Mre11 is essential for the maintenance of chromosomal DNA in vertebrate cells

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Yeast Mre11 functions with Rad50 and Xrs2 in a complex that has pivotal roles in homologous recombination (HR) and non-homologous end-joining (NHEJ) DNA double-strand break (DSB) repair pathways. Vertebrate Mre11 is essential. Conditionally, *MRE11* null chicken DT40 cells accumulate chromosome breaks and die upon Mre11 repression, showing frequent centrosome amplification. Mre11 deficiency also causes increased radiosensitivity and strongly reduced targeted integration frequencies. Mre11 is, therefore, crucial for HR and essential in mitosis through its role in chromosome maintenance by recombinational repair. Surprisingly perhaps, given the role of Mre11 in yeast NHEJ, disruption of NHEJ by deletion of *KU70* greatly exacerbates the effects of *MRE11* deficiency, revealing a significant Mre11-independent component of metazoan NHEJ.

Keywords: chromosomal aberration/double-strand break repair/DT40/homologous recombination/Mre11

Introduction

Double-strand breaks (DSBs) occur frequently during DNA replication and are induced by ionizing radiation. There are at least two repair pathways for DSBs: non-homologous end-joining (NHEJ) and homologous recombination (HR)-mediated recombinational repair. NHEJ joins DSBs without regard to sequence homology and requires the Ku proteins. Repair of DSBs by HR

requires the presence of homologous duplex DNA genome, and genes from elsewhere in the *RAD52* epistasis group in yeast (Shinohara *et al.*, 1992). These two pathways are highly conserved from yeast to humans (Boulton and Jackson, 1996a,b; Siede *et al.*, 1996; Bezzubova *et al.*, 1997; Essers *et al.*, 1997). Whereas vertebrate cells appear to favor an NHEJ pathway for DSB repair, HR is the primary mechanism of DSB repair in yeast. In fact, most genes for HR were identified by their role in the repair of ionizing radiation-induced DNA damage in yeast (Shinohara *et al.*, 1992). Although the NHEJ and HR pathways are essentially independent of one another, the genes *MRE11*, *RAD50* and *XRS2* are involved in both pathways in *Saccharomyces cerevisiae* (Haber, 1998).

The initial steps in DSB repair by HR involve processing of DNA ends to produce 3' single-strand tails, the substrates for homologous pairing and strand invasion (White and Haber, 1990). Rad51 and Rad54, among others, appear to act during the homologous pairing stage of the reaction which follows exonucleolytic processing by forming a nucleoprotein filament along the 3' single-stranded tails. The identity of the nuclease (or nucleases) in this processing step has remained elusive, but recent studies have suggested Mre11 as a candidate for this activity (Furuse *et al.*, 1998; Paull and Gellert, 1998, 1999; Trujillo *et al.*, 1998; Usui *et al.*, 1998; Moreau *et al.*, 1999). Yeast *mre11*, *rad50* and *xrs2* mutants exhibit a very similar phenotype characterized by ionizing radiation sensitivity, delayed processing of DSBs generated by the HO nuclease, retarded growth rates, elevated levels of spontaneous mitotic recombination between heteroalleles in diploids and defects in telomere maintenance and the formation of meiosis-specific DSBs (Ajimura *et al.*, 1993; Boulton and Jackson, 1998). Ogawa and colleagues (Johzuka and Ogawa, 1995; Usui *et al.*, 1998) have shown that Mre11 interacts physically with both Rad50 and Xrs2, which is consistent with the similarity between these mutant phenotypes.

The NHEJ pathway joins the two ends of a DSB through a process that is largely independent of terminal DNA sequence homology and produces junctions that can vary in their sequence composition. NHEJ in *S.cerevisiae* requires a large number of genes, including *RAD50*, *XRS2*, *MRE11*, *KU70*, *KU80* and *LIG4* (Boulton and Jackson, 1996a,b; Moore and Haber, 1996; Teo and Jackson, 1997). Whereas the Mre11–Rad50–Xrs2 complex appears to participate in recombinational repair as a nuclease, it is not clear how these proteins are involved in NHEJ (Haber, 1998). In addition, the involvement of Mre11 in NHEJ has not been demonstrated in vertebrate cells.

Mre11 and Rad50 are highly conserved proteins also found in *Schizosaccharomyces pombe* (Tavassoli *et al.*, 1995), mouse and human cells (Petrini *et al.*, 1995). Recently, the human homolog of *XRS2*, *NBS1*, was found

Fig. 1. Generation of *MRE11*^{-/-} clones. (A) Schematic representation of partial restriction map of the *GdMRE11* locus, the two gene-disruption constructs and the configuration of the targeted loci. Black boxes indicate the positions of exons. Relevant *EcoRI* restriction enzyme sites are shown. (B) Southern blot analysis of wild-type (+/+), heterozygous mutant (+/-) and homozygous mutant (-/-) clones. *EcoRI*-digested genomic DNA was hybridized with the probe DNA shown in (A). (C) Western blot analysis of chicken Mre11 expression. Cell extracts were separated by SDS-PAGE and transferred to a filter. Mre11 was visualized by incubation with rabbit anti-human Mre11 serum (Dolganov *et al.*, 1996) and subsequent binding of a labeled second antibody. (D) Suppression of Mre11 expression from the transgene. Whole-cell lysates were prepared from *MRE11*^{-/-} cells at the indicated times following the addition of tetracycline. Western blot analysis was performed as in (C).

to be mutated in Nijmegen breakage syndrome (NBS; Carney *et al.*, 1998; Matsuura *et al.*, 1998; Varon *et al.*, 1998), the symptoms of which include a defect in the cellular DNA-damage response, radiation sensitivity and immunodeficiency (Weemaes *et al.*, 1981; van der Burgt *et al.*, 1996). To date, the precise role of Mre11 in higher eukaryotes has not been clarified because depletion of Mre11 is lethal in animal cells (Xiao and Weaver, 1997). To investigate the role of Mre11 in vertebrate cells, we conditionally constructed Mre11-null clones by generating *MRE11*^{-/-} cells expressing a tetracycline (tet)-repressible chicken *MRE11* (*GdMRE11*) transgene, using the hyper-recombinogenic chicken B-cell line DT40 (Buerstedde and Takeda, 1991). Phenotypic analysis of these mutant clones revealed that Mre11 plays an important role in the recombinational repair of spontaneous DSBs during DNA replication, as well as in the repair of DSBs induced by ionizing radiation.

