Xrcc2 is required for genetic stability, embryonic neurogenesis and viability in mice

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Repair of DNA damage by homologous recombination has only recently been established as an important mechanism in maintaining genetic stability in mammalian cells. The recently cloned *Xrcc2* gene is a member of the mammalian *Rad51* gene family, thought to be central to homologous recombination repair. To understand its function in mammals, we have disrupted *Xrcc2* in mice. No *Xrcc2*^{-/-} animals were found alive, with embryonic lethality occurring from mid-gestation. *Xrcc2*^{-/-} embryos surviving until later stages of embryogenesis commonly showed developmental abnormalities and died at birth. Neonatal lethality, apparently due to respiratory failure, was associated with a high frequency of apoptotic death of post-mitotic neurons in the developing brain, leading to abnormal cortical structure. Embryonic cells showed genetic instability, revealed by a high level of chromosomal aberrations, and were sensitive to γ -rays. Our findings demonstrate that homologous recombination has an important role in endogenous damage repair in the developing embryo. *Xrcc2* disruption identifies a range of defects that arise from malfunction of this repair pathway, and establishes a previously unidentified role for homologous recombination repair in correct neuronal development.

Keywords: apoptosis/embryonic lethality/knockout mouse/neuronal development/*Rad51*-like gene

Introduction

Accurate repair of the genome underpins the correct development and genetic stability of multicellular organisms. Several pathways of DNA repair in mammals have been recognized, but the influence of repair by homologous recombination has only recently been highlighted (Kanaar *et al.*, 1998; Liang *et al.*, 1998). Homologous recombination repair (HRR) entails the invasion of an undamaged DNA molecule by a damaged molecule of identical or very similar sequence, followed by resynthesis of the damaged region using the undamaged molecule as a template. A sister chromatid may be used as the template for repair, or less frequently the paternal and maternal copies of chromosomes provide the required homology. HRR is important for the repair of severe types of DNA damage, such as double-strand breaks, and allows the replacement of damaged regions without loss or alteration

of base sequence. However, there are other pathways that can rejoin double-strand breaks in DNA. In mammalian cells, DNA end-joining processes, which do not rely on sequence homology (non-homologous end joining, NHEJ), have an important role in break repair but do not ensure its fidelity (Jeggo, 1998).

HRR has been studied extensively in the yeast *Saccharomyces cerevisiae*, where the *RAD52* group of genes controls this pathway. These genes specify proteins including Rad51p, which is a homologue of the bacterial recA protein and is central to the DNA pairing and strand invasion steps of HRR (Shinohara and Ogawa, 1995; Baumann and West, 1998; Thacker, 1999a). Additionally, the Rad51-like proteins Rad55p and Rad57p form a heterodimer that promotes homologous pairing reactions (Sung, 1997). Other proteins such as Rad52p and Rad54p interact with Rad51p and stimulate recombination. In yeast, none of the HRR genes is essential for survival, but their mutation gives rise to sensitivity to DNA-damaging agents such as X-rays (Game, 1993).

Mammalian counterparts of the *RAD52* group of genes, including *RAD51*, *RAD52* and *RAD54*, have been identified in the last few years (Shinohara *et al.*, 1993; Muris *et al.*, 1994; Kanaar *et al.*, 1996), and recently a number of novel *RAD51*-like genes have been found (Thacker, 1999b). We recently identified the human *RAD51*-like gene *XRCC2* by positional cloning, making use of its ability to complement a DNA damage-sensitive hamster cell line, *irs1* (Tambini *et al.*, 1997). The *irs1* line is sensitive to various DNA-damaging agents, including X-rays, UV light and mitomycin-C (Jones *et al.*, 1987), and shows elevated frequencies of spontaneous and radiation-induced gene mutation, chromosomal damage and chromosomal missegregation (Tucker *et al.*, 1991; Thacker *et al.*, 1994; Griffin *et al.*, 2000). Importantly, *irs1* has also been shown to be defective in recombination of homologous substrates arranged tandemly in genomic DNA (Johnson *et al.*, 1999; A.M.George and J.Thacker, unpublished). Cloning of the full-length cDNA (Cartwright *et al.*, 1998; Liu *et al.*, 1998) has allowed preliminary functional analysis of the *XRCC2* gene product, to show that it interacts (Braybrooke *et al.*, 2000; Schild *et al.*, 2000) and forms a heterodimer (Braybrooke *et al.*, 2000) with the product of another *Rad51*-like gene, *RAD51L3*. In multiple alignments, the *Xrcc2* protein has greatest homology to Rad55p (Thacker, 1999b), and, therefore, it is possible that *Xrcc2* acts with its partner in the same way as the yeast Rad55p–Rad57p heterodimer to facilitate Rad51-dependent recombination processes.

