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Homologous and non-homologous recombination differentially affect DNA damage repair in mice

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Ionizing radiation and interstrand DNA crosslinking compounds provide important treatments against cancer due to their extreme genotoxicity for proliferating cells. Both the efficacies of such treatments and the mutagenic potential of these agents are modulated by the ability of cells to repair the inflicted DNA damage. Here we demonstrate that homologous recombination-deficient *mRAD54*$^{-/-}$ mice are hypersensitive to ionizing radiation at the embryonic but, unexpectedly, not at the adult stage. However, at the adult stage *mRAD54* deficiency dramatically aggravates the ionizing radiation sensitivity of severe combined immune deficiency (*scid*) mice that are impaired in DNA double-strand break repair through DNA end-joining. In contrast, regardless of developmental stage, *mRAD54*$^{-/-}$ mice are hypersensitive to the interstrand DNA crosslinking compound mitomycin C. These results demonstrate that the two major DNA double-strand break repair pathways in mammals have overlapping as well as specialized roles, and that the relative contribution of these pathways towards repair of ionizing radiation-induced DNA damage changes during development of the animal.

Keywords: DNA-dependent protein kinase/DNA double-strand breaks/DNA end-joining/DNA interstrand crosslinks/ionizing radiation

Introduction

DNA double-strand breaks (DSBs) are extremely genotoxic DNA lesions, because they cause problems for DNA transcription, replication and segregation. Improper processing of DSBs gives rise to chromosomal instability that can result in carcinogenesis through activation of proto-oncogenes or inactivation of tumor suppressor genes. DSBs are caused by exogenous sources such as ionizing radiation, and by endogenous sources such as radicals generated during metabolic processes. In addition, it is likely that a predominant source of DSBs in dividing cells is the process of DNA replication itself (Kogoma, 1997; Cox, 1998; Haber, 1999). The adverse effects of DSBs have triggered the evolution of multiple pathways for their repair. The two major pathways are homologous recombination and DNA end-joining (Kanaar *et al.*, 1998; Tsukamoto and Ikeda, 1998). The fundamental difference between these pathways is their dependence on DNA homology and accuracy of repair. In general, homologous recombination ensures accurate repair by using the undamaged sister chromatid or homologous chromosome as a template. DNA end-joining, on the other hand, uses no or extremely limited sequence homology to rejoin ends in a manner that need not be error free. Both major pathways can be divided into subpathways that can result in different outcomes of DSB repair and might require common as well as subpathway-specific genes. Homologous recombination includes single-strand annealing, gene conversion and break-induced replication (Paques and Haber, 1999). DNA end-joining includes precise end-joining and microhomology-directed end-joining (Critchlow and Jackson, 1998).

Mutants in the *Saccharomyces cerevisiae RAD52* epistasis group display hypersensitivity to ionizing radiation and are defective in DSB repair through homologous recombination (Petes *et al.*, 1991; Game, 1993; Smith and Nicolas, 1998). Key proteins in the *RAD52* epistasis group are Rad51, Rad52 and Rad54. Biochemical analyses have shown that the central events of recombination, homologous DNA pairing and strand exchange are mediated by Rad51 (Sung, 1994; Bianco *et al.*, 1998), and that both the Rad52 and Rad54 proteins can stimulate the recombination activities of the Rad51 protein (Sung, 1997; New *et al.*, 1998; Petukhova *et al.*, 1998; Shinohara and Ogawa, 1998). The functional significance of DSB repair through homologous recombination is underscored by the conservation of the *RAD52* pathway from fungi to humans. Mammalian homologs of *RAD51*, *RAD52* and *RAD54* have been identified (Petrini *et al.*, 1997; Baumann and West, 1998; Kanaar *et al.*, 1998) and the human Rad51, Rad52 and Rad54 proteins have been shown to possess similar activities to their yeast counterparts (Baumann *et al.*, 1996; Mortensen *et al.*, 1996; Reddy *et al.*, 1997; Benson *et al.*, 1998; Petukhova *et al.*, 1998, 1999; Shinohara and Ogawa, 1998; Sugiyama *et al.*, 1998; Swagemakers *et al.*, 1998; Tan *et al.*, 1999). While homologous recombination is the predominant DSB repair pathway in bacteria and yeast, DNA end-joining is believed to be the principal DSB repair pathway in vertebrate cells (Kanaar *et al.*, 1998). However, experiments using cells containing a combination of mutations effecting both DNA repair pathways revealed that homologous recombination and DNA end-joining can both contribute to repair of ionizing radiation-induced DNA damage in *S.cerevisiae*, *Drosophila melanogaster* and chicken cells (Siede *et al.*,

