Introduction

Bloom’s syndrome (BS), which is caused by mutations in the BLM gene, is characterized by a predisposition to a wide variety of cancers. BS cells exhibit elevated frequencies of sister chromatid exchanges (SCEs), interchanges between homologous chromosomes (mitotic chiasmata), and sensitivity to several DNA-damaging agents. To address the mechanism that confers these phenotypes in BS cells, we characterize a series of double and triple mutants with mutations in BLM and in other genes involved in repair pathways. We found that XRCC3 activity generates substrates that cause the elevated SCE in blm cells and that BLM with DNA topoisomerase IIIα suppresses the formation of SCE. In addition, XRCC3 activity also generates the ultraviolet (UV)- and methyl methanesulfonate (MMS)-induced mitotic chiasmata. Moreover, disruption of XRCC3 suppresses MMS and UV sensitivity and the MMS- and UV-induced chromosomal aberrations of blm cells, indicating that BLM acts downstream of XRCC3.

Biochemical analyses indicated that BLM demonstrates G4 DNA unwinding, branch migration, and canonical DNA helicase activity (Bachrati and Hickson, 2003). More importantly, BLM with DNA topoisomerase IIIα (TOP3α) was shown to resolve double Holliday junctions (HJs) to yield noncrossover products (Wu and Hickson, 2003). BLM interacts physically with several proteins involved in various aspects of DNA metabolism, such as RAD51 and WRN (Yu et al., 1996; Kitao et al., 1999). In addition, BLM is a component of the BRCA1-associated genome surveillance complex (Y. Wang et al., 2000), which contains BRCA1, ATM, MRE11, RAD50, NBS1, MSH2, MSH6, and RFC, and a complex containing the five Fanconi anemia complementation proteins (FANCA, FANCG, FANCC, FANCE, and FANCF), RPA, TOP3α, and BLAP75 (Meetei et al., 2003; Yin et al., 2005). BLAP75 was recently shown to stimulate the dissolution of double HJs by BLM and TOP3α (Raynard et al., 2006; Wu et al., 2006).

Despite the accumulation of biochemical data on the activities and binding partners of BLM, little is known about its biological functions. Recently, we have reported a possible involvement of BLM and TOP3α in the dissolution of sister chromatids during the late stage of DNA replication using corresponding...
gene-disrupted chicken DT40 cells (Seki et al., 2006a). However, in BS cells, the molecular basis of the elevated frequencies of SCE, interchanges between homologous chromosomes, and their sensitivity to several DNA-damaging agents is not well understood. To address the mechanism that confers these BS cell phenotypes and to understand the functions of BLM in the cell, we used chicken DT40 cells to establish and characterize double and triple blm mutants bearing mutations in other genes involved in various repair pathways, including homologous recombination (XRCC3, RAD52, and RAD54). Based on our data, we discuss BLM function and propose an error-free lesion bypass mechanism that involves XRCC3 and BLM.

Results

Generation of various BLM-deficient and XRCC3-deficient cell lines

To investigate the function of BLM under DNA damage–inducing conditions, we generated double and triple mutants of BLM bearing mutations in genes of the RAD52 epistasis group (RAD52, RAD54, and XRCC3) that are relevant for homologous recombination, and in genes involved in postreplication repair (RAD18) and nonhomologous end-joining (KU70) using the chicken B cell line DT40 (Bezzubova et al., 1997; Takata et al., 1998, 2001; Yamaguchi-Iwai et al., 1998; Yamashita et al., 2002). We generated five mutants with XRC gene–disrupted (xrc3) cells containing human XRCC3 (hXRCC3) and EGFP transgenes, which can be deleted by activating the Cre recombinase with 4-hydroxy tamoxifen (Ishiai et al., 2004). Although growth of hXRCC3-complemented xrc3 cells was slightly slower than that of wild-type cells, the sensitivities of the cells to methyl methanesulfonate (MMS) and UV were indistinguishable from those of wild-type cells (unpublished data). Thus, we considered hXRCC3-complemented xrc3 cells to be equivalent to wild-type cells. A scheme for the systematic generation of the various double and triple mutants from xrc3 + hXRCC3 (“wild-type”) cells is shown in Fig. 1A. Gene disruption was confirmed by RT-PCR (Fig. 1B) and genomic PCR (not depicted). Generation of rad52/blm, ku70/blm, and rad18/blm cells is shown in Fig. 1C and described in Materials and methods.