Disruption of the *Rad51* gene in mice was shown to give early embryonic lethality (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996), perhaps because of its key role in HRR and the need for high-fidelity repair in replicating cells of the

Fig. 1. Disruption of *Xrcc2*. (A) Diagram of the wild-type *Xrcc2* genomic locus, targeting construct and the targeted locus. Homologous recombination of the targeting vector with the *Xrcc2* locus results in the replacement of exon III with a neomycin cassette, and the introduction of a *Bam*HI site. Relevant *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Xba*I (X) and *Xho*I (Xh) restriction sites, and the position of the probe used for Southern blot analysis are shown. Exons II and III are indicated by numbered solid boxes. (B) Genomic DNA from ES cell colonies digested with *Bam*HI, and analysed by Southern blotting and hybridization with the probe indicated in (A). Truncation of wild-type 11.1 kb fragment to 6.2 kb confirms correct targeting of an allele. +/+ and +/- represent the genotypes of wild-type (*Xrcc2*^{+/+}) and targeted (*Xrcc2*^{+/-}) ES lines, respectively. (C) RT-PCR on total RNA extracts from embryos using *Xrcc2* exon II to exon III specific primers, with primers to *Gapdh* as a control. *Xrcc2*^{+/+} and *Xrcc2*^{+/-}, but not *Xrcc2*^{-/-}, generated a band corresponding to *Xrcc2* mRNA. (D) A representative example of PCR genotyping for a litter of E8.5 embryos from an *Xrcc2*^{+/-} intercross. PCR primers detect *neo* or exon III in the same reaction, giving products of 280 and 545 bp, respectively. Control lanes shown are a targeted ES cell line (ES +/-), wild-type genomic DNA (129Sv) and a plasmid constructed to mimic the targeted locus (pX2); -/- denotes homozygous disruption of *Xrcc2*. (E) Double targeting of *Xrcc2* in ES cells shown by Southern blot analysis of *Xho*I-digested genomic DNA. HM-1 ES cells (lane 1) underwent sequential gene targeting steps, first with an *Hprt*-based vector (lane 2) and then, following confirmation of correct targeting of an *Xrcc2* allele, with the *neo*-based construct as in (A). Both strategies introduce discernible *Xho*I restriction sites, reducing a wild-type fragment from 26.5 kb to 4.2 or 3.4 kb for *Hprt* or *neo*, respectively. All targeted clones isolated following transfection with the *neo* vector (shown for four clones; lanes 3, 4, 5 and 6) had re-targeted the already disrupted allele ($n = 8$).

developing embryo. However, other important HRR genes, such as *Rad52* and *Rad54*, do not give embryonic lethality when disrupted (Essers *et al.*, 1997; Rijkers *et al.*, 1998). Given the potential facilitating function of *Xrcc2* in HRR, the consequences of loss of *Xrcc2* in mice could not be predicted. Therefore, we have assessed the effects of a targeted disruption of the mouse *Xrcc2* gene and, surprisingly, found that *Xrcc2* has a significant role in ensuring normal embryonic development, especially in the developing nervous system.

Results

Targeted mutagenesis of mouse *Xrcc2*

Disruption of *Xrcc2* in 129/Sv/Ev embryonic stem cells was achieved as illustrated in Figure 1A. Homologous recombination of the targeting vector with the *Xrcc2* gene results in the introduction of a neomycin-resistance cassette deleting exon III, the largest exon of *Xrcc2* (86% of the coding region). This deleted region encodes the nucleotide binding motifs, which are conserved in the RecA/Rad51 gene family and essential for their activity (Shinohara *et al.*, 1992), and the whole 3' end of the gene

including poly(A) signal sites (Cartwright *et al.*, 1998). Correctly targeted ES clones were identified by Southern blot analysis (Figure 1B); these were used for blastocyst injections to yield chimeric offspring and transmission through the germ line after crossing with C57BL/6 females.

Disruption of *Xrcc2* results in embryonic lethality

Mice heterozygous for the *Xrcc2* disruption (*Xrcc2*^{+/-}) appear indistinguishable from wild-type littermates at 9 months. However, heterozygote intercrosses failed to produce mice homozygous for the mutation (*Xrcc2*^{-/-}) in >300 offspring (Table I). *Xrcc2*^{+/-} and *Xrcc2*^{+/+} were found in normal Mendelian ratios of ~2:1, indicating that *Xrcc2*^{-/-} is lethal.

To confirm the effectiveness of the gene disruption, RNA was isolated from embryonic tissue of wild-type, *Xrcc2*^{+/-} or *Xrcc2*^{-/-} embryos. RT-PCR analysis (Figure 1C) showed the absence of full-length *Xrcc2* transcript in *Xrcc2*^{-/-} embryos.

The stage of development attained by *Xrcc2*^{-/-} embryos was determined through the analysis of litters from timed matings between heterozygotes. Embryos were dissected

Table I. Genotypes of progeny from *Xrcc2*^{+/-} intercrosses

Age (days)	Litters (decidua)	<i>Xrcc2</i> genotype: live (dead)			Resorbed	% -/-
		+/+	+/-	-/-		
E9.5	5 (46)	13	18 (1)	14	0	100
E10.5	8 (66)	18	35	8 (3)	2	45
E11.5	5 (49)	12 (1)	22 (2)	4 (4)	4	35
E12.5	3 (34)	6 (2)	15 (1)	2 (7)	1	29
E14.5–15.5	12 (107)	21	54 (1)	6 (8)	17	24
E18.5	9 (77)	16	37	5 (1)	18	28
3 weeks	50	95	228	0	NA	0

The numbers of live embryos for each genotype at the indicated stages of development are shown. Genotypes in parenthesis are for necrotic or partially resorbed embryos where sufficient yolk sac tissue was available to allow genotyping; the numbers of decidua where maternal resorption was complete are indicated. % -/- denotes the number of viable *Xrcc2*^{+/-} embryos observed as a percentage of that predicted by Mendelian law. NA, not available.

