Homology-directed DNA Repair, Mitomycin-C Resistance, and Chromosome Stability Is Restored with Correction of a Brca1 Mutation

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ABSTRACT

Chromosomal breaks occur spontaneously as a result of normal DNA metabolism and after exposure to DNA-damaging agents. A major pathway involved in chromosomal double-strand break repair is homologous recombination. In this pathway, a DNA sequence with similarity to a damaged chromosome directs the repair of the damage. The protein products of the hereditary breast cancer susceptibility genes, BRCA1 and BRCA2, interact with the Rad51 protein, a central component of homologous repair pathways. We have recently shown that this interaction is significant by demonstrating that Brca1- and Brca2-deficient cells are defective in homology-directed chromosomal break repair. We confirm that Brca1-deficient embryonic stem (ES) cells are defective in gene targeting and homology-directed repair of an 1-Sce 1-induced chromosome break. The phenotypic paradigm that defines homology-directed repair mutants is extended to these Brca1-deficient cells by the demonstration of 100-fold sensitivity to the interstrand cross-linking agent mitomycin-C and spontaneous chromosome instability. Interestingly, although chromosome aberrations were evident, aneuploidy was not observed. Repair phenotypes are partially restored by expression of a Brca1 transgene, whereas correction of one mutated Brca1 allele through gene targeting fully restores mitomycin-C resistance and chromosome stability. We conclude that the inability to properly repair strand breaks by homology-directed repair gives rise to defects in chromosome maintenance that promote genetic instability and, it is likely, tumorigenesis.

INTRODUCTION

Individuals who carry mutations in the hereditary breast cancer gene, BRCA1, are predisposed to early-onset breast and ovarian cancer. Although the relationship to cancer predisposition remains speculative, the BRCA1 protein has been implicated in multiple cellular functions associated with the DNA damage response. These functions include DNA repair (1–3), the cellular response to DNA damage via upstream damage-signaling proteins, and transcriptional regulation of proteins involved in the downstream response to DNA damage. After treatment of cells with DNA damaging agents, BRCA1 is phosphorylated by the signaling proteins ATM, ATR, and Chk2 (4–6) and appears to regulate the expression of genes farther downstream, such as p21, p53, and GADD45 (7–9). Biochemical analysis of nuclear extracts demonstrates that the BRCA1 protein is part of a large complex of proteins involved in damage recognition and repair, which has led to the hypothesis that BRCA1 acts as a scaffold to coordinate the cellular response (10).

One type of damage to which mouse and human BRCA1-deficient cells respond abnormally is the DNA DSB.4 Chromosome DSBs arise from both endogenous processes and exogenous agents such as IR, causing cell death or genomic alterations if not repaired appropriately. BRCA1 mutant human cells (i.e., HCC1937 cancer cells) are hypersensitive to IR, and this sensitivity is partially relieved (2–3×) by expression of wild-type BRCA1. Of note, significant IR sensitivity remains in the BRCA1-complemented HCC1937 cells (3). Brca1 mutant mouse cell lines, such as the 236.44 ES cell line, are also sensitive to IR, as are Brca1 mutant embryos (1,11). In mammalian cells two major repair pathways, homologous recombination and NHEJ, prevent deleterious outcomes after treatment of cells with agents that cause DSBs. The association of Rad51, the eukaryotic homologue of the Escherichia coli RecA protein, with BRCA1 in immunoprecipitable complexes, as well as the observed subnuclear localization of BRCA1 with Rad51 in IR-induced foci (12,13), is highly suggestive of a role for BRCA1 in homology-directed repair (HDR). However, after IR treatment of cells, BRCA1 also colocalizes with the Rad50 complex, which, in yeast, has a role in NHEJ as well as in HDR, raising the possibility of a role for BRCA1 in this DSB repair pathway (14).

We have recently examined DSB repair at the molecular level in the Brca1−/− 236.44 ES cell line to determine the nature of the repair defect. This cell line was constructed through consecutive rounds of gene targeting such that both Brca1 alleles are mutated by deletion of the 5′ end of exon 11 (15). The targeted alleles are hypomorphs, inasmuch as they express a truncated product arising from an alternatively spliced transcript that skips exon 11 (16). In addition to being hypersensitive to IR, the 236.44 cell line is hypersensitive to cisplatin, has reduced Rad51 focus formation after cisplatin treatment, and is defective in transcription-coupled repair of oxidative damage (1,17). By examining repair of an endonuclease-generated DSB in this cell line, we demonstrated that HDR is reduced, though NHEJ is not impaired but, rather, slightly elevated (2). Because DSB repair mutants often exhibit spontaneous chromosome instability, there was a concern that additional genomic changes had occurred in the Brca1−/− 236.44 cells, such that the observed repair defects are not fully attributable to loss of wild-type Brca1. Partial complementation of these cells by the reintroduction of wild-type Brca1 has, in fact, not demonstrated a clear restoration of homologous recombination (16).

