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Transcription-associated recombination is independent of XRCC2 and mechanistically separate from homology-directed DNA double-strand break repair

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ABSTRACT

It has previously been shown that transcription greatly enhances recombination in mammalian cells. However, the proteins involved in catalysing this process and the recombination pathways involved in transcription-associated recombination (TAR) are still unknown. It is well established that both the BRCA2 protein and the RAD51 paralog protein XRCC2 are required for homologous recombination. Here, we show that the BRCA2 protein is also required for TAR, while the XRCC2 protein is not involved. Expression of the XRCC2 gene in XRCC2 mutated *irs1* cells restores the defect in homologous recombination repair of an I-SceI-induced DNA double-strand break, while TAR is unaffected. Interestingly, the XRCC2-deficient *irs1* cells are also proficient in recombination induced at slowed replication forks, suggesting that TAR is mechanistically linked with this recombination pathway. In conclusion, we show that TAR depends on BRCA2 but is independent of XRCC2, and that this recombination pathway is separate from that used to repair a two-ended DNA double-strand break.

INTRODUCTION

Transcription-associated recombination (TAR) is conserved in all cellular organisms investigated, from bacteria to mammals (1). The mechanisms and underlying causes of TAR are still largely unknown, as are the proteins catalysing this process. Studies in *Saccharomyces cerevisiae* has shown that a number of factors influence TAR; for instance DNA damage appears to have a synergistic

effect with transcription on the levels of recombination (2). It has also been shown in *S. cerevisiae* that RNA polymerase I-dependent transcription can result in collision with the replication machinery leading to replication fork blockage and recombination in rDNA repeat copies (3). RNA polymerase II transcription can also impair the replication fork progression, which results in an increased recombination (4). A similar increase in recombination levels can be observed in *S. cerevisiae* strains with impaired transcription elongation owing to mutations in the THO/TREX complex (5,6). The THO/TREX complex is active at the interface between transcription and messenger ribonucleoprotein (mRNP) metabolism, and as a consequence of the mRNP biogenesis defect in THO/TREX mutants an impaired replication fork progression could be observed, which appear to stimulate TAR (7). Nickoloff and Reynolds (8) were first to show that transcription stimulated homologous recombination in mammalian cells, using heteroallelic neomycin genes. TAR in mammalian cells has been shown to also be associated with replication and restricted to cells in the S phase of the cell cycle (9). The available data thus suggest that TAR is likely involved in bypassing an active RNA polymerase, but details of the recombination mechanisms and proteins involved in the process remain elusive.

Homologous recombination (HR) can be induced by DNA double-strand breaks (DSBs) or at replication forks (10–12). There are two major pathways by which DSBs can be repaired; non-homologous end-joining (NHEJ) or homologous recombination (HR). NHEJ is a fast and error-prone repair pathway that involves ligation of free ends (13), while homologous recombination is slow and error-free if an intact DNA molecule, usually the sister chromatid, is used in the repair (14,15). Several proteins, e.g. BRCA2, RAD51 and five RAD51 paralogs,

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have been shown to be essential for efficient homologous recombination repair of DSBs in mammalian cells (16–19), and mutations in these genes often results in cellular sensitivity to ionizing radiation. Chinese hamster cells sensitive to ionizing radiation have been isolated (20) and it was later found that one of the five RAD51 paralog genes, *XRCC2*, correct the ionizing radiation sensitive hamster cell line irs1 (21). The precise function of *XRCC2* is unknown, but it has been shown to associate with three other RAD51 paralogs; *RAD51B*, *RAD51C* and *RAD51D* (22). This complex is called BCDX2 and has been shown to bind single-stranded DNA as well as single-stranded regions of DNA and to facilitate *RAD51* filament formation (22). Cell lines deficient in *XRCC2* are viable, but exhibit chromosomal aberrations, sensitivity to cross-linking agents, a mild sensitivity to γ -radiation (20) and defective *RAD51* foci formation (18).

