 ± 2.2) than the parental line (8.6 ± 4.3). There was also a statistically significant difference ($p < 0.001$) between the two cell lines in the distribution of SCEs per metaphase (Fig. 3). For example, the percentage of metaphases with 10 or more SCEs was 2.4% for the hRAD51-K133R expressing cell line compared with 35% for the parental line. Thus, expression of the hRAD51-K133R protein in otherwise wild-type cells leads to a reduced rate of observable spontaneous SCE.

Expression of hRAD51-K133R Inhibits HDR of a Chromosomal DSB While Not Affecting Overall DSB Repair—Because the initiating events that lead to SCEs and the repair of lesions generated by MMC and IR are uncertain, we next wanted to address the effect of hRAD51-K133R expression on the repair of a defined lesion. In these experiments, we compared the parental cell line and the hRAD51-WT and hRAD51-K133R expressing lines for the repair of an endonuclease-generated chromosomal DSB using the reporter DR-GFP (Fig. 4*A*) (27, 28). In this reporter, which is integrated at the *hprt* locus, a DSB is generated by expressing the rare-cutting endonuclease I-*Sce*I, whose 18-bp recognition site has been integrated into a gene (*SceGFP*) encoding green fluorescent protein, in such a way that it disrupts the gene. Repair of the I-*Sce*I break by gene conversion gives rise to a functional *GFP* gene when directed by the downstream *GFP* repeat, *iGFP*, which is an internal *GFP* gene fragment truncated at both ends. This HDR event can be scored in individual cells as green fluorescence, using flow cytometric analysis (Fig. 4*B*), as established previously by analysis of genomic DNA of purified green fluorescent cells (23, 28). By using this assay, transfection of the I-*Sce*I expression vector into the parental cell line and hRAD51-WT expressing cell line resulted in 2.2 and 2.1% GFP-positive cells (Fig. 3*C*), respectively, similar to wild-type cell lines analyzed previously (23, 27). In contrast, transfection with the I-*Sce*I expression vector in the hRAD51-K133R expressing cell line resulted in only 0.44% GFP-positive cells, ~5-fold less than the other two lines (Fig. 4*C*). These results indicate that the hRAD51-K133R expressing cell line is deficient at HDR.

Repair of the I-*Sce*I-induced break can occur by HDR, as described above, or it can occur by other repair pathways that do not give rise to GFP-positive cells (28). These other pathways include NHEJ, which would result in small insertions or deletions around the I-*Sce*I site, and SSA, which would result in a homologous deletion between the *GFP* repeats, forming a 3′-truncated *GFP* gene (Fig. 5*A*). To determine whether expression of the hRAD51-K133R protein specifically reduces HDR or more globally affects DSB repair, we measured overall DSB repair. Because loss of the I-*Sce*I site is a common feature of the repair products from these three pathways, we quantified total DSB repair as the percent of *SceGFP* genes that have lost the I-*Sce*I site.

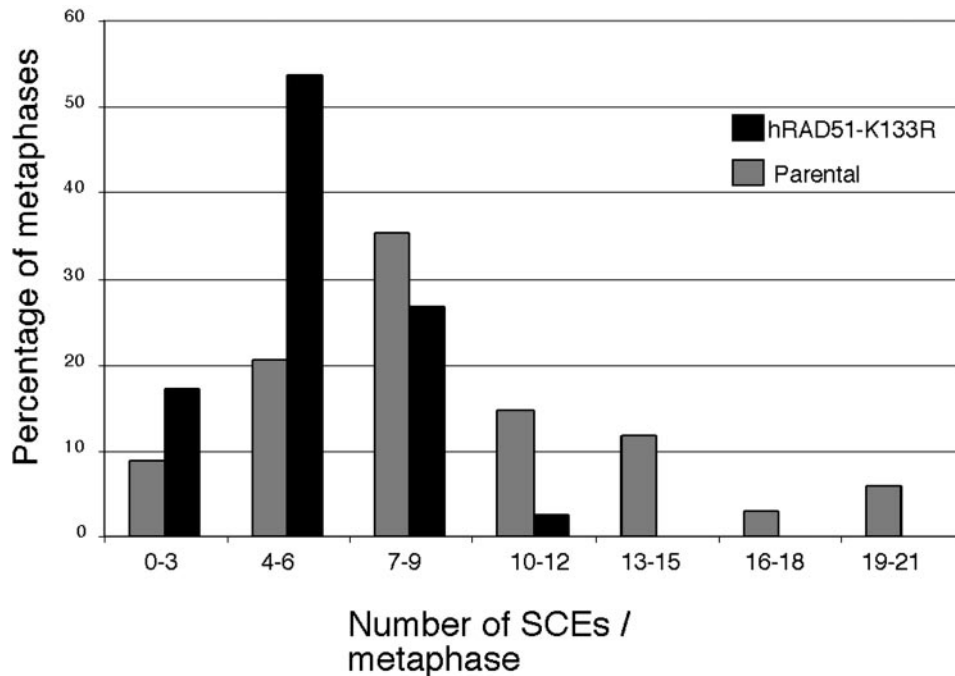The percent of I-*Sce*I site loss was determined by amplifying