1996; Takata *et al.*, 1998; Kooistra *et al.*, 1999). Genes involved in DNA end-joining include *XRCC4*, *Ku70*, *Ku80* and *DNA-PK$_{cs}$* (Critchlow and Jackson, 1998; Jeggo, 1998; Lieber, 1999). The *DNA-PK$_{cs}$* gene, encoding the catalytic subunit of DNA-dependent protein kinase (DNA-PK), is defective in severe combined immune deficiency (*scid*) mice (Blunt *et al.*, 1995; Kirchgessner *et al.*, 1995; Peterson *et al.*, 1995). The *scid* mutation confers immunodeficient and ionizing radiation hypersensitive phenotypes due to deficiencies in processing DSB intermediates during development of the immune system and repair of ionizing radiation-induced DNA damage (Schuler and Bosma, 1989; Fulop and Phillips, 1990; Biedermann *et al.*, 1991; Hendrickson *et al.*, 1991; Smith and Jackson, 1999).

We have shown previously that *mRAD54$^{-/-}$* embryonic stem (ES) cells are hypersensitive to ionizing radiation and the DNA interstrand crosslinking agent mitomycin C. The *mRAD54$^{-/-}$* ES cells display a reduced level of homologous recombination compared with wild-type ES cells (Essers *et al.*, 1997). These results have revealed that in addition to DNA-PK-mediated DNA end-joining, homologous recombination can contribute to the repair of ionizing radiation-induced DNA damage in mammalian cells. In contrast to homozygous disruption of other genes implicated in DSB repair through homologous recombination, including *mRAD51*, *BRCA1* and *BRCA2* (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996; Sharan *et al.*, 1997; Zhang *et al.*, 1998; Moynahan *et al.*, 1999), disruption of *mRAD54* results in viable mice (Essers *et al.*, 1997). Thus, *mRAD54$^{-/-}$* mice provide the opportunity to study the biological relevance of homologous recombination in mammalian DNA damage repair.

Results and discussion

mRAD54$^{-/-}$ mice are hypersensitive to ionizing radiation at the embryonic but not the adult stage

To determine whether the ionizing radiation hypersensitive phenotype of *mRAD54$^{-/-}$* ES cells was also displayed by *mRAD54$^{-/-}$* mice, we treated 2- to 4-month-old *mRAD54*-proficient and -deficient mice with different doses of ionizing radiation. Four groups of five *mRAD54$^{-/-}$* and five *mRAD54$^{+/-}$* mice were irradiated with doses of 6, 7, 7.5 and 8 Gy, respectively. Unexpectedly, only one *mRAD54$^{-/-}$* mouse irradiated with 7 Gy died within 3 weeks, whereas none of the mice irradiated with 6 Gy and 7.5 Gy died. Irradiation with 8 Gy was lethal for all mice, irrespective of *mRAD54* status (data not shown).

We subsequently investigated whether this dramatic difference in ionizing radiation hypersensitivity between *mRAD54$^{-/-}$* ES cells and mice might be due to a difference in genetic background between the ES cells and the mice. The *mRAD54$^{-/-}$* ES cells are derived from a 129 mouse strain (Essers *et al.*, 1997), while the *mRAD54$^{-/-}$* mice were C57Bl6/129 hybrids. Possibly, the difference in genetic background could mask the effect of *mRAD54* disruption in the mice. To test this hypothesis, we isolated *de novo* ES cells from the *mRAD54$^{-/-}$* C57Bl6/129 mice. The C57Bl6/129-derived *mRAD54$^{-/-}$* ES cells showed a sensitivity to ionizing radiation similar to 129 *mRAD54$^{-/-}$* ES cells (Figure 1A). Expression of the human *RAD54* (*hRAD54*) cDNA in the C57Bl6/129 *mRAD54$^{-/-}$* ES cells corrected the ionizing radiation sensitivity of the cells to the level of 129 *mRAD54*-proficient ES cells. We conclude that *mRAD54$^{-/-}$* mice are not hypersensitive to ionizing radiation and that this lack of ionizing radiation hypersensitivity compared with that of *mRAD54$^{-/-}$* ES cells is not due to a difference in genetic background. Interestingly, opposite results have been obtained for *DNA-PK$_{cs}$*. *DNA-PK$_{cs}^{-/-}$* ES cells are not ionizing radiation hypersensitive,