Here, we investigated the connection between homologous recombination and TAR using well-characterized Chinese hamster cell lines, irs1 and V-C8, deficient in *XRCC2* and *BRCA2*, respectively (21,23). We find that *BRCA2* defective cells are deficient in TAR, showing that TAR employs homologous recombination proteins. Surprisingly, the *XRCC2* defective irs1 cells are still able to carry out TAR, in spite of being defective in homologous recombination of a DSB induced by ionizing radiation or a restriction endonuclease. This genetically separates TAR from DSB-induced homologous recombination. Furthermore, we show that *XRCC2*-deficient irs1 cells are also proficient in thymidine-induced recombination, which altogether suggest that TAR is employing a similar recombination mechanism to bypass replication blocks as for lesions produced by thymidine treatment.

MATERIALS AND METHODS

Cell culture

All cell lines used in this study are derived from V79 Chinese hamster cells, with an additional mutation in *BRCA2* (V-C8 and V-C8TofZM4), *XRCC2* (irs1TofZM14 and irs1TofZM15) or with a partial duplication in the *hprt* gene S8TofZM5 (Table 1). The cells were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and 1% PEST (penicillin–streptomycin). To select for the desired clones and to keep the inserted genes in the cells, 0.25 mg/ml of zeocin and 120 U/ml hygromycin were added to the medium. In order to keep the transcription of the recombination substrate off during growth, 1 μ g/ml of doxycycline was also added to the medium. The cells were cultured in an incubator at 37°C with 5% CO₂.

Transfection

The Chinese hamster cell lines were stably transfected with a recombination substrate using electroporation. The plasmids were purified from *Escherichia coli*. Fifteen micrograms of plasmid DNA were used for each transfection. The plasmid DNA was diluted in 50 μ l dH₂O and mixed with 7.5×10^6 cells in an electroporation cuvette. The cells were transfected using the voltage 2.5 kV/cm and the

Table 1. Genotype and origin of Chinese hamster cell lines used in this study

Cell line	Genotype	Defect/modification	Origin	References
irs1TofZM14,15	<i>XRCC2</i> ⁻	<i>XRCC2</i> ⁻ , deficient in homologous recombination, carrying TAR substrate	V79	(37)
S8TofZM5	WT	Wild-type Chinese hamster, partial duplication in <i>hprt</i> gene, carrying TAR substrate	V79	(38)
V-C8	<i>BRCA2</i> ⁻	<i>BRCA2</i> ⁻ , deficient in homologous recombination	V79	(23)
V-C8TofZM4	<i>BRCA2</i> ⁻	<i>BRCA2</i> ⁻ , deficient in homologous recombination, carrying TAR substrate	V79	(23)
V79	WT	Wild-type Chinese hamster	Lung	(23)

capacitance 25 μ F. The electroporated cells were aliquoted in the amounts 20, 50 and 100 μ l on Petri-dishes and incubated for 2 days before the appropriate selective agent was added. After the colonies had formed, 30 colonies of varying sizes were picked and transferred to culture plates for further cultivation. Transient transfection was performed using Lipofectamine 2000 from Invitrogen according to the manufacturer's protocol.

Luciferase assay

For measuring luciferase activity individual clones were trypsinised and counted and 2×10^5 cells from each clone were incubated with and without doxycycline for 24 h prior to lysis. After the incubation the cells were rinsed twice with Dulbecco's PBS w/o Ca and Mg. All PBS was removed and 500 μ l of lysis buffer was added to each well. The plates were shaken for 20 min in order to lyse the cells and the luciferase activity was measured using an illuminator. Clones exhibiting low background and high inducibility in the luciferase assay were selected for further experiments.

Recombination assay

For measuring recombination 1×10^6 cells were seeded onto four Petri-dishes. To two of the dishes, 10 μ l (1 mg/ml) of doxycycline was added to repress expression from the *P*_{bi-1} promoter. In the case of I-SceI-induced recombination, the cells were transiently transfected using Lipofectamine after 24 h. After an additional 24 h the cells were plated on cloning and selection dishes to determine survival and recombination, respectively. On the cloning dishes, 500 cells were seeded and on the selection dishes 300 000 cells were seeded. After 24 h, 100 μ l of G418 was added. When colonies had formed (10 days for cloning and 15 days for selection), the cells were stained, using methylene blue in methanol (4 g/l), and counted. The recombination frequency was calculated as the number of recombinants formed in relation to survival. Thus, differential cloning efficiencies are compensated for in the recombination assay. Recombination assays while

over-expressing wild-type XRCC2 was also performed. For these assays, 20 µg of XRCC2 plasmid (24) was used for each 10-cm Petri-dish. One microgram of I-SceI plasmid was used for each Petri-dish in order to induce a DSB in the recombination substrate. Transient transfections were performed using Lipofectamine 2000 according to the manufacturer's protocol.