Results

Generation of *MRE11*^{-/-} and *MRE11*^{-/-}/*KU70*^{-/-} clones

Targeted integration of the *MRE11* deletion constructs (Figure 1A) disrupts the reading frame of the *MRE11*

sequence from amino acids 98 to 385. Targeting events were examined by Southern blot analysis of *EcoRI*-digested DNA for the appearance of the diagnostic 5 kb band, as well as the disappearance of the wild-type 7 kb band (Figure 1B). We generated two *MRE11*^{-/-} clones conditionally expressing either chicken Mre11 (*GdMRE11*) or carrying a His₆ tag at its N-terminus (*His-GdMRE11*). Western blotting with anti-Mre11 antiserum (Dolganov *et al.*, 1996) showed that the level of Mre11 expression in these transgenes was 10- to 20-fold higher than that of endogenous Mre11 (Figure 1C). The level of transgene expression was reduced by 10-fold 12 h after the addition of tet, and became undetectable at 24 h (Figure 1D). RT-PCR revealed that the *GdMRE11* transcript level was reduced by two orders of magnitude 3 days after the addition of tet (data not shown). Since cells expressing the two *MRE11* transgenes showed essentially the same phenotype, we show data from the clone carrying the wild-type *GdMRE11* transgene. We generated *MRE11*^{-/-}/*KU70*^{-/-} clones from an *MRE11*^{-/-} clone expressing the wild-type transgene.

Proliferative properties of Mre11-deficient cells

The proliferative properties of wild-type, *MRE11*^{-/-} cells and *MRE11*^{-/-}/*KU70*^{-/-} cells in the absence of tet (Mre11⁺) were monitored using growth curves and cell-cycle analysis. The growth curve of the *MRE11*^{-/-} Mre11⁺ cells was indistinguishable from that of wild-type cells, which divided approximately every 8 h. In contrast, *MRE11*^{-/-}/*KU70*^{-/-} Mre11⁺ clones grew slightly more slowly than wild-type cells, as shown in Figure 2A. Fluorescence-activated cell sorting (FACS) analysis showed that the cell-cycle pattern was essentially the same in these three types of cells [Figure 2B, compare wild-type with tet(-), Mre11⁺].

Next, we examined cell growth and viability following the addition of tet to the medium. *MRE11*^{-/-} cells were capable of proliferating for several rounds of the cell cycle after the depletion of Mre11 (Figure 2A). A marked accumulation of Mre11-deficient cells at the G₂/M phase began 4 days after the addition of tet, followed by cell death at day 5 (Figure 2B). Five days after the addition of tet, the proportion of *MRE11*^{-/-} cells at G₂/M was 42%, whereas the mitotic index was 5.8% (data not shown), indicating an accumulation of cells at the G₂ phase rather than in mitosis. An earlier onset of cell death was observed in *MRE11*^{-/-}/*KU70*^{-/-} cells following tet addition (Figure 2A and B).

Mre11-deficient cells exhibit spontaneous chromosomal breaks before cell death

To investigate the cause of cell death, we analyzed the chromosomes of mitotic cells, as described previously for Rad51-deficient cells (Sonoda *et al.*, 1998). For up to 3 days after the repression of Mre11, the karyotype of *MRE11*^{-/-} cells was normal, which was in agreement with the normal kinetics of their proliferation. The level of spontaneous chromosomal breaks increased significantly 4 days after the addition of tet (Table I), when the cells began to accumulate at the G₂ phase (Figure 2B). The level of chromosomal aberrations was further increased at day 5, when the cells began to die. Thus, the presence of unrepaired DSBs in Mre11-deficient cells may result

Fig. 2. Proliferative characteristics of $MRE11^{-/-}$ and $MRE11^{-/-}/KU70^{-/-}$ cells. **(A)** Growth curves corresponding to the indicated cell cultures in the absence and presence of tetracyclin (tet). The data shown are the average results from two separate clones for each genotype. **(B)** Representative cell-cycle distribution of the indicated cell cultures as measured by BrdU incorporation and DNA content in flow cytometry analysis. After the indicated cells were treated with tet, they were pulse-labeled with BrdU for 10 min and subsequently stained with FITC-anti-BrdU to detect BrdU incorporation (vertical axis, logarithmic scale) and propidium iodide to detect total DNA (horizontal axis, linear scale). The upper gate identifies cells incorporating BrdU (S phase), the lower-left gate identifies G_1 cells and the lower-right gate displays G_2/M cells. The numbers show the percentages of cells falling in each gate.

Table 1. Spontaneous chromosomal aberrations

Genotypes of cells analyzed	Chromosome		Chromatid		Exchanges	Total aberrations ($\pm$ SE)
	Breaks	Gaps	Breaks	Gaps		
Wild-type	0.25	0.25	0.25	1.25	0	2 ± 0.7
$MRE11^{-/-}$						
Day 0	0	0	0.5	0.5	0	1 ± 0.7
Day 3	0	1	0	2	0	3 ± 2
Day 4	17	5	0	1	0	23 ± 5
Day 5	42	7	1.5	0	6	56 ± 5
$MRE11^{-/-}/KU70^{-/-}$						
Day 0	0	0	1	0	0	1 ± 1
Day 3	8	0	0	0	0	8 ± 4
Day 4	26	4	6	2	0	38 ± 9
Day 5	38	12	12	2	4	68 ± 12

Data are presented as the number of aberrations per 100 cells. The actual number of cells analyzed was 400 for wild-type, 200 for $MRE11^{-/-}$ cells without tet treatment, and 50 each for the other types of cells. If the numbers of cells analyzed and total chromosomal aberrations are defined as N and x , respectively, the number of total aberrations per cell $\pm$ SE is calculated as $x/N \pm \sqrt{x/N}$, based on the Poisson distribution of spontaneous chromosomal aberrations we observed previously (Sonoda *et al.*, 1998).

in the activation of a G_2/M checkpoint, as well as in subsequent cell death. The spontaneous chromosomal breaks observed were mostly of the chromosome type, where both sister chromatids are broken at the same site.

