

BRCA1 Regulates RAD51 Function in Response to DNA Damage and Suppresses Spontaneous Sister Chromatid Replication Slippage: Implications for Sister Chromatid Cohesion, Genome Stability, and Carcinogenesis

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Abstract

The breast/ovarian cancer susceptibility proteins BRCA1 and BRCA2 maintain genome stability, at least in part, through a functional role in DNA damage repair. They both colocalize with RAD51 at sites of DNA damage/replication and activate RAD51-mediated homologous recombination repair of DNA double-strand breaks (DSB). Whereas BRCA2 interacts directly with and regulates RAD51, the role of BRCA1 in this process is unclear. However, BRCA1 may regulate RAD51 in response to DNA damage or through its ability to interact with and regulate MRE11/RAD50/NBS1 (MRN) during the processing of DSBs into single-strand DNA (ssDNA) ends, prerequisite substrates for RAD51, or both. To test these hypotheses, we measured the effect of BRCA1 on the competition between RAD51-mediated homologous recombination (gene conversion and crossover) versus RAD51-independent homologous recombination [single-strand annealing (SSA)] for ssDNA at a site-specific chromosomal DSB within a DNA repeat, a substrate for both homologous recombination pathways. Expression of wild-type BRCA1 in BRCA1-deficient human recombination reporter cell lines promoted both gene conversion and SSA but greatly enhanced gene conversion. In addition, BRCA1 also suppressed both spontaneous gene conversion and deletion events, which can arise from either crossover or sister chromatid replication slippage (SCRS), a RAD51-independent process. BRCA1 does not seem to block crossover. From these results, we conclude that (a) BRCA1 regulates RAD51 function in response to the type of DNA damage and (b) BRCA1 suppresses SCRS, suggesting a role for this protein in sister chromatid cohesion/alignment. Loss of such control in response to estrogen-induced DNA damage after BRCA1 inactivation may be a key initial event that triggers genome instability and carcinogenesis. (Cancer Res 2005; 65(24): 11384-91)

Introduction

Germ line mutations in the *BRCA1* and *BRCA2* genes predispose humans to breast and ovarian cancers (1). Tumors derived from predisposed individuals show loss of heterozygosity for the wild-

type allele with retention of the inherited, cancer-eliciting, mutant allele, indicating that *BRCA1* and *BRCA2* act as tumor suppressor genes. *BRCA1* and *BRCA2* genes encode large, unrelated nuclear proteins that act as regulators of DNA repair, transcription, and the cell cycle in response to DNA damage, although their precise role in these processes is unclear and the mechanisms underlying tissue specificity of their tumor-suppressive property remain speculative (2–4).

Common to BRCA1 and BRCA2 is the activation of sister chromatid recombination, a homologous recombination process in which a chromatid that suffers a double-strand break (DSB) during replication uses neighboring, undamaged sister chromatid as a template for repair (2, 3). Initial evidence of such a role derives from the observation that BRCA1 and BRCA2 both relocate with the essential component of homologous recombination, RAD51, to sites of DNA damage and replication forks marked by proliferating cell nuclear antigen (2). In addition, BRCA1- and BRCA2-deficient cells exhibit a deficit in RAD51-mediated homologous recombination repair of site-specific chromosomal DSBs and hypersensitivity to DNA-damaging agents that induce DSBs (5). Moreover, replication forks stalled at DNA damage collapse into DSBs in BRCA2-deficient cells (6). Convincingly, BRCA1- and BRCA2-deficient mouse or human cells sustain spontaneous chromatid-type aberrations, including chromosome and chromatid breaks as well as triradial and quadriradial chromosomes, markers of aberrant mitotic S-phase recombination that are typical of the human cancer predisposition diseases Bloom's syndrome, ataxia telangiectasia, and Fanconi's anemia mutated in *BLM*, *ATM*, and *FANC* genes, respectively (3, 7).

Whereas in this ménage à deux BRCA2 regulates both intracellular localization and function of RAD51 (8–10), the role of BRCA1 is unclear, however. RAD51, the equivalent of bacterial RecA, is functional as a polymer, made up of hundreds of monomers that coat single-strand DNA (ssDNA) and form a nucleoprotein filament that invades and pairs with an intact homologous DNA duplex (11). This DNA strand exchange reaction enables the invading 3' ssDNA end to prime new DNA synthesis, leading to the formation of a homologous recombination intermediate that can either disassemble (i.e., the newly synthesized strand can be displaced and anneal with the noninvading 3' ssDNA end to elicit noncrossover gene conversion only) or be processed to a Holliday junction intermediate to yield gene conversion with or without crossover (12–15). In mitotic cells, homologous recombination can occur almost exclusively by gene conversion and is considered to be error free when it involves perfectly aligned sister chromatids (16). However, homologous recombination can also be deleterious when it takes place between repetitive sequences, and in excess, it

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can promote genome instability and cause genetic diseases and cancer (7, 12, 17).

BRCA2 directly binds RAD51 and controls the assembly and disassembly of RAD51 nucleoprotein filament (8). BRCA1 might not directly regulate RAD51, because interaction between BRCA1 and RAD51 is indirect and stoichiometrically negligible (18). However, BRCA1 may regulate RAD51 function in response to DNA damage or through its interaction with other proteins involved in homologous recombination. In response to replication fork arrest or DNA damage, BRCA1 is bound and hyperphosphorylated by ATM and ATM-related kinase ATR (4). However, whether or how these modifications of BRCA1 affect RAD51 function remains obscure. BRCA1 also interacts with RAD50 and its partners MRE11 and NBS1, and in response to DNA damage, BRCA1 and MRN complex colocalizes with other homologous recombination proteins at sites of DNA damage and replication (19, 20). BRCA1 apparently functions as a regulator of the MRN complex (21). The MRE11 subunit possesses nuclease activity, which resects flush ends of DSBs to generate ssDNA tracts, and BRCA1 directly binds DNA and inhibits this MRE11 activity presumably to regulate the length and the persistence of ssDNA generation at sites of DNA damage (22). Because ssDNA is a substrate for RAD51, BRCA1 might play an essential role in RAD51 recombination by modulating MRE11 activity. The MRN complex also functions in nonhomologous end joining (NHEJ) of DSB ends (23). Consistent with this, BRCA1-deficient cells exhibit a deficit in both gene conversion repair (24, 25) and microhomology-mediated NHEJ repair of site-specific chromosomal DSBs (26).