In the current study, we have examined repair phenotypes of the Brca1 mutant 236.44 cell line as well as derivative cell lines in which wild-type Brca1 is reexpressed as a result of either complementation from a Brca1 transgene or correction of one of the Brca1 hypomorphic alleles. Using a recombination reporter assay recently developed for the analysis of Brca1 mutant cells, we show that the 236.44 cell line has impaired HDR of an induced chromosome break and reduced gene targeting, and we confirm that wild-type Brca1 expression in this cell line restores levels of both types of homologous recombination. We also demonstrate that Brca1 deficiency results in chromosome instability in ES cells, although not the dramatic instability detected in MEFs. Moreover, Brca1-deficient cells exhibit exquisite sensitivity to the interstrand cross-linking agent mitomycin-C.

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4 The abbreviations used are: DSB, double-strand break; IR, ionizing radiation; ES, embryonic stem; NHEJ, nonhomologous end-joining; HDR, homology-directed repair; MEF, mouse embryonic fibroblasts; Tg, transgene; pBS, pBluescript; pur6, purumycin-resistant; hyg R, hygromycin-resistant; DR-GFP, direct repeat-green fluorescent protein; neo, neomycin phosphotransferase; hprt, hypoxanthine phosphoribosyl transferase; XRCC, X-ray cross-complementing repair.
cross-linking agent mitomycin-C, suggesting that this or related DNA-damaging agents may have a highly favorable therapeutic ratio. Notably, restoration of Brca1 by transgene complementation was less effective at reversing repair defects than correction of a defective Brca1 allele by gene targeting, presumably because of the difficulty in expressing a physiologically relevant amount of Brca1.

MATERIALS AND METHODS

DNA Manipulations. The p59xDR-GFP6 pim1-targeting vector was previously described (18). The targeting fragments were obtained by XhoI digestion. The pBBxAs transgene and the puromycin-resistant Brca1+/−/− cell line containing the Brca1 cDNA was a gift of Beverly Koller, University of North Carolina, Chapel Hill, NC (16). The plasmid was linearized before transfection. The pBrca1+S1neo Brca1 allele was digested with NheI-SpeI, and religated to create pBrca1/puro allele, thereby deleting an internal 2.4-kb NheI Brca1 fragment. pBS was digested with EcoRV-HincII and religated to delete the EcoRV site. pBrca1/puro allele was digested with EcoRI to obtain a 7.6-kb Brca1 fragment. NotI linker sequences were added after Klenow treatment and ligated to NotI digested pBS/S1 neo to create pBrca1/puro. A blunt-ended hyg-1 fragment (19) was inserted into a unique EcoRV site in intron 9 of pBrca1/puro, creating pBrca1+RVS1neo. Orientation of the hyg cassette was running in the opposite orientation of Brca1. S1 neo was completed by digestion of pBrca1+RVS1neo with NheI and ligation with a 2.4-kb NheI Brca1 fragment that had been deleted in an earlier cloning step. Multiple-restriction digests verified the correct orientation of the insert. S1 neo was linearized by digestion with NotI before gene targeting.

Cell Transfections and Nucleic Acid Analysis. For stable transfection of pBBxAs, Brca1+/− ES cells were cotransfected by electroporation at 800 V/3 μF with 75 μg of the linear fragment from pBBxAs and 25 μg of phosphoglycerate kinase-puromycin expression plasmid with subsequent selection after 24 h in 1.0 μg/ml puromycin. 2 μg/ml clones were picked and expanded 12–16 days later. Southern blots of genomic DNA from these clones were hybridized with a Brca1 probe. For gene targeting, ES cells were electroporated with 75 μg of linear targeting fragment from p59xDR-GFP6 with subsequent selection after 48 h in hygromycin 110 μg/ml and puromycin 1.0 μg/ml hygR and purR clones were picked and expanded 10–12 days later. Southern blots of genomic DNA from these clones were digested with HincII and hybridized with a radiolabeled pim1 probe. For Brca1 correction by gene targeting, Brca1+/− ES cells were electroporated with 75 μg of linear pBrca1+S1neo Brca1 allele was digested with HincII and hybridized with a 3′ 1.2-kb Brca1 probe. For DBS repair assays, actively growing cells were electroporated at 250 V/960 μF with 30–50 μg of pCBrAnSce (20), mock DNA, or pNZE-CAG (21) and plated in nonselective media. Cells were trypsinized at 45–48 h and analyzed by flow cytometry. Data were analyzed with Lysis software. For Brca1 expression, total RNA was extracted from actively growing cells by RNAzol (Biotecx Laboratories) treatment. Northern analysis was performed by standard techniques.

Mitomycin-C Clonogenic Survival Assays. For mitomycin-C survival assays, cells were exposed to various mitomycin-C doses for 4 h and then rinsed three times in PBS. The cells were replated and allowed to grow undisturbed for 8–10 days. The colonies were stained and counted. Survival experiments were performed in triplicate.

Metaphase Spreads and Karotype Analysis. For karyotype analysis, cells were cultured with 0.05 μg/ml colcemid (Life Technologies, Inc.) for 1.5 h, then trypsinized and resuspended in hypotonic saline (0.075 M KCl) at 37°C for 10 min. The cells were fixed in 3:1 mix of methanol and acetic acid. Fixed cell suspensions were transferred to glass slides and allowed to air-dry. Metaphase spreads were stained with 4% Giemsa in PBS for 20 min.