We have previously shown that HR fulfills an essential role in the repair of spontaneously occurring chromosome breaks in proliferating cells of higher eukaryotes (Sonoda *et al.*, 1998, 1999; Takata *et al.*, 1998). Since Mre11 is involved in HR in yeast, a defect in HR may be sufficient

to account for the appearance of chromosomal breaks in Mre11-deficient DT40 cells. To investigate the capability of HR in Mre11-deficient cells, we measured their gene-targeting frequencies. A gene-targeting construct for the *OVALBUMIN* locus (G418^R; Buerstedde and Takeda, 1991) was transfected into $MRE11^{-/-}$ cells 3 days after the addition of tet, when the cells were still capable of proliferating with normal kinetics. The cells were incubated with tet for another 12 h, before being plated in

tet-free media containing G418 in 96-well plates. The elimination of tet from the medium resulted in the re-expression of the *MRE11* transgene and rescued 20–30% of the *MRE11*^{-/-} cells that had been incubated with tet for 3 days. Southern blot analysis revealed that none of the 57 G418^R transfectants rescued from transiently Mre11-deficient cells had performed targeted integration, whereas targeting events were observed in 31 of 35 wild-type transfectants and in 24 of 46 *MRE11*^{-/-} Mre11⁺ transfectants (tet⁻). This finding reveals that HR is indeed severely impaired in Mre11-deficient cells. *MRE11*^{-/-}/*KU70*^{-/-} Mre11⁻ cells displayed only slightly higher levels of chromosomal aberrations than *MRE11*^{-/-} Mre11⁻ cells (Table I), suggesting that the NHEJ pathway does not play a major role in maintaining chromosomal integrity. This is in agreement with there being no increase in the level of spontaneous chromosomal aberration in cells deficient in NHEJ (Kemp and Jeggo, 1986; Darroudi and Natarajan, 1987; Takata *et al.*, 1998).

Mre11 deficiency causes elevated radiosensitivity

To investigate HR in Mre11-deficient cells further, we assessed the capacity of HR-mediated repair of DSBs induced by γ -radiation. A colony-survival assay showed that the DSB repair capacity of Mre11-expressing *MRE11*^{-/-} and *MRE11*^{-/-}/*KU70*^{-/-} cells (tet⁻) was essentially the same as that of wild-type and *KU70*^{-/-} cells (Figure 3A). We were unable to measure the radiosensitivity of Mre11-deficient cells using a simple colony-survival assay because of their limited growth capacity. Thus, we tried to evaluate the capability of DSB repair by measuring the percentage of cells that enter the next round of the cell cycle after γ -irradiation (Figure 5B) and the level of γ -irradiation-induced chromosome aberrations (Table II).

To measure the recovery of cycling cells, we carried out cell-cycle analysis by FACS on wild-type cells over time after γ -irradiation. Upon irradiation, cells accumulated in G₂/M, but not in G₁ (Figure 3B, 6 and 9 h). The lack of a G₁/S checkpoint may be caused by the absence of p53 expression in DT40 cells (data not shown), as consistently observed in other chicken cell lines (Ulrich *et al.*, 1992). Subsequently, a fraction of the cells passed through the G₂/M checkpoint and started to enter the next S phase at 12 h after γ -irradiation. At this time, *RAD54*^{-/-} (HR-deficient) and *KU70*^{-/-} (end-joining-deficient) exhibited a marked reduction in the percentages of cells in S phase compared with wild-type cells, i.e. 16% for *RAD54*^{-/-}, 29% for *KU70*^{-/-} and 34% for wild-type cells (Figure 3B). Thus, the extent of cell-cycle recovery at 12 h after γ -irradiation may reflect the capability of DSB repair. *MRE11*^{-/-} cells were γ -irradiated after treatment with tet for 3 days, and cultured with tet for another 12 h for subsequent FACS analyses. The cell-cycle recovery of such *MRE11*^{-/-} Mre11⁻ cells was consistently poor after various doses of γ -irradiation, whereas *MRE11*^{-/-} Mre11⁺ cells (tet⁻) show radiosensitivities, as measured by cell-cycle recovery, similar to that of wild-type cells (Figure 5B). This finding clearly shows an elevated radiosensitivity of Mre11-deficient cells.

The FACS analyses revealed that the kinetics of appearance of cells entering the next round of the cell cycle after γ -irradiation was comparable between wild-type and Mre11-deficient cells (data not shown), suggesting that

Fig. 3. Increased radiosensitivity of *MRE11*^{-/-} Mre11⁻ cells. (A) A colony-survival assay. The fractions of colonies surviving after γ -radiation compared with non-irradiated controls of the same genotype are shown on the y-axis in a logarithmic scale. (B) Cell-cycle distribution after 2 Gy of γ -irradiation. Wild-type cells were BrdU pulse-labeled at the indicated times after 2 Gy of γ -irradiation. The numbers given on the boxes indicate the percentages of gated events. The numbers given on the boxes indicate the percentages of cells in S phase. (C) Radioresistant DNA-synthesis assay. The relative amounts of DNA synthesis after increasing doses of γ -radiation compared with non-irradiated controls are shown. (D) Entry of the indicated clones into mitosis without and after γ -irradiation. Colcemid was added at the time of γ -irradiation and irradiated cells were incubated for the times indicated, followed by the measurement of mitotic index.

the Mre11 deficiency did not impair the S- and G₂/M-phase checkpoints. To analyze the S-phase checkpoint, we measured radiation-induced inhibition of DNA synthesis 1 h after γ -irradiation. We found that Mre11-deficient cells were indistinguishable from wild-type cells with respect to the suppression of DNA synthesis by γ -irradiation (Figure 3C). To examine the G₂/M checkpoint, we measured the mitotic index over time after γ -irradiation of Mre11-deficient cells cultured with tet for 3 days. Irradiated and non-irradiated cells were treated with colcemid, a microtubule-disrupting agent that can trap cells in mitosis for several hours (Bunz *et al.*, 1998). The fractions of mitotic cells in the non-irradiated wild-type and