Given that ssDNA is also a substrate for single-strand annealing (SSA), a RAD51-independent, nonconservative homologous recombination pathway that repairs DSBs within DNA repeats (12, 13), we reasoned that by measuring the effect of BRCA1 on the competition between gene conversion and SSA for ssDNA we could gain insight into the role of BRCA1 in the RAD51 pathway. The premise is that a role of BRCA1 only in the processing of DSB ends would presumably affect gene conversion and SSA equally, whereas an additional role of BRCA1 in activating RAD51 would favor gene conversion over SSA as has been reported with BRCA2 (9, 27, 28). To discriminate between these two possibilities, we employed a well-characterized homologous recombination assay system that can monitor distinct homologous recombination pathways in a BRCA1-deficient background (29). Recently, by using this assay system, we found that BRCA2 not only activates RAD51 pathway following chromosomal DSBs but also suppresses it in the absence of such DNA damage, providing evidence of a dual role for this protein in regulating RAD51 function in response to the type of DNA damage (9). In addition, BRCA2 seemed to suppress sister chromatid replication slippage (SCRS), suggesting a role for this protein in sister chromatid cohesion/alignment (9).

Here, we report that in this assay system BRCA1 acts like BRCA2, supporting the notion that both proteins work in concert to protect against estrogen-induced DNA damage and maintain both chromosome structure and number.

Materials and Methods

DNA manipulations, cell lines, culture, and DNA transfections. The recombination reporter plasmid pCAdir was described previously (Fig. 1; refs. 9, 29). The human breast cancer cell lines MCF-7 and HCC1937 originated from the American Type Culture Collection (Manassas, VA). To generate the parental HCC1937 cell lines d55 and d56, cells were electro-

porated at 250 V/960 μ F with 5 μ g linear pCAdir (9, 29). The electroporated cells were subjected to hygromycin (Hyg) selection (75 μ g/mL) 48 hours later, and several Hyg-resistant (Hyg^R) cell clones were picked and amplified individually for PCR and Southern blot analyses to identify intact, single-copy integration events. Single-copy integration events were deemed necessary not only to facilitate analysis of recombination products but also to prevent silencing of the homologous recombination reporter gene by the adjacent transcription units (30).

The parental cell lines were stably transfected with a pCR3-*Neo*-based expression vector containing wild-type *BRCA1* (31) or with an empty control vector by using the Effectene Transfection Reagent kit (Qiagen, Valencia, CA). Forty-eight hours later, the transfected cells were cultured in medium containing G418 (450 μ g/mL). Neomycin-resistant (G418^R) cell clones were picked and amplified individually for qualitative *BRCA1* expression analysis by reverse transcription-PCR (RT-PCR) and Western blotting.

Reverse transcription-PCR. RNA from individual cell clones was extracted with Trizol reagent and transcribed to cDNA using reverse transcriptase and primers (hexamers) of the SuperScript First-Strand Synthesis System RT-PCR kit (Invitrogen, Carlsbad, CA). The primer pairs employed for PCR (pCR3-forward 5'-CGACTCACTATAGGGAG-3' and *BRCA1*-reverse 5'-ATACCTTTGGATGATAGA-3') amplify the 377-bp fragment of wild-type *BRCA1* cDNA only, whereas 18S-forward (5'-TGAGGCCATGATTAAGAGGG-3') and 18S-reverse (5'-CGCTGAGCCAGTTCAGTGAG-3') amplify a 642-bp fragment of 18S cDNA of rRNA. PCR amplifications were undertaken with the Expand High-Fidelity PCR System kit (Boehringer Mannheim, Indianapolis, IN) for 30 cycles (32).

Protein manipulations. Proteins were extracted and quantified as described previously (9, 29). Protein extract (50 μ g) was separated on 5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ), and probed with mouse anti-*BRCA1* antibody (Ab1, Oncogene, Boston, MA), which recognizes wild-type *BRCA1* and the truncated *BRCA1* protein.

Homologous recombination frequency and rate. For spontaneous homologous recombination, 1×10^6 to 5×10^6 cells were subjected to puromycin (Puro; 0.5 μ g/mL) selection 24 hours after plating. Homologous recombination frequency was assessed by dividing the number of Puro-resistant (Puro^R) colonies by the number of cells plated for selection. Because homologous recombination between direct repeats can delete the *Hyg* gene, the cells were maintained for 1 to 2 weeks without Hyg before Puro selection.

The homologous recombination rate was determined in two independent experiments as described previously (9, 29). Briefly, 10 or 12 independent cultures (1-100 cells) of either parental d55 or its *BRCA1*-expressing derivative, the d55/*BRCA1*-4 cell line, were plated in parallel and cultured to confluence. Cells were trypsinized and counted, and a portion was taken for plating efficiency estimation. The remaining cells were plated under Puro selection, and the resulting Puro^R colonies were used to calculate the frequency of homologous recombination. The homologous recombination rate was calculated from these frequencies by a fluctuation test.

For I-*SceI*-induced homologous recombination experiments, the vectors pBactin-*SceI* and pFRED25, which express the meganuclease I-*SceI* and green fluorescent protein (GFP) respectively, were cotransfected into 1×10^5 to 4×10^5 cells with the Eugene 6 reagent kit (Boehringer Mannheim). In parallel experiments, the same number of cells was transfected with pFRED25 and pMC1neo, the latter to correct for DNA content as a control for I-*SceI* efficiency (spontaneous homologous recombination). GFP-expressing cells were counted 48 hours after transfection to examine the efficiency of transfection and normalize the homologous recombination frequencies. Puro selection was done 10 days after transfection. The frequency of I-*SceI*-induced homologous recombination was calculated by dividing the number of Puro^R colonies by the number of GFP-expressing cells (9).