RESULTS

Partial Complementation of the Gene-targeting Defect in Brca1+/− ES Cells by Brca1 Transgene Expression. Gene targeting is a measure of the ability of cells to homologously integrate transfected DNA into genetic loci containing sufficient sequence homology to the transfected DNA. Deficiencies in gene targeting, attributable to cellular defects in homologous recombination, have been reported for Brca1, Brca2, and Rad54 mutant cell lines, although the precise relationship between homologous integration and HDR is not well understood (2, 18, 22). To investigate both homologous...
recombination pathways, i.e., gene targeting and HDR of a targeted chromosomal break, the DR-GFP reporter substrate was subcloned into the \textit{pim1} gene-targeting vector, p59x, creating p59xDR-GFP6 (Fig. 1A; Ref. 18). Gene targeting with the p59x vector is highly efficient, because the selectable hygromycin resistance gene (\textit{hyg}^R) is fused in-frame to \textit{pim1} coding sequences such that expression of \textit{hyg}^R is dependent on either targeting to the \textit{pim1} locus on chromosome 17 or a fortuitous nonhomologous integration adjacent to promoter sequences of another locus (23). The p59xDR-GFP6 vector additionally contains an intact puromycin resistance gene (\textit{pur}^R), which can also be used to select for vector integration events.

In our initial report examining homologous recombination in the \textit{Brca1}^+/− 234.64 cell line, we determined that gene targeting was reduced 13- and 23-fold relative to the control \textit{Brca1}^+/− cell line, as measured at two distinct genomic loci, \textit{Rb} and \textit{pim1}, respectively (2). To confirm that the defect in gene targeting was attributable to the loss of wild-type \textit{Brca1}, we electroporated the linearized p59xDR-GFP6 targeting fragment into a \textit{Brca1}^+/−,\textit{Tg}^− cell line that is derived from the \textit{Brca1}^+/− line, but which expresses full-length mouse \textit{Brca1} from a randomly integrated DNA at ~10–20% normal \textit{Brca1} levels (Ref. 16 and data not shown). Doubly resistant \textit{hyg}^R/\textit{pur}^R colonies were selected, and genomic DNA from individual colonies was analyzed by Southern blotting using a \textit{pim1} probe located outside the targeting fragment (Fig. 1A). Efficient gene targeting was observed in the \textit{Brca1}^+/− cells, with 94% of \textit{hyg}^R/\textit{pur}^R clones correctly targeted (Fig. 1B; Table 1). However, homologous integrations were rarely detected in the \textit{Brca1}^+/− cells, with only 6% of the \textit{hyg}^R/\textit{pur}^R clones correctly targeted, which is a 16-fold lower frequency of gene targeting as compared with \textit{Brca1}^+/− cells. This diminished ability to gene target is comparable with the 23-fold lower frequency we originally reported at the \textit{pim1} locus in the \textit{Brca1}^+/− cells. The small improvement in gene targeting may be related to the more stringent combined hygromycin and puromycin selection used in the targeting of the p59xDR-GFP6 fragment, because we observed that background colonies observed during hygromycin selection of \textit{Brca1}^−/− cells were significantly reduced with the combined selection. The addition of puromycin to the hygromycin selective media for \textit{Brca1}^+/− and \textit{Brca1}^−/− cells did not alter or improve upon the gene targeting frequencies obtained with hygromycin alone (data not shown). The addition of puromycin to the \textit{Brca1}^+/−,\textit{Tg}^− would not effect gene targeting, because these cells were selected for \textit{pur}^R during the integration of the \textit{Brca1} transgene (16).

In the \textit{Brca1}^+/−,\textit{Tg}^− cells, gene targeting was partially restored, whereby homologous integrants were obtained at a frequency of nearly 62% (Fig. 1B and Table 1). This is a 10-fold improvement in gene targeting as compared with the \textit{Brca1}^−/− mutant cells. However, when compared with the \textit{Brca1}^+/− cells, a 1.6-fold decreased targeting efficiency remained in the transgene corrected cell line, suggesting only partial complementation of the gene targeting defect by expression from the \textit{Brca1} transgene.

In this experiment we also examined gene targeting in parental \textit{Brca1}^+/− cells, because loss of a single \textit{BRCA1} allele in human cell lines has been implicated in sensitivity to DNA-damaging agents (24). Similar to the \textit{Brca1}^+/− cells, 97% of \textit{hyg}^R/\textit{pur}^R clones were correctly targeted. Thus, mouse cells heterozygous for a \textit{Brca1} mutation have no detectable defect in gene targeting as compared with \textit{Brca1}^+/− ES cells. In addition, these results imply that the \textit{Brca1} exon 10–12 splice variant that is expressed in the \textit{Brca1}^+/− cells has no detectable dominant-negative effect on gene targeting.