RAD51 foci

To score for Rad51 foci, cells were treated with ionizing radiation or thymidine or left untreated as a control. For cells that were transiently transfected with a plasmid containing XRCC2, transfection was performed as described previously. Irradiated cells were treated with 5 Gy and left in an incubator for 5 h in order for foci to appear. For the thymidine treatment, the cells were treated with 2 mM of thymidine for 24 h. For Rad51 foci detection, 5×10^4 cells per slide were seeded onto sterilized cover slides. The next day the slides were either irradiated or treated with thymidine. After the treatment the cells were fixed with 3% paraformaldehyde containing 0.1% Triton X-100. The cells were then rinsed with PBS containing 0.1% TX-100 2×10 min. After rinsing the cells were permabilized with PBS + 0.3% TX-100 for 10 min. The cells were then blocked with PBS + 3% BSA for 40 min. After blocking, the primary antibody, h92 Santa Cruz anti RAD51 polyclonal rabbit diluted 1:1000 in PBS + 3% BSA was added and the cells were left at 4°C overnight. The next day, the cells were rinsed with PBS containing 0.1% TX-100. After rinsing the cells were permabilized with PBS + 0.3% TX-100 for 10 min. After permabilization, the secondary antibody was added; donkey anti rabbit alexa 555 from Invitrogen, diluted 1:500 in PBS + 3% BSA. The slides were incubated in the dark for 1 h. The cells were then rinsed with PBS containing 0.1% TX-100 2×10 min and permabilized with PBS + 0.3% TX-100 for 10 min. They were then rinsed with PBS for 5 min and stained with ToPro from Invitrogen for 30 min. The slides were then rinsed with PBS for 5 min and mounted with anti fade kit and sealed with nail polish. The slides were coded to make scoring impartial. Cells with more than 10 foci were scored as positive and 200 cells per slide were scored.

Western blot

To confirm that transient transfection with wild-type XRCC2 increased the levels of the XRCC2 protein in the cells, western blots were performed. An antibody against XRCC2 diluted 1:200 was used. An antibody against α -tubulin diluted 1:1000 was used as a loading control. The proteins were extracted using lysis buffer and the protein concentration was measured by using the Coomassie (Bradford) Protein Assay Kit from Pierce. A standard western blot protocol was used and the proteins were separated on a NuPage Novex 10% Bis-Tris gel from Invitrogen and then blotted onto a PVDF membrane. The proteins were visualized using SuperSignal Western blotting kit from Pierce.

RESULTS

BRCA2 is required for TAR

We have previously constructed a substrate to investigate the effect of transcription on homologous recombination (9). Briefly, the construct contains two non-functional neomycin resistance genes; one *neo* repeat has a 18-bp long recognition site for I-SceI inserted, which introduces a stop codon that truncates the gene product, and the second *neo* repeat has a 3' deletion and is called 5'*neo* (Figure 1A). A functional *neo*^R gene can be regained either through gene conversion (GC) or sister chromatid exchange (SCE), but not through single-strand annealing. The truncated *neo* repeat is under the control of a bi-directional inducible promoter based on a Tet-Off gene expression system. Using this approach, the levels of transcription over this *neo* repeat can be measured using the expression levels of the luciferase gene, simultaneously expressed in the opposite direction in the vector (9).

Here, we transfected wild-type, XRCC2 and BRCA2 mutated Chinese hamster cells with both the Tet-regulatory vector pTetOffZeo and the recombination construct pBI-LMScI and selected for individual clones resistant to zeocin and hygromycin, to obtain cells with both vectors stably integrated into the genome. Withdrawal of doxycycline from the media resulted in increased luciferase activity in both wild-type (S8TofZM5) and BRCA2 defective (V-C8TofZM4) cells (Figure 1B), showing that BRCA2 does not influence the inducibility of transcription on the recombination substrate.