PCR analysis of puromycin-resistant colonies. Genomic DNA from individual Puro^R cell clones was extracted and 400 ng were subjected to PCR as described previously (32). The primer pairs employed in PCR

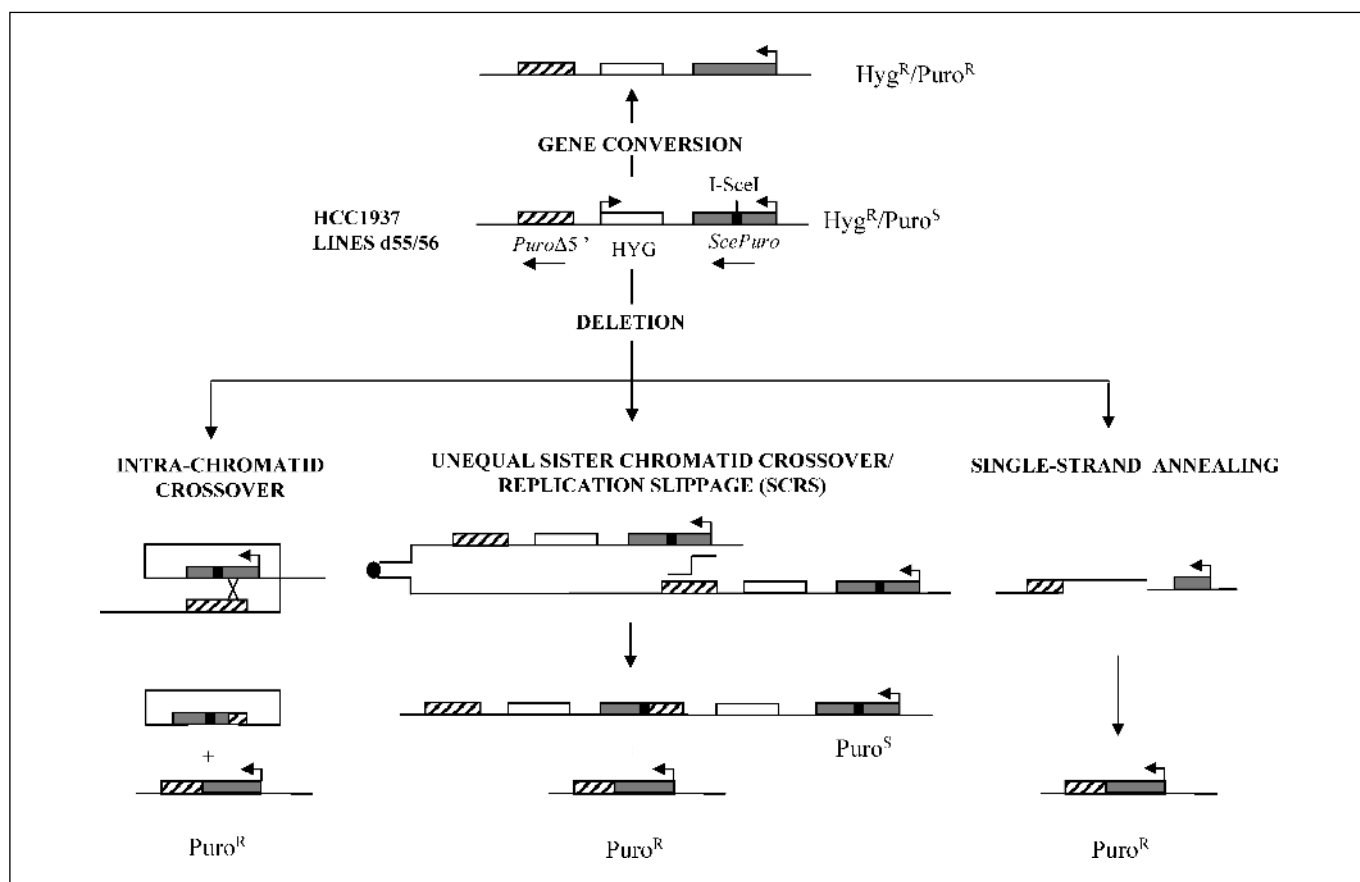


Figure 1. Chromosomal homologous recombination between direct repeats. The HCC1937 cell lines d55 and d56 carry, in their genome, a direct repeat of two inactive *Puro* genes separated by the *Hyg* gene. They are Hyg^R but *Puro* sensitive (Puro^S). A spontaneous or a DSB-induced homologous recombination event reconstitutes a functional *Puro* gene through loss of the *I-SceI* site and gain of the *EagI/BssHIII* sites, restoring resistance to *Puro* (Puro^R). Because the *I-SceI* insertion mutation in *ScePuro* entails deletion of the *EagI* and *BssHIII* sites, only homologous recombination events between the two *Puro* cassettes restore a functional *ScePuro* gene. A gene conversion event restores one functional *Puro* gene without affecting the overall structure of the locus. A deletion event, which can result from either crossover, SSA, or SCRS, removes one copy of the *Puro* cassettes together with the intervening *Hyg* gene.

(Hyg4419 5'-GCTGTGTAGAAGTACTCGCCG-3' and pUC469 5'-TGACCAT-GATTACGCCAAGCT-3') amplify a 2,324-bp gene conversion fragment, whereas pUC315 (5'-AAAGGGGGATGTGCTGCAAGGCGA-3') and pUC469 amplify the 1,863-bp deletion fragment (9).