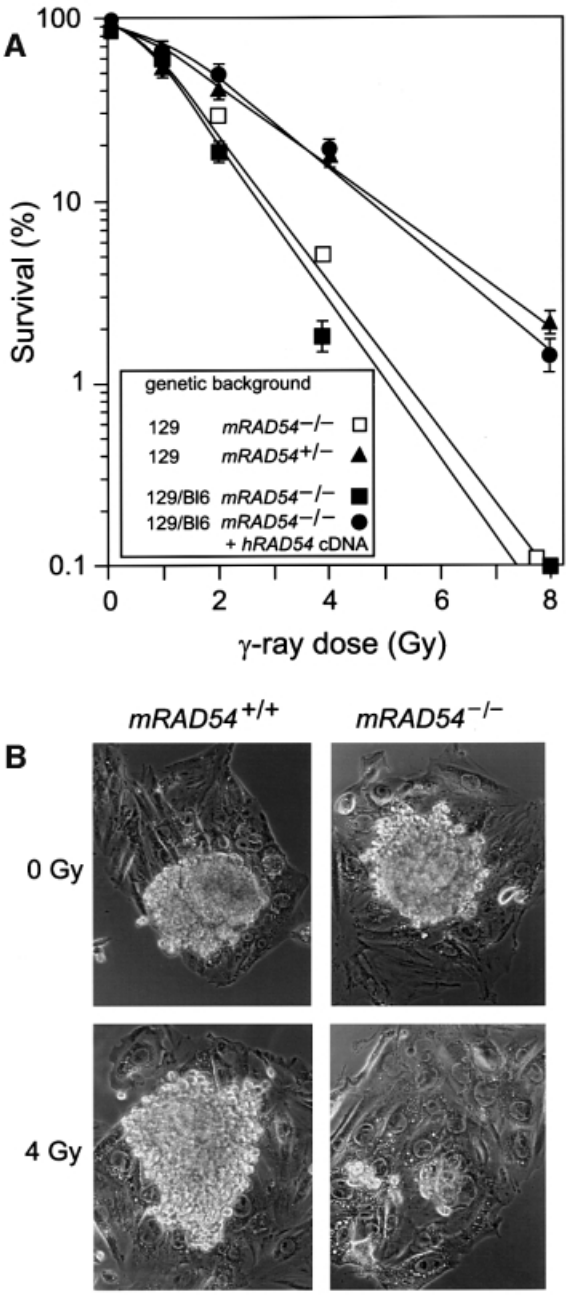

Fig. 1. Effect of ionizing radiation on ES cells and day 3.5 embryos. (**A**) Effect of ionizing radiation on 129 and C57Bl6/129 *mRAD54*-proficient and -deficient ES cells. The percentage of surviving cells as measured by their colony-forming ability is plotted as a function of ionizing radiation dose. The 129 *mRAD54*-deficient ES cells were generated by gene targeting (Essers *et al.*, 1997). The C57Bl6/129 *mRAD54*-deficient ES cells were isolated *de novo*. A *RAD54*-proficient derivative of this ES cell line was generated by selecting a cell line that stably expressed an *hRAD54* cDNA construct. (**B**) After isolation, day 3.5 embryos were irradiated with an ionizing radiation dose of 0 and 4 Gy and cultured for 10 days. Outgrowth of the inner cell mass and trophoblast cells from the embryos was determined.

Table I. The absence of mRad54 results in ionizing radiation hypersensitive embryos

Outgrowth of inner cell mass from day 3.5 embryos		
Dose (Gy)	Genotype	
	mRAD54$^{+/+}$	*mRAD54*$^{-/-}$
0	100% (3/3)	93% (14/15)
4	77% (10/13)	<7% (0/13)

Quantitation of the results shown in Figure 1B. *mRAD54*-proficient and -deficient embryos were treated with an ionizing radiation dose of 0 or 4 Gy and cultured for 10 days. The percentage of embryos showing outgrowth of the inner cell mass was obtained by dividing the number of embryos showing outgrowth of the inner cell mass and the trophoblast cells by the number of embryos showing outgrowth of the trophoblast cells only. Absolute numbers are shown in parentheses.

whereas *scid* mice are (Biedermann *et al.*, 1991; Gao *et al.*, 1998).

Next, we determined whether, in contrast to the adult stage, *mRAD54* deficiency resulted in ionizing radiation hypersensitivity at the embryonic stage. We isolated *mRAD54*$^{-/-}$ and *mRAD54*$^{+/+}$ embryos at day 3.5 of gestation. Embryos were treated with ionizing radiation doses of 0 or 4 Gy and subsequently cultured for 10 days (Figure 1B). Without irradiation the percentage of embryos showing outgrowth of the inner cell mass and trophoblast cells was similar for both wild-type and mutant embryos (Table I). After exposure to 4 Gy the percentage of wild-type embryos showing outgrowth of the inner cell mass was slightly reduced. However, after 10 days the inner cell mass of all 13 irradiated *mRAD54*$^{-/-}$ embryos was completely ablated. The presence of trophoblast cells provided a control for initial attachment of the embryos to the culture dish. Thus, in contrast to adult *mRAD54*$^{-/-}$ mice, *mRAD54*$^{-/-}$ embryos are hypersensitive to ionizing radiation.