FIG. 3. **Cells expressing hRAD51-K133R have a reduced number of spontaneous SCEs per metaphase compared with wild-type cells.** Metaphase spreads for the parental cell line and the hRAD51-K133R expressing line were analyzed. A total of 34 and 42 metaphases were analyzed from the parental cell line and the hRAD51-K133R expressing line, respectively, for the number of SCEs per metaphase. Shown are the percent of metaphases in each group of *0–3*, *4–6*, *7–9*, *10–12*, *13–15*, *16–18*, and *19–21* SCEs per metaphase, for both the parental cell line and the hRAD51-K133R expressing line.

the *SceGFP* gene from genomic DNA of cells transfected with the I-*Sce*I expression vector, using primers that flanked the I-*Sce*I site, and then cleaving the amplified product with I-*Sce*I (Fig. 5*A*). Reconstruction experiments, using defined concentrations of cells that have lost the I-*Sce*I site, have demonstrated that there is a direct correlation between the percentage of cells known to have lost the I-*Sce*I site and the percent I-*Sce*I site loss as determined by PCR analysis (data not shown). By using this analysis, transfection of the I-*Sce*I expression vector into each of the parental cell lines and the hRAD51-WT and hRAD51-K133R expressing lines was found to result in ~10% I-*Sce*I site loss, although untransfected cells did not exhibit I-*Sce*I loss (Fig. 5*B*). To attempt to differentiate repair pathways, we also cleaved the amplified products by *Bcg*I, as HDR and SSA products are both cleavable by this enzyme (Fig. 5*A*). For each cell line, the *Bcg*I-cleavable products were ≤2% of the total PCR products, which is at or below the limit of detection for this assay (data not shown). Thus, although HDR is readily quantifiable by GFP fluorescence, HDR and SSA are difficult to quantitate using the PCR assay. As NHEJ products are not cleavable by *Bcg*I, this result indicates that NHEJ predominates among the DSB repair pathways that are detectable with this substrate.

The efficiency of HDR relative to that of total repair was then estimated by dividing the percentage of GFP-positive cells by the percent I-*Sce*I site loss (Fig. 5*C*). From the analysis of at least four independent transfections for each cell line, the hRAD51-K133R expressing cell line exhibited a significant decrease in the percentage of HDR relative to I-*Sce*I site loss, compared with both the parental cell line (5.8-fold decrease) and hRAD51-WT expressing cell line (5.3-fold decrease). These data indicate that expression of hRAD51-K133R, in otherwise wild-type cells, results in a specific defect in HDR rather than an overall deficiency in DSB repair.

Expression of a BRC Repeat from BRCA2 Exacerbates the HDR Deficiency Caused by Expression of hRAD51-K133R— Because the hRAD51-K133R protein has been shown previ-

ously (26) to catalyze DNA strand exchange *in vitro*, it is possible that disruption of strand exchange is not the mechanism by which the hRAD51-K133R protein inhibits HDR. A prediction of this hypothesis is that if strand exchange were still proficient in the hRAD51-K133R expressing cell line, inhibition of strand exchange would exacerbate the HDR defect in this line. RAD51 nucleoprotein filament formation, a prerequisite of strand exchange, has been shown recently to be inhibited by a peptide from a region of the human BRCA2 protein that contains 8 copies of an amino acid sequence termed a BRC repeat (31, 42). Specifically, a 69-amino acid peptide of BRCA2 containing the third BRC repeat (BRC3, amino acids 1415–1483) has been shown to inhibit RAD51 nucleoprotein filament formation, whereas a mutant peptide, mBRC3, with a deletion of 7 amino acids that are highly conserved among BRC repeats (ΔPhe^{1428}–Lys^{1434}), does not inhibit this process (31). Also, expression of another BRC repeat, BRC4, has been shown to inhibit gene targeting (43) and RAD51 irradiation-induced focus formation (44).

To test the effect of the BRC3 peptides on repair of the DSB in DR-GFP, we co-transfected the I-*Sce*I expression vector with vectors we generated for the expression of either BRC3 or mBRC3, each with an N-terminal nuclear localization signal. HDR events were scored as green fluorescence, as described above. In these experiments, expression of BRC3 in the parental cell line reduced the number of GFP-positive cells by 2-fold, whereas expression of mBRC3 had no effect (Fig. 6). Expression of BRC3 in the hRAD51-WT expressing cell line had a smaller effect, reducing the number of GFP-positive cells 1.5-fold, with expression of mBRC3 again having no effect. Expression of BRC3 in the hRAD51-K133R expressing cell line also reduced the number of GFP-positive cells, in this case 6-fold below the already reduced level, such that HDR is decreased 31-fold relative to the parental cell line (*i.e.* 2.2 *versus* 0.07%).