Table II. Ionizing radiation-induced chromosomal aberrations

Genotypes of cells analyzed	γ dose (Gy)	Chromosome		Chromatid		Exchanges	Total aberrations ($\pm$ SE)
		Breaks	Gaps	Breaks	Gaps		
Wild-type	0.3	0	1	2	3	0	6 $\pm$ 2
<i>KU70</i> ^{-/-}	0.3	2	0	2	2	0	6 $\pm$ 2
<i>RAD54</i> ^{-/-}	0.3	6	1	20	8	1	36 $\pm$ 6
<i>KU70</i> ^{-/-} / <i>RAD54</i> ^{-/-}	0.3	12	11	130	18	1	172 $\pm$ 13
<i>MRE11</i> ^{-/-} Mre11 ⁺	0.3	1	1	15	4	2	23 $\pm$ 5
<i>MRE11</i> ^{-/-} / <i>KU70</i> ^{-/-} Mre11 ⁺	0.3	9	9	19	1	2	40 $\pm$ 6
<i>MRE11</i> ^{-/-} Mre11 ⁻	0.3	12	4	12	10	2	40 $\pm$ 9
<i>MRE11</i> ^{-/-} / <i>KU70</i> ^{-/-} Mre11 ⁻	0.3	92	8	48	4	2	154 $\pm$ 20

The number of aberrations per 100 cells is presented. At least 50 mitotic cells were analyzed in each case. The number of total aberrations per cell $\pm$ SE is calculated as $x/N \pm \sqrt{x/N}$ as in Table I.

Mre11-deficient cells increased constantly with time after the addition of colcemid to the medium (Figure 3D). After the irradiation of wild-type cells with 2 Gy of γ -rays, most of the cells did not enter mitosis for several hours. Similarly, irradiation of the Mre11-deficient cells with 2 Gy of γ -rays strongly suppressed movement of the cells toward mitosis (Figure 3D). Furthermore, an efficient G₂ arrest was also observed in Mre11-deficient cells irradiated with 0.3 Gy of γ -rays, which dose impeded cell-cycle progression at 12 h post-irradiation in Mre11-deficient cells to the same extent as did 2 Gy in wild-type cells (Figure 3D). These observations strongly suggest that the elevated radiosensitivity is caused by a defect in DSB repair, but not by impaired S or G₂/M checkpoints.

A defect in recombinational repair accounts for the increased radiosensitivity of Mre11-deficient cells

In addition to analyzing the recovery of cycling cells after γ -irradiation, we measured the level of chromosomal aberrations after γ -radiation (Table II). When asynchronous cells are irradiated, cells in the late S to G₂ phase are expected to enter mitosis within 3 h of γ -irradiation. We have previously shown that the HR pathway is used preferentially over the end-joining pathway for DSB repair during the late S to G₂ phase in DT40 cells (Takata *et al.*, 1998). Thus, DSB repair by HR can be estimated by measuring the level of chromosomal breaks up to 3 h after γ -irradiation of asynchronous cells. This notion is verified by the following previous data: the total chromosomal aberrations per 100 cells 0–3 h after 2 Gy of γ -irradiation were 26 for wild-type, 35 for Ku70-deficient cells and 280 for Rad54-deficient cells (Takata *et al.*, 1998), indicating that the majority of DSBs were indeed repaired by HR involving Rad54. To assess the capability of HR-mediated DSB repair, we measured the levels of induced chromosome aberrations by comparing *MRE11*^{-/-} Mre11⁺ cells (tet⁻) and *MRE11*^{-/-} Mre11⁻ cells incubated with tet for 3 days, when these two types of cells proliferate with the same rate. The level of chromosomal aberrations induced in *MRE11*^{-/-} Mre11⁺ cells was slightly higher than in wild-type cells (Table II), although *MRE11*^{-/-} Mre11⁺ and wild-type cells were indistinguishable with regard to radiosensitivity measured using a colony-survival assay (Figure 3A) and the recovery of the cell cycle (Figure 5B). Thus, overexpression of Mre11 may interfere somewhat with recombinational

repair. Nonetheless, chromosome analysis revealed that the level of chromosomal aberrations in Mre11-deficient cells after treatment with tet for 3 days was significantly higher than in *MRE11*^{-/-} Mre11⁺ cells (Table II). It should be noted that, at this point, the Mre11-deficient cells were capable of proliferating with normal kinetics and did not exhibit an increased level of spontaneous chromosomal aberrations (Table I). This finding indicates that a defect in HR possibly causes their elevated radiosensitivity when it does not result in the appearance of spontaneous chromosomal aberrations.

To analyze DSB repair by HR further, the kinetics of Rad51 focus formation after γ -irradiation were monitored in Mre11-deficient cells. Rad51 can catalyze the pairing and strand transfer of homologous DNA strands *in vitro*, which requires the assembly of higher order multimers of Rad51 on single-strand DNA (Kowalczykowski and Eggleston, 1994; Sung and Robberson, 1995). Single-strand tails form at the sites of DSBs *in vivo* and these tailed species accumulate in *rad51* mutants, suggesting that they are normal substrates for Rad51 assembly (Shinohara *et al.*, 1992). The assembly of Rad51 in recombinational intermediate molecules may be assessed by immunostaining subnuclear foci of Rad51 in mitotic vertebrate cells, mouse spermatocyte nuclei (Scully *et al.*, 1997b; Plug *et al.*, 1998) and meiotic nuclei of *S.cerevisiae* (Shinohara *et al.*, 1997; Gasior *et al.*, 1998). Rad51 foci can be induced by treatment with DNA-damaging agents but are also found in untreated cells during S phase (Haaf *et al.*, 1995; Tashiro *et al.*, 1996; Maser *et al.*, 1997; Scully *et al.*, 1997a; Bishop *et al.*, 1998; Plug *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1998). To assess recombinational repair in Mre11-deficient cells, we γ -irradiated *MRE11*^{-/-} cells treated with tet for 3 days, and found no pronounced difference between Mre11⁻ and Mre11⁺ cells with respect to the kinetics of Rad51 focus formation or their intensity (data not shown). Furthermore, Rad51 foci were induced by radiation in *MRE11*^{-/-} Mre11⁻ cells even 5 days after the addition of tet (Figure 4). These findings are perhaps surprising because Mre11 has been shown to affect the formation of single-strand tails at the sites of DSBs, particularly in HR during meiosis (Sugawara and Haber, 1992; Tsubouchi and Ogawa, 1998; Usui *et al.*, 1998; Moreau *et al.*, 1999). Thus, other nuclease(s) may be responsible for the resection of DSB ends induced by γ -radiation prior to Rad51 focus formation.