Disruption of mRAD54 augments the ionizing radiation hypersensitivity of scid mice

The lack of a severe ionizing radiation hypersensitivity of *mRAD54*$^{-/-}$ mice compared with *mRAD54*$^{-/-}$ ES cells could be explained if *mRAD54* ceases to function after a certain developmental stage; either because its function is restricted to embryonic cells or because homologs of *mRAD54* might be able to substitute for its function later during development (Dresser *et al.*, 1997; Klein, 1997; Shinohara *et al.*, 1997; Hiramoto *et al.*, 1999). Alternatively, in contrast to the embryonic stage, the role of *mRAD54*-dependent DSB repair could be masked by the DNA end-joining pathway at later developmental stages. To discriminate between these possibilities, the ionizing radiation sensitivity of *scid* mice and *scid/mRAD54*$^{-/-}$ mice was compared. Consistent with previous observations (Biedermann *et al.*, 1991), half of the *scid/mRAD54*$^{+/-}$ mice died after irradiation with 3 Gy, while all mice survived a dose of 2 Gy. In contrast, even at the low irradiation dose of 2 Gy all *scid/mRAD54*$^{-/-}$ mice died within 2 weeks (Figure 2). Therefore, *scid/mRAD54*$^{-/-}$ mice are extremely radiosensitive, even more so than *Ku80*$^{-/-}$ and *ATM*$^{-/-}$ mice (Barlow *et al.*, 1996; Nussenzweig *et al.*, 1997). We conclude that *mRAD54* is

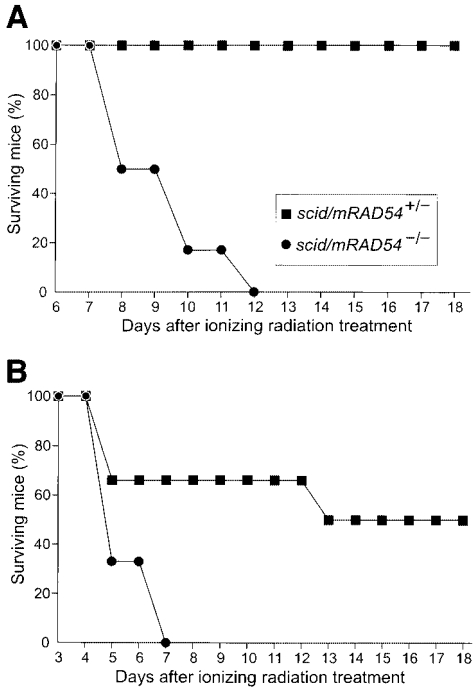

Fig. 2. Ionizing radiation sensitivity of *scid/mRAD54*$^{+/-}$ and *scid/mRAD54*$^{-/-}$ mice. Shown are survival curves of two groups of six *scid/mRAD54*$^{+/-}$ and six *scid/mRAD54*$^{-/-}$ mice after irradiation with 2 Gy (**A**) and 3 Gy (**B**), respectively. Each curve represents three male and three female mice.

functional in adult mice and that its role in radioprotection is masked by the DNA-PK-dependent DNA end-joining pathway.

To determine whether the extreme radiosensitivity of the *scid/mRAD54*$^{-/-}$ mice was due to global radiation toxicity or to selective toxicity of specific organs, *scid/mRAD54*$^{+/-}$ and *scid/mRAD54*$^{-/-}$ mice were irradiated with 2 Gy and tissues were examined histologically at 0.12, 1, 2, 3, 4, 9 and 11 days post-irradiation. No histological abnormalities were found in the majority of tissues, including brain, heart, kidney, liver and lung. A complete list of tissues investigated can be found in Materials and methods. Moderate to severe radiation-induced damage in the form of apoptotic cells was detected in stomach, colon, jejunum, ileum and rectum. These tissues completely recovered from the radiation effects after 9 days, although the kinetics of recovery, as measured by bromodeoxy uridine (α-BrdU) incorporation, was delayed in the absence of *mRAD54* (data not shown). In contrast, a dramatic difference in the effect of the irradiation on the bone marrow of *scid/mRAD54*$^{+/-}$ and *scid/mRAD54*$^{-/-}$ mice was observed (Figure 3). Two days after ionizing radiation exposure an equally severe depletion of cells in the bone marrow of *mRAD54*-proficient and -deficient mice was apparent. The cellularity of the bone marrow of *scid/mRAD54*$^{+/-}$ mice completely recovered, as was evident from the hyper-proliferation 9 days after the irradiation (Figure 3). In contrast, the bone marrow of *scid/mRAD54*$^{-/-}$ mice remained devoid of cells even at 11 days postirradiation. Similar effects on cellular depletion and lack of recovery to those observed in the bone marrow were seen in the spleen (data not shown). We conclude that *scid/mRAD54*$^{-/-}$ mice do not display a global