To determine whether the effect of BRC3 expression on the percent of GFP-positive cells is because of a specific loss of HDR events rather than decreased total DSB repair, we examined

A.

hprtDRGFP

B.

C.
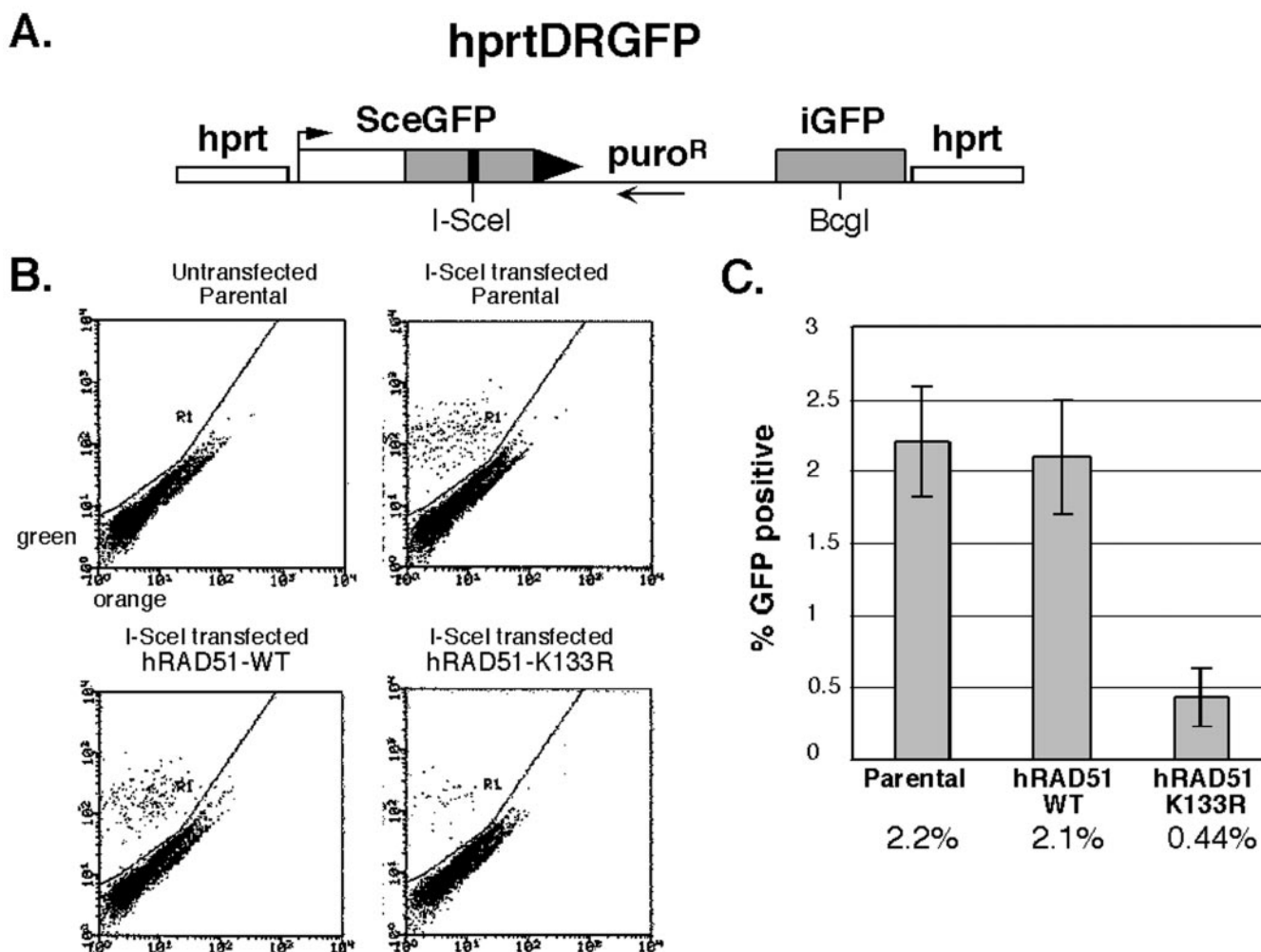
Fig. 4. **Cell lines expressing hRAD51-K133R exhibit defects in HDR of a chromosomal DSB.** *A*, the hprtDRGFP reporter substrate. The recombination reporter DR-GFP was targeted to the *hprt* locus. *SceGFP* is a *GFP* gene that contains the I-*Sce*I site within the coding region. Cleavage of this I-*Sce*I site *in vivo* can result in HDR directed by the downstream *GFP* repeat, *iGFP*. HDR by gene conversion without crossing over restores functionality to the *GFP* gene, which can be scored by green fluorescence. *B*, flow cytometric analysis of ES cells containing the targeted hprtDRGFP reporter. Shown are plots of green fluorescence on the *y axis* and orange fluorescence on the *x axis* for 10,000 cells of the parental cell line and hRAD51-WT and hRAD51-K133R expressing lines transfected with an I-*Sce*I expression vector. A plot of untransfected parental cells is also shown. The GFP-positive population is denoted by the R1 gate as those cells that fluoresce more green than orange in contrast to the GFP-negative population that fluoresces equally green and orange. *C*, the percentage of GFP-positive cells from the parental cell line and hRAD51-WT and hRAD51-K133R expressing lines transfected with an expression vector for I-*Sce*I. Each percentage shown is the mean of four independent transfections, and *error bars* represent the S.D.