Fig. 2. *Xrcc2*^{-/-} embryos display growth retardation and morphological defects. *Xrcc2* null (-/-) and wild-type littermates (+/+) are shown at (A) E9.5, (B) E11.5, (C) E14.5 and (D) E18.5 stages of development. An additional smaller *Xrcc2*^{-/-} fetus, from a separate litter, illustrates the extremities of size observed at E18.5. (E) *Xrcc2*^{-/-} E11.5 embryo with failed neural tube closure, denoted by asterisks. (F) *Xrcc2*-null E14.5 embryo with exencephaly. (G and H) The lungs of *Xrcc2*-null neonates fail to inflate. In comparison with wild-type littermates (G), alveolar size is reduced in haematoxylin–eosin-stained lungs of *Xrcc2*^{-/-} (H) neonates.

at various stages of gestation and genotype determined by PCR of yolk-sac tissue (Figure 1D). The results of genotyping are summarized in Table I, with embryo stages shown in Figure 2. Until E8.5 (E = days post-coitum), the development of *Xrcc2*^{-/-} embryos was overtly normal. At E9.5, while embryos were recovered at the frequencies predicted by Mendelian law, *Xrcc2*^{-/-} embryos were reduced in size by comparison with littermates. The retardation in growth and development was increasingly apparent at later stages, with *Xrcc2*^{-/-} embryos also displaying consistent morphological abnormalities including dorsal truncation and a stunted tail, and cranial defects. Additional, more severe cranial malformation was evident in three of 10 *Xrcc2*^{-/-} embryos at E11.5–14.5. These

included an E11.5 embryo with incomplete closure of the neural tube and an E14.5 embryo with exencephaly, a phenotype likely to arise from the former defect (Figure 2E and F). No such abnormalities were evident in wild-type or *Xrcc2*^{+/-} littermates.

Loss of viability of *Xrcc2*^{-/-} embryos, indicated by necrosis and resorption, occurred from E10.5 during the stages of organogenesis, with survival reduced at E12.5 to ~30% of that expected (Table I). A corresponding increase in the number of non-surviving, partially resorbed *Xrcc2*^{-/-} embryos was observed. There was no further reduction in viability by E18.5, indicating that the majority of *Xrcc2*^{-/-} embryos reaching E12.5 were likely to survive to birth. Observations at birth showed that, while initially alive and

attempting to breathe, *Xrcc2*^{-/-} neonates (*n* = 3) failed to survive, with cyanosis and death within 20 min, while wild-type and heterozygous littermates flushed pink and were breathing strongly. No live or dead *Xrcc2*^{-/-} offspring were found beyond post-natal day 1, suggesting rapid cannibalization of deceased or runted mutants.

Anatomical and histological examination of the *Xrcc2*^{-/-} neonates indicated all organs to be present, although reduced in size when compared with wild-type or *Xrcc2*^{+/-} littermates. The lungs of post-natal *Xrcc2*^{-/-} pups showed a reduction in alveolar volume when compared with wild-type littermates, indicating a failure to inflate with air (Figure 2G and H). There were no apparent cardiovascular abnormalities or defects in liver, thymus, spleen or kidneys, although in one *Xrcc2*^{-/-} neonate, haemorrhaging was evident in the brain. Therefore, it appears likely that lethality in *Xrcc2*^{-/-} mice results from respiratory failure in the transition from fetus *in utero* to neonate at birth.

***Xrcc2*^{-/-} cells fail to proliferate in culture**

ES cells were subjected to sequential gene targeting steps: the first to disrupt one allele with *Hprt* and then the other with *neo*. As shown by Southern blot analysis, all correct targeting events constituted a re-targeting of the already disrupted allele (*n* = 8) (Figure 1E). Given an equal chance of targeting either allele, the absence of double-targeted (*Xrcc2*^{-/-}) cells is highly significant (*p* < 0.005). Furthermore, in contrast to cells derived from *Xrcc2*^{+/+} or *Xrcc2*^{+/-} littermates, primary embryonic fibroblast cells isolated from E10.5–E15.5 *Xrcc2*^{-/-} embryos (*n* = 6) underwent premature senescence with complete growth arrest at or before passage 4 in culture (data not shown). Together these results indicate that *Xrcc2* is essential for normal proliferation in culture.