Fig. 4. Immunofluorescence staining of Rad51. Wild-type, *MRE11*^{-/-} *Mre11*⁺ cells and *MRE11*^{-/-} *Mre11*⁻ cells treated with tet for 5 days were analyzed 8 h after 8 Gy of γ -irradiation.

Mre11-independent end-joining

Since *Mre11* plays an essential role in NHEJ in *S. cerevisiae* (Moore and Haber, 1996; Boulton and Jackson, 1998), the capability of the end-joining pathway in *Mre11*-deficient DT40 cells was investigated. We compared the radiosensitivity of *MRE11*^{-/-} *Mre11*⁻ and *MRE11*^{-/-} *KU70*^{-/-} *Mre11*⁻ cells by analyzing cell-cycle recovery at 12 h after γ -irradiation and also by measuring chromosomal aberrations induced 0–3 h after γ -radiation, which were treated with tet for 3 days. Furthermore, we compared the radiosensitivity of *KU70*^{-/-}, *MRE11*^{-/-} *Mre11*⁺ and *MRE11*^{-/-} *Mre11*⁻ cells synchronized at G₁ to early S phase, where the Ku-dependent end-joining pathway plays a dominant role in DSB repair (Takata, 1998).

As expected, the level of recovery of *MRE11*^{-/-} *Mre11*⁺ cells was significantly higher than that of *MRE11*^{-/-} *KU70*^{-/-} *Mre11*⁺ cells, and expression of a chicken *KU70* transgene in *MRE11*^{-/-} *KU70*^{-/-} cells restored the level of their recovery to that of *MRE11*^{-/-} cells (Figure 5B). Remarkably, even in the absence of *Mre11*, *MRE11*^{-/-} *KU70*^{-/-} cells exhibited significantly higher radiosensitivity than *MRE11*^{-/-} cells (Figure 5B). Similarly, analysis of radiation-induced chromosome aberrations revealed that after 0.3 Gy of γ -irradiation, *MRE11*^{-/-} *KU70*^{-/-} cells displayed a 3.3-fold higher level of aberrations than *MRE11*^{-/-} cells. This difference is comparable with the 4.7-fold higher level of chromosome aberrations seen in *RAD54*^{-/-} *KU70*^{-/-} cells compared with *RAD54*^{-/-} cells (Table II). Thus, *MRE11*^{-/-} *Mre11*⁻ cells were significantly more radioresistant than *MRE11*^{-/-} *KU70*^{-/-} *Mre11*⁻ cells, indicating the presence of *Mre11*-independent end-joining activity in *MRE11*^{-/-} *Mre11*⁻ cells. We measured the radiosensitivity of *Mre11*-deficient cells using a colony-formation assay. Upon the addition of tet, we irradiated *Mre11*-deficient cells at day 2.5 and further incubated the cells with tet before they were plated onto tet-free methylcellulose plates at day 3. Cells were synchronized with nocodazole as described previously (Takata *et al.*, 1998) before γ -irradiation. The radiosensitivities of *MRE11*^{-/-} *Mre11*⁺ and *MRE11*^{-/-} *Mre11*⁻ cells synchronized at the G₁ to early S phase were indistinguishable with respect to radiosensitivity, although *Ku70*-deficient cells exhibited extremely high radiosensitivity at this cell-cycle phase (Figure 5C). Thus, *Ku70*-dependent DSB repair appears to work in the absence of *Mre11*.

Chromosomal aberrations are associated with abnormal mitotic divisions

We analyzed the gross morphology of mitotic *MRE11*^{-/-} cells treated with tet for 5 days, at which time the cells began to die. Double immunostaining with γ -tubulin (a component of centrosomes) and α -tubulin (a component of microtubules) revealed that about half the mitotic *MRE11*^{-/-} cells at day 5 contained multiple centrosomes (Figure 6F and G), whereas no morphological abnormality was observed in *MRE11*^{-/-} *Mre11*⁺ cells. Furthermore, the additional centrosomes were frequently associated with spindles, resulting in the formation of multiple poles (Figure 6H). Consequently, many of the mutant cells segregated their chromosomes abnormally during mitosis. This missegregation can cause unequal nuclear division, leading to the formation of multiple nuclei within a single cell (Figure 6I, J and K). It is known that such abnormal mitosis is frequently observed in γ -irradiated mammalian cells (Mantel *et al.*, 1999), as observed in wild-type DT40 cells irradiated with 8 Gy of γ -radiation, although less frequently (data not shown). Similarly, Xu *et al.* (1999) reported that multiple centrosomes were found in a substantial fraction of mouse cells deficient in exon 11 of the *BRCA1* gene, which is involved in DNA repair (Gowen *et al.*, 1998). These observations suggest that the presence of chromosome aberrations may cause multiple centrosomes through unknown mechanisms. The gross morphological abnormality observed in mitotic *Mre11*-deficient cells may reflect the presence of various types of chromosomal aberrations in addition to the cytologically visible aberrations.

Discussion

We generated *MRE11*^{-/-} cells carrying a chicken *MRE11* transgene under the control of the tet-repressible promoter. After repression of the *MRE11* transgene, elevated radiosensitivity and the reduction of HR, examined using targeted integration efficiencies, were observed, even when the cells were able to proliferate normally. Subsequently, spontaneous chromosomal aberrations and abnormal mitotic divisions were observed before cell death. These findings reveal an important role for *Mre11* in recombinational repair and chromosome integrity in vertebrate cells. In contrast, *Mre11*-deficient DT40 cells appeared to

Fig. 5. Increased radiosensitivity of *MRE11*^{-/-}/*KU70*^{-/-} *Mre11*⁻ cells compared with *MRE11*^{-/-} *Mre11*⁺ cells. (A) Cell-cycle distribution after 0.3 Gy of γ -irradiation. *MRE11*^{-/-}/*KU70*^{-/-} and *MRE11*^{-/-} cells treated with tet for 3 days (*Mre11*⁺) were BrdU pulse-labeled 12 h after 0.3 Gy of γ -irradiation. The numbers given in the boxes indicate the percentages of cells in S phase. (B) Cell-cycle recovery 12 h after γ -irradiation. The percentages of cells in S phase 12 h after γ -irradiation relative to the percentages of unirradiated cells in S phase are displayed on the y-axis. (C) Ku70-dependent DSB repair in *Mre11*-deficient cells. A colony-survival assay of cells synchronized in G₁ to early S phase. The fractions of colonies surviving after γ -radiation compared with non-irradiated controls of the same genotype are shown on the y-axis in a logarithmic scale. *MRE11*^{-/-} *Mre11*⁺ cells were irradiated 2.5 days after the addition of tet, and further incubated with tet before being plated on tet-free methylcellulose medium.

possess end-joining activity and can implement S and G₂ arrests after damage. Thus, *Mre11* deficiency causes a strikingly more severe phenotype than NBS, despite the association of *Mre11* with Nbs1 (Carney *et al.*, 1998). This observation indicates that NBS1 is dispensable for some of the functions of *Mre11*.

