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Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination

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The repair of DNA double-strand breaks is essential for cells to maintain their genomic integrity. Two major mechanisms are responsible for repairing these breaks in mammalian cells, non-homologous end-joining (NHEJ) and homologous recombination (HR)^{1,2}: the importance of the former in mammalian cells is well established³, whereas the role of the latter is just emerging. Homologous recombination is presumably promoted by an evolutionarily conserved group of genes termed the *Rad52* epistasis group^{4–11}. An essential component of the HR pathway is the strand-exchange protein, known as RecA in bacteria⁸ or Rad51 in yeast⁶. Several mammalian genes have been implicated in repair by homologous recombination on the basis of their sequence homology to yeast Rad51 (ref. 11): one of these is human *XRCC2* (refs 12, 13). Here we show that *XRCC2* is essential for the efficient repair of DNA double-strand breaks by homologous recombination between sister chromatids. We find that hamster cells deficient in *XRCC2* show more than a 100-fold decrease in HR induced by double-strand breaks compared with the parental cell line. This defect is corrected to almost wild-type levels by transient transfection with a plasmid expressing *XRCC2*. The repair defect in *XRCC2* mutant cells appears to be restricted to

recombinational repair because NHEJ is normal. We conclude that *XRCC2* is involved in the repair of DNA double-strand breaks by homologous recombination.

Similar to yeast mutants that affect DNA double-strand break (DSB) repair by HR⁶, hamster cells that lack *XRCC2* are hypersensitive to ionizing radiation (about 2-fold) and crosslinking agents (60- to 100-fold), and show an increase in chromosome instability^{13,14}. In contrast to yeast, all characterized mammalian DSB-repair mutants have been found to be defective in NHEJ. Thus, the role of *XRCC2* in DNA repair is unclear. To determine whether the hamster cell line *irs1*, which is deficient in *XRCC2* (refs 12, 13), can repair DSBs by HR, we used a novel recombination reporter substrate SCneo (Fig. 1a). SCneo contains two nonfunctional copies of the neomycin phosphotransferase (*neo*) gene. One copy, designated 3' *neo*, is a 5' truncation of the *neo* gene¹⁵. The second copy, designated S2neo, is mutated at an *NcoI* site by deletion of 4 base pairs (bp) of *neo* gene coding sequence and insertion of the 18-bp site for the rare-cutting *I-SceI* endonuclease¹⁶. The two *neo* genes are in direct orientation and are separated by a functional hygromycin

Figure 1 Recombination reporter substrate SCneo. **a**, Structure of SCneo and predicted HR products. The *neo* probe is indicated. X/H, *XhoI/HindIII*; X/H/N, *XhoI/HindIII/NcoI*; X/H/B/I, *XhoI/HindIII/BamHI/I-SceI*. **b**, Southern blot analysis of SCneo cell lines. Each cell line contains a single copy of SCneo, except the parental cell line 4-13 which contains two copies. **c**, Southern blot analysis of cell lines 4-18 (V79) and 8-3 (*irs1*) and G418^R recombinants derived from them. Genomic DNA was digested with *XhoI/HindIII* to distinguish STGC (4.0 kb) and LTGC/SCE (7.3 kb). Recombinants with both fragments probably underwent two recombination events.

resistance gene (*hyg^R*). SCneo was stably integrated into the genome of the *irs1* mutant line and its radioresistant parental line V79 by selecting for *hyg^R* cells. Clones containing an intact SCneo (two each for V79 and *irs1*) were verified by Southern blotting (Fig. 1b). In each of the clones, SCneo integrated at a different genomic location (data not shown).

In cell lines containing SCneo, DSBs introduced into the chromosome by *I-SceI* could be repaired by HR to restore a *neo⁺* gene. Wild-type cell lines transfected with the *I-SceI* expression vector pCMV3xnl*s-I-SceI* underwent HR at a frequency of $1-2 \times 10^{-3}$ per plated cell, more than 100-fold higher than cells transfected with the control plasmid (Fig. 2). The actual HR frequency is even higher, as equal sister-chromatid HR events are undetectable in our assay. This large induction of recombination by a chromosomal DSB is consistent with what has previously been reported in other systems¹⁷. In stark contrast, the *XRCC2*-deficient *irs1* cell lines had a much reduced frequency of recombinational repair, in the range of $2-6 \times 10^{-6}$. The recombination defect observed in the mutant lines can be attributed to the defect in *XRCC2*, as cotransfection of pCMV3xnl*s-I-SceI* with pXR2, a human *XRCC2* expression vector, resulted in nearly wild-type recombination levels (about 5×10^{-4} ; Fig. 2). *XRCC2* expression is evidently promoting DSB-induced recombination, because transfection of pXR2 alone is not sufficient to increase recombination (data not shown). In contrast to *XRCC2*, expression of human *Rad51* did not correct the HR defect in *XRCC2* (R.D.J. and M.J., unpublished results).