***Xrcc2*^{-/-} blastocysts are hypersensitive to γ radiation**

We have shown previously that an established line of *Xrcc2*-deficient hamster cells (*irs1*) is ~3-fold more sensitive to ionizing radiation than wild-type V79 hamster cells (Jones *et al.*, 1987). Therefore, we assessed the impact of disrupting the mouse *Xrcc2* gene on sensitivity to radiation using blastocysts isolated at E3.5, following *Xrcc2*^{+/-} intercrosses. Blastocysts are composed of two cell types: highly proliferative cells in the inner cell mass (ICM) and relatively quiescent trophoblast cells. The blastocysts were given 4 Gy of γ -rays before culture *in vitro* for 6 days, when the area of the developing ICM was measured. The genotypes of littermates were determined at the end of this period by PCR. All blastocysts attached successfully, and with the exception of one irradiated *Xrcc2*^{-/-} blastocyst there was outgrowth of the ICM. As shown in Table II, mean ICM areas for untreated control blastocysts were equivalent for each genotype. Following irradiation, the mean ICM areas for both *Xrcc2*^{+/+} and *Xrcc2*^{+/-} blastocysts were reduced to approximately half, while that of *Xrcc2*^{-/-} mice was reduced to one-fifth of the unirradiated ICM (significantly different from the reduction in *Xrcc2*^{+/+} and *Xrcc2*^{+/-}). Representative photographs of control and irradiated blastocysts are shown in Figure 3A–D. These data suggest that, as in cell culture, the loss of *Xrcc2* confers a moderate level of radiation sensitivity in the mouse.

Table II. *In vitro* culture of blastocysts after γ -radiation

Dose (Gy)	Inner cell mass area (mm ²)		
	<i>Xrcc2</i> genotype		
	+/+	+/-	-/-
0	0.067 ± 0.012 <i>n</i> = 6	0.054 ± 0.004 <i>n</i> = 9	0.064 ± 0.008 <i>n</i> = 7
4	0.035 ± 0.006 <i>n</i> = 7	0.033 ± 0.003 <i>n</i> = 12	0.013 ± 0.004 ^a <i>n</i> = 7

^aSignificantly different (*p* < 0.01) from irradiated +/+ and +/- groups on a Kruskal–Wallis test.

***Xrcc2*^{-/-} cells have a high level of chromosomal instability**

The proliferative capacity of embryonic cells may suffer if the loss of *Xrcc2* leads to genetic instability. To check the integrity of chromosomes, disassociated embryonic cells were examined immediately after placing the cells into culture medium. Chromosomal aberration frequencies were scored in ~100 cells from E15.5 littermates of *Xrcc2*^{-/-}, *Xrcc2*^{+/-} and wild-type embryos. Very high frequencies of breaks and exchanges were seen in the *Xrcc2*^{-/-} cells (approximately two per cell), while very few (~0.02 per cell) were observed in cells from heterozygotes and wild-type embryos (Table III). The majority of the aberrations involved chromatids, indicating that these were occurring in the current division and that the embryonic cells were experiencing persistent genetic instability (chromatid-type aberrations will become chromosome-type following cell division). Some of the chromatid exchanges in *Xrcc2*^{-/-} cells were very complex, as illustrated in Figure 3E. Chromosome-type aberrations were also identified using three-colour fluorescence *in situ* hybridization (FISH) to show rearrangements such as reciprocal translocations, which may be transmissible through somatic cell divisions (Figure 3F).

A role for *Xrcc2* in the prevention of apoptosis, especially in the developing nervous system

To investigate the cellular pathology linked to the morphological defects and lethality associated with *Xrcc2* disruption, we used the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labelling) assay as an indicator of apoptotic death. At E9.5, there was a marked elevation of apoptotic cells throughout *Xrcc2*^{-/-} embryonic tissues when compared with littermate or stage-matched wild-type embryos. Cell death in *Xrcc2*^{-/-} embryos was highest in caudal and dorsal regions, and developing areas of the brain as shown in Figure 4.

At E11.5 and later stages, extensive cell death was detected in *Xrcc2*^{-/-} embryos, although this was largely confined to the developing central and peripheral nervous system. TUNEL-positive cells were observed in the spinal cord, midbrain, hindbrain and forebrain in *Xrcc2*^{-/-} embryos, with TUNEL staining localized to pyknotic nuclei as expected (Figures 5 and 6). In other organs, including the lungs, heart and liver, abnormal numbers of apoptotic cells were not observed (data not shown).

Neurogenesis in normal embryos is a spatially and temporally regulated process: in normal cortex it

Fig. 3. *Xrcc2*^{-/-} embryonic cells are hypersensitive to radiation and have a high level of chromosomal aberrations. Representative photographs of blastocysts cultured for 6 days following either no irradiation [(A) *Xrcc2*^{+/+} and (B) *Xrcc2*^{-/-}] or γ -irradiation (4 Gy) [(C) *Xrcc2*^{+/+} and (D) *Xrcc2*^{-/-}]. ICM, inner cell mass; TGC, trophoblast giant cells. (E) Complex chromatid exchange involving at least six chromosomes, including chromosomes 2 (yellow) and 4 (green); centromeres are stained pink. (F) Reciprocal chromosome exchange involving chromosomes 4 (green) and 11 (red); centromeres are stained pink.