***Mre11* is required for recombinational repair of spontaneously occurring and radiation-induced DNA lesions in vertebrate cells**

Possible explanations for the appearance of spontaneous chromosome breaks include an impaired checkpoint, the

erosion of telomeres and a defect in DSB repair. A defect in the checkpoint is known to cause chromosome instability, as manifested in NBS. However, NBS cells exhibit chromatid-type breaks only occasionally (Taalman *et al.*, 1989; Chrzanowska *et al.*, 1995), whereas *Mre11* deficiency causes chromosome-type breaks in the majority of cells, resulting in massive cell death. Furthermore, γ -irradiation appears to stimulate the S and G₂/M checkpoints in *Mre11*-deficient cells with the same efficiency as in wild-type cells (Figure 3C and D). Thus, a defect in the checkpoints does not account for frequent chromosome-type breaks causing cell death. Erosion of telomeres might cause chromosomal aberrations through the formation of dicentric chromosomes, followed by their breakage during the metaphase–anaphase transition (Smith *et al.*, 1992). Such chromosomal breakage should result in the separation of broken pieces of a dicentric chromosome because attaching kinetochore microtubules may pull the two pieces apart, whereas broken pieces of a chromosome mostly remained in proximity in *Mre11*-deficient cells. Furthermore, neither a reduction in telomere size nor dicentric chromosomes were detectable in *Mre11*-deficient cells up to 5 days after the addition of tet (data not shown). These observations indicate that telomere erosion does not account for the spontaneous chromosome breaks in *Mre11*-deficient cells. Concerning a defect in DSB repair, a defect in HR may account for the appearance of spontaneous chromosomal breaks, as manifested in Rad51- and Rad54-deficient DT40 cells (Sonoda *et al.*, 1998; Takata *et al.*, 1998). In addition, a defect in HR in *Mre11*-deficient cells is clearly shown by their reduction in gene-targeting efficiency. Thus, a defect in DSB repair by HR is a possible cause of chromosome-type breaks in *Mre11*-deficient cells.

The appearance of spontaneous chromosome breaks in *Mre11*-deficient DT40 cells suggests the occurrence of DSBs during DNA replication and the subsequent recombinational repair of the DSBs. This conclusion has also been suggested in *Escherichia coli* and *S.cerevisiae* (Zou and Rothstein, 1997; Kobayashi *et al.*, 1998; Seigneur *et al.*, 1998). In addition, the presence of active recombinational repair of such DSBs in vertebrate cells has been suggested by the appearance of Rad51 foci in S-phase nuclei (Haaf *et al.*, 1995; Tashiro *et al.*, 1996), spontaneous sister chromatid exchanges (~10 per mitotic human cell), which are mediated by HR (Sonoda *et al.*, 1999), and spontaneous chromosomal breaks observed in HR-defective Rad51-, Rad54-, Xrcc2- and Xrcc3 cells (Liu *et al.*, 1998; Sonoda *et al.*, 1998; Takata *et al.*, 1998). Conceivably, in *Mre11*-deficient yeast cells spontaneously occurring DNA lesions are not processed appropriately during recombinational repair, resulting in poor mitotic growth associated with elevated rates of spontaneous mitotic recombination (Johzuka and Ogawa, 1995). These observations consistently show the important role of recombinational repair involving *Mre11* in the maintenance of chromosomal DNA.

***Mre11* deficiency impairs HR repair but not Ku70-dependent end-joining**

Mre11-deficient cells exhibited elevated radiosensitivity even when they proliferated without displaying spontaneous chromosomal aberrations. Since they showed

Fig. 6. Centrosome amplification in Mre11-deficient cells. (A–H) Cells were immunostained with γ -tubulin for centrosomes (green), α -tubulin for centrosomes (red) and DAPI-stained for DNA (blue). Right- and left-hand photographs in each figure are the same cell but displayed differently (C–K). Wild-type interphase (A and B) and mitotic (C) cells, both containing two centrosomes. The transition from metaphase (D) to anaphase (E) of wild-type cells. (F–K) Mre11-deficient cells treated with tet for 5 days exhibit gross morphological abnormality. (F) Interphase cell with four centrosomes. (G) Mitotic cell with four centrosomes. (H) A cell stained with α -tubulin reveals that amplified centrosomes can nucleate spindle poles. (I–K) Multiple centrosomes might cause abnormal segregation of chromosomes during mitosis. The left-hand photographs are stained with DAPI for DNA (white). (I) Four centrosomes might result in cytokinesis of two daughter cells each carrying two nuclei. (J) An interphase cell exhibits three nuclei. (K) A mitotic cell with genetic material left in the midbody as a result of abnormal segregation of chromosomes. All cells are shown at the same scale; bar = 10 μ m.

apparently normal S and G₂/M checkpoints (Figure 3C and D), defective DSB repair possibly explains this elevated radiosensitivity. Defective DSB repair may be caused by impaired HR, as suggested by the defective gene-targeting capability. In addition, defective HR repair may explain the dramatic increase in the radiosensitivity of *MRE11*^{−/−}/*KU70*^{−/−} cells compared with *MRE11*^{−/−} cells, which is similar to the marked difference in radiosensitivity between *RAD54*^{−/−}/*KU70*^{−/−} and *RAD54*^{−/−} cells (Takata *et al.*, 1998). Furthermore, as discussed above, the appearance of many chromosome-type breaks after irradiation at G₂ supports a defect in HR repair in the absence of Mre11.

