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DNA repair gene *Ercc1* is essential for normal spermatogenesis and oogenesis and for functional integrity of germ cell DNA in the mouse

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SUMMARY

Ercc1 is essential for nucleotide excision repair (NER) but, unlike other NER proteins, Ercc1 and Xpf are also involved in recombination repair pathways. *Ercc1* knockout mice have profound cell cycle abnormalities in the liver and die before weaning. Subsequently *Xpa* and *Xpc* knockouts have proved to be good models for the human NER deficiency disease, xeroderma pigmentosum, leading to speculation that the recombination, rather than the NER deficit is the key to the *Ercc1* knockout phenotype. To investigate the importance of the recombination repair functions of Ercc1 we studied spermatogenesis and oogenesis in *Ercc1*-deficient mice. Male and female *Ercc1*-deficient mice were both infertile. *Ercc1* was expressed at a high level in the testis and the highest levels of Ercc1 protein occurred in germ cells following meiotic crossing over. However, in *Ercc1* null males some