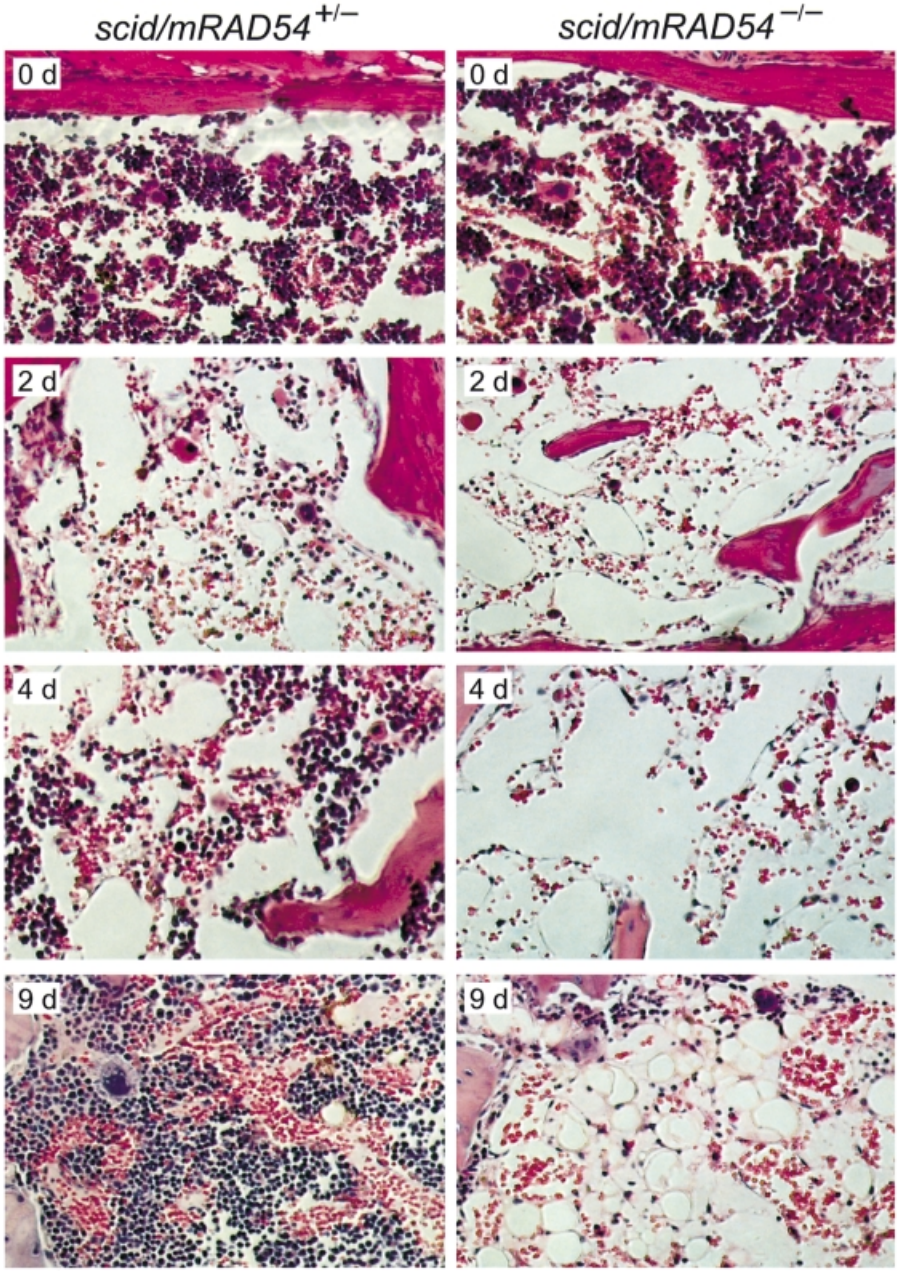

Fig. 3. Histological appearance of bone marrow after ionizing radiation exposure. Two-month-old *scid/mRAD54*$^{+/-}$ and *scid/mRAD54*$^{-/-}$ mice were irradiated with 2 Gy and euthanized at 0.12, 1, 2, 3, 4, 9 and 11 days postirradiation. Haematoxylin/eosin-stained sections of bone marrow before (day 0) and postirradiation (day 2, 4 and 9) are shown. Two days after irradiation (2 d), the depletion of cells seen in the bone marrow of *mRAD54*-proficient and -deficient *scid* mice was comparable. Four days after irradiation, the cellularity of the bone marrow of *scid/mRAD54*$^{+/-}$ mice was recovering (4 d). In contrast, no recovery was observed in the bone marrow of *scid/mRAD54*$^{-/-}$ mice (4 d and 9 d).

radiation toxicity, rather their extreme radiosensitivity results from effects of bone marrow failure.