Transcription on the recombination substrate enhanced recombination levels in S8TofZM5 cells 3.7-fold (Figure 1C), which is in agreement with what was reported earlier for other wild-type hamster cells (9). However, transcription did not induce recombination in BRCA2 defective V-C8TofZM4 cells (Figure 1C). This is expected as the BRCA2 protein is vital for RAD51-mediated recombination (17), and in particular the gene conversion events produced by transcription (9,25). Here, we confirm the homologous recombination defect in BRCA2 defective V-C8 cells and show that RAD51 foci cannot form following either ionizing radiation [which has been shown before (16)] or following thymidine treatments (Figure 1D).

Transcription associated recombination is independent of XRCC2

We also studied TAR in XRCC2 mutated *irs1* cells carrying the Tet-regulatory vector pTetOffZeo and the recombination construct pBI-LMScI. Transcription was efficiently increased over the recombination substrate following removal of doxycycline from the media, as determined by measuring the luciferase activity (Figure 2A). Surprisingly, both the *irs1*TofZM14 and *irs1*TofZM15 clones showed a 3.8- and 3.3-fold increase in TAR following removal of doxycycline, respectively (Figure 2B), indicating a functional recombination pathway. This was unexpected as the XRCC2-defective *irs1* clone earlier has been shown to be defective in homology directed repair of