\textbf{HDR of a Chromosomal DSB in \textit{Brca1}^+/− ES Cells Is Also Partially Restored by \textit{Brca1} Transgene Expression.} The DR-GFP reporter substrate (21), which was incorporated at the \textit{pim1} locus during targeting of the p59xDR-GFP6 vector, measures HDR of an endonuclease-induced DSB by a gene conversion mechanism. It is composed of two differentially mutated \textit{GFP} genes

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Genotype & Gene-targeting efficiency & FD \footnote{FD, fold decrease.} \\
& (no. targeted clones/total & \\
& analyzed) & \\
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\textit{Brca1}^+/− & 96.7\% (58/60) & 1.0 \\
\textit{Brca1}^+/− & 94.1\% (48/51) & 1.0 \\
\textit{Brca1}^−/− & 5.9\% (2/34) & 16.4 \\
\textit{Brca1}^+/−,\textit{Tg}^− & 61.9\% (52/84) & 1.6 \\
\hline
\end{tabular}
\caption{Gene-targeting efficiency at the \textit{pim1} locus}
\end{table}

Cells were electroporated with linearized p59x-DR-GFP6 and selected in hygromycin (110 \textmu g/ml) and puromycin (1 \textmu g/ml).
oriented as direct repeats and separated by the purR gene (Fig. 2A). The SceGFP gene is a GFP gene that is mutated by 11 bp substitutions to contain the 18 bp recognition sequence for the I-Sce I endonuclease. Downstream of SceGFP is the 0.8-kb GFP fragment iGFP, which is a wild-type GFP gene truncated at both its 5′ and 3′ ends. Expression of I-Sce I in cells that have the DR-GFP substrate integrated into their genome results in a DSB in the chromosome at the position of the I-Sce I site. Repair of the induced DSB in SceGFP by a non-crossover gene conversion with iGFP reconstructs a GFP+ gene, expression of which can be scored by cellular fluorescence. Although other DSB repair events at the I-Sce I site are possible, they are not detected, because the 11-bp substitutions in the SceGFP gene cannot be restored to the wild-type GFP sequence except through a templated gene conversion event. Molecular analysis of the DR-GFP substrate in sorted GFP-positive cells after I-Sce I expression has verified that cellular green fluorescence, as measured by flow cytometry, results from repair by gene conversion (18, 21).

To analyze HDR in cell lines with various Brca1 genotypes, several of the p59xDR-GFP6 targeted clones for each genotype were electroporated with the I-Sce I expression vector pCBASce to transiently express the I-Sce I endonuclease. Electroporated cells were typically examined 48 h later by flow cytometry. GFP-positive cells were undetected or rarely detected (<0.01%) in any of the cell lines in the absence of I-Sce I expression (data not shown), indicating that spontaneous intrachromosomal gene conversion is rare. After transfection with pCBASce, however, GFP-positive cells were readily detected in the Brca1+/+ and Brca1+/− cells, indicating robust HDR of the induced DSB. The absolute frequency of GFP positive cells varied between experiments, with a range of 1–4% positive cells, although there was no detectable difference between the Brca1+/+ and Brca1+/− cells when normalized to each other within a particular experiment (Fig. 2B). As with gene targeting, therefore, HDR of an induced chromosomal break is not diminished in ES cells containing only one wild-type Brca1 allele. Proficient HDR in the Brca1+/− cells also indicates that the exon 10–12 splice variant arising from the mutated Brca1 allele does not exhibit a dominant-negative effect on the wild-type Brca1 allele.

The Brca1−/− cells exhibited many fewer GFP-positive cells after I-Sce I expression, such that there was a 6-fold decrease in HDR relative to the Brca1+/+ and Brca1+/− cell lines (Fig. 2B). This decrease in gene conversion measured by GFP expression is comparable with the previously reported 5–6-fold decrease in gene conversion measured by a PCR-based assay with a neo substrate (2). The transgene-complemented Brca1+/−/Tgs cell lines, however, showed a 2-fold increase in HDR over the Brca1−/− cells. Thus, HDR was increased by wild-type Brca1 expression, although, as with gene targeting, it was not completely restored.

Electroporations were also performed with a GFP expression vector to verify that the different cell lines had a similar transfection efficiency. The GFP expression vector that was electroporated, pCAG-NZE, uses the same control elements to express GFP as the pCBASce vector uses to express I-Sce I. Electroporated cells were examined by flow cytometry. No difference in GFP expression, which ranged from 44–58%, was detected between any of the Brca1+/+, Brca1+/−, Brca1−/−, and Brca1−/−/Tgs+ targeted cell clones (data not shown), indicating that the transfection efficiency was not appreciably different for these lines.

Brca1-deficient Cells Are Hypersensitive to the DNA Interstrand Cross-linking Agent Mitomycin-C. The first recognized mammalian HDR mutants, the isr1 and isr1SF hamster cell lines deficient for the Rad51-related proteins XRCC2 and XRCC3, respectively, show moderate sensitivity to IR, as do Brca1-deficient cells, but an extreme sensitivity to mitomycin-C (25). Mitomycin-C produces several types of DNA damage, one of which is an interstrand DNA cross-link. This lesion could potentiate DSBs through replication fork blockage, producing an intermediate that is much more dependent for repair on HDR than on NHEJ, although the exact mode of repair is unknown (26). To investigate whether Brca1 deficiency also results in mitomycin-C sensitivity, clonogenic survival assays were performed in the Brca1+/+, Brca1+/−, Brca1−/−, and Brca1−/−/Tgs+ cell lines after exposure to increasing doses of mitomycin-C.