SCneo allows the detection of two recombination products (Fig. 1a). One product results from a short tract gene conversion (STGC) in which the *neo* sequences immediately surrounding the DSB in the *S2neo* gene are repaired without changing the overall architecture of the SCneo reporter (Fig. 1a). In an STGC event, repair can occur from the 3' *neo* gene located on either the same chromatid or the sister chromatid. The second predicted recombination product results in expansion of the SCneo reporter from two to three *neo*-containing repeats, either as a result of a long tract gene conversion (LTGC) with the sister chromatid or a sister-chromatid exchange (SCE) event (Fig. 1a). As shown in Fig. 1c and Table 1, both products are recovered in equal proportions from the mutant and wild-type cell lines, showing that loss of *XRCC2* reduces both STGC and LTGC/SCE recombination.

Mammalian cells possess a potent end-joining activity which repairs DSBs using little or no homology³. To determine whether *XRCC2* affects all DSB-repair processes rather than HR specifically, we used a polymerase chain reaction (PCR) based assay that simultaneously detects both NHEJ and HR (Fig. 3a; ref. 2). A DSB was induced at the SCneo substrate by transfection of the *I-SceI* expression vector, and genomic DNA was isolated immediately after transfection as a control, and after 48 h. As shown in Fig. 3b, the product amplified from genomic DNA recovered immediately after transfection (0 h) was cleaved by *I-SceI*. By contrast, much of the product amplified from genomic DNA recovered 48 h after

Table 1 Summary of DSB-induced recombination products

Cell line	No. STGC events	No. LTGC or SCE events
V79		
4-13	17	8
4-18	6	16
Total	23	24
<i>irs1</i>		
8-3	16	8
8-5	4	11
Total	20	19

transfection was not cleavable by *I-SceI*, indicating recovery of DSB-repair products. To identify HR (*NcoI*⁺) and NHEJ (*NcoI*⁻/*I-SceI*⁻) repair products, the resulting PCR products were digested with *NcoI* and/or *I-SceI* (Fig. 3b). In the wild-type cell lines, the *NcoI*⁺ fragments are readily detectable in the 48 h samples, as is the *NcoI*⁺/*I-SceI*⁻ band, indicating robust homologous and nonhomologous DSB repair. In the *XRCC2*-deficient cell lines, the *NcoI*⁺ fragments are significantly reduced (Fig. 3b), although they can be restored by complementation (data not shown). This confirms that loss of *XRCC2* results in a defect in HR. Unlike the HR product, the *NcoI*⁺/*I-SceI*⁻ NHEJ products are readily detectable in the *XRCC2* mutant, indicating that loss of *XRCC2* does not affect nonhomologous repair. Thus, the repair defect in the *XRCC2*-deficient cell line can be attributed to a defect in HR, rather than a global defect in DSB repair. Wild-type V79 cells exhibit an increased resistance to radiation during S-phase, whereas *irs1* cells do not¹⁸. Taken together, our results strongly indicate that S-phase radiation resistance in wild-type cells is due to sister-chromatid recombination, with the *irs1* cells having a defect in this type of DSB repair.

Loss of *Rad51* in mice results in early embryonic lethality¹⁹, and cells recovered from the *Rad51*^{-/-} mutant embryos contain chromosomal abnormalities²⁰. This indicates that recombinational repair may be critical for chromosome integrity and cell proliferation. *XRCC2* in the *irs1* cell line is essentially a null allele¹³, and cells lacking *XRCC2* show only a mild growth defect in culture, and are not completely defective for HR (Fig. 1c); therefore, *XRCC2* is not essential for recombinational repair or cell viability. As the mammalian *Rad51* protein has the greatest identity to yeast *Rad51* and *E. coli* *RecA*¹¹, and can catalyse strand exchange *in vitro*²¹, *Rad51* is probably the central strand-transferase protein in the cell. We speculate that *XRCC2*, which has only 20% sequence identity to *Rad51*, and other *Rad51*-related proteins may promote, but are not essential for, *Rad51* activity. This may also be true of other recombination proteins (for example, *Rad54* and *Rad52*)^{5,7}; consistent with the *Rad51*-related proteins modifying *Rad51* function, these proteins interact with one another^{13,22}, similar to the interactions observed between yeast *Rad51* and the *Rad55* or *Rad57* proteins^{23,24}, which promote *Rad51* activity *in vitro*²⁵.