Results

Stable expression of wild-type BRCA1 in HCC1937 recombination reporter cell lines. The human breast carcinoma cell line HCC1937, which derives from a germ line *BRCA1* mutation carrier, shows decreased DSB repair efficiency that can be partially restored by expressing an exogenous wild-type *BRCA1* allele (25, 33, 34) but not mutant, cancer-eliciting *BRCA1* alleles (34). HCC1937 cells synthesize a truncated BRCA1 protein, the product of a cancer-eliciting mutant allele (*5382insC*), at a very low level, but no wild-type protein (18, 25).

To study the effect of the absence of intact BRCA1 on RAD51-mediated homologous recombination, we expressed wild-type BRCA1 in two HCC1937 recombination reporter cell lines, d55 and d56 (Fig. 1). These were transfected with pCR3-*BRCA1* or pCR3 (31), a G418^R-borne vector encoding or not wild-type BRCA1 protein. G418^R cell clones were amplified in parallel and subjected to qualitative analyses for *BRCA1* expression at both mRNA and protein levels (Fig. 2A and B). The different BRCA1-expressing derivative lines, listed in Fig. 2 and Table 1, manifested no major

changes in growth rate or plating efficiency relative to G418^R, parent lines transfected with the empty vector (refs. 24, 25, 34; data not shown). The BRCA1 expression level in complemented cells seemed to be lower than in MCF-7 cells (Fig. 2B; data not shown).

BRCA1 decreases spontaneous homologous recombination.

The HCC1937 reporter cell lines carry, in their genome, a single, intact copy of a homologous recombination construct, pCAdir, containing a direct repeat of two inactive *Puro*^R genes separated by the *Hyg*^R gene (Fig. 1). Deleting *EagI* and *BssHIII* restriction sites and inserting one *I-SceI* cleavage site inactivated the full-length *Puro* gene, whereas the wild-type *Puro* gene contained an inactivating 5' deletion. A homologous recombination event between the two *Puro* cassettes would reconstitute a functional *Puro* gene through loss of the *I-SceI* and gain of the wild-type *EagI/BssHIII* sites, restoring resistance to the drug *Puro* in a colony assay (9, 29).

First, we determined whether spontaneous homologous recombination (i.e., without treatment of cells with exogenous DNA-damaging agents) is affected by BRCA1. Single G418^R/*Hyg*^R HCC1937 cell clones and BRCA1-expressing derivatives, listed in Table 1, were grown in parallel and subjected to *Puro* selection 2 to 3 weeks later. *Puro*^R colonies were counted to assess the frequency of spontaneous homologous recombination as the number of

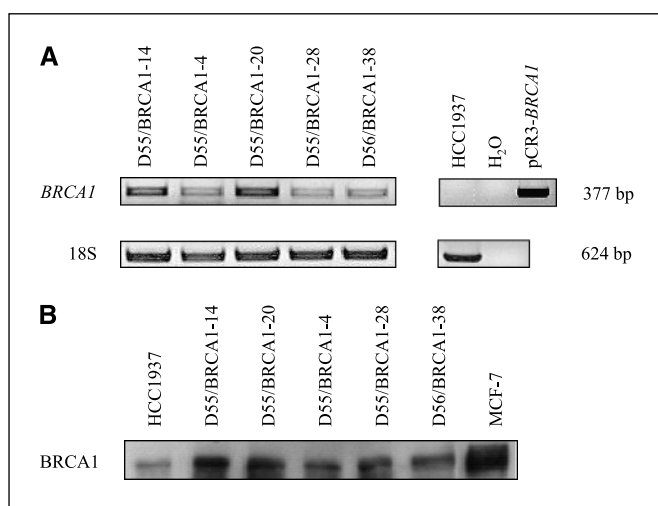


Figure 2. Stable expression of BRCA1 in HCC1937 recombination reporter lines. **A**, qualitative RT-PCR was carried out with a primer pair that is specific for wild-type *BRCA1* mRNA to amplify a 377-bp fragment in the expressing cell lines, whereas a primer pair specific for rRNA chosen as a control for RT-PCR amplified a 624-bp fragment in both BRCA1-expressing and nonexpressing cell lines. DNA of pCR3-*BRCA1* was used as a positive control, whereas a G148^R-HCC1937 cell line and water were employed as negative controls. **B**, Western blot analysis of protein extracts with an antibody directed against the NH₂ terminus of full-length and truncated BRCA1 proteins.

Puro^R colonies divided by the number of cells plated 24 hours before selection. BRCA1-expressing cell clones exhibited 4- to 7-fold frequency decreases relative to their parents (Table 1). We also ascertained the homologous recombination rate (homologous recombination events/cell/generation) of the parental clone d55 ($1.41 \pm 0.34 \times 10^{-6}$) and its BRCA1-expressing derivative, the d55/BRCA1-4 cell clone ($2.90 \pm 0.62 \times 10^{-7}$). BRCA1 diminished the rate by 5-fold. These results reveal that HCC1937 cells exhibit a spontaneous hyperrecombination phenotype as BRCA2-deficient cells (9, 27).

BRCA1 decreases both spontaneous gene conversion and deletion events without affecting the processing of recombination intermediates. The use of direct DNA repeats as homologous recombination substrates provides a measure of both intrachromatid and unequal sister chromatid interactions that can result in two types of products: gene conversion and deletion (Fig. 1; refs. 9, 13 and references therein). Gene conversion associated or not with crossover depends on RAD51. Gene conversion keeps the structure of a locus intact and thus can arise from either intrachromatid or unequal intersister chromatid interactions. Whereas an intrachromatid crossover event leads to the deletion of 1 DNA repeat and the intervening sequences as a circle (Fig. 1), an unequal sister chromatid crossover culminates in one chromatid with deletion and the sister chromatid with triplication of DNA repeat and duplication of the intervening sequences (Fig. 1). However, such unequal sister chromatid exchange events can also arise spontaneously from SCRS, a RAD51-independent process (Fig. 1).