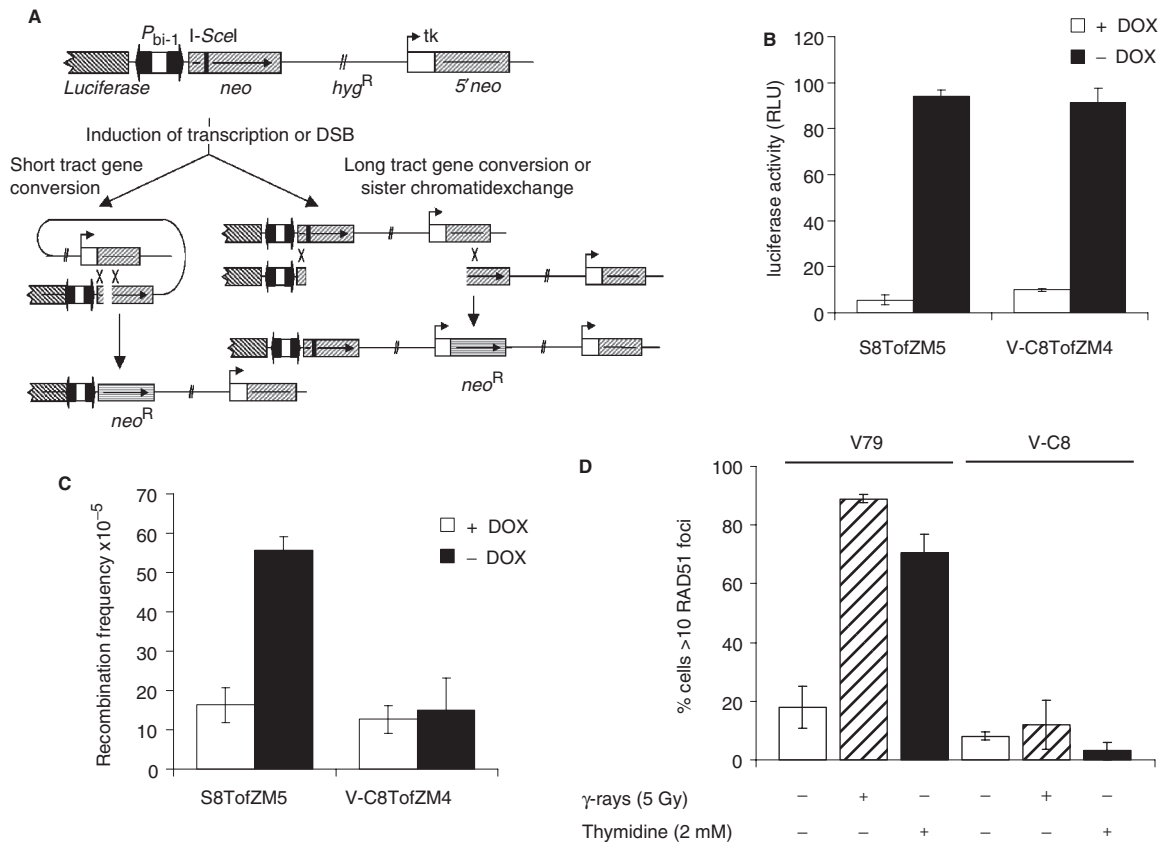


Figure 1. BRCA2 is required for transcription-associated recombination. (A) Transcription-associated recombination is monitored between a mutated *neo^R* and a truncated *neo^R* gene, which after homologous recombination can revert to a functional *neo^R* gene that can be selected with G418. Recombination can be induced either by enhanced transcription after removal of doxycycline (DOX) or after induction of a DSB at the I-SceI site following transient transfection with the pCMV3xnlS-I-SceI vector. A functional neomycin resistance gene can be regained following short tract gene conversion or long tract gene conversion or sister chromatid exchange (see ref. 9 for details). (B) The inducibility at the bidirectional *P_{bi-1}* promoter is determined by measuring luciferase activity in S8TofZM5, VC-8TofZM4, both with stably integrated copies of both the pTetoffZeo and pBI-LMscI vectors (9). (C) The recombination frequency was monitored as reversion to a functional neomycin resistant gene following removal of doxycycline, which increases transcription at the recombination substrate. Transcription enhanced recombination in wild-type S8TofZM5 ($P < 0.01$ in *t*-test), but not in BRCA2 defective V-C8TofZM4 ($P > 0.05$ in *t*-test). (D) The number of cells with RAD51 foci was scored in BRCA2 defective V-C8 and parental V79 cell line 5 h after irradiation with 5 Gy or following 24 h 2 mM thymidine treatments. The numbers of cells with foci increased in wild-type cells following both treatments ($P < 0.01$ in *t*-test), but not in BRCA2 defective V-C8 cells following any treatment ($P > 0.05$ in *t*-test). The average and standard deviation of three independent experiments is depicted in all experiments.

DSBs (26,27) and to be defective in RAD51 foci formation following ionizing radiation treatment (18,19).

To fully test if XRCC2 is not involved in TAR, we transiently transfected *irs1*TofZM14 cells with the wild-type XRCC2 in the pIRESneo2 vector and determined expression using western blot (Figure 3A). We found that expression of XRCC2 did not influence TAR levels (Figure 3B), confirming that the XRCC2 protein or XRCC2 protein level have no function in TAR.

TAR is mechanistically distinct from homologous recombination repair of DSBs

The *irs1* cells used here have previously been reported to be defective in DSB-induced homologous recombination repair of an I-SceI-induced DSB (27). We found that transfection of the *irs1* cell line with an XRCC2 expression vector did not influence TAR, which could be explained by XRCC2 not being involved in TAR, or alternatively, that the XRCC2 expressed in the

*irs1*TofZM14 is non-functional. As our recombination construct contains an I-SceI site, we tested if the XRCC2 expressed in *irs1*TofZM14 cells could complement the reported deficiency in homologous recombination. To test this, we transiently transfected *irs1*TofZM14 cells with both the pCMV3xnlS-I-SceI vector and/or XRCC2 (24) with or without doxycycline. We found that the XRCC2 vector efficiently reverts the defect in homology directed DSB repair in *irs1*TofZM14 cells (Figure 4A); showing that the over-expressed XRCC2 protein is functional. Furthermore, we find that the effect of TAR is additive, which is in agreement with earlier findings that transcription does not influence the homology directed repair of a DSB (9,28,29). Interestingly, we find that homologous recombination is induced by an I-SceI-induced DSB also in non-complemented *irs1*TofZM14 cells ($P < 0.05$), while the original report showed no induction of recombination in the same *irs1* cells (27). The data presented here suggests that *irs1* cells are defective in homologous recombination repair of I-SceI-induced