DNA-repair processes are essential in maintaining chromosomal structure and genetic integrity. The significance of these processes is

Figure 2 *XRCC2*-deficient *irs1* cells have severely reduced levels of DSB repair by HR. Transfection of wild-type V79 cell lines with the *I-SceI* expression vector increases HR more than 100-fold compared with transfection of the control plasmid pCMV-*lacZ*. By contrast, mutant *irs1* cell lines show little or no increase in HR. DSB-induced HR is substantially restored in the mutant cell lines by transient transfection of an *XRCC2* expression vector (pXR2) with the *I-SceI* expression vector. The variability observed within each pair of cell lines is probably due to position effects.

emphasized by links between defects in DNA-repair pathways and human disease and malignancy²⁶. Cancer cells frequently contain abnormal genomes exhibiting chromosomal rearrangements and aneuploidy. Thus it seems likely that failure to repair a DSB or its illegitimate repair may contribute to a cell's progression towards malignancy. The demonstration that the products of the hereditary breast cancer genes interact with Rad51 (ref. 27) indicates that recombinational repair may be disrupted in cells from these patients, and that loss of HR may promote tumorigenesis. A number of genes potentially involved in HR have been identified in mammalian cells because of their homology with yeast genes. However, the difference in severity of phenotype between yeast and mammalian cells that carry mutations in those genes (for example, Rad51 (refs 19, 20) and Rad52 (ref. 7) makes it vital to analyse recombination in mammalian cell mutants. We have demonstrated the involvement of a mammalian repair protein in recombinational repair of DNA damage; it will be important to determine whether XRCC2 and other genes involved in recombinational repair are tumour suppressor genes, as are the hereditary breast cancer genes. □

Methods

Plasmids

SCneo was constructed from IRneo, which is identical to DRneo¹⁵ except that the 3' *neo* gene is in reverse orientation (M.J., unpublished results). IRneo was modified by the insertion of a double-stranded oligonucleotide containing *Cla*I and *Bam*HI sites into its *Xho*I site. This oligonucleotide regenerated a single *Xho*I site and inserted *Cla*I and *Bam*HI sites upstream of the promoter of *S2neo*. This plasmid was digested with *Cla*I, which contains the *S2neo* gene on a 1.1-kb *Cla*I fragment, and then re-ligated with the *S2neo* fragment to obtain a plasmid with *S2neo* in the opposite orientation to that present in IRneo, designated SCneo. The I-SceI expression vector, pCMV3xns-I-SceI, is a modified version of pCMV-I-SceI (ref. 28) that contains a triplicated nuclear localization signal fused to I-SceI (ref. 29). Expression of I-SceI is not toxic to cultured mammalian cells²⁸. pXR2 was constructed by digesting pcDNA-XR2 (ref. 13) with *Sma*I and *Bst*BI to remove a 900-bp fragment containing the entire *neo* coding sequence. XRCC2 expression does not alter the transient expression of cotransfected genes, as *lacZ* expression is not affected when pCMV-*lacZ* is cotransfected with pXR2 (data not shown).

DNA manipulations

Southern blot analysis was performed using 8 µg genomic DNA according to standard procedures. The probe was a 1.1-kb *Xho*I–*Hind* III fragment containing the complete *neo*

gene¹⁶. PCR was done as described², using genomic DNA that has been precleaved with I-SceI *in vitro* to reduce the amplification of I-SceI⁺ *neo* genes.

Cell transfections

Transfections were done by mixing 1.6×10^7 cells suspended in PBS with 30 µg uncut plasmid and electroporating at 250V, 960 µF. Drug selections were begun 24 h after transfection. To establish cell lines with a stably integrated SCneo reporter substrate, cells were electroporated with the SCneo plasmid and selected in media containing 0.5 mg ml⁻¹ hygromycin. *hyg*^R colonies were screened by Southern blot analysis for intact incorporation of the SCneo reporter substrate. Recombination frequencies were determined by transfection of SCneo-containing cell lines with the indicated plasmid, followed by selection in media containing 1 mg ml⁻¹ G418. Cells were grown for 10–12 days and surviving G418^R colonies were stained with a 10% Giemsa solution for colony counts. Of the G418^R colonies obtained in these transfections, 95% were also *hyg*^R.

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Figure 3 XRCC2-deficient *irls1* cells have normal levels of NHEJ. **a**, PCR-based assay of DSB repair. The primers amplify both products of HR occurring by either STGC or LTGC/SCE and most NHEJ events that contain small deletions and insertions (Δ , +). **b**, DSB repair in the wild-type and XRCC2-deficient cell lines. DSB-induced HR is reduced in the XRCC2 mutant, as indicated by the lack of detectable *Nco*I PCR product; however, NHEJ is robust in both the wild-type and XRCC2 mutant cell lines, as seen by the presence of the *Nco*I/I-SceI band.