To determine which homologous recombination pathway was more affected by BRCA1, we analyzed independent, spontaneous homologous recombination events that derived from several independent cultures by plating cells at a lower density. A single Puro^R colony was picked from each culture and analyzed by PCR (Fig. 3). Of 47 spontaneous homologous recombination events, 13

(28%) represented gene conversion and the remaining 34 (72%) represented deletion events (Table 2). Such a predominance of spontaneous deletion events over gene conversion events in the absence of a functional BRCA1 could be due to either increased crossover events or SCRS events or both. Although there is yet no evidence of a role of BRCA1 in crossover suppression, BRCA1 shows high affinity for branched DNA structures (22) and interacts with the DNA helicase BLM (20) that acts on Holliday junctions to suppress crossover and promote gene conversion (35). If BRCA1 were to act during this function of BLM, the proportion of spontaneous deletion events would be expected to decrease and that of gene conversion events to increase in the presence of wild-type BRCA1. However, no apparent difference in the proportion of gene conversion and deletion events was observed between parental cell clones and BRCA1-expressing derivatives (Table 2). It remains possible that BRCA1 will suppress a yet unidentified homologous recombination pathway that leads exclusively to crossover events. Whereas current models of homologous recombination invoke that gene conversion and crossover can arise from the processing of the same Holliday junction intermediate (12, 13, 36), there is evidence that gene conversion and crossover can be regulated differently during meiosis in the yeast *Saccharomyces cerevisiae* (37). Whether similar mechanisms also operate in mammals is not known, but BRCA1 has been shown to promote rather than suppress crossover during spermatogenesis in mice (38). These data argue against a role of BRCA1 in crossover suppression. Therefore, we conclude that BRCA1 expression in HCC1937 cells decreased both spontaneous RAD51-mediated homologous recombination and RAD51-independent SCRS events.

BRCA1 promotes homologous recombination repair of chromosomal double-strand breaks. Direct evidence that BRCA1 promotes homologous recombination repair of

Table 1. Effect of BRCA1 on spontaneous homologous recombination

Cell lines	Homologous recombination frequency*	Ratio [†]
d55	$1.0 \pm 0.33 \times 10^{-5}$	
d55/BRCA1-4	$1.5 \pm 0.71 \times 10^{-6}$	5.4
d55/BRCA1-14	$1.4 \pm 0.28 \times 10^{-6}$	7.4
d55/BRCA1-20	$1.9 \pm 0.13 \times 10^{-6}$	6.9
d55/BRCA1-21	$2.2 \pm 0.71 \times 10^{-6}$	4.6
d55/BRCA1-28	$2.3 \pm 0.14 \times 10^{-6}$	4.4
d56	$3.5 \pm 1.1 \times 10^{-6}$	
d56/BRCA1-20	$7.0 \pm 1.41 \times 10^{-7}$	5.0
d56/BRCA1-24	$5.0 \pm 3.54 \times 10^{-7}$	7.0
d56/BRCA1-38	$5.0 \pm 0.07 \times 10^{-7}$	7.0

*Homologous recombination frequency represents the mean of three independent experiments for each cell line and is determined from the number of Puro^R cell clones relative to the total number of viable cells plated 24 hours before selection. Parent and BRCA1-expressing G418^R derivative lines were plated in parallel.

[†]Ratio (parent/BRCA1-expressing derivative lines) indicates homologous recombination fold decrease in BRCA1-expressing cells relative to their parents.

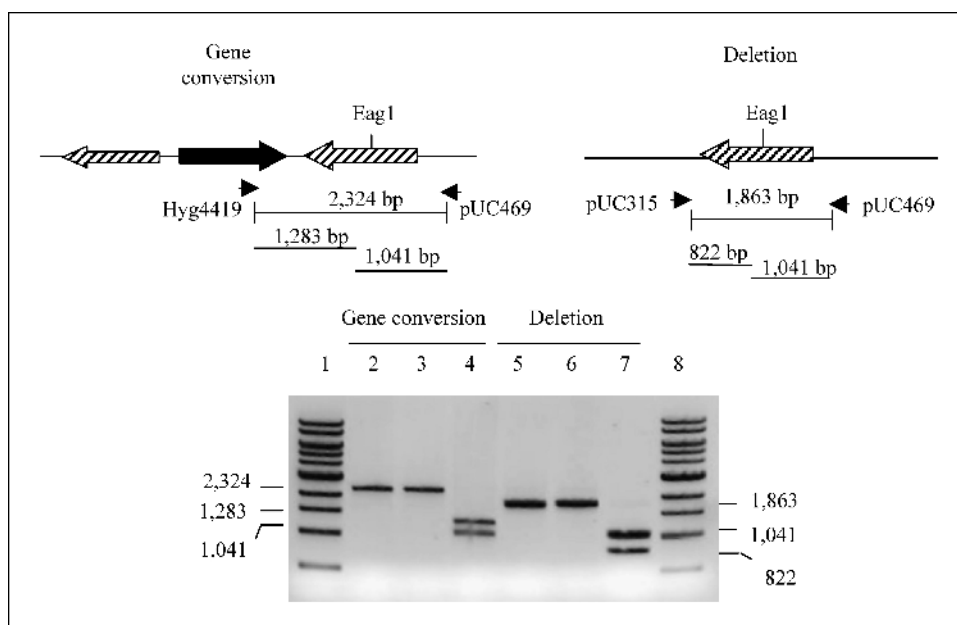


Figure 3. PCR analysis of homologous recombination products. The primer pair Hyg4419/pUC469 amplifies a gene conversion fragment of 2,324 bp (lane 2), which is resistant to I-SceI (lane 3) but can be cut with *EagI* into 1,283- and 1,041-bp fragments (lane 4). The primer pair pUC315 and pUC469 amplifies a deletion (crossover/SSA/SCRS) fragment (lane 5), which is resistant to I-SceI (lane 6) but can be cut with *EagI* into 822- and 1,041-bp fragments (lane 7). Lanes 1 and 8, a 1-kbp ladder.