Figure 1. Generation of double and triple mutants of BLM with mutations in other genes involved in DNA repair pathways. (A) Schematic representation of the generation of several mutants in a conditional xrc3 background. (B) RT-PCR analysis of total RNA from the indicated mutants in the conditional xrc3 background. [a–c] Each mutant cell line was examined for the expression of hXRCC3, WRN, RAD54, and RECQL1 mRNA. RECQL1 was amplified as a control. (d and e) Each mutant cell line was examined for the expression of hXRCC3, WRN, BLM, and RECQL1 mRNA. (C) Disruption of BLM in the indicated single gene mutants. (a) Schematic representation of the generation of mutants. (b–d) RT-PCR analysis of total RNA from the indicated mutants.
Elevated SCE in blm cells depends on XRCC3

A characteristic feature of BS cells is a high incidence of SCE. A possible explanation for this property is that BLM with TOP3α dissolves double HJs in a manner that does not produce crossovers, and that the BLM defect results in crossovers that are detected as SCE. Double HJs are formed by the activities of proteins involved in homologous recombination such as RAD51 (Ira et al., 2003; Wu and Hickson, 2003). Thus, we investigated the frequency of SCE in relevant mutant cells. As shown in Fig. 2 A, the SCE frequency in xrcc3 cells was lower than in xrcc3+/hXRCC3 cells, and the elevated SCE frequency in xrcc3/blm+/hXRCC3 ("blm") cells was greatly reduced by deletion of hXRCC3 (Fig. 2 A, bottom). We previously reported that disruption of RAD54 considerably reduces the frequency of SCE in blm cells (W. Wang et al., 2000). As expected, the xrcc3/blm/rad54+/hXRCC3 ("blm/rad54") cells generated in this study showed a lower SCE frequency than xrcc3/blm+/hXRCC3 cells (Fig. 2 B). In contrast, disruption of RAD52 did not affect the increased frequency of SCE in blm cells (Fig. 2 C).

We also examined the functional relationship between BLM and TOP3α in the suppression of SCE. As TOP3α-depleted cells exhibit lethality, we previously generated top3α and top3α/blm cells carrying a mouse Top3α transgene placed under the control of the doxycyclin-repressible promoter (Seki et al., 2006a). The top3α cells ceased to grow within 3 d after the addition of doxycyclin, and they showed an increase in SCE frequency 2 d after the treatment, as similarly observed for blm cells (Fig. 2 D). Moreover, the SCE frequency in top3α/blm cells 2 d after doxycyclin addition was almost the same as that of blm cells, indicating that TOP3α functions with BLM to suppress the formation of SCE. Notably, disruption of TOP3β did not increase the SCE frequency (Fig. 2 E).

BLM and XRCC3 belong to the same DNA-repair or damage-tolerance pathway

To identify the pathway in which BLM functions under DNA damage–inducing conditions, we performed colony survival assays of various mutant cells in the presence of MMS. Double mutant ku70/blm and rad18/blm cells were more sensitive to MMS than either single mutant (Fig. 3 A). The same tendency was also seen in rad52/blm cells.

Interestingly, the MMS sensitivity of xrcc3/blm+/hXRCC3 cells was partially suppressed by disruption of XRCC3 (Figs. 3 B, a,
and S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200702183/DC1). All clones of \(\text{xrcc3/blm}\) cells derived from the same parental \(\text{xrcc3/blm+hXRCC3}\) cells showed almost the same sensitivity to MMS (unpublished data), which excluded the possibility that the suppression of MMS sensitivity was caused by mutations occurring during mutant isolation. Thus, BLM appears to function downstream of XRCC3 under damage-inducing conditions.

It has been reported that \(\text{wrn/blm}\) cells show synergistic or additive increases in sensitivity to genotoxic agents including MMS, compared with either single mutant (Imamura et al., 2002), suggesting that BLM and WRN perform nonoverlapping functions. The MMS sensitivity of \(\text{xrcc3/wrn}\) cells was higher than that of either single mutant (Fig. 3 B, b), suggesting that WRN functions independently of XRCC3 in response to MMS-induced damage. However, the MMS sensitivity of \(\text{xrcc3/wrn/blm}\) triple mutant cells was not higher than that of \(\text{xrcc3/wrn/blm+hXRCC3}\) (“\(\text{wrn/blm}\)”) or \(\text{xrcc3/wrn}\) cells (Fig. 3 B, c), indicating that BLM and XRCC3 function in the same pathway, even in the \(\text{wrn}\) background.