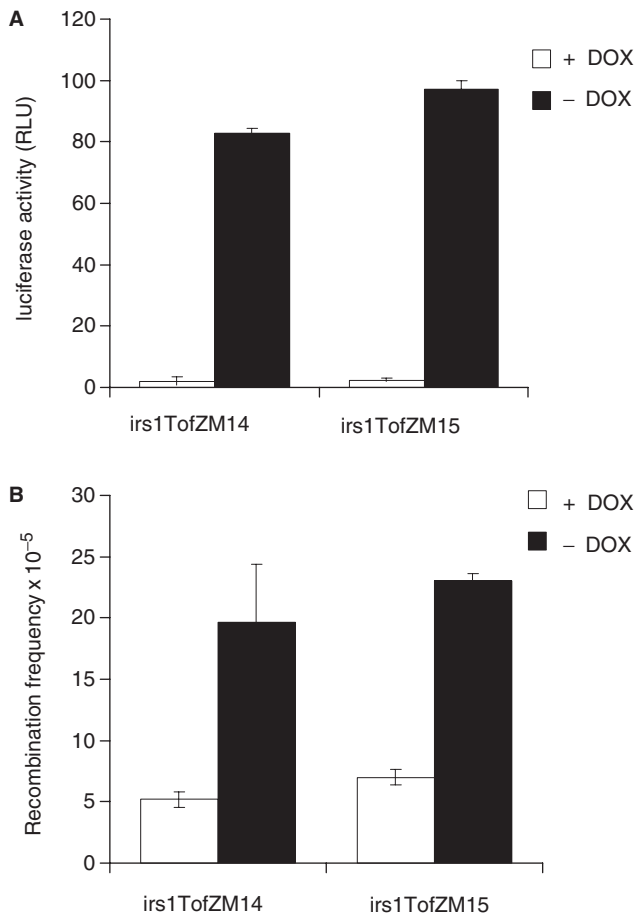


Figure 2. XRCC2 defective *irs1* cells are proficient in transcription-associated recombination. (A) The inducibility at the bidirectional P_{bi-1} promoter is determined by measuring luciferase activity in *irs1TofZM14* and *irs1TofZM15* cells, both with stably integrated copies of both the pTetoffZeo and pBI-LMscI vectors (9). (B) The recombination frequency was monitored as reversion to a functional neomycin resistance gene following removal of doxycycline, which increases transcription at the recombination substrate. Transcription enhanced recombination in both XRCC2 defective clones *irs1TofZM14* and *irs1TofZM15* ($P < 0.01$ in *t*-test). The average and standard deviation of three independent experiments are depicted.

DSBs and are at the same time proficient in TAR. Thus, there might be a sub-pathway of homologous recombination repair in *irs1* cells that is still functional. It was previously reported that *irs1* cells are proficient in thymidine-induced RAD51 foci, but defective in hydroxyurea and γ -ray-induced RAD51 foci (30). Thymidine is an agent that depletes only the (dCTP) pool in cells, as a consequence of a negative feedback mechanism of the R1 subunit of ribonucleotide reductase that follows high (dTTP) levels (31). The consequence is that the progression of the replication fork is slowed down during replication, which results in a unique replication lesion, with no DSBs, that is separate from those produced by hydroxyurea. Hydroxyurea directly inhibits ribonucleotide reductase, depleting all (dNTP) pools resulting in stalled replication forks and DSB formation (32). Thymidine efficiently induces homologous recombination at replication forks (32), which likely involves a template switching mechanism (33).