To assess the capability of end-joining in Mre11-deficient cells we analyzed their radiosensitivity at G₁ to early S (Figure 5C), and compared the radiosensitivity of *MRE11*^{−/−} and *MRE11*^{−/−}/*KU70*^{−/−} cells (Figure 5B; Table II). Since the NHEJ and HR pathways play redundant roles in DSB repair at late S to G₂, cells deficient in both pathways are profoundly more radiosensitive than cells deficient in either pathway (Takata *et al.*, 1998). Thus, if Mre11 deficiency causes a defect in HR, but not in NHEJ, *MRE11*^{−/−}/*KU70*^{−/−} Mre11^{−/−} cells, which would be deficient in the NHEJ and HR pathways, should be significantly more radiosensitive than *MRE11*^{−/−} Mre11^{−/−} cells. The data consistently indicated the presence of a Ku-dependent pathway in the absence of Mre11, most clearly in the G₁–early S phase. Efficient end-joining activity in Mre11^{−/−} DT40 cells may be surprising, given the essential role of Mre11/Rad50/Xrs2 in NHEJ in *S.cerevisiae*. This finding may suggest that an extremely low amount of Mre11 is sufficient for proficient end-joining. Alternatively, Mre11 is not a unique pathway for end-joining in vertebrate cells. We postulate that the latter interpretation is more likely

because a *S.pombe* mutant defective in Rad32, a homolog of Mre11, exhibits a nearly normal level of end-joining (K.Manolis and P.Jeggo, personal communication). Thus, the role of Mre11 in end-joining may differ depending on the species. Nonetheless, it is not clear that Mre11-deficient cells possess the full activity of end-joining. For technical reasons, we were unable to measure the activity of end-joining directly by, for example, analyzing V(D)J recombination.

Mre11 may be required for processing DSB ends for HR

DSB repair by HR requires the presence of homologous sequences elsewhere in the genome, e.g. a homologous chromosome or a sister chromatid. In yeast, the HR pathway can repair induced DSBs by using the other intact homologous chromosome as well as the sister chromatid (Moore and Haber, 1996). In contrast, the contribution of allelic recombination to DSB repair is relatively low in vertebrate cells, whereas the HR pathway involving Rad54 may repair a chromatid DSB by using the intact sister chromatid as a template (Takata *et al.*, 1998). Accordingly, after the induction of chromatid breaks by γ -irradiation in the late S to G₂ phase, Rad54 deficiency results in an increased level of chromatid-type breaks rather than chromosome-type breaks (Takata *et al.*, 1998; Figure 7). It is thus surprising that γ -irradiation of Mre11-deficient cells in the late S to G₂ phase induces a substantial fraction of chromosome-type breaks (Table II). This finding suggests that, in the absence of Mre11, chromatid breaks induced by the γ -irradiation of late S to G₂ phase cells are converted into chromosome-type breaks, where both sister chromatids are broken at the same sites (Figure 7).

Fig. 7. Model of HR-mediated repair of spontaneous DSBs and those induced by γ -irradiation. Chemical and base damage of genomic DNA is usually repaired by the base-excision repair pathway. When a replication fork encounters such damage, which is shown by a closed triangle, replication of the damaged strand is arrested, possibly resulting in a chromatid break. Similarly, γ -irradiation of cells at late S to G₂ causes chromatid breaks, which are associated with chemical adducts at their broken ends. The absence of DSB repair would result in chromatid-type breaks in mitotic cells. If homologous recombination of a chromatid break with the sister chromatid is initiated, there are three possible outcomes: abortive homologous pairing resulting in a chromatid-type break, inappropriate processing of the recombinational intermediate molecule resulting in a chromosome-type break, as observed in *Mre11*-deficient cells, or successful recombinational repair.

Conceivably, in γ -irradiated *Mre11*-deficient cells, broken chromatids may be able to form Holliday junctions (Holliday, 1964) by interacting with the other intact chromatid. Subsequently, the intermediate molecules are not processed appropriately, resulting in DSBs in both chromatids. However, it remains to be determined whether *Mre11* is involved after, as well as prior to, Holliday junction formation. The absence of a single-strand resection at DSBs by *Mre11* might result in the impaired processing of Holliday junctions if *Mre11* is required to remove chemical adducts induced by γ -irradiation, as is essential for removing Spo11p, a type II topoisomerase covalently attaching to broken ends during HR in yeast meiosis (Usui *et al.*, 1998; Moreau *et al.*, 1999). This is in agreement with the finding that although *Mre11*-deficient cells are highly sensitive to γ -irradiation and MMS, which often chemically modify broken DNA ends, they are proficient in repairing the HO endonuclease-induced DSBs (Tsubouchi and Ogawa, 1998).

This model might also explain the appearance of spontaneous chromosome-type breaks in Rad51- and *Mre11*-deficient cells (Sonoda *et al.*, 1998), where DSBs occur on a chromatid during DNA replication (Zou and Rothstein, 1997; Kobayashi *et al.*, 1998; Seigneur *et al.*, 1998) and, subsequently, abnormal HR of the broken chromatid with the intact sister chromatid results in the induction of a DSB on both sister chromatids (Figure 7).

Materials and methods

Plasmid constructs

A chicken *MRE11* (GdMRE11) partial cDNA fragment was amplified by RT-PCR using chicken testis RNA with degenerate primers designed from amino acid sequences conserved (GNHDD and WGHEHC) between ScMRE11 and hMRE11. Subsequent 5' and 3' RACE with chicken testis RNA were carried out to isolate cDNA containing the entire open reading frame (ORF) of GdMRE11. To construct the *MRE11* expression plasmid, the GdMRE11 ORF was inserted into an expression vector containing a tet-repressible promoter, pUHG10-3 (a gift from Prof. H. Bujard, Heidelberg, Germany). Approximately 13 kb of part of the chicken genomic *MRE11* locus were isolated from DT40 genomic DNA using long-range PCR. To construct chicken *MRE11* disruption plasmids, ~5 kb of genomic DNA corresponding to amino acids 98–385 of GdMre11 were replaced with hisD- or bsr-selection marker cassettes (Sugawara *et al.*, 1997) to make *MRE11*-his or *MRE11*-bsr, respectively.