chromosomal DSBs was obtained by using the *I-SceI* system (24, 25, 39). To analyze the effect of BRCA1 on chromosomal DSB repair, the parental lines and their BRCA1-expressing derivatives were transiently cotransfected with p β actin-*SceI* vector, which expresses *I-SceI*, and a GFP-expressing vector was employed to evaluate transfection efficiency. When the *I-SceI* site in the full-length *Puro* gene is cleaved, homologous recombination would repair the DSB (9). No difference in cell viability following transfection was noted between BRCA1-expressing and non-expressing cell lines (refs. 24, 25, 34, 39; data not shown). Cotransfected cells were maintained in Hyg-free medium, and *Puro* was added to the medium 10 days later. *I-SceI* expression increased the frequency of *Puro*^R colony formation in all cell lines, but BRCA1-expressing lines exhibited 4- to 9-fold increments relative to their parents (Table 3). This is similar to the 5-fold increase in *I-SceI*-induced homologous recombination in mouse embryonic stem cells or HCC1937 cells complemented with wild-type BRCA1 (24, 25, 39).

BRCA1 activates RAD51 function. BRCA1 promotes gene conversion repair of *I-SceI*-induced chromosomal DSBs (24, 25), but its role in this RAD51-mediated process is unclear. Although BRCA1 colocalizes with both RAD51 and BRCA2 in S-phase nuclei and in cells treated with DNA-damaging agents, the majority of

BRCA1 does not physically associate with BRCA2 and RAD51 (18). However, BRCA1 interacts physically with the MRN complex (19, 20). Whereas MRN is involved in DSB ends processing into ssDNA at an early step in gene conversion repair, RAD51 is involved at a later step. Thus, BRCA1 may promote gene conversion repair by regulating MRN, RAD51, or both. Direct repeat assay systems can be used to discriminate between these possibilities. Following a DSB at direct repeats, gene conversion competes with SSA for ssDNA (13). SSA anneals complementary ssDNA ends flanking a DSB, culminating in the deletion of one DNA repeat and the intervening sequences (Fig. 1), and becomes highly efficient in cells that are deficient in RAD51 function (9, 13, 27, 28). Thus, a role of BRCA1 in RAD51-mediated homologous recombination solely in the processing of DSB ends would affect gene conversion and SSA equally, whereas a role for BRCA1 in activating RAD51 function would favor gene conversion over SSA.

Previous studies could not determine the contribution of BRCA1 in SSA and gene conversion in BRCA1-deficient backgrounds, as the homologous recombination assay systems employed monitored only deletion events (39) or only gene conversion events (24, 25). To determine which DSB repair pathway was more affected by BRCA1, we analyzed the structure of DSB repair products (*Puro*^R colonies) by PCR (Fig. 3). With the parental cell

Table 2. Effect of BRCA1 on homologous recombination pathways

Cell lines	Spontaneous homologous recombination		<i>I-SceI</i> homologous recombination	
	Gene conversion (%)	Crossover/SCRS (%)	Gene conversion (%)	Crossover/SSA (%)
d55/56 (BRCA1 ⁻)	13/47 (28)	34/47 (72)	12/37 (32)	25/37 (68)
d55/56 (BRCA1 ⁺)	5/20 (25)	15/20 (75)	39/75 (52)	36/75 (48)

NOTE: Gene conversion and crossover require RAD51, whereas SCRS and SSA do not (see text). The shift of *I-SceI*-induced crossover/SSA to gene conversion in BRCA1-expressing cells compared with parental cells is significant ($P = 0.039$).

Table 3. Effect of BRCA1 on I-SceI-induced homologous recombination

Cell lines	Homologous recombination frequency*		Ratio [†] of I-SceI/spontaneous
	Spontaneous homologous recombination	I-SceI homologous recombination	
d55	$1.0 \pm 0.14 \times 10^{-5}$	$8.2 \pm 1.13 \times 10^{-5}$ (8.2)	
d55/BRCA1-4	$3.0 \pm 0.21 \times 10^{-6}$	$1.7 \pm 0.50 \times 10^{-4}$ (56.7)	6.9
d55/BRCA1-14	$1.0 \pm 0.71 \times 10^{-6}$	$7.3 \pm 2.12 \times 10^{-5}$ (73.0)	8.9
d55/BRCA1-28	$2.0 \pm 0.57 \times 10^{-6}$	$1.3 \pm 0.03 \times 10^{-4}$ (65.0)	7.9
d56	$5.0 \pm 0.71 \times 10^{-6}$	$1.7 \pm 0.03 \times 10^{-4}$ (34)	
d56/BRCA1-20	$2.0 \pm 0.71 \times 10^{-6}$	$2.7 \pm 0.14 \times 10^{-4}$ (135)	3.9
d56/BRCA1-38	$2.0 \pm 1.41 \times 10^{-6}$	$4.2 \pm 0.04 \times 10^{-4}$ (210)	6.1

*Homologous recombination frequency represents the mean of three independent experiments for each cell line. I-SceI frequency was calculated from the number of Puro^R colonies relative to the number of cells expressing GFP (see Materials and Methods). Puro selection was applied 10 days after transfection of cells with I-SceI-expressing vector or GFP-expressing control vector for spontaneous homologous recombination. Numbers in parentheses represent fold increase in homologous recombination frequencies after I-SceI expression.