In contrast to what we found for XRCC3, we previously observed that \(\text{blm/rad}\) cells show higher sensitivity to genotoxic agents, including MMS (W. Wang et al., 2000), compared with either single mutant. \(\text{xrcc/blm/rad54+hXRCC3}\) cells were similarly more MMS sensitive than either single mutant (Fig. 3 C, a). In \(S.\text{cerevisiae}\), proteins belonging to the RAD52 epistasis group, such as RAD51, RAD54, RAD55, and RAD57, are involved in recombinational repair (Sung et al., 2000). Thus, it is possible that XRCC3, a RAD51 paralogue in higher eukaryotic cells, functions in a recombinational repair pathway involving RAD54.

Therefore, we examined the MMS sensitivity of \(\text{xrcc3/rad54}\) and \(\text{xrcc3/blm/rad54}\) cells. The MMS sensitivity of the \(\text{xrcc3/rad54}\) cells was higher than that of either single mutant (Figs. 3 C, b, and S1 B), and the sensitivity of \(\text{xrcc3/blm/rad54}\) cells was almost

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**Figure 3.** Survival curves of mutant cells exposed to MMS. [A–C] Cells were treated with the indicated concentrations of MMS. Colonies were counted after 7–14 d, and the percent survival was determined relative to the number of colonies of untreated cells. Representative data are shown. The error bars indicate SD. The differences in the duplicated data were often so minute that the SD was hidden by the symbols in the figures.
the same as that ofxrcc3/blm/rad54+hXRCC3 cells (Fig. 3 C, c). Thus, the genetic data obtained here are compatible with the notion that BLM and XRCC3 function in the same DNA repair or damage tolerance pathway after MMS treatment, but probably not in the canonical recombinational repair pathway.

To investigate the functional relationship between BLM and XRCC3, we examined RAD51 focus formation after exposure to MMS (unpublished data). After MMS treatment, an increase in the number of cells exhibiting RAD51 foci was observed in bothxrcc3/blm+hXRCC3 andxrcc3+hXRCC3 cells. However, xrcc3 andxrcc3/blm cells showed little increase in the number of RAD51 foci after MMS exposure. In contrast, MMS-induced RAD51 focus formation was observed in rad52 and rad52/blm cells. These results indicated that RAD51 focus formation does not correlate with survival after exposure to MMS.

Chromosomal aberrations in blm, xrcc3, and xrcc3/blm cells induced by exposure to MMS

To understand the mechanism of the suppression of MMS sensitivity inxrcc3/blm+hXRCC3 cells after disruption ofXRCC3, we examinedxrcc3/blm, rad52/blm, and related cell lines for a variety of chromosomal aberrations (the types of chromosome aberrations analyzed are presented in Fig. S2, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200702183/DC1). As shown in Fig. 4 A, the number of chromosomal aberrations increased 12 h after exposure to MMS in all cells examined; xrcc3/blm+hXRCC3 cells had a higher number of chromosome aberrations thanxrcc3+hXRCC3 andxrcc3 cells, and the defect inxrcc3/blm+hXRCC3 cells was suppressed by deletion ofhXRCC3.

In contrast, a slight increase in chromosomal aberrations was observed inrad52/blm cells compared with either single mutant after exposure to MMS (Fig. 4 B), indicating that disruption ofXRCC3 but notRAD52 specifically suppresses the defect inblm cells.

Elevation of MMS-induced mitotic chiasmata inblm cells

We next focused our attention on chromatid exchanges. Although we observed chromatid exchanges between nonhomologous chromosomes or different regions of homologous chromosomes (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200702183/DC1), the majority of chromatid exchanges observed after exposure to MMS involved homologous chromosomes, which is a typical feature of chicken DT40 cells (Fig. 4 C). This type of chromosomal aberration is called a mitotic chiasma because it resembles the chiasma structure seen in meiosis. A slight increase of mitotic chiasma was observed inxrcc3/blm+hXRCC3 cells compared withxrcc3+hXRCC3 cells (Fig. 4 D, a). This phenotype is reminiscent of the increased interchanges between homologous chromosomes in BS cells (Chaganti et al., 1974). As shown in Fig. 4 D, MMS-induced mitotic chiasmata inxrcc3+hXRCC3 andxrcc3/blm+hXRCC3 cells were almost completely suppressed by deletion ofhXRCC3 (Fig. 4 D, a), whereas disruption ofRAD52 had no effect on the formation of mitotic chiasma (b). In chromosomes forming mitotic chiasma, it is generally held that the events of homologous recombination, but not the separation of the recombinant chromosomes linked by sister chromatid cohesion, have been completed (Fig. S3 B; Huttner and Ruddle, 1976; Therman and Kuhn, 1981). If this were the case, RAD54 would also be likely to be required for the formation of mitotic chiasmata. Indeed, RAD54 was required for mitotic chiasma formation in the presence or absence ofBLM (unpublished data).