Hypersensitivity was seen at all mitomycin-C doses in the Brca1−/− cells as compared with the wild-type and heterozygous Brca1 cell lines (Fig. 3). The extent of sensitivity to mitomycin-C was dose dependent. At a mitomycin-C concentration of 0.5 μM, which was approximately the LD50 for wild-type cells, the Brca1−/− cells exhibited a >100-fold increased sensitivity (Fig. 3). No difference in sensitivity to mitomycin-C in the Brca1+/+ and Brca1+/− cell lines was observed at this or other doses. Consistent with the homologous recombination assays, the Brca1−/−/Tgs+ cell lines revealed an intermediate sensitivity to mitomycin-C.

In an attempt to restore wild-type levels of mitomycin-C resistance to the Brca1−/− cells, we constructed additional transgene-complemented clones. Brca1+/− cells were electroporated with the selectable purR gene and the pBBpAX expression vector, which expresses mouse Brca1 from 4 kb of Brca1 upstream sequences and has a bovine growth hormone polyadenylation signal. This is the same vector previously used to construct the Brca1−/−/Tgs+ cell line (16). PurR clones were analyzed by Northern blotting for Brca1 expression, and, although some clones did not express Brca1, others had levels comparable with the Brca1−/−/Tgs+ cell line (data not shown). Two of these Brca1-expressing clones were tested in a mitomycin-C clonogenic survival assay along with a purR clone that did not express Brca1. Whereas the nonexpressing clone demonstrated the same hypersensitivity to mitomycin-C as did the parental Brca1−/− cell line, the Brca1-expressing clones demonstrated a similar intermediate level of mitomycin-C sensitivity that was seen for the Brca1−/−/Tgs+ cell line (data not shown). Thus, we were unable to fully restore wild-type levels of mitomycin-C resistance by transgene complementation.

Correction of the Brca1 Exon 11 Deletion through Gene Targeting. The incomplete restoration of homologous recombination and mitomycin-C resistance in the Brca1 transgene-complemented cell
lines could be attributable to inadequate expression of Brca1 or undetected alterations in the Brca1 transgene upon integration. It is also formally possible that genomic changes occurred in the Brca1−/− cell line that contributed to the observed repair defects. This was a particular concern, given the dramatic chromosomal instability observed in MEFs harboring a similar Brca1 mutation.

To better address the dependence of the repair defects on Brca1-deficiency, a gene-targeting fragment was constructed to correct the exon 11 deletion mutation in the Brca1−/− 236.44 cell line (Fig. 4A). The Brca1 alleles in this line are disrupted by replacement of the 3′ end of intron 10 and 1.5 kb from the 5′ end of exon 11 with neo and hprt selectable marker genes. To correct one of these alleles, a targeting fragment was constructed in which the hygR gene was inserted into intron 9 of wild-type murine Brca1 genomic sequences. Gene targeting of this fragment, termed S1Rhgy, at either Brca1 allele would therefore restore an intact exon 11, giving rise to a Brca1+/− allele. In wild-type ES cells, we found that the S1Rhgy fragment yielded a high targeting efficiency, such that 75–90% of the hygR clones had homologously integrated this fragment at the Brca1 locus (data not shown). Thus, although gene targeting is substantially reduced in the Brca1−/− cell line, the high targeting efficiency of this fragment suggested that we would be able obtain homologous integrations with sufficient screening of hygR clones.

As the Brca1−/− cell lines with the integrated DR-GFP substrate were already hygR, the parental Brca1−/− 236.44 cell line was electroporated with the S1Rhgy targeting fragment. HygR clones were analyzed by Southern blotting using a Brca1 probe located 3′ to the targeting fragment (Fig. 4A). Two of 159 hygR clones yielded EcoRV fragments indicative of correction of the neo allele (Fig. 4B). Whereas most of the clones gave two distinct fragments for the hprt and neo alleles (12.3 and 5.9 kb, respectively), as in the parental Brca1−/− cell line, in the two targeted clones, E4 and H7, the neo fragment was replaced with a fragment expected for a targeted Brca1+ R hprt allele (11.3 kb). Because of the similarity in size of the hprt and neo alleles, the blots were probed with an hprt fragment to determine whether the hprt allele was gene-targeted. In each of the clones, the hprt allele was still present, indicating that none of the other 157 clones was targeted (data not shown).