Table III. Chromosomal aberrations in embryonic cells at E15.5 from littermates of crosses between heterozygous (*Xrcc2*^{+/-}) mice

Embryo genotype	Cell number	Number of chromosomal aberrations						
		Gaps	Breaks	Isochromatid breaks	Exchanges	Triradials	Rings	Complexes
+/+	134	1	1					
+/-	103		1					
+/-	101	1		1				
-/-	77	3	38	14	82	14	2	11
-/-	64	2	20	11	69	14		7

Aberrations are classified according to Savage (1976).

commences at E11–12.0, peaks at around E12.5 and E13.5, and then decreases to lower levels by E18 (Bayer and Altman, 1991). This pattern was reflected in the TUNEL staining of developing lateral cortex of the forebrain of *Xrcc2*^{-/-} embryos (Figure 6). Neurons arise from mitotic progenitor cells in the ventricular zone (VZ),

and newly formed post-mitotic neurons initially migrate to form the primordial plexiform layer (PPL); at later stages this migration occurs through an intermediate zone (IZ) to form the cortical plate (CP), the future cortical grey matter. Extensive cell death was first observed in the migrating cells at E12.5 (Figure 6B) and increased until E13.5–14.5,

Fig. 4. Extensive cell death in *Xrcc2*^{-/-} E9.5 embryos (sagittal sections) as shown by TUNEL staining (green). (A and B) midbrain and (C and D) dorsal region at the level of the forelimb of stage-matched *Xrcc2*^{+/+} (A and C) and *Xrcc2*^{-/-} embryos (B and D).

with large numbers of apoptotic cells apparent in the VZ and IZ (Figure 6D). By E16.5, cell death was substantially reduced (Figure 6F), continuing at gradually lower levels to E18.5 (data not shown). Few TUNEL-staining cells were detected in littermate controls over the same period (Figure 6A, C and E). Neurogenesis in the hindbrain precedes that of the forebrain, commencing at E11.0 and proceeding to completion by E14.0 (Nornes and Carry, 1978). Correspondingly, extensive cell death was detected in the hindbrain of *Xrcc2*^{-/-} embryos at E11.5–12.5, but had ceased by E14.5 (data not shown). No increased TUNEL staining was seen in mature neurons or in the cerebellum, where development extends into the post-natal period.

To confirm the identity of apoptotic cells, we stained E12.0 *Xrcc2*^{-/-} sections of forebrain precortex with TUNEL reagents and a TUJ1 antibody specific to β 3-tubulin, an early marker of post-mitotic neurons (Lee *et al.*, 1990). In the frontal cortex (Figure 7A and B), TUNEL staining occurs within the VZ and at the boundary with the PPL where the majority of TUJ1-staining neurons are found. In the septal area of the developing cortex (Figure 7C and D), neurogenesis is more advanced and TUNEL-positive cells localize predominantly within the PPL and to a lesser extent in the VZ. Similar data were obtained for the hindbrain (not shown). Therefore, the extensive apoptosis in *Xrcc2*^{-/-} embryos occurred within the early post-mitotic neuronal cell population, during or shortly following the completion of migration to the PPL layer.

At E14.5–16.5 (Figure 6) and E18.5 (data not shown), the thickness of the cortical plate in *Xrcc2*^{-/-} embryos is reduced when compared with wild-type littermates. Therefore, it is evident that the extensive cell death results in a severely abnormal cortical structure.

Discussion

Early embryonic death, apoptosis and genetic instability

Disruption of the *Xrcc2* gene in mice leads to embryonic lethality, but the mutant embryos appeared normal up to E8.5. Thereafter, retardation in growth and development occurred, and by E12.5 about three-quarters of the mutant embryos were dead. The majority of *Xrcc2*^{-/-} embryos that survived this period remained viable until respiratory failure at birth.

Early embryonic death is found for some other gene disruptions affecting HRR. Lethality arising from the disruption of *Rad51*, a central gene in the homologous recombination–repair pathway, occurs even earlier than for *Xrcc2*^{-/-} embryos (before E8.5) (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996). Disruption of the *Brca1* and *Brca2* genes, which have been associated with *Rad51* and HRR activity (Venkitaraman, 1999), generally leads to death before E9.5 (Hakem *et al.*, 1998). These findings show that some HRR genes are critical for survival at early stages of development, probably because they perform crucial roles in combating DNA damage, particularly in the rapidly proliferating cells of the early embryo. Severe impairment of embryonic cell proliferation is characteristic of the *Rad51* and *Brca* gene disruptions, whereas the loss of *Xrcc2* does not appear to compromise proliferation significantly in isolated blastocysts (Table II) or embryos up to E8.5. *Xrcc2* disruption may have less impact than *Rad51* or *Brca* gene disruptions because its role in HRR is to facilitate the process (see Introduction). However, at mid-gestation, corresponding to the onset of organogenesis, sufficient cellular loss (or loss in specific tissues) may have occurred in many *Xrcc2*^{-/-} embryos to compromise their viability. Those surviving this phase have morpho-

Fig. 5. Extensive cell death in the developing CNS of *Xrcc2*^{-/-} E11.5–12.5 embryos. TUNEL staining (DAB) in (A and B) the spinal cord at E12.5, (C and D) the midbrain at E12.5, and (E and F) the hindbrain at E11.5 in sagittal sections of *Xrcc2*^{+/+} (A, C and E) and *Xrcc2*^{-/-} (B, D and F) littermates. Nuclei are counterstained with haematoxylin. V, fourth ventricle; VZ, ventricular zone; ML, mantle layer.

logical defects (truncation, exencephaly) attributable to significant but not critical cell loss sustained in mid-gestation. Interestingly, the recent disruption of another *Rad51*-like gene, *Rad51L3*, which has been found to physically associate with *Xrcc2* (see Introduction), similarly leads to mid-gestation lethality and truncation of the caudal tail region (Pittman and Schimenti, 2000).