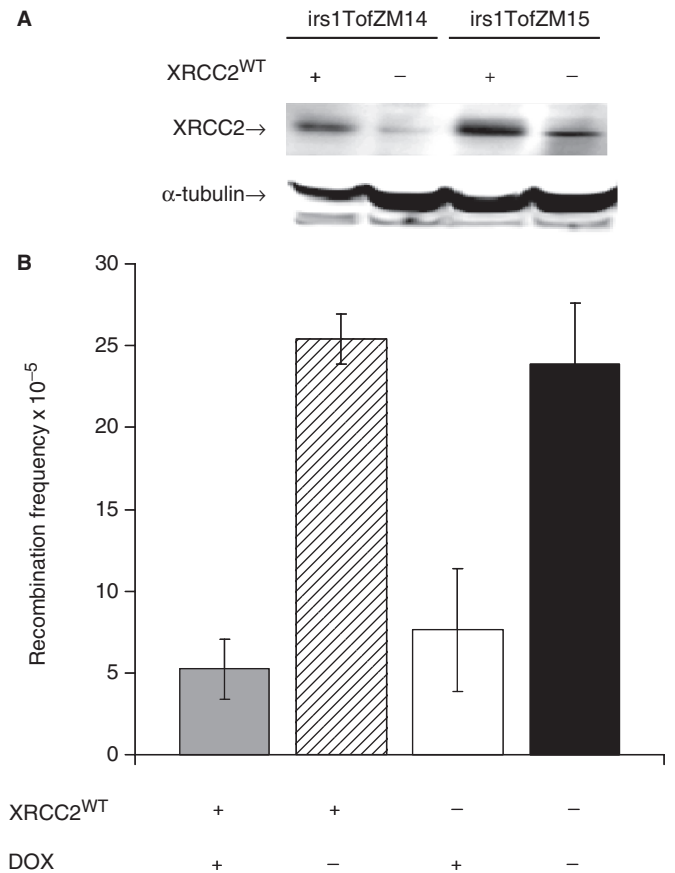


Figure 3. XRCC2 does not influence transcription-associated recombination. (A) *irs1TofZM14* and *irs1TofZM15* cells were transiently transfected with a plasmid expressing wild-type XRCC2 (XRCC2^{WT}). The expression levels were verified using western blot. (B) The influence of XRCC2 expression on transcription-associated recombination was monitored in *irs1TofZM14* cells as the reversion to G418 resistant clones among 10^5 surviving cells. The average and standard deviation of three independent experiments are depicted.

Here, we tested if also the *irs1TofZM14* cells are proficient in thymidine-induced RAD51 foci as this may explain the proficiency in TAR. We found that the *irs1TofZM14* cells were defective in RAD51 foci induced by ionizing radiation as compared to wild-type cells and proficient in RAD51 foci formation induced by thymidine (Figure 4B), which altogether suggests that the *irs1TofZM14* cells are still proficient in a sub-pathway of homologous recombination repair at replication forks. The deficiency in RAD51 foci formation following ionizing radiation was reverted by transient transfection with a plasmid containing wt XRCC2, while not altering the number of RAD51 foci following thymidine treatment, confirming that the XRCC2 deficiency is responsible for the lack of RAD51 foci following ionizing radiation in *irs1TofZM14* cells.

DISCUSSION

TAR is a phenomenon present in all investigated cellular organisms but, in spite of this, the proteins involved in catalysing TAR are unknown. Here, we show that

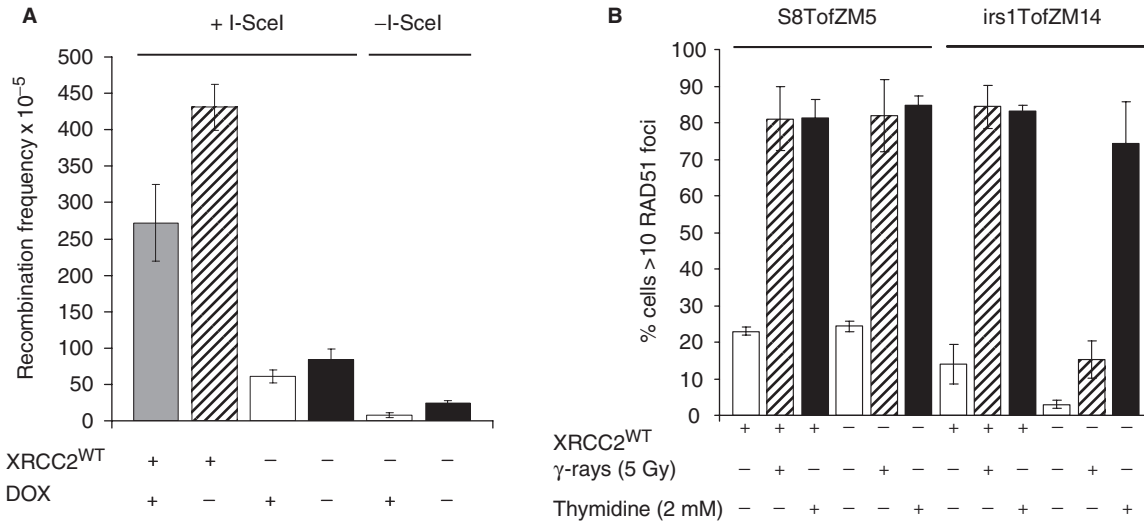


Figure 4. XRCC2 is required for homologous recombination. (A) The influence of transcription and XRCC2 on homologous recombination triggered by an I-SceI-induced DSB following transient transfection of the pCMV3xnlS-I-SceI vector was monitored in *irs1TofZM14* cells as the reversion to G418 resistant clones among 10³ surviving cells. (B) RAD51 foci were visualized in wild-type (S8TofZM5) or XRCC2 defective (*irs1TofZM14*) cells 5 h following γ -ray treatment (5 Gy) or following a 24-h thymidine (2 mM) treatment. Cells containing >10 RAD51 foci were counted as positive. The average and standard deviation of three independent experiments are depicted.