UV sensitivity and increased chromosomal aberrations ofblm cells are suppressed by disruption ofXRCC3

As described in the previous section, we analyzedblm cells andblm-related double mutant cells after exposure to MMS. However, MMS induces a variety of DNA lesions including base alkylation and generation of single- and double-strand breaks. Thus, we examined these cells after irradiation with UV light that generates a specific lesion, thymine dimers. We also examined the sensitivity of these cells to x rays because x rays and MMS are reported to induce double-strand breaks. As shown in Fig. 5 A (a),xrcc3/blm+hXRCC3 cells were as sensitive to x rays asxrcc3+hXRCC3 cells. The sensitivity ofxrcc3/blm cells to x rays was not higher than that of either single mutant. Note that the x-ray sensitivity ofxrcc3 cells was very mild compared with that ofatm cells, which are sensitive to ionizing radiation (Takao et al., 1999). Similar results were previously reported withxrcc3+DT40 cells (Takata et al., 2001; Yonetani et al., 2005). However, hamster irsSF cells carrying a mutation inXRCC3 are reportedly sensitive to ionizing radiation (Liu et al., 1998; Hinz et al., 2005). The relatively high rate of recombination inDT40 cells may account for this inconsistency.

In contrast to their ionizing radiation sensitivity,xrcc3/blm+hXRCC3 cells were mildly UV sensitive compared withxrcc3+hXRCC3 cells, and this sensitivity was suppressed by deletion ofhXRCC3 to the level ofxrcc3 cells (Fig. 5 A, b). Chromosomal aberrations increased gradually during incubation after UV irradiation (Fig. 5 B, a). Details of various types of chromosome aberration are shown in Fig. S2 C. Chiasmata began to appear 6 h after UV irradiation and their frequency increased thereafter (Fig. 5 B, b). This type of chromosomal aberration was increased inblm cells. Deletion ofhXRCC3 suppressed various types of chromosome aberrations inxrcc3/blm+hXRCC3 cells to the level seeninxrcc3 cells. This especially concerned chromosome-type aberrations that manifest gaps or breaks at the same positions on sister chromatids (Figs. 5 C [a] and S2 D). The induction ofmitotic chiasma by UV irradiation inxrcc3/blm+hXRCC3 andxrcc3+hXRCC3 cells was almost completely suppressed by deletion ofhXRCC3 (Fig. 5 C, b).

Discussion

Cells derived from BS patients exhibit elevated levels of SCE, interchange between homologous chromosomes, and sensitivity to several DNA-damaging agents. In this paper, we performed a systematic genetic analysis of mutant chicken DT40 cells to explore the function ofBLM and identify a putative mechanism underlying the phenotype ofBS cells.

We demonstrated thatXRCC3 andRAD54 are required for the elevated levels of SCE and MMS- or UV-induced mitotic...
chiasmata observed in \textit{blm} cells. The MMS sensitivity of \textit{blm} cells was partially suppressed by disruption of \textit{XRCC3}, but not by disruption of \textit{RAD52}, \textit{RAD54}, \textit{WRN}, \textit{RAD18}, or \textit{KU70}. The suppression of \textit{blm}-associated phenotypes upon disruption of \textit{XRCC3}, particularly cell viability and increased chromosomal aberrations, was clearly evident in response to UV irradiation. Thus, the increased frequency of SCE, the sensitivities to MMS and UV, and the elevated frequency of mitotic chiasmata are caused by the function of \textit{XRCC3}.

\textbf{BLM and TOP3α negatively regulate the formation of SCE by acting on substrates generated by XRCC3}

It is noteworthy that BLM interacts with RAD51D as well as RAD51 (Wu et al., 2001; Braybrooke et al., 2003). To determine the relationship between BLM and RAD51 in SCE formation, we constructed \textit{rad51/blm + hRAD51} cells carrying a tetracycline-repressible human \textit{RAD51} gene. Because \textit{RAD51} is essential for cell viability, we could not assay the sensitivity of \textit{rad51/blm}...
cells to DNA-damaging agents. We measured spontaneous SCE frequency under conditions of reduced hRAD51 expression. Under these conditions, we found that the elevated SCE frequency in blm cells was reduced considerably (Seki et al., 2006b). Next, to examine whether RAD51D functions like XRCC3, we generated rad51d/blm cells and found that, like XRCC3, RAD51D is required for the increased SCE frequency in blm cells (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200702183/DC1).