In accordance with the timing of lethality, we observed apoptosis throughout *Xrcc2*^{-/-} embryos at E9.5, although by about E12.5 it was largely confined to post-mitotic cells in neurological tissue. Given the role of *Xrcc2* in DNA repair, it is likely that an accumulation of unrepaired or misrepaired damage triggers apoptosis. It is important to note that, unlike some other repair pathways, HRR is a mechanism for high-fidelity repair of damage such as DNA double-strand breaks (see Introduction). Consistent with loss of this pathway, there is a highly elevated frequency of chromosomal aberrations in *Xrcc2*^{-/-} embryonic cells (Table III). The occurrence of chromatid aberrations in *Xrcc2*^{-/-} cells is indicative of persistent genetic instability, and isochromatid breaks may signify failed recombination between sister chromatids in post-replicative cells. In agreement with our earlier findings with an established line of *Xrcc2*-deficient hamster cells, *irs1* (Jones *et al.*, 1987), we found a moderate increase in the sensitivity of *Xrcc2*^{-/-} embryonic cells to ionizing radiation (Table II). Thus, exposure to a DNA strand break-inducing agent augments the response to endogenous damage. However, while the *Xrcc2*-deficient *irs1* line is viable, we found that mouse embryonic cells

Fig. 6. Extensive apoptosis in *Xrcc2*^{-/-} embryos reflects the spatial and temporal pattern of neurogenesis. TUNEL staining for sagittal sections of the developing cortex of the forebrain at (A and B) E12.5, (C and D) E14.5 and (E and F) E16.5 for wild-type (A, C and E) and *Xrcc2*^{-/-} (B, D and F) embryos. At E16.5, arrows indicate TUNEL-positive nuclei. V, lateral ventricle; VZ, ventricular zone; PPL, primordial plexiform layer; IZ, intermediate zone; CP, cortical plate.

Fig. 7. Apoptosis in early post-mitotic neurons of *Xrcc2*^{-/-} embryos. Sagittal section of (A and B) the frontal cortex and (C and D) the septal area of the developing forebrain in an E12.0 *Xrcc2*-null embryo, stained with propidium iodide (A and C), and double stained with TUNEL reagents (green) and the anti-β3-tubulin antibody, TUJ1 (red) (B and D). TUNEL-positive nuclei are localized predominantly within the newly generated, TUJ1-staining neuronal cell population of the PPL layer. V, lateral ventricle; VZ, ventricular zone; PPL, primordial plexiform layer.

senesced prematurely, and that mouse ES cells with both alleles of *Xrcc2* disrupted could not be recovered. Similar findings have been reported for disruptions of both *Rad51* (Lim and Hasty, 1996) and *Brca2* (Sharan *et al.*, 1997). These failures in mouse primary cells are likely to be due to the presence of effective checkpoint genes acting as a barrier to cell cycle progression in the presence of persistent DNA damage (Lim and Hasty, 1996; Hakem *et al.*, 1997). In this context, it will be interesting to assess the effects of *Xrcc2* disruption on a checkpoint-defective genetic background (e.g. *Tp53*^{-/-}); this may allow the birth of viable offspring, and an assessment of the influence of concomitant genetic instability on cancer incidence.

Defective neurogenesis and perinatal lethality

The elimination of a few per cent of neuronal cells normally accompanies cellular proliferation in mammalian neurogenesis (Thomaidou *et al.*, 1997), but the amount of apoptosis in *Xrcc2*^{-/-} embryos considerably exceeds the normal level. In recent studies of mouse embryos in which evolutionarily conserved genes of the NHEJ repair pathway were disrupted, it has also been found that elevated levels of neuronal apoptosis occur at E12.5 onwards. Importantly, the extent of apoptosis depends on which of the NHEJ genes is disrupted: a massive increase in apoptosis of post-mitotic neurons occurs in ligase IV- and XRCC4-deficient embryos, but more subtle increases occur in Ku70- and Ku80-deficient

embryos (Barnes *et al.*, 1998; Frank *et al.*, 1998; Gao *et al.*, 1998; Gu *et al.*, 2000). Ligase IV- and XRCC4-deficient embryos have genotypes in Mendelian ratios up until about E15 but do not survive to birth, while Ku70 and Ku80 deficiencies allow survival to give viable, although growth-retarded mice. Adult Ku70-deficient mice have also been reported to suffer from aganglionosis in the gut, similar to that found in the congenital disorder of the enteric nervous system known as Hirschsprung syndrome (Li *et al.*, 1998). Severe apoptotic damage to the nervous system may therefore be a cause of embryonic lethality (Copp *et al.*, 1990; Gu *et al.*, 2000), while less severe damage is compatible with life. In support of this link, mice doubly deficient in p53 and either ligase IV or XRCC4 show abrogation of the neurological defects and increased survival (Frank *et al.*, 2000; Gao *et al.*, 2000).

The finding of cell lethality in the developing nervous system of embryos with disrupted NHEJ genes led to the suggestion that the NHEJ pathway may be particularly important for neurogenesis, perhaps because there is no back-up from other repair pathways such as HRR (Gu *et al.*, 2000). However, our data now show that HRR as exemplified by the function of *Xrcc2* is important in ensuring correct neuronal development, since the loss of this pathway leads to defects similar to the loss of components of NHEJ. Neurological defects were not observed in *Rad51* and *Brca* gene disruptions, presumably because the embryos died before such assessments could

be made. However, there is one report of *Brca1* disruption where the embryos survived to E11–13 and developmental abnormalities including exencephaly were reported (Gowen *et al.*, 1996).

It is unclear at present why post-mitotic neurons in particular are adversely affected. This cell type may be especially sensitive to apoptotic stimuli (Gao *et al.*, 1998), or there may be increased levels of oxidative damage in these cells leading to the formation of both single- and double-strand breaks in DNA (Barnes *et al.*, 1998). The idea that there is an increased level of DNA breakage in certain parts of the developing brain has led to the further speculation that the NHEJ pathway has a specific role in creating genetic diversity in neurons: it has been proposed that NHEJ can rejoin DNA breaks at defined genomic sites, in a similar way to the involvement of this pathway in the generation of antigen-recognition diversity in the immune system (Chun and Schatz, 1999; Gilmore *et al.*, 2000). This speculation is made less attractive by our findings, at least in terms of a specific role for NHEJ, since another repair pathway (HRR) seems to be equally important in the repair of this damage. This conclusion is supported by a very recent study on the effects of disruption of the gene encoding DNA polymerase β ($\text{pol}\beta$), a component of the DNA base-excision repair pathway. Null $\text{pol}\beta$ embryos suffer defective neurogenesis and perinatal lethality associated with respiratory failure (Sugo *et al.*, 2000). The similarity of findings for null $\text{pol}\beta$ and *Xrcc2*^{-/-} embryos also supports the idea that unrepaired/misrepaired DNA damage can affect neurological development in ways that predict later failures in specific organs, such as the lungs.

Taken with other recent work, our findings indicate that unrepaired or misrepaired DNA damage arising from the loss of individual components of several different repair pathways can lead to the apoptosis observed in the developing nervous system and the failure to survive at birth. Further, the severity of the phenotype in mice may relate to the specific role of the disrupted gene, although functional redundancy among genes may play a role in mitigating severity. In this context it will be interesting to look for subtle alterations of the nervous system in mice with other HRR gene disruptions, such as *Rad52* (Rijkers *et al.*, 1998) and *Rad54* (Essers *et al.*, 1997), where these survive to birth.

Materials and methods

Construction of the targeting vector

A 17 kb genomic fragment containing exons II and III of murine *Xrcc2* was isolated from a 129/Sv mouse genomic library (Lambda FixII; Stratagene) by screening with a human *Xrcc2* cDNA probe. Following restriction mapping and the determination of intron–exon boundaries, fragments were subcloned into pBluescript SK(+) (Stratagene). To generate a targeting vector, 1.6 kb *HindIII*–*EcoRI* and 4.5 kb *EcoRI*–*XbaI* fragments, from 5' and 3' to exon III, respectively, were inserted into pBTII SK(+) (Stratagene) flanking either side of a PGK-*neo* cassette, with a PGK-*HSVtk* gene positioned upstream of the short arm. *Neo* and *tk* markers were from pPNT (Tybulewicz *et al.*, 1991).

Targeting of ES cells and generation of germ-line chimeras

MAC3 ES cells (M.Maconochie, unpublished) derived from mouse strain 129/Sv/Ev were cultured as described previously (Ramirez-Solis *et al.*, 1993). *NotI*-linearized targeting vector was introduced into ES cells by electroporation, and colonies surviving G418 (180 $\mu\text{g}/\text{ml}$ active ingredient) and gancyclovir (1 μM) selection were screened by

Southern blot analysis of *Bam*HI-digested genomic DNA; a 1.8 kb *XhoI*–*Bst*XI fragment 5' to the region of targeting was used as a probe. To generate chimeras, a correctly targeted ES clone was injected into C57Bl/6 blastocysts followed by transfer to pseudopregnant foster mothers (C57Bl/6 $\times$ CBA F₁ hybrid). Resultant high-grade agouti-marked chimeric offspring were mated to C57Bl/6 females, and germ-line transmission was demonstrated by the presence of agouti coat colour in the F₁ offspring. The presence of a disrupted *Xrcc2* allele was confirmed by Southern blot analysis. The animal studies described were carried out under the guidance issued by the Medical Research Council in 'Responsibility in the Use of Animals for Medical Research' (July, 1993) and Home Office Project Licence 30/1544.

Double targeting of *Xrcc2* in ES cells

HM-1 ES cells (kindly supplied by Drs D.Melton and J.McWhir, Edinburgh University) were initially targeted with a vector based on that shown in Figure 1A, with the *neo* selection cassette replaced by the *Hprt* mini-gene (containing an internal *XhoI* restriction site) (Selfridge *et al.*, 1992). Selection following electroporation was in medium containing hypoxanthine (100 μM), aminopterin (0.4 μM) and thymidine (16 μM) (Life Technologies) and gancyclovir (2 μM). An *Xrcc2*^{+/-} cell line was re-electroporated in a second step with the *neo*-based vector (Figure 1A). Selection was with G418 (250 $\mu\text{g}/\text{ml}$) and gancyclovir (2 μM). Gene disruption was determined by Southern blot analysis of *XhoI*-digested genomic DNA in each case.

In vitro culture and irradiation of blastocysts

Ionizing radiation sensitivity of embryos was determined as described previously (Sharan *et al.*, 1997). Blastocysts from *Xrcc2*^{+/-} intercrosses were isolated at E3.5 and washed in M16 medium (Sigma) before exposure to γ -radiation (⁶⁰Co, 4 Gy at 1.4 Gy/min). Blastocysts were cultured for 6 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum, 0.1 mM sodium pyruvate, 100 μM β -mercaptoethanol, 2 mM glutamine, 100 μM non-essential amino acids, and antibiotics. Inner cell mass area was determined by confocal microscopy after cell staining with 3,3'-dihexyloxycarbonyl iodide.

PCR genotyping of blastocysts and embryos

Genotype determination of *Xrcc2* was performed on cell lysates prepared by digestion of either blastocysts, whole embryos or yolk sacs in lysis buffer (50 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.5% Tween-20, 0.5 $\mu\text{g}/\text{ml}$ Proteinase K) for 0.5–2 h at 55°C followed by Proteinase K inactivation at 95°C for 5 min. Three primers were used in the PCR reaction. A single 5' sense primer to the *Xrcc2* intron upstream of exon III (5'-GGTTCATCTTGTGCTTTTGTGTGTTTA-3'), and 3' antisense primers to exon III (5'-TCTGTTTTCCTCCGTTCTCTCTG-3') and the *Pgk-1* promoter of the *neo* cassette (5'-GCATGCTCCAGACTGCCTTGG-3') were used to detect wild-type and mutant alleles, respectively. Amplification for 30–35 cycles at 94°C for 35 s, 58°C for 1 min and 72°C for 45 s gave fragments of 545 bp (wild type) and 280 bp (mutant), separated by electrophoresis through a 1.5% agarose gel. Wild-type (Wt) mouse genomic DNA (129Sv) and a plasmid (pX2) containing *neo* and *Xrcc2* sequences mimicking a targeted locus were used as controls.

Chromosome analysis

Tissue from E15.5 embryos was macerated and cells disaggregated by incubation in trypsin. Cells were incubated overnight in DMEM–15% fetal calf serum, then exposed to colcemid (0.05 $\mu\text{g}/\text{ml}$) for 2 h, trypsinized and incubated in hypotonic solution (1:1, 0.075 M KCl:0.034 M trisodium citrate) for 15 min at 37°C. Cells were fixed in 3:1 ethanol:acetic acid and dropped onto slides, before staining with Giemsa for cytogenetic analysis. FISH used chromosome-specific probes for chromosomes 2 [fluorescein isothiocyanate (FITC)-labelled and biotinylated in a ratio of 3:1], 4 (FITC-labelled) and 11 (biotinylated), together with a cy3-labelled pan-centromeric probe (Cambio, UK).

Histological analysis and TUNEL assay

Embryos were fixed in 4% paraformaldehyde, paraffin embedded and serially sectioned (4 μm). Sections for TUNEL assay were dewaxed and permeabilized according to standard procedures, and an *in situ* Cell Death Detection Kit used as per the manufacturer's instructions (Boehringer Mannheim). Samples were analysed by fluorescent microscopy or following immunostaining with alkaline phosphatase-conjugated anti-fluorescein antibody (Boehringer Mannheim) and reaction with 3,3'-diaminobenzidine (DAB). Nuclei were counterstained with propidium iodide or haematoxylin. Immunostaining of post-mitotic neurons was

performed using monoclonal anti- β -tubulin antibody TUJ1 (BabCO) followed by Texas-Red-conjugated horse anti-mouse IgG (Vector Laboratories).

RT-PCR analysis

Total RNA was extracted from embryonic tissue (E15.5) using TRIzol reagent (Life Technologies). The mRNA in 5 μ g total RNA was reverse transcribed using oligo(dT)₁₂₋₁₈ primers and Superscript II reverse transcriptase (Life Technologies), according to the supplier's protocol. PCR to determine the presence of *Xrcc2* mRNA was performed using a 5' sense, exon II-specific primer (5'-GAAGGCAGAAGCTCGTTG-AAAGAA-3') and 3' antisense primer to exon III (5'-CAGGGCTTC-CAGGGAGTGC-3'). PCR conditions were 94°C for 45 s, 53°C for 1 min and 72°C for 1 min, for 31 cycles. As a control for reverse transcription, the following primers were used for amplification of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) cDNA: 5' sense primer, 5'-GAG-AAACCTGCCAAGTATGATGAC-3'; 3' antisense primer, 5'-TGA-TGGTATTCAAGAGAGTAGGGAG-3'. The products of amplification, 354 and 421 bp for *Xrcc2* and *Gapdh* cDNAs, respectively, were separated by electrophoresis on a 1.5% agarose gel.

Acknowledgements

We thank Terry Hacker, Nicola Powles, Stuart Townsend and the Harwell Transgenic Facility for technical support; David Papworth for statistical analysis; Ruth Arkell for advice; and the European Commission for partial financial support (contract F14P-CT950010).

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*Received September 7, 2000; revised November 1, 2000;
accepted November 2, 2000*