[†]Ratio indicates fold increase in BRCA1-expressing cells relative to their parents.

clones d55 and d56, of 37 Puro^R colonies analyzed, 32% (12 of 37) represented gene conversion and 68% (25 of 37) represented deletion events. Such a predominance of SSA over gene conversion in HCC1937 cells is a defining feature of BRCA2-deficient cells, indicating a defect in RAD51 pathway (9, 27, 28). In addition, with the BRCA1-expressing derivative lines, 52% (39 of 75) represented gene conversion and only 48% (36 of 75) represented deletion events (Table 2). Such a significant shift from SSA to gene conversion together with the 4- to 9-fold increase in the frequency of I-SceI-induced homologous recombination in BRCA1-expressing derivative lines (Table 3) indicate that BRCA1 promotes DSB repair by both gene conversion and SSA but plays a greater role in gene conversion. However, as homologous recombination repair involving direct repeats occurs almost exclusively by gene conversion in wild-type cells (refs. 16, 29 and references therein), the partial shift to gene conversion events in BRCA1-expressing cell clones could reflect their partial complementation (Fig. 2B). From these results, we conclude that the role of BRCA1 in RAD51-mediated recombination is not limited only to the processing of DSB ends but also to activate RAD51.

Discussion

This is the first study that employed a direct repeat assay system to monitor distinct homologous recombination pathways in a BRCA1-deficient background. An additional characteristic feature of the assay system is that the homologous recombination reporter gene escapes repression from the adjacent, dominant marker gene in both BRCA1-expressing and nonexpressing cell clones (ref. 29; data not shown). Efficient expression of reporter genes is as important as DNA strand exchange when measuring homologous recombination in this type of assay systems. It has been consistently shown with various organisms that in established cell clones, which had been initially selected for the expression of a marker gene, the adjacent reporter gene was usually repressed (reviewed in ref. 30). This silencing phenomenon, known as “transcriptional interference” or “promoter suppression” occurs at the transcriptional level, is *cis*-acting, epigenetic, and heritable and can be associated with alterations of chromatin structure. Given that BRCA1 regulates DNA trans-

cription and alters chromatin structure (4, 23), an interference/suppression-free homologous recombination assay system was deemed necessary for this study.

In this assay system, BRCA1 not only promotes gene conversion repair of chromosomal DSBs but also suppresses spontaneous gene conversion events, providing evidence of a dual role for this protein in regulating RAD51 recombination according to the type of DNA damage. In addition, following chromosomal DSBs, BRCA1 promotes both gene conversion and SSA but greatly enhances gene conversion, providing the first direct evidence that BRCA1 activates RAD51. Moreover, BRCA1 suppresses spontaneous deletion events by SCRS, suggesting a role for this protein in sister chromatid cohesion and/or alignment. Given that these roles of BRCA1 have also been shown for BRCA2 (9), and BRCA1- and BRCA2-deficient cells exhibit similar chromosomal abnormalities, BRCA1 and BRCA2 may act in concert to regulate such processes (Fig. 4; ref. 9).

It is noteworthy that the spontaneous hyperrecombination phenotype of BRCA1/BRCA2-deficient human cells (this study; ref. 9) and BRCA2-deficient hamster cells (27) has not been reported with BRCA1/BRCA2-deficient mouse cells. Although the exact reason for this remains to be elucidated, differences between species or the assay systems employed can be mentioned. However, a likely explanation may be that unlike human BRCA1-deficient HCC1937 and BRCA2-deficient CAPAN-1 cells or hamster cells, which are null for BRCA1 and BRCA2 and grow in culture, equivalent mouse cells are not viable (5). The engineered mouse *BRCA1/BRCA2* mutant genes tested for recombination are clearly hypomorphs (partial loss of function), expressed at normal levels, and their protein products still localize normally to the nucleus where they interact and sequester RAD51, whereas in cells with a spontaneous hyperrecombination phenotype the *BRCA1/BRCA2* mutant alleles either are expressed at a very low level or the truncated protein products are sequestered in the cytoplasm (5).

The spontaneous hyperrecombination phenotype of BRCA1/BRCA2-deficient cells is also a defining feature of the human cancer predisposition diseases ataxia telangiectasia, Bloom's syndrome, and Fanconi's anemia with similar chromosomal abnormalities (7), although the underlying mechanisms may be different (see Discussion in ref. 9). Spontaneous hyperrecombination has the

potential to trigger chromosomal instability and tumorigenesis in the presence of functional DNA damage response that would otherwise kill BRCA1/BRCA2-incident tumor cells in the presence of unrepaired DSBs (2, 3). Whereas hypergene conversion would increase the loss of heterozygosity when acting between two heteroalleles or between a gene and a pseudogene (cited in refs. 9, 29), increased SCRS/crossover events between misaligned sister chromatids would generate gross chromosomal rearrangements, such as deletions, duplications, and triplications of large DNA segments (Fig. 4). Such rearrangement events between repetitive DNA elements have been reported to occur at many chromosomal loci (7, 17), including *BRCA1* and *BRCA2* genes (40, 41). SCRS/crossover could also culminate in acentric and dicentric (quadriradial) chromosomes when involving inverted repeats or heterologous chromosomes. Quadriradial chromosomes are presumably unstable and can undergo breakage-fusion cycles, which, in DSB repair-deficient cells, would yield triradials and broken chromosomes. Chromosomal rearrangements could also lead to improper regulation of gene expression that could, in turn, result in inactivation of tumor suppressor genes or activation of oncogenes. Thus, hyperrecombination would be expected to increase the load of such mutational events. This would allow incipient tumor cells with broken chromosomes to bypass the proliferation block, grow, and accumulate further chromosomal rearrangements by error-prone DSB pathways (Fig. 4).