Biochemical studies of BLM showed that it has DNA helicase and branch migration activities. More importantly, it has been reported that BLM and TOP3α can dissolve double HJs to form noncrossover products in vitro (Wu and Hickson, 2003). Thus, it is possible that double HJs are formed as a result of the ability of XRCC3, RAD51D, RAD51, and RAD54 to deal with lesions and that these structures are dissolved by BLM and TOP3α in a manner that does not result in crossovers (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200702183/DC1).

Therefore, defects in BLM, TOP3α, or both cause an increase in crossing over when HJs are resolved by certain nucleases, resulting in an increase in SCE.

A possible mechanism for the increase of mitotic chiasmata in blm cells after exposure to MMS or UV

Mitotic chiasmata are the visual manifestation of crossover recombination involving homologous chromosomes (Fig. S3 B). The increase of mitotic chiasmata in blm cells seems to be caused by a defect in a function of BLM/TOP3α that resolves recombination intermediates and prevents their crossing over. However, it is also possible that the increased incidence of mitotic chiasmata in blm cells is caused by an increase in lesions leading to homologous recombination, because the elevated...
level of mitotic chiasmata is very close to the elevated level of chromosomal aberrations.

The classical symmetrical quadriradial chromosomes often observed in human BS cells are identical to the mitotic chiasma observed in the current study. A mitotic chiasma is a product of homologous recombination between homologous chromosomes. In fact, crossover recombination events involving homologous chromosomes have been observed in BS cells that carry different mutations in the two alleles of $BLM$, as indicated by the appearance of the wild-type $BLM$ gene during cell culture (Ellis et al., 1995). The recombination between homologous chromosomes associated with crossovers may cause a loss of heterozygosity, which may underlie the predisposition to cancer observed in BS patients.

A possible mechanism for the suppression of the MMS and UV sensitivity of $blm$ cells by disruption of $XRCC3$

In a previous paper, we demonstrated that $blm$/rad54 double mutant cells are more sensitive to MMS than either of the corresponding single mutants (Fig. 3; W. Wang et al., 2000). $XRCC3$, RAD51B/C/D, and $XRCC3$ are RAD51 paralogues. In this paper, we found that disruption of $XRCC3$ but not $RAD51D$ (Fig. S4) suppresses the sensitivity of $blm$ cells to both MMS and UV irradiation, suggesting that BLM functions downstream of $XRCC3$. However, $XRCC3$ disruption exerted only a partial effect on MMS sensitivity, indicating that BLM also functions in an $XRCC3$-independent pathway. It is noteworthy that $XRCC3$ and $RAD51D$ play similar but independent roles in the response to DNA damage (Yonetani et al., 2005). Thus, the discrepancy between the effects of the $XRCC3$ deletion and the $RAD51D$ deletion on the DNA damage sensitivity of $blm$ cells could be caused by certain differences derived from these independent roles.

Mutant $xrcc3$/rad54 cells showed higher sensitivity to MMS than either of the corresponding single mutants. One possible model that could explain the data is shown in Fig. 6 A. RAD51-mediated homologous recombination is probably initiated by many homologous recombination-related proteins and RAD51 paralogues, including $XRCC3$. The functions of the RAD51 paralogues could be supported by the RAD52 backup system (Fujimori et al. 2001), and the search and invasion of homologous regions by the DNA associated with RAD51 filaments could be supported by RAD54/RAD54B. HJs could be dissolved by BLM/TOP3 to avoid the formation of crossovers. As $XRCC3$ reportedly resolves HJs, it seems possible that $XRCC3$ would also function in the resolution of HJs (Liu et al., 2004) with the formation of crossovers. Accordingly, in the absence of BLM/TOP3, $XRCC3$ would resolve HJs, resulting in increased SCE. This conjecture is supported by the observation that no high incidence of SCE is observed in $blm$/xrcc3 cells in the absence of both BLM and $XRCC3$. The higher sensitivity of the $xrcc3$/rad54 and $xrc3$/rad54 cells to MMS and UV compared with that of each mutant alone could be also explained by this model, assuming that RAD54B acts as a backup system in these circumstances. In addition, the suppression of the high SCE frequency and increased sensitivities of $blm$ cells to MMS and UV by the $XRCC3$ disruption could also be explained by this model if $XRCC3$ functions upstream of BLM. However, this model does not necessarily explain all the data obtained in this study.