Because insertions into the intron of a gene can sometimes disrupt its expression, we determined whether Brca1 mRNA was restored in the targeted clones by performing Northern blot analysis. Brca1−/− cells were found to express the wild-type 7.2-kb Brca1 mRNA (Fig. 5), which was not present in the Brca1−/− cells (data not shown). Rather, a 3.9-kb transcript was seen in the mutant cells that resulted from the exon 10–12 splice. In the E4 and H7 Brca1−/− hygR clones, the 7.2-kb mRNA was observed, indicating restoration of Brca1 expression (Fig. 5). Quantitation of Brca1 expression in these clones indicated that mRNA levels were similar to the Brca1−/− cells. A third hygR clone, H10, that was derived from the S1Rhgy targeting experiment but which had randomly integrated the fragment, was also examined for Brca1 expression. As expected, only the 3.9-kb exon

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**Fig. 4.** Correction of the Brca1−/− cells through gene targeting. A, a portion of genomic Brca1 at exons 10–12. The genomic structure of the gene-targeted alleles in the Brca1−/− cells shows the disruption of exon 11 by hprt on one allele and neo on the other allele. The targeting fragment, S1Rhgy, has an intact exon 11 with a hyg gene inserted at an EcoRV site within intron 9 of Brca1 genomic sequences. Gene targeting of the S1Rhgy fragment to the Brca1 locus in the Brca1−/− cells can restore wild-type Brca1 to one allele to create a Brca1−/+ allele. B, Southern blot analysis of hygR clones after transfection with the S1Rhgy fragment. Genomic DNA from 159 hygR clones was digested with EcoRV and hybridized to a radio-labeled 3′ Brca1 probe as indicated in A. Two Brca1−/+ alleles, E4 and H7, were identified which contained the 11.3-kb S1Rhgy Brca1 fragment, indicative of correction of the Brca1− neo allele with the Brca1−/+ allele.

**Fig. 5.** Expression of Brca1 is restored in Brca1−/+ alleles. Northern blot analysis was performed with 10 μg of total RNA obtained from Brca1−/−, Brca1−/+R, and Brca1−/+ cells. Endogenous Brca1 expression results in a 7.2-kb mRNA, which is observed in the Brca1 heterozygote C6 clone and the two Brca1−/+R corrected E4 and H7 clones. The Brca1−/− H10 clone is a hygR clone that was obtained from the S1Rhgy gene-targeting experiment in which the fragment had integrated randomly into the genome and therefore did not correct the Brca1 mutation. This cell line does not express the 7.2-kb Brca1 mRNA, but it does express the 3.9-kb Brca1 exon 10–12 splice variant. Quantitation of mRNA by Phospho-image analysis indicated that the gene-corrected and heterozygote cells expressed wild-type Brca1 at similar levels. The slightly slower mobility for the H7 Brca1 mRNA was not seen consistently. A Brca1 probe containing the sequence from exons 9–11 was used for detection. The blot was rehybridized with a β-actin probe to confirm equal RNA loading.
RESTORATION OF REPAIR, RESISTANCE, AND STABILITY IN BRCA1

Fig. 6. Mitomycin-C resistance is restored with correction of the Brca1 mutation. In addition to the Brca1+/+, Brca1+/−, Brca1−/−, and Brca1−/−, 785+ cell lines, the two corrected Brca1+/+ cells were treated with mitomycin-C at various doses. Clonogenic survival assays were performed as described in the legend to Fig. 3. Mitomycin-C resistance is restored to near wild-type levels in the gene-corrected E4 and H7 Brca1−/−, 785+ cell lines. Depicted are the various Brca1 genotypes.

10–12 variant mRNA was observed in the H10 clone, similar to the parental Brca1−/− cells (Fig. 5).

To determine whether restoration of Brca1 expression in the Brca1−/−, 785+ cells led to normal levels of mitomycin-C resistance, the E4 and H7 clones were analyzed in clonogenic survival assays together with the control cells. Unlike the transgene complemented lines, restoration was nearly complete in both of the gene-corrected E4 and H7 Brca1−/−, 785+ cell lines. Depicted are the various Brca1 genotypes.

Table 2 Spontaneous gross chromosomal aberrations in mouse ES cell lines with different Brca1 genotypes

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* Int del, interstitial deletion; ter del, terminal deletion; f, fragment; trans, translocation.
* Repetitive aberration (number of repetitive aberrations/number of metaphases with repetitive aberrations).
* Random integrant derived from gene correction experiment.

DISCUSSION

These results provide direct evidence that cells containing a Brca1 mutation have impaired homologous recombination, as measured by we examined metaphase spreads of ES cell lines carrying different Brca1 genotypes to determine whether these cells have chromosomal abnormalities. Unlike Brca1-deficient MEFs, Brca1−/− ES cells did not exhibit pronounced aneuploidy (data not shown). The average chromosome number in the Brca1−/− cells was 40, which was not significantly different from the wild-type and Brca1+/− cell lines. However, chromosome instability was evident in the Brca1-deficient cells, as characterized by an increase in the number of chromosome aberrations (Table 2). The control cell lines expressing wild-type Brca1, i.e., Brca1+/+, Brca1+/−, and corrected Brca1−/−, 785+ cell lines, each had a similar low frequency of observed chromosome aberrations, ~3–4% of metaphases. By contrast, the Brca1 mutant cells had a substantially increased number, with ~20% of metaphases containing aberrations. Spontaneous chromosomal instability is significantly higher (P = 0.0079) in Brca1−/− cells as compared with Brca1+/+, Brca1+/−, and Brca1−/−, 785+ cells. The increase was attributable to a number of different chromosomal abnormalities, including chromatid breaks and exchanges and chromosome breaks, deletions, and translocations. As seen with other assays, the Brca1−/−, 785+ cells fell between mutant and wild-type cells. They had ~2-fold fewer aberrations than Brca1−/− cells (P = 0.043), but 2–3-fold more aberrations than wild-type cells, in accordance with partial correction of the Brca1 defect in these cells. This contrasted with the gene-targeted E4 and H7 clones that were fully corrected.