Cell culture, DNA transfections and irradiation

Cells were cultured in RPMI-1640 medium supplemented with 10^{-5} M β -mercaptoethanol, penicillin, streptomycin, 10% fetal calf serum (FCS) and 1% chicken serum (Sigma, St Louis, MO) at 39.5°C. DNA transfections and selection were performed as described previously (Buerstedde and Takeda, 1991). Tetracyclin hydrochloride (1 μ g/ml tet; Sigma, St Louis, MO) was used to suppress the expression of the *MRE11* transgene. To measure cell-cycle, phase-specific radiation sensitivity, cells were synchronized with nocodazole (Sigma) as described previously (Takata *et al.*, 1998). To enrich cells at prometaphase, cells were cultured for 7 h in medium containing 0.5 μ g/ml nocodazole, then washed three times with pre-warmed medium. Cells were irradiated 4 h after nocodazole treatment when cells were at G₁ and early S. Irradiated cells were incubated for another 12 h before plating onto methylcellulose plates. *MRE11*^{-/-} cells were irradiated when they had been incubated with tet for 2.5 days. They were subsequently incubated with tet for 12 h before being washed three times with pre-warmed medium and plated onto tet-free methylcellulose plates. The colony-forming efficiency of non-irradiated *Mre11*-deficient cells was 10–20%. The percentage of cell survival was calculated assuming that the cell number did not change during this 12 h period. γ -irradiation was performed using ¹³⁷Cs (0.02 Gy/s; Gammacell 40, Nordion, Kanata, Ontario, Canada).

Western blot analysis

Cells (10^6) were washed with phosphate-buffered saline (PBS) and lysed in 20 ml SDS lysis buffer (25 mM Tris-HCl pH 6.5, 1% SDS, 0.2 M dithiothreitol, 0.1% Bromophenol Blue, 5% glycerol). Following boiling, aliquots (routinely 50%) were subjected to 7.5% SDS-PAGE. After transfer to a nitrocellulose membrane, proteins were detected by polyclonal rabbit anti-human *Mre11* or Rad51 polyclonal sera (Tashiro *et al.*, 1996; Carney *et al.*, 1998) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (Amersham Pharmacia, London, UK) using Super Signal™ CL-HRP Substrate System (Pierce, Rockford, IL).

Cell-cycle analysis

Cells were labeled for 10 min with 20 mM bromodeoxyuridine (BrdU; Amersham, Buckinghamshire, UK). They were then harvested and fixed at 4°C overnight with 70% ethanol, and incubated as follows: (i) in 4 N HCl, 0.5% Triton X-100 for 30 min at room temperature; (ii) in fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Pharmingen, San Diego, CA) for 1 h at room temperature; (iii) in 5 mg/ml propidium iodine in PBS. Between each incubation, cells were washed with PBS containing 2% bovine serum albumin (BSA) and 0.1% sodium azide. Subsequent flow cytometric analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA). Fluorescence data were displayed as dot-plots using the Cell Quest software (Becton Dickinson).

DNA synthesis assays

Mre11-deficient cells were treated with tet for 3 days before γ -irradiation. Cells plated at 2.3×10^5 /ml in complete medium were irradiated with γ -rays. After 1 h incubation, cells were labeled with 1 μ Ci/ml of [³H]thymidine for 15 min. Cells in 4 ml of medium were fixed by adding 2 ml of a freshly prepared 3:1 mixture of methanol:acetic acid. Each sample was spotted onto filter paper strips (Ready filter™ with Xtalscint™; Beckman, Fullerton, CA). Each filter strip was washed sequentially with a 3:1 mixture of methanol:acetic acid, 5% trichloroacetic acid (TCA) and ethanol at each step and dried using an infrared

lamp. Filter strips were placed into scintillation vials. Averages and standard deviations of the c.p.m. values were determined from triplicate samples.

Chromosome aberration analysis and morphological analysis of mitotic cells

Karyotype analysis was performed as described previously (Sonoda *et al.*, 1998). For the morphological analysis of chromosome aberrations, cells were treated with colcemid for 3 h to enrich mitotic cells. The mitotic index was measured after the fixation of cells using the same method as for karyotype analysis, followed by taking photographs of fixed cells and counting cells containing distinct chromosomes.

To analyze the morphology of mitotic cells, chicken DT40 cells were fixed with 100% methanol (−30°C) for 10 min, washed twice with PBS and incubated in a blocking buffer, 1% BSA in PBS, for at least 1 h at room temperature. The cells were then incubated with monoclonal antibodies (mAb) against α -tubulin (TAT1; a gift of Dr Keith Gull, University of Manchester, UK; 1:50 dilution) or γ -tubulin (Sigma; 1:500 dilution) for 20 h at 4°C, washed twice with PBS and incubated with Cy-2-conjugated goat anti-mouse IgG antibody (Amersham; 1:100 dilution) for 4 h at room temperature. After washing twice with PBS, the cells were incubated with PBS containing 1 mg/ml 4',6-diamidino-2-phenylindole (DAPI) and 2.5% 1,4-diazabicyclo-2,2,2-octane (DABCO), and were analyzed by fluorescence microscopy as described below. Details of the microscope system have been described previously (Haraguchi *et al.*, 1997). In brief, fluorescence microscope images were obtained with an Olympus IX-70 inverted microscope using an oil-immersion objective lens (PlanApo 60 $\times$, NA = 1.4) and high-selectivity filters for Hoechst 33342, Cy-2 and Texas Red (Chroma Technology, Brattleboro, UT). Serial optical section data (15–30 focal planes at 0.5 mm intervals) for DNA, α -tubulin or γ -tubulin were collected on a Peltier-cooled charge-coupled device (Photometrics, Tucson, AZ), and processed computationally using a three-dimensional deconvolution method (Agard *et al.*, 1989).

Measurement of Rad51 foci

Formaldehyde-fixed cells were permeabilized with 0.1% NP-40 (Sigma) and incubated with rabbit anti-human Rad51 antiserum (Tashiro *et al.*, 1996). Staining was visualized by FITC-conjugated anti-rabbit IgG (Santa Cruz) and cells were mounted under Slow Fade solution (Molecular Probes, Eugene, OR). All images were taken by confocal microscopy (MRC-1024, Bio-Rad, Cambridge, MA) and processed using Adobe Photoshop version 4.0J.

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