That BRCA1 and BRCA2 suppress SCRS suggests a role for these two proteins in sister chromatid cohesion/alignment. Sister chromatid cohesion, established during the S and G₂ phases of the cell cycle, is also essential to homologous recombination repair of DSBs, chromosome segregation, cell division, survival, and maintenance of genome stability (42). How BRCA1 and BRCA2

function in sister chromatid cohesion/alignment remains to be elucidated, but these proteins may act as regulators rather than components of the cohesin complex; cohesin is not required for intrachromosomal gene conversion or the resection of DSB ends (cited in ref. 42). BRCA1 may act together with ATM, ATR, MRN, or H2AX (4) that have been reported to be also required for the recruitment of cohesin complex to sites of DNA damage (42). In addition, BRCA1 is required for the recruitment of activated ATM to sites of DNA damage and for cohesin phosphorylation by ATM (43). Sister chromatids remain connected by cohesin until the transition from G₂ to M, and cohesin is dissociated from chromosomes in the prophase in a Polo-like kinase (PLK1)-promoted process (44). BRCA1 and BRCA2 both seem to be involved in G₂-M cell cycle checkpoints (4), and PLK1, which has been implicated in the regulation of several mitotic events, including entry into mitosis, centrosome separation, metaphase-to-anaphase transition, and cytokinesis, binds and hyperphosphorylates BRCA2 during G₂-M, and BRCA1 regulates PLK1 (45–47). BRCA2 also associates with the spindle checkpoint kinase BUBR1 that monitors mitotic checkpoints and chromosome segregation events (48). Furthermore, previous work has shown that BRCA2-deficient cells exhibit dysfunction of the spindle assembly checkpoints (49) and aberrant cytokinesis (50). BRCA1- and BRCA2-deficient cells also undergo centrosome amplification and aneuploidy (2, 3). Although distinct mechanisms can be invoked for such abnormalities (51), a loss of or a decrease in sister chromatid cohesion might be the trigger.

In conclusion, our data support the idea that BRCA1 and BRCA2 act in concert to maintain chromosome structure and number intact. In this model, BRCA1 and BRCA2 maintain alignment of sister chromatids, ensure appropriate chromosome

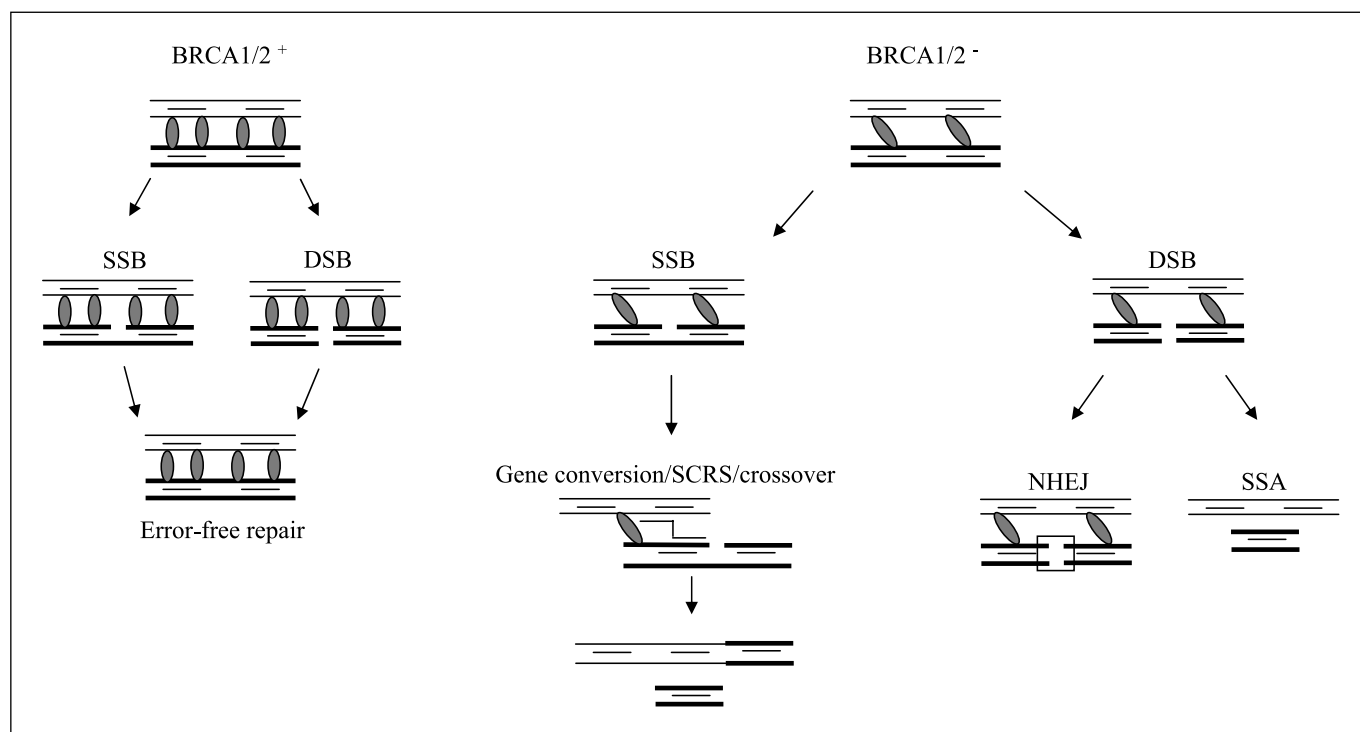


Figure 4. A model for BRCA1 and BRCA2 in genome stability maintenance. In normal cells, BRCA1 and BRCA2 keep the sister chromatids aligned and allow error-free repair of SSB and DSBs (see text; ref. 9). In BRCA1/BRCA2-deficient cells, loss of sister chromatid cohesion results in aberrant chromosome segregation spontaneous hyperrecombination (gene conversion with or without crossover and SCRS), and DSB repair by error-prone pathways (NHEJ and SSA). These pathways can take place within a chromatid or an interchromatid or between heterologous chromosomes (see text for more details).

segregation, and regulate RAD51 recombination in response to the type of DNA damage (9). Given that BRCA1/BRCA2-deficient cells are impaired in DSB repair, their spontaneous hyperrecombination phenotype cannot be induced by DSBs but rather is consistent with single-strand breaks (SSB) as the initiating lesions (36). Because estrogen metabolites can adduct DNA and cause SSBs as well as genome instability (52), estrogen-induced hyperrecombination may underlie tissue specificity of the tumor-suppressive properties of BRCA1 and BRCA2 and the increased risk of breast and ovarian cancers (53). Such a risk could be modulated by regimens that would decrease or block the genotoxic effects of estrogen metabolites (54).

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