However, there is another explanation for the suppression of SCE and $blm$ cell sensitivity to DNA-damaging agents by the $XRCC3$ disruption. Specific DNA damage produced by defects in BLM/TOPO could require $XRCC3$ to initiate unnecessary homologous recombination that could lead to increased sensitivity to DNA-damaging agents or an increase in SCE. If there were no such initiation, the recombination induced by the defect in $XRCC3$ wouldn’t cause cells to die or produce SCE.

We prefer the alternative model shown in Fig. 6 B to explain the suppression of the sensitivity of $blm$ cells to DNA-damaging agents by $XRCC3$ disruption. Interestingly, in fission yeast, the deletion of the human RAD51D or $XRCC2$ (another RAD51 parologue) homologues $rdl1$ or $rlp1$ suppresses the UV and MMS sensitivity of an $rhl1$ disruptant, the $S. pombe$ BLM homologue deletion mutant (Martin et al., 2006). Moreover, it is noteworthy that mutation of $SGS1$, the budding yeast homologue of $BLM$, leads to RAD51-dependent accumulation of cruciform structures when replication forks encounter DNA lesions on the template strand (Liberi et al., 2005; Branzei et al., 2006). Based on this observation, a model has been proposed in which stalled or failed replication forks are converted by RAD51 to intermediates that possess a pseudo double HJ, which is subsequently dissolved by Sgs1 and Top3 to restore the replication fork. This model proposes a novel, error-free lesion bypass system. In the context of this model, our results suggest that $XRCC3$ is involved in the formation of pseudo double HJs, which are
dissolved by BLM and TOP3α, resulting in the restoration of replication forks (Fig. 6 B). The failure to dissolve pseudo double HJs in bhm cells stabilizes these structures and results in DNA breaks, which manifest themselves as chromosomal aberrations. Disruption of XRCC3 suppresses the formation of detrimental HJs in the absence of normal BLM function, leading to suppression of MMS- and UV-induced chromosomal aberrations. XRCC3, but not RAD54, is required for slowing replication fork progression after exposure to DNA-damaging agents, such as UV and cisplatin (Henry-Mowatt et al., 2003). Although the mechanism underlying this phenomenon has not been addressed, this model provides a putative explanation. Upon activation of the XRCC3/BLM-dependent error-free lesion bypass pathway, progression of the replication fork halts until the repair process is completed. In the absence of XRCC3, replication proceeds, but not RAD54, is required for slowing replication fork progression after exposure to DNA-damaging agents, such as UV and cisplatin (Henry-Mowatt et al., 2003). Although the mechanism underlying this phenomenon has not been addressed, this model provides a putative explanation. Upon activation of the XRCC3/BLM-dependent error-free lesion bypass pathway, progression of the replication fork halts until the repair process is completed. In the absence of XRCC3, replication proceeds, bypassing DNA lesions and generating gaps, as observed in yeast cells. Remaining lesions are bypassed or repaired by translesion synthesis or other mechanisms (Lopes et al., 2006). Recently, it has been reported that the MMS and UV sensitivity of rad18 cells is also suppressed by disruption of XRCC3 (Szuts et al., 2006). Our results indicate that BLM and RAD18 function in a different pathway upon exposure to MMS (Fig. 3 A). Thus, elucidation of the relationship between the XRCC3–BLM and XRCC3–RAD18 pathways should provide a more comprehensive view of DNA lesion–avoidance systems involving XRCC3, BLM, and RAD18.

### Materials and methods

#### Cell culture and DNA transfection

Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% chicken serum (Sigma-Aldrich), and 100 μg kanamycin/ml at 39.5°C. For gene targeting, 107 DT40 cells were electroporated with 30 μg of linearized targeting constructs using a Gene Pulser apparatus (Bio-Rad Laboratories) at 550 V and 25 μF. Drug-resistant colonies were selected in 96-well plates. Genomic DNA was isolated from drug-resistant clones. Gene disruption was confirmed by RT-PCR.

For generating double or triple mutants of xrcc3 with mutations in BLM, WRN, and/or RAD54, these genes were disrupted in conditional xrcc3 cells as described in Fig. 1. After gene disruption, the hXRCC3 expression cassette was excised by Cre recombinase activated by 4-hydroxytamoxifen (Ishiai et al., 2004). The BLM gene was also disrupted in rad52, ku70, and rad18 cells. The cell strains used in this study are summarized in Table I.