It is plausible that chromosome aneuploidy and multiple aberrations within a cell are better tolerated in a differentiated cell type and extended further when cell checkpoints are eliminated. However, when cells responsible for self-renewal incur chromosome insults, such as the ES cell, it is possible that cell death pathways predominate to limit the propagation of this instability. Although increased cell death was not formally evaluated in this study, a striking small-colony morphology was typically exhibited in the Brca1−/− cell lines as compared with the cell lines that contained a wild-type Brca1 allele (data not shown). This small-colony morphology was eliminated by correction in the Brca1−/−, 785+ cell lines (data not shown). Cell cycle distribution was evaluated previously (2, 17) and was found to be similar between the Brca1−/− and Brca1+/− cell lines and therefore was not likely to be a factor in the etiology of small colonies.

Table 2 Spontaneous gross chromosomal aberrations in mouse ES cell lines with different Brca1 genotypes

<table>
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<tr>
<th>Brca1 genotype and cell lines</th>
<th>Metaphases analyzed</th>
<th>Metaphases with aberrations (%)</th>
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gene targeting and, importantly, HDR of a chromosome break. Moreover, *Brca1* deficiency results in mitomycin-C sensitivity, which is more profound than the previously published IR and cisplatin sensitivities (1, 17). A substantial increase in gross chromosomal aberrations was also observed, although aneuploidy was not evident. These latter defects of cross-linking agent sensitivity and genomic instability are fully attributable to *Brca1* deficiency, and it is probable that the HDR defect in this cell line is also completely attributable to *Brca1* deficiency.

Mammalian cell lines identified to have substantially impaired HDR defects, *i.e.*, the *XRCC2* and *XRCC3* mutants, were originally characterized on the basis of their weak IR sensitivity but substantial mitomycin-C sensitivity and a high frequency of both spontaneous and induced chromosomal aberrations (25, 29). The repair phenotypes arising from *Brca1* mutation described in this report are similar to those found in classical HDR mutants, and recent results suggest that similar phenotypes arise from *Brca2* mutation (30). These phenotypes may define the paradigm for HDR mutants and provide a contrast to NHEJ mutants, for which severe IR sensitivity and little or no cross-linking agent sensitivity is observed (26, 31). Induced chromosomal aberrations are also frequent in NHEJ mutants, although spontaneous instability is not uniformly observed in cells with reduced NHEJ (32–35). An additional emerging characteristic that may be attributable to defects in HDR is that of centrosome abnormalities, which have been observed in *XRCC2, XRCC3, BRCA1,* and *BRCA2* mutant cells, in contrast with the lack of abnormalities in cells defective for NHEJ (27, 34, 36, 37). Thus, a proportion of gross chromosome abnormalities may result from chromosome missegregation. Although aneuploidy was not evident in *Brca1*-deficient ES cells, it is commonly observed in other HDR mutant cells.

Gross chromosomal instability is observed in MEFs and tumor cells derived from mice harboring *Brca1* hypomorphic alleles (11, 27, 28). However, as shown here, ES cells harboring similar hypomorphic *Brca1* alleles have chromosome instability that is much less pronounced, suggesting that MEFs (as well as tumors) may be inherently prone to accumulate damage. The ES cell ultimately is required to generate all tissues of the adult organism and therefore may be exquisitely sensitive to a very low threshold of genetic change. As the cell cycle distribution of the mutant ES cells is normal, increased cell death may eliminate cells with unrepaired damage. Recently it has been shown that although p53 accumulates during stalled DNA replication, it is functionally impaired, suggesting repression of the p53 response during S-phase arrest (38). One possibility for the lack of aneuploidy is that p53 function is less tightly regulated during replication blocks in ES cells, resulting in the elimination of cells when unrepaired chromosome breaks are encountered.

We found that correction of a mutant *Brca1* allele led to complete rescue of the repair phenotypes, yet transgene expression of *Brca1*, even from a *Brca1* promoter, led to only partial complementation. It remains uncertain why transgene rescue was incomplete; however, these results point to the difficulty of ectopic expression and suggest that a larger genomic region is necessary for proper regulation of *Brca1* gene expression. Previously, transgene expression was shown to be insufficient for even partial restoration of normal gene-targeting efficiency in the *Brca1* mutant cell line (16). It is possible that targeting at loci tested in this previous study had a more stringent requirement for normal *Brca1* levels than the *pim1* locus. Nevertheless, *Brca1* transgene expression led to a robust 10-fold improvement in *pim1* targeting, which was confirmed by molecular analysis.

We expect that HDR of a chromosomal DSB is more physiologically relevant than gene targeting. In HDR assays, *Brca1* transgene expression led to increased recombination, improving it to an intermediate level as seen for the other repair phenotypes. DSBs arising from exogenous sources like I-Sce I are potent inducers of recombination between sister-chromatids, which are templates for repair after DNA replication (39). DSBs may also arise during normal S-phase progression in mammalian cells from replication fork disruption, such that HDR is required to restart the fork for a proper completion of replication as in *E. coli* (40). Consistent with a similar role in mammalian cells is the colocalization of BRCA1 with PCNA after hydroxyurea treatment of cells (13).

Mice and cells harboring a *Brca1* mutation that deletes exon 11 are considered to be hypomorphic but not null for *Brca1*, because null alleles cause early embryonic lethality, whereas mice with an exon 11 deletion can survive at low frequency on a p53 background (41). These mice have underdeveloped mammary glands, as do mice with an exon 11 deletion specifically targeted to breast tissue, consistent with impaired cellular proliferation (28, 41). It is possible that extended viability results from residual HDR, because, even if diminished, we find that HDR is not completely abrogated by the exon 11 deletion. Although the role of nuclear foci is uncertain, Rad51 foci are also not completely abrogated in this cell line, consistent with residual HDR (17).

Spliced isoforms of *BRCA1* found *in vivo* include *BRCA1*Δ11b and *BRCA1*Δ672–4095 (42, 43). Some lack the nuclear localization signals that are present in the 5′ end of exon 11 and are predominantly located in the cytoplasm (42, 43). The cytoplasmic localization of these products would seem to be predictive of a HDR defect, as improper cellular localization of BRCA2 and, surprisingly, Rad51 has been implicated as the mechanism for defective HDR observed in cells with a *BRCA2* mutation (44). The exon 11 splice variants would still be expected to interact with other proteins, such as BARD1 or other BRCA1 RING-interacting proteins and the BRCT interacting proteins, as these interactions involve NH2- and COOH-terminal sequences, respectively, rather than the central exon 11 encoded sequences (45). Nevertheless, functions arising from these interactions may be compromised through sequestration of these interacting proteins in the cytoplasm. The recently described BRCA1 nuclear export sequence suggests that this protein does not reside solely in the nucleus but may shuttle between the cytoplasm and nucleus (46). However, a direct functional analysis of these endogenous spliced products has not been performed.

Heterozygosity for a *BRCA1* mutation in humans results in a predisposition to early-onset breast and ovarian cancer. This predisposition is incurred when the remaining wild-type allele is lost or mutated, as is the case for classical tumor suppressor genes. A phenotype for the heterozygote state has been inferred for some tumor suppressor gene mutations through a dominant-negative phenotype (47, 48). Evidence for loss of tumor suppression solely from haploinsufficiency is less well documented (49, 50). A heterozygote phenotype for viability or tumorigenesis has not been reported in mice with targeted *Brca1* alleles, and thus far human tumors derived from *BRCA1* mutation carriers consistently reveal a loss of the wild-type allele. However, subtle changes in ovary and breast morphology have been described in mice heterozygous for either *Brca1* or *Brca2* mutations (51). Furthermore, it has been reported that cells from *BRCA1* and *BRCA2* mutation carriers are more radiosensitive than cells from wild-type individuals (24). In our analysis of the *Brca1*−/− cells, there was no decrease in the ability to gene target, repair a DSB by gene conversion, or maintain genetic integrity or mitomycin-C resistance. However, only partial complementation of repair defects was found with low transgene expression, which measured 10–20% of wild-type *Brca1* expression. Therefore, the possibility remains that a significant reduction in the expression of *BRCA1* or the presence of dominant-negative
truncated alleles may impart functional consequences that predispose to tumorigenesis, especially under conditions of increased DNA damage or increased proliferation.

We have found that Brca1<sup>-/-</sup> cells exhibit nearly 100-fold sensitivity as compared with the Brca1<sup>+/+</sup> cells after treatment with mitomycin-C at the LD<sub>50</sub> for wild-type cells. Recently, it was shown that in Saccharomyces cerevisiae, the pathways used in the repair of cisplatin-induced interstrand cross-links were cell cycle dependent.

The intermediate in the repair of the interstrand cross-links in dividing cells is a DSB that is repaired by homologous recombination (52). Agents that produce DNA interstrand cross-links are some of the most effective antitumor agents in clinical use, although their use is often limited by the toxicity incurred in normal cells. If the preference for HDR in cycling mammalian cells is preserved, it suggests that the exquisite sensitivity of Brca1-deficient cells to interstrand cross-linking agents may provide an extremely favorable therapeutic ratio.

Recombination proteins may provide an extremely favorable therapeutic ratio in HDR in cycling mammalian cells is preserved, it suggests that the cells is a DSB that is repaired by homologous recombination (52).

ACKNOWLEDGMENTS

We thank Dr. Beverly Koller (University of North Carolina, Chapel Hill, Chapel Hill, NC) for providing the BRCA1<sup>-/-</sup> 256.4 ES and BRCA1<sup>+/+</sup> Igt cell lines.

REFERENCES

Homology-directed DNA Repair, Mitomycin-C Resistance, and Chromosome Stability Is Restored with Correction of a Brca1 Mutation

Mary Ellen Moynahan, Tracy Y. Cui and Maria Jasin