Adult mRAD54$^{-/-}$ *mice are hypersensitive to mitomycin C*

The demonstration that the contribution of *mRAD54* towards repair of ionizing radiation-induced DNA damage is revealed in a *scid* background eliminates the possibility that *mRAD54* expression is shut off at a certain developmental stage (Figure 2). Our observation generates the question of whether disruption of *mRAD54* in an otherwise repair-proficient background causes hypersensitivity of adult mice towards DNA-damaging agents other than ionizing radiation. A critical difference between the homologous recombination-deficient *mRAD54*$^{-/-}$ ES cells and DNA end-joining-deficient *scid* cells is their sensitivity to mitomycin C. While *mRAD54*$^{-/-}$ ES cells are hypersensitive to mitomycin C, cells derived from *scid* mice are not (Biedermann *et al.*, 1991; Hendrickson *et al.*, 1991; Essers *et al.*, 1997). Therefore, we investigated the mitomycin C sensitivity of *mRAD54*-proficient and -deficient mice. After peritoneal injection of 10 and 7.5 mg of mitomycin C per kg body weight, half of the *mRAD54*$^{-/-}$ female and male mice died within 2 weeks (Figure 4A and C). All *mRAD54*$^{+/+}$ mice survived the treatment. Enhanced sensitivity and shorter latency periods in *mRAD54*$^{-/-}$ mice

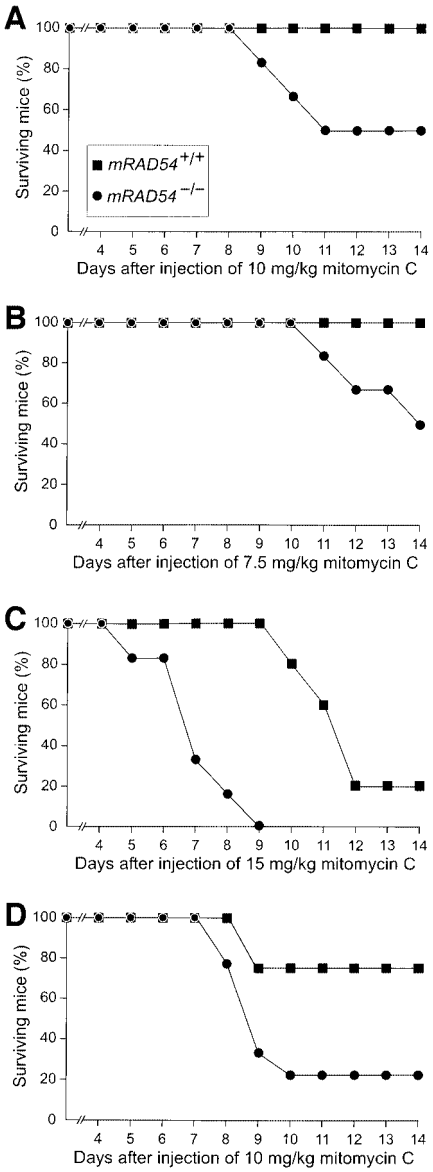

Fig. 4. Mitomycin C sensitivity of *mRAD54*$^{-/-}$ mice. Shown are survival curves of *mRAD54*$^{+/-}$ and *mRAD54*$^{-/-}$ mice after a single intraperitoneal injection of the amount of mitomycin C indicated. (**A**) Survival curve of six *mRAD54*$^{+/-}$ female and six *mRAD54*$^{-/-}$ female mice after injection at day 0 with a dose of 10 mg/kg mitomycin C. (**B**) Survival curve of five *mRAD54*$^{+/-}$ female and six *mRAD54*$^{-/-}$ female mice after injection with 15 mg/kg mitomycin C. (**C**) Survival curve of eight *mRAD54*$^{+/-}$ male and nine *mRAD54*$^{-/-}$ male mice after injection with 7.5 mg/kg mitomycin C. (**D**) Survival curve of six *mRAD54*$^{+/-}$ male and eight *mRAD54*$^{-/-}$ male mice after injection with 10 mg/kg mitomycin C.

were seen at higher mitomycin C doses (Figure 4B and D). We conclude that *mRAD54*$^{-/-}$ mice are hypersensitive to mitomycin C.

The bone marrow is a major target for mitomycin C-inflicted damage *in vivo*. Therefore, we tested whether mitomycin C treatment differentially affected cells in the blood of *mRAD54*$^{+/-}$ and *mRAD54*$^{-/-}$ mice using the peripheral blood micronucleus assay. The presence of micronuclei in polychromatic erythrocytes provides a measure of chromosomal aberrations. Two-month-old *mRAD54*$^{-/-}$ and *mRAD54*$^{+/-}$ mice were injected with 1.0, 2.5 or 5.0 mg/kg mitomycin C. This treatment resulted in