the efficiency of HDR relative to I-*Sce*I site loss for each of these transfections, using the PCR-based assay described above. In these experiments, co-expression of I-*Sce*I with either BRC3 or mBRC3 in each of the cell lines resulted in ~10% I-*Sce*I site loss, similar to that obtained by transfection of the I-*Sce*I expression vector alone (Fig. 7*A*). Subsequently, the percent HDR relative to I-*Sce*I site loss was computed by dividing the percentage of GFP-positive cells by the percent I-*Sce*I site loss for each individual transfection (Fig. 7*B*). In the parental cells, the percent HDR relative to I-*Sce*I site loss was reduced 1.8-fold with BRC3 expression, whereas such expression had little or no effect on the hRAD51-WT expressing cell line. In cells expressing hRAD51-K133R, the percent HDR relative to I-*Sce*I site loss was reduced 3.1-fold with BRC3 expression. Therefore, co-expression of hRAD51-K133R and BRC3 led to an overall 18-fold reduction in HDR relative to I-*Sce*I site loss compared with the parental cell line. Expression of mBRC3, by contrast, had no effect on HDR relative to I-*Sce*I site loss in any of the cell lines.

Thus, whether measured directly by the number of GFP-positive cells or relative to total DSB repair leading to I-*Sce*I

site loss, expression of BRC3 reduces HDR in the hRAD51-K133R expressing cell line as well as the parental cell line. By contrast, the excess RAD51 protein in the hRAD51-WT expressing line appears to interfere with the inhibitory effect of BRC3.

DISCUSSION

RAD51 is an essential gene in vertebrate cells (16–18) and has been implicated in tumor suppression through its interactions with the breast cancer susceptibility genes *BRCA1* and *BRCA2* (5, 21). To begin to investigate the function of RAD51 in mammalian DNA repair, we used a dominant negative approach, expressing the ATP hydrolysis-defective hRAD51-K133R protein in mouse ES cells that retain expression of the endogenous wild-type RAD51 protein. We found that expression of hRAD51-K133R impairs multiple types of DNA repair, *i.e.* HDR of a chromosomal DSB, repair leading to SCE, and repair of damage arising both from the cross-linking agent MMC and from IR. In contrast, overall DSB repair, predominantly NHEJ, was not affected by expression of hRAD51-K133R. Although it is formally possible that reduced HDR and
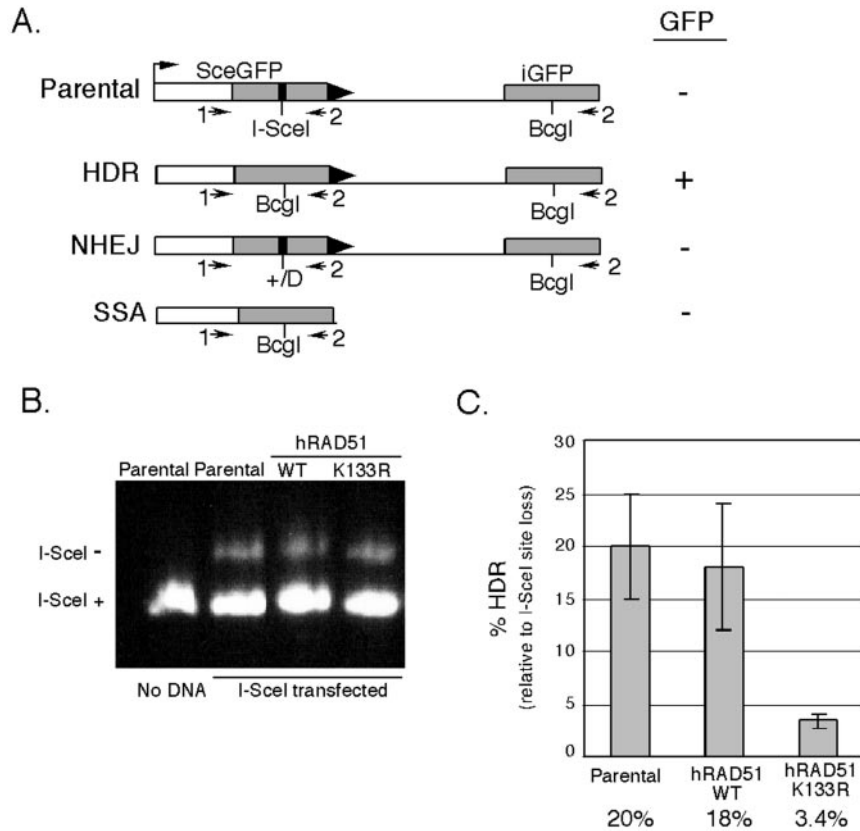
FIG. 5. **DSB repair is not globally reduced in the hRAD51-K133R expressing cell line.** *A*, DSB repair products that have lost the I-*Sce*I site after I-*Sce*I expression. Shown are diagrams of the parental DR-GFP substrate along with the products of three pathways of DSB repair that result in I-*Sce*I site loss: HDR, NHEJ, and SSA. Loss of the I-*Sce*I site in cells transfected with an I-*Sce*I expression vector is quantified using PCR of genomic DNA with *primers 1* and *2* (*arrows*). *B*, analysis of I-*Sce*I site loss by PCR amplification of *SceGFP*. PCR amplification of the genomic region surrounding the I-*Sce*I break site in *SceGFP* results in a 725-bp product that is cleaved by I-*Sce*I into two fragments of 546 and 179 bp. After repair of the DSB by HDR, NHEJ, or SSA, the ~725-bp product is not cleaved by I-*Sce*I. Shown are PCR products digested with I-*Sce*I and resolved on agarose gels from the parental cell line and hRAD51-WT and hRAD51-K133R expressing lines transfected with an expression vector for I-*Sce*I. The ~725- and 546-bp fragments are denoted as the *I-Sce*I− and *I-Sce*I+ products, respectively. To determine the percent of the PCR product from each cell population no longer digestible by I-*Sce*I, the ~725- and 546-bp fragments were quantified using a Bio-Rad Chemidoc 2000 system, and the quantity of the 546-bp band was corrected to compensate for weaker staining due to reduced length. Quantification by this method was validated by analyzing mixed concentrations of genomic DNA samples from pure populations of I-*Sce*I+ and I-*Sce*I− cells (data not shown). *C*, the percent HDR relative to I-*Sce*I site loss. This percentage was determined for each cell line by dividing the percentage GFP-positive cells by percent I-*Sce*I site loss after transfection of the I-*Sce*I expression vector. Each percentage shown is the mean of four independent transfections, and *error bars* represent the S.D.