#### RT-PCR analysis

Total RNA was isolated using TRIzol (Invitrogen) and converted to cDNA with Superscript III (Invitrogen). A part of each gene was amplified with Ex Taq polymerase. Primers were used to amplify hXRCC3 (sense, 5′-GAATTTGGATCTGTTGACCAGCTGAGTCAC-3′; antisense, 5′-GAGCTGCGTCGCTCGGAAGGCAGCTGATAG-3′), BLM (sense, 5′-ACAGGCTGAGTGCTCTCTGCTG-3′; antisense, 5′-CTACAGATTTGGAAGGGAGGC-3′), XRCC3 (sense, 5′-CAGTGAAATTGTAACTCAGCAT-3′; antisense, 5′-CTCAGATCTGGAAGTGG-3′), and XRCC5 (sense, 5′-CTGCGCCAGAAGGGGGGCGGAGG-3′; antisense, 5′-CTGCGCCAGAAGGGGGGCGGAGG-3′). Generation of double or triple mutants was confirmed by PCR using primers specific to each gene.

### Table I. DT40 strains used in this study

| Genotype | Disrupted gene (selective marker) | Expression plasmid | Reference |
|----------|-----------------------------------|--------------------|-----------|
| bhm      | BLM (His/Bsr)                     |                    | W. Wang et al., 2000 |
| wrn      | WRN (His/Bsr)                     |                    | Imamura et al., 2002 |
| rad52    | RAD52 (His/Bsr)                   |                    | Yamaguchi-Ikawa et al., 1998 |
| ku70     | KU70 (His/Bsr)                    |                    | Takata et al., 1998 |
| rad18    | RAD18 (His/Hyg)                   |                    | Yamaohata et al., 2002 |
| top3α    | TOP3α [Neo/Hyg]                   | hXRCC3: Neo        | Seki et al., 2006a |
| top3β    | TOP3β [Puro/Bsr]                  | hXRCC3: Neo        | Seki et al., 2006a |
| atm      | ATM (Neo/Puro)                    | hXRCC3: Neo        | Seki et al., 2006a |
| rad52/blm| RAD52 (His/Bsr), BLM (Hyg/Puro)  | hXRCC3: Neo        | This study |
| ku70/blm | KU70 (His/Bsr), BLM (Hyg/Neo)     | hXRCC3: Neo        | This study |
| rad18/blm| RAD18 (His/Hyg), BLM (Bsr/Neo)   | hXRCC3: Neo        | This study |
| top3α/blm| TOP3α [Neo/Hyg], BLM (Puro/Bsr)  | FLAG-mTOP3α: Hyg  | Seki et al., 2006a |
| top3β/blm| TOP3β [Puro/Bsr], BLM (Neo/His)  | hXRCC3: Neo        | Seki et al., 2006a |
| xrcc3    | XRCC3 [His/Bsr]                   | hXRCC3: Neo        | Ishiai et al., 2004 |
| xrcc3/bhm| XRCC3 [His/Bsr], BLM (Eco/Puro)  | hXRCC3: Neo        | Ishiai et al., 2004 |
| xrcc3/blm| XRCC3 [His/Bsr], BLM (Eco/Puro)  | hXRCC3: Neo        | Ishiai et al., 2004 |
| xrcc3/wrn| XRCC3 [His/Bsr], WRN (Eco/Puro)  | hXRCC3: Neo        | Ishiai et al., 2004 |
| xrcc3/wrn/bhm| XRCC3 [His/Bsr], WRN (Eco/Puro) | hXRCC3: Neo      | This study |
| xrcc3/wrn/bhm| XRCC3 [His/Bsr], WRN (Eco/Puro) | hXRCC3: Neo      | This study |
| xrcc3/ rad54 | XRCC3 [His/Bsr], RAD54 (Hyg/Puro) | hXRCC3: Neo      | This study |
| xrcc3/ rad54 | XRCC3 [His/Bsr], RAD54 (Hyg/Puro) | hXRCC3: Neo      | This study |
| xrcc3/ rad54 | XRCC3 [His/Bsr], RAD54 (Hyg/Puro) | hXRCC3: Neo      | This study |
| xrcc3/ rad54 | XRCC3 [His/Bsr], RAD54 (Hyg/Puro) | hXRCC3: Neo      | This study |
| xrcc3/ rad54 | XRCC3 [His/Bsr], RAD54 (Hyg/Puro) | hXRCC3: Neo      | This study |

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**Cell culture and DNA transfection**

Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% chicken serum (Sigma-Aldrich), and 100 μg kanamycin/ml at 39.5°C. For gene targeting, 107 DT40 cells were electroporated with 30 μg of linearized targeting constructs using a Gene Pulser apparatus (Bio-Rad Laboratories) at 550 V and 25 μF. Drug-resistant colonies were selected in 96-well plates. Genomic DNA was isolated from drug-resistant clones. Gene disruption was confirmed by RT-PCR.