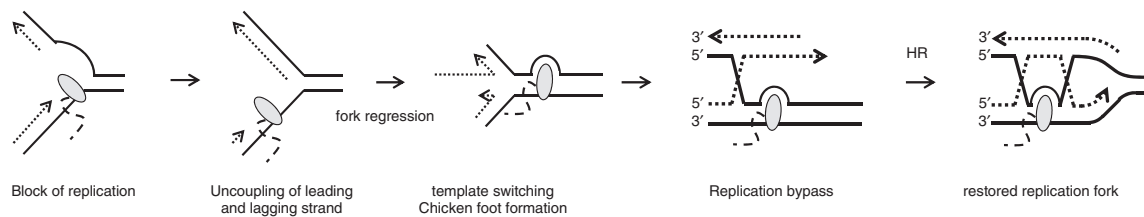


Figure 5. Model how template switching at replication forks may be used for the DNA polymerase to bypass the transcription machinery. The leading and lagging strand of replication may uncouple as the leading strand of DNA polymerase is stalled by the RNA polymerase. The replication fork may then regress and use template switching to bypass the RNA polymerase. Following the bypass the DNA end is used for replication restart using homologous recombination, which would account for the transcription-associated recombination reported in this study. This model explains how the RNA polymerase may be over taken by the DNA polymerase, as well as explaining how the XRCC2 allele in *irs1* cells are proficient for both thymidine and transcription-induced recombination.

BRCA2 is required for TAR, which strongly support that TAR involves RAD51-mediated strand invasion (34). This is an expected finding and therefore, it is more surprising to find that the XRCC2 protein is not required for TAR. The XRCC2 protein was earlier found to be important in catalysing DSB-induced homologous recombination and RAD51 foci formation after ionizing radiation (18,19,27). Here we report that the *irs1* cell line is able to catalyse TAR and that overexpression of a wild-type XRCC2 protein does not influence TAR levels, which altogether show that XRCC2 is not involved in TAR. As *irs1* cells are functional for TAR, while defective for DSB-induced homologous recombination, these experiments clearly show that the recombination induced following transcription is functionally different than homologous recombination induced by an I-SceI-induced DSB. This finding is of biological importance as the two-ended DSB formed by ionizing radiation or an endonuclease likely is a rare event *in vivo*. However, the discrepancy of the XRCC2 gene involved in one pathway, but not another, is anticipated as homologous recombination is involved in repairing several different types of lesions (33).

Using the *irs1TofZM14* and *irs1TofZM15* cell lines, we now have a system to genetically separate TAR from ordinary DSB-induced homologous recombination repair. Interestingly, it was reported that the *irs1* cell line is partially proficient for thymidine-induced homologous recombination, while defective for DSB or hydroxyurea-induced homologous recombination (30), which we can confirm to be the case also in the *irs1* cells used here. In speculation, it is possible that the thymidine-induced and transcription-induced homologous recombination occur with similar mechanisms in mammalian cells, a mechanism that appears to be largely distinct from DSB-induced homologous recombination. We have shown in several reports that thymidine causes a unique substrate for homologous recombination, which may involve template switching to bypass a lesion (33,34,35,36). The DNA polymerase is about 20 times faster than RNA polymerase II and would need to over take the RNA polymerase not to be slowed down. The model for thymidine-induced recombination may also explain how the RNA polymerase could be bypassed during replication (Figure 5). There is already strong support for TAR being connected with replication in both mammals and yeast (3,9) and it has been

shown in yeast that recombination is induced when a replication fork hits an elongating RNA polymerase (4). Also, in mammalian cells TAR is restricted to the S-phase of the cell cycle and relies on a rapidly moving replication fork (9).

In conclusion, we show a differential involvement of the BRCA2 and XRCC2 proteins in catalysing TAR, demonstrating that homology directed DSB repair and TAR employ distinct recombination pathways.

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