the other DNA repair phenotypes we observed arise from disruption of some other function of RAD51 than ATP hydrolysis, these results implicate ATP hydrolysis by RAD51 as being important for efficient levels of certain types of DNA repair in mammalian cells.

Although RAD51 has been shown to play a central role in recombination reactions in many organisms (15), the effect of a defined mutation in *RAD51* on HDR in mammalian cells has not been reported previously, even in a dominant negative context. The ~5-fold reduction in HDR of the I-*Sce*I endonuclease-generated DSB we found in the hRAD51-K133R expressing cells is similar to that seen in ES cells with hypomorphic mutations of *Brca1* and *Brca2* (22–25), although *Rad54* null ES cells have a much milder HDR defect (45). Gene-targeting defects also arise from expression of the hRAD51-K133R mutant, as reported previously in chicken DT40 cells (26) and as suggested in our experiments by the decreased efficiency with which we obtained targeted ES cell clones. However, mutations in homologous recombination genes can have very different effects on gene targeting and HDR. Notably, *Rad54* and *Brca1* mutations have been shown to have much larger fold effects on gene targeting than on HDR (22, 45), whereas a *Brca2* mutation gives a more profound HDR defect (23). Mutations in NHEJ genes also differentially affect gene targeting and HDR

(27, 46). Thus, the various DSB repair proteins in mammalian cells appear to have distinct roles in different recombination reactions, with those proteins having major roles in HDR also being critical for cellular and organismal viability (*i.e. Rad51*, *Brca1*, and *Brca2*).

In yeast, spontaneous interchromosomal recombination has been shown to be defective in cells expressing an ATP hydrolysis mutant of RAD51 (36), although in this case it is not clear if the initiating event is a DSB. In ES cells, we also found that spontaneous SCE is reduced in cells expressing the hRAD51-K133R protein. SCE events may be somewhat distinct mechanistically from HDR of an endonuclease-generated DSB, because SCEs are predicted to be promoted by one-ended DSBs that arise during DNA replication (20), in contrast to the two-ended DSBs that arise from I-*Sce*I endonuclease cleavage (27). Thus, ATP hydrolysis by RAD51 appears to be important for mechanistically different homologous recombination processes.