For generating double or triple mutants of XRCC3 with mutations in BLM, WRN, and/or RAD54, these genes were disrupted in conditional xrcc3 cells as described in Fig. 1. After gene disruption, the hXRCC3 expression cassette was excised by Cre recombinase activated by 4-hydroxytamoxifen (Ishiai et al., 2004). The BLM gene was also disrupted in rad52, ku70, and rad18 cells. The cell strains used in this study are summarized in Table I.

**RT-PCR analysis**

Total RNA was isolated using TRIzol (Invitrogen) and converted to cDNA with Superscript III (Invitrogen). A part of each gene was amplified with Ex Taq polymerase. Primers were used to amplify hXRCC3 (sense, 5′-GAATTTGGATCTGTTGACCAGCTGAGTCAC-3′; antisense, 5′-GAGCTGCGTCGCTCGGAAGGCAGCTGATAG-3′), BLM (sense, 5′-ACAGGCTGAGTGCTCTCTGCTG-3′; antisense, 5′-CTACAGATTTGGAAGGGAGGC-3′), XRCC3 (sense, 5′-CAGTGAAATTGTAACTCAGCAT-3′; antisense, 5′-CTCAGATCTGGAAGTGG-3′), and XRCC5 (sense, 5′-CTGCGCCAGAAGGGGGGCGGAGG-3′; antisense, 5′-CTGCGCCAGAAGGGGGGCGGAGG-3′). Generation of double or triple mutants was confirmed by PCR using primers specific to each gene.
Measurement of spontaneous SCE
5 × 10^5 cells were cultured for two cycles in a medium containing 10 μM BrdU and pulsed with 0.1 μg/ml colcemid for 2 h. The cells were harvested and treated with 75 mM KCl for 12 min at room temperature and fixed with methanol-acetic acid (3:1) for 30 min. The cell suspension was dropped onto wet glass slides and air dried. The cells on the slides were incubated with 10 μg/ml Hoechst 33258 stain in phosphate buffer, pH 6.8, for 20 min and rinsed with MacIlvaine solution (164 mM Na2HPO4, 16 mM citric acid, pH 7.0). The cells were exposed to a black light (352 nm) at a distance of 1 cm for 30 min, incubated in 2× SSC (0.3 M NaCl and 0.03 M sodium citrate) at 58°C for 20 min, and stained with 3% Giemsa solution (Merck) for 25 min.

Measurement of sensitivity to MMS, UV, and x-rays.
To determine MMS sensitivity, 4 × 10^5 cells were inoculated into 60-mm dishes containing various concentrations of MMS in a medium supplemented with 1.5% (wt/vol) methocellose, 15% fetal bovine serum, and 1.5% chicken serum. Colonies were counted onto wet glass slides, air dried, and stained with 3% Giemsa solution, pH 6.8, for 25 min, and the cells were examined by light microscopy.

Detection of chromosomal aberrations.
Cells were treated with 0.1 μg/ml colcemid the last 2 h to increase metaphase-arrested cells and were harvested at the indicated time points. Harvested cells were treated with 75 mM KCl for 12 min at room temperature and fixed with methanol-acetic acid (3:1) for 30 min. The cell suspension was dropped onto wet glass slides, air dried, and stained with 3% Giemsa solution, pH 6.8, for 25 min, and the cells were examined by light microscopy.

To enumerate MMS- and UV-induced chromosomal aberrations, cells were cultured in the presence of 10 μM BrdU. Incorporation of BrdU into sister chromatids was used to discriminate first and second metaphase, and only chromosomal aberrations occurring during first metaphase were counted.

All images of mitotic chiasmata were collected with a camera (Cool- SNAP; Photometrics) mounted on a microscope (BX50F; Olympus). CoolSNAP version 1.2.0 (Roper Scientific) was used for image acquisition.

Online supplemental material
Fig. S1 shows survival curves of various cell lines exposed to MMS. Fig. S2 shows the classification of chromosomal aberrations. Fig. S3 shows mitotic classes. Fig. S4 shows characterization of rad51b/dm1 cells. Fig. S5 is a schematic model of SCE formation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200702183/D1C.

This work was supported by grants-in-aid for scientific research on priority areas from the Ministry of Education, Culture, Sports, Science and Technology.

Submitted: 28 February 2007
Accepted: 6 September 2007

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