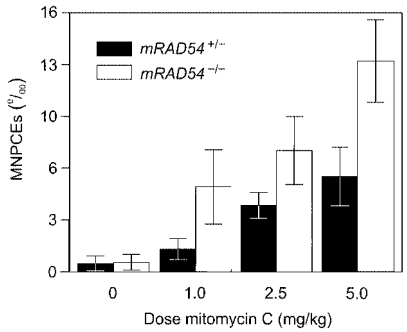

Fig. 5. Induction of micronuclei by mitomycin C in polychromatic erythrocytes. *mRAD54*$^{+/-}$ and *mRAD54*$^{-/-}$ mice were intraperitoneally injected with a single dose of 1.0, 2.5 and 5.0 mg/kg bodyweight mitomycin C. At 24 h before and 48 h after mitomycin C treatment, 25 µl of peripheral blood were collected by orbita puncture. For each animal, 1000 polychromatic erythrocytes were observed and the number of cells with micronuclei was recorded. Plotted are the number of micronuclei-containing polychromatic erythrocytes (MNPCEs) per 1000 polychromatic erythrocytes. Data points represent an average from four independently treated animals. The standard error of the mean is indicated.

dose-related increases in the frequency of micronuclei-containing polychromatic erythrocytes (Figure 5). Before the mitomycin C treatment micronuclei-containing polychromatic erythrocyte levels were similar in *mRAD54*$^{-/-}$ and *mRAD54*$^{+/-}$ mice. Consistent with the mitomycin C hypersensitivity of *mRAD54*$^{-/-}$ mice, the crosslinking agent induced significantly higher levels of micronuclei-containing polychromatic erythrocytes in *mRAD54*$^{-/-}$ mice compared with *mRAD54*$^{+/-}$ mice (Figure 5).

Overlapping and specialized roles of homologous recombination and DNA end-joining in mice

Our results show that *mRAD54*-mediated homologous recombination and DNA-PK-mediated DNA end-joining have overlapping roles in providing ionizing radiation resistance in mice. At the cellular level it has been shown that both DSB repair pathways contribute to repair of ionizing radiation-induced DNA damage in a specialized immunological cell-type derived from the chicken (Takata *et al.*, 1998). For metazoan embryos similar results have been reported using *D.melanogaster* (Kooistra *et al.*, 1999). The pivotal insight gained from the study presented here is that in mammals the relative contribution of the two major DSB repair pathways, homologous recombination and DNA end-joining, changes during development of the animal.

While homologous recombination provides protection against ionizing radiation-induced DNA damage in embryos its contribution in adults is not detected, unless DNA end-joining is disabled (Figures 1 and 2). In this regard the mouse embryo resembles *S.cerevisiae* cells (Siede *et al.*, 1996). We consider three possible reasons for these findings. First, the major contribution of homologous recombination to ionizing radiation resistance early in mouse development, as opposed to in the adult animal, might be due to the greater efficiency of homologous recombination in rapidly dividing cells because of the availability of the sister chromatid as a repair template in the S and/or G_2 phases of the cell cycle (Sonoda *et al.*, 1999) or because the presence of active components of the DNA replication machinery is necessary to complete

homologous recombination efficiently. A second reason for a preference of homologous recombination over DNA end-joining for repair of a DSB might be found in the difference in repair fidelity between the pathways. Accurate repair, ensured by homologous recombination but not by DNA end-joining, might be more important for cells early in development compared with terminally differentiated somatic cells. Inaccurate repair could be more easily tolerated by differentiated somatic cells because a large fraction of their genome is no longer functional. For a similar reason homologous recombination could be the preferred mechanism of DSB repair in a unicellular organism such as *S.cerevisiae* because most of its genome contains coding information.

A third reason for the existence of at least two separate DSB repair pathways is their specialized function. Although their functions can overlap for the processing of certain types of DNA damages such as those produced by ionizing radiation, they also have pathway-specific functions. DNA end-joining is involved in processing the DSB intermediates required for proper immunoglobulin and T-cell receptor gene expression (Critchlow and Jackson, 1998; Jeggo, 1998; Lieber, 1999), whereas *mRAD54*-mediated homologous recombination is not (Essers *et al.*, 1997). In contrast, our experiments show that *mRAD54* protects adult mice from the deleterious effects of the interstrand DNA crosslinking agent mitomycin C (Figures 4 and 5), while experiments with cells derived from *scid* mice suggest that *DNA-PK*$_{cs}$ does not appear to contribute to the repair of mitomycin C-induced DNA damage (Biedermann *et al.*, 1991; Hendrickson *et al.*, 1991). The underlying reason for these observations might be the greater versatility of homologous recombination compared with DNA end-joining. DNA end-joining has evolved to deal specifically with DSBs. Homologous recombination on the other hand can also repair DNA lesions that do not necessarily involve a DSB intermediate, as is thought to be the case during interstrand DNA crosslink repair in *Escherichia coli* (Friedberg *et al.*, 1995). Although some interstrand DNA crosslinks are processed into DSBs (Vock *et al.*, 1998), it is not certain whether this is the case for a mitomycin C-induced interstrand DNA crosslink. Even if it was processed into a DSB, the DNA end-joining pathway will not be optimally suited for its repair. While homologous recombination can bypass a crosslink starting from a single DSB to one side of the crosslink, DNA end-joining cannot. The latter pathway will require more or less simultaneously produced DSBs on either side of the crosslink before it can be repaired in an error-prone manner.