Although the efficiency of HDR in the hRAD51-K133R expressing cells is reduced, it was not completely abolished. The residual HDR in the hRAD51-K133R expressing cells could be RAD51-independent, because RAD51-independent pathways have been identified in *S. cerevisiae* (47–49). However, this residual HDR appears to be RAD51-dependent given that it is substantially reduced by expression of a peptide that disrupts
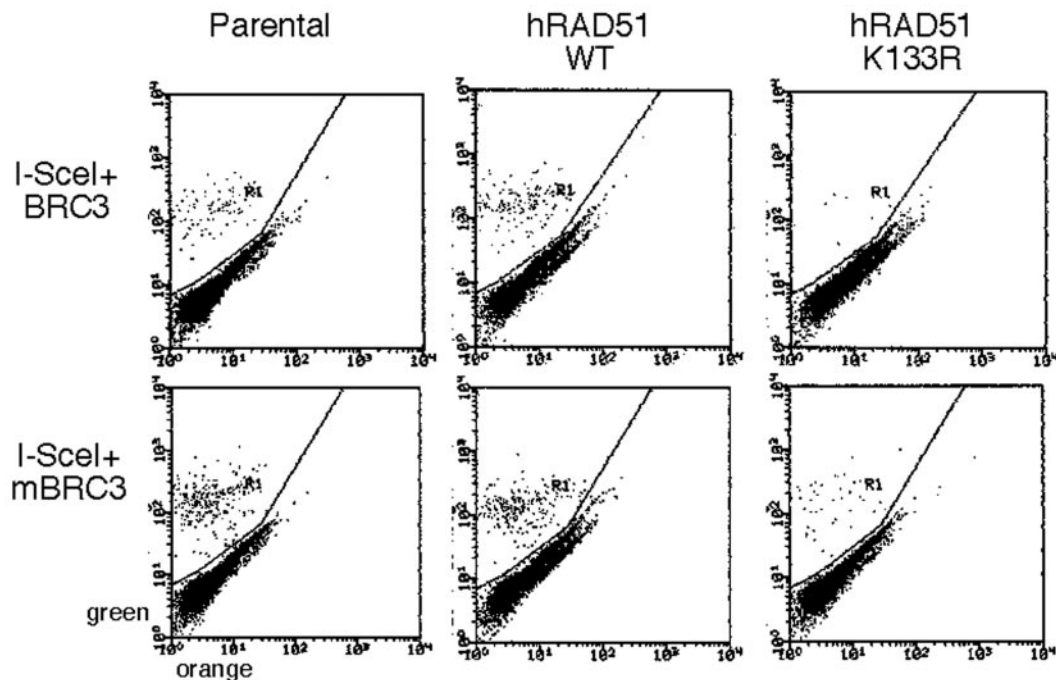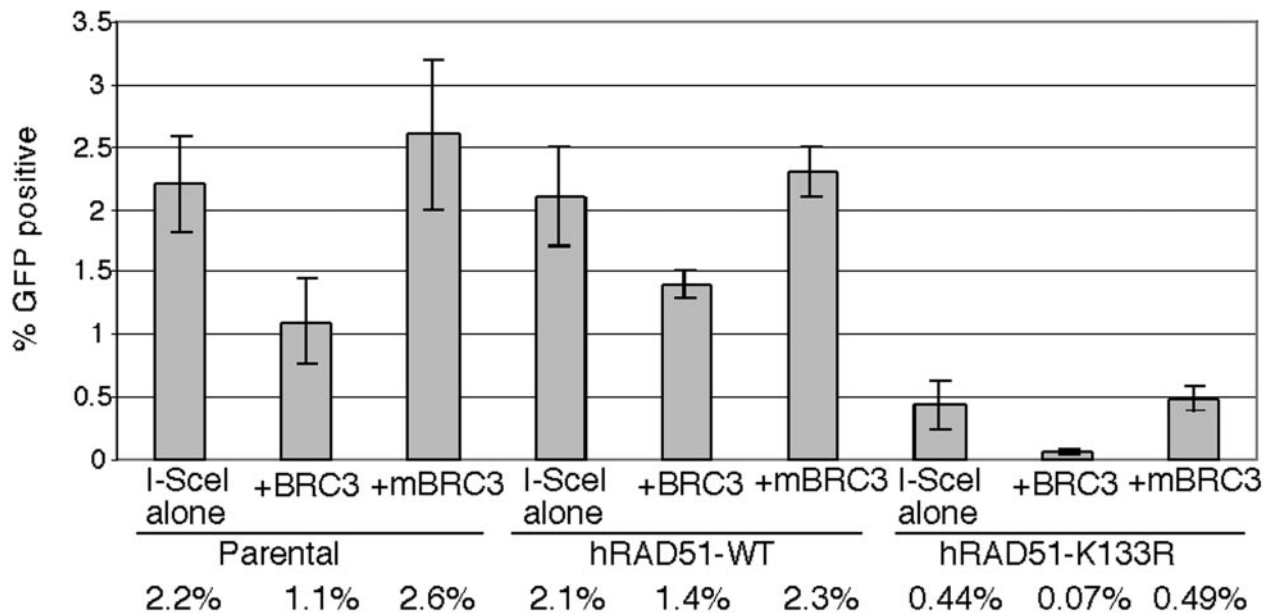
FIG. 6. **Cell lines expressing BRC repeat 3 from human BRCA2 exhibit defects in HDR of a chromosomal DSB.** *A*, flow cytometric analysis of the parental cell line and hRAD51-WT and hRAD51-K133R expressing lines transfected with an expression vector for I-*Sce*I in combination with expression vectors for either BRC3 or mBRC3. Shown are plots of green fluorescence on the *y axis* and orange fluorescence on the *x axis* for 10,000 cells for these transfections. *B*, percent of GFP positive cells from the transfections of the I-*Sce*I expression vector alone or in combination with expression vectors for BRC3 or mBRC3. Each percentage shown is the mean of four independent transfections, and *error bars* represent the S.D.

an early step in RAD51-mediated strand exchange, *i.e.* a BRC repeat, BRC3, from BRCA2. BRC3 interacts with RAD51 *in vitro* (50, 51) and prevents its association with DNA into nucleoprotein filaments (31), which is a prerequisite for RAD51-mediated strand exchange (15). These results suggest that the hRAD51-K133R expressing cells could be relatively proficient at strand exchange, because of strand exchange activity of the hRAD51-K133R protein itself (26) or residual activity from the endogenous wild-type protein. Other HDR processes could nevertheless require ATP hydrolysis; for example, ATP hydrolysis by RAD51 has been shown to cause dissociation of RAD51 from

complexes with DNA (52). This dissociation could be important for completing recombination or for turnover of the protein to monomers, enabling them to function in subsequent reactions.

Nevertheless, we cannot rule out that ATP hydrolysis by RAD51 in mammalian cells has effects upon strand exchange during HDR *in vivo*. ATP hydrolysis by yeast Rad51 has been shown to be dispensable for efficient and extensive strand exchange (53, 54). Despite these observations, it is formally possible that the human RAD51-K133R protein could be defective for extensive strand exchange, because the strand exchange activity of this protein has only been tested using short
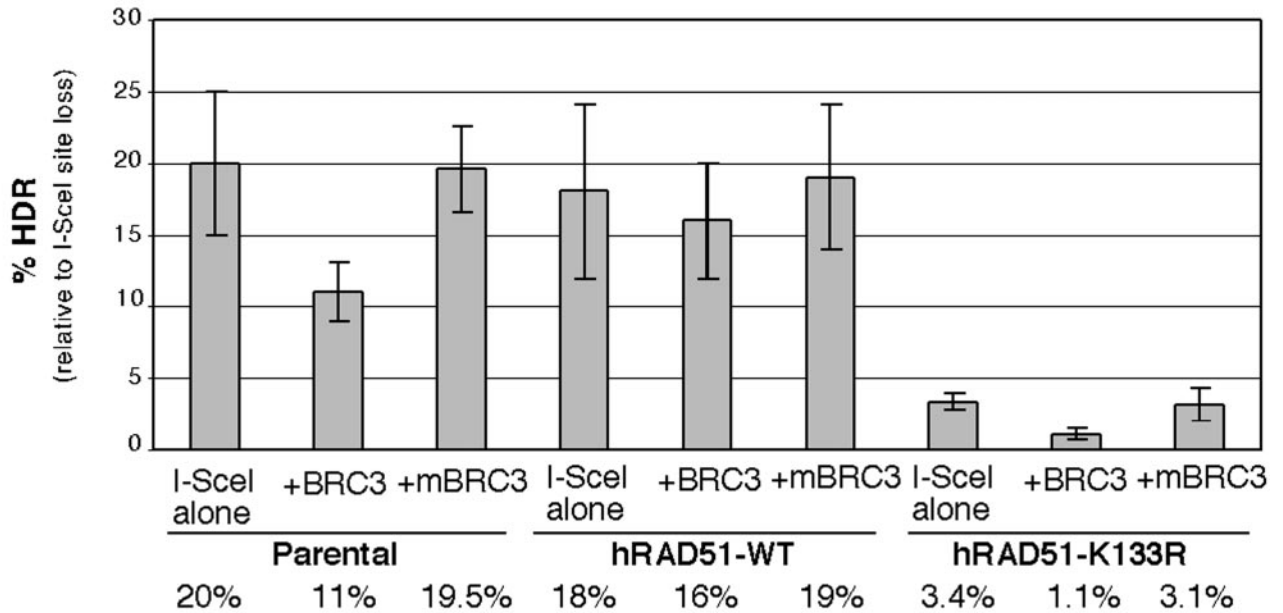
A.
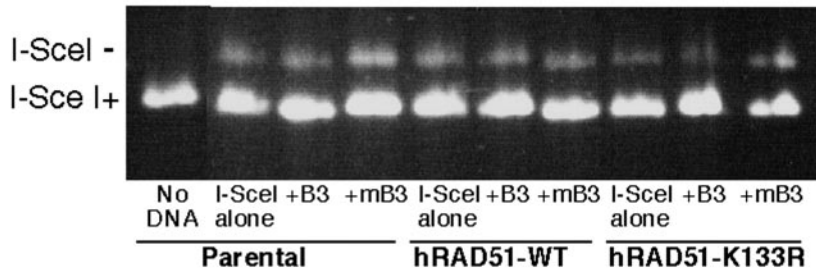
B.

FIG. 7. **DSB repair is not globally reduced with BRC3 expression.** *A*, determination of percent I-*Sce*I site-loss after I-*Sce*I expression. Shown are PCR products digested with I-*Sce*I and resolved on agarose gels from parental cell line and hRAD51-WT and hRAD51-K133R expressing lines transfected with an expression vector for I-*Sce*I alone or in combination with expression vectors for either BRC3 or mBRC3. The *I-SceI+* and *I-SceI−* products are labeled as in Fig. 5B. The percent of the PCR product from each cell population no longer digestible by I-*Sce*I was determined as in Fig. 5B. *B*, percent HDR relative to I-*Sce*I site loss from the transfections of the I-*Sce*I expression vector alone or in combination with expression vectors for BRC3 or mBRC3. Each percentage shown is the mean of four independent transfections, and *error bars* represent the S.D.

oligonucleotide DNA molecules (26). It is also important to consider that wild-type RAD51 protein is also present in the cells expressing hRAD51-K133R, such that wild-type and mutant protein could form mixed RAD51 nucleoprotein filaments which may have intermediate activities during extensive strand exchange *in vivo*. In general, the *in vivo* requirements for strand exchange could be different from those *in vitro*, due to the presence of other factors and where chromosomes are the substrates for recombination.

Although chicken DT40 cells that express hRAD51-K133R as the only RAD51 in the cell are no more sensitive to IR than wild-type chicken cells (26), we found that the hRAD51-K133R expressing ES cells are hypersensitive to IR. Mouse ES cells and chicken DT40 cells may therefore have different requirements for RAD51 in the repair of IR damage. Despite this disparity between the mouse and chicken cell lines, the phenotypes of the hRAD51-K133R expressing mouse cells are similar to that of other mammalian HDR mutants, such as *XRCC2*, *XRCC3*, and *BRCA1*, which have increased sensitivity to MMC as well as to IR (22, 39, 40, 55, 56). Although not examined in our study, sensitivity to the alkylating agent MMS has also been shown in *S. cerevisiae* to be affected by ATP hydrolysis defects of RAD51, as tested both in a dominant negative context and when expressed at normal levels in a *rad51* null

mutant (36–38). These results again point to a role for ATP hydrolysis by RAD51 during DNA repair, although it is important to note that overexpression of an ATP hydrolysis-defective mutant in yeast will suppress the MMS sensitivity of a *rad51* null mutant (54).

Because disruption of *RAD51* results in cellular lethality in mammalian cells (17, 18), it has been difficult to discern its role in DNA repair. In this report, we have begun to examine the effect of defined *RAD51* mutations on DNA repair in mammalian cells using a dominant negative approach. Although we have successfully achieved moderate levels of hRAD51-K133R expression in otherwise wild-type cells, it is still unclear whether the ATP hydrolysis mutant could substitute for wild-type RAD51 in mammalian cells, as it does in chicken cells. Our attempts to express the mutant at higher levels have thus far been unsuccessful,[2] suggesting that viability of the hRAD51-K133R expressing ES cells may be dependent upon sufficient levels of expression of the wild-type protein to counteract the effects of the mutant.

A dominant negative approach to study RAD51 function has been reported previously (57, 58) to be successful in mamma-

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[2] J. M. Stark and M. Jasin, unpublished results.

RAD51 Mutation and DNA Repair in Mammalian Cells

lian cells using a mouse Rad51 fusion protein containing an N-terminal extension from the yeast Rad51 protein. The effect of this fusion protein could conceivably be linked to altered ATP hydrolysis by RAD51 because, like the hRAD51-K133R mutant, it reduces HDR. However, it seems more likely that the N-terminal extension interferes with some other aspect of RAD51 function, because spontaneous SCE was only slightly diminished in cells expressing the fusion protein (57, 58), and these cells are not sensitive to IR (57), unlike cells expressing the hRAD51-K133R protein. The expectation in these previously reported dominant negative experiments and those described here is that the mutant RAD51 proteins interact with the wild-type protein to form mixed complexes or even mixed nucleoprotein filaments with DNA. However, it is also possible that the mutant proteins disrupt DNA repair by the wild-type protein by interacting directly with recombination intermediates or by associating with other DSB repair proteins.

In addition to an essential function for cellular viability, a role for RAD51 in tumor suppression is suggested by the interaction of RAD51 with the breast and ovarian tumor suppressors BRCA1 and BRCA2 (21), which are known to promote HDR and gene targeting (22–25, 43), and also by the findings that single nucleotide polymorphisms and mutations in *RAD51* are associated with breast tumors (5). Further analysis of RAD51 function, either by the dominant negative approach described here or with knock-in mutations, will be essential for a thorough understanding of RAD51 function in DNA repair, genome stability, and tumor suppression.

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