In summary, we have demonstrated that *mRAD54*$^{-/-}$ mice are not hypersensitive to ionizing radiation, while *mRAD54*$^{-/-}$ embryos are. However, at the adult stage *mRAD54* deficiency exacerbates the ionizing radiation sensitivity of *scid* mice. In contrast, regardless of developmental stage, *mRAD54*$^{-/-}$ mice are hypersensitive to mitomycin C. These results suggest that the relative contribution of two major DSB repair pathways, homologous recombination and DNA end-joining, can differ depending on mammalian developmental stage (i.e. cell type) and on the specific type of DNA damage.

Materials and methods

Cell culture

ES cells, isolated from a 129 mouse strain, were cultured and electroporated as described (Weeda *et al.*, 1997). To isolate *de novo* C57Bl6/129 ES cells (Hogan *et al.*, 1994) *mRAD54*$^{-/-}$ blastocysts were isolated from a C57Bl6/129 *mRAD54*$^{-/-}$ cross. These *mRAD54*$^{-/-}$ mice were littermates from an *mRAD54*$^{+/-}$ cross, obtained by backcross three of an *mRAD54* chimera to C57Bl6 mice. The *PGK-hRAD54* construct (Swagemakers *et al.*, 1998) was electroporated into C57Bl6/129 *mRAD54*$^{-/-}$ ES line *mRAD54*$^{307neo/neo}$ (Essers *et al.*, 1997). Both disrupted *mRAD54* alleles in this line contain the neomycin selectable marker gene. The *PGK-hRAD54* construct was co-electroporated with a plasmid carrying a puromycin selectable marker. Puromycin resistant clones were isolated and screened for hRad54 expression by immunoblot analysis as described (Essers *et al.*, 1997; Swagemakers *et al.*, 1998).

Generation of scid/mRAD54$^{+/-}$ and scid/mRAD54$^{-/-}$ mice

mRAD54$^{+/-}$ mice (backcross three of a 129 *mRAD54* chimera to C57Bl6 mice; Essers *et al.*, 1997) were bred with homozygous mutant C.B.-17 *scid* mice (Jackson Laboratories) to obtain F1 *mRAD54*$^{+/-}$/*scid*$^{+/-}$ offspring. The *scid* mutation was fixed by crossbreeding F1 animals. Homozygosity for the *scid* mutation was confirmed by determining the level of the IgM subclass in the serum as described (Essers *et al.*, 1997). *mRAD54* status was determined by PCR analysis of tail DNA.

DNA damage sensitivity assays

The sensitivity of ES cells to increasing doses of ionizing radiation was determined by measuring their colony-forming ability after irradiation with a ^{137}Cs source (Essers *et al.*, 1997). To determine the ionizing radiation sensitivity of mice, 2- to 4-month-old animals were exposed to ionizing radiation from a ^{137}Cs source. Single doses between 2 and 8 Gy were used. Ionizing radiation sensitivity of day 3.5 embryos was determined as described (Sharan *et al.*, 1997; Luo *et al.*, 1999). To determine the mitomycin C sensitivity of mice, 2-month-old *mRAD54*$^{+/+}$ and *mRAD54*$^{-/-}$ animals were injected intraperitoneally with 7.5, 10 or 15 mg/kg bodyweight mitomycin C. After exposure to ionizing radiation or mitomycin C, mice were kept in sterile isolators and observed for 28 and 14 days, respectively. After these periods surviving animals were euthanized. In none of the assays employed to date was a difference observed between *mRAD54*$^{+/+}$ and *mRAD54*$^{+/-}$ cells and mice (data not shown).

Micronucleus assay

Two-month-old *mRAD54*$^{+/-}$ and *mRAD54*$^{-/-}$ animals were injected intraperitoneally with 1.0, 2.5 or 5.0 mg/kg bodyweight mitomycin C. At 24 h before and 48 h after mitomycin C treatment, 25 µl of peripheral blood were collected by orbita puncture. The blood was placed on an acridine orange-coated glass slide, covered with a coverslip, and allowed to stain (Hayashi *et al.*, 1990). Erythrocytes with a red fluorescing reticulum in the cytoplasm were observed. One thousand erythrocytes were observed per animal by fluorescence microscopy within a few days of slide preparation. The number of cells with micronuclei displaying greenish yellow fluorescence was recorded.

Pathological analysis

Collected tissues were fixed in 10% buffered formalin, embedded in paraffin blocks, sectioned, and stained using standard methods, including haematoxylin/eosin staining and α-BrdU immunohistochemistry. Sections were examined and photographed using a light microscope. After irradiation with 2 Gy no histological abnormalities were found in adrenal gland, bone, bladder, brain, cartilage, heart, kidney, liver, lung, pancreas, pituitary gland, prostate, thyroid, tongue, salivary gland, seminal vesicles, skeletal muscle, skin and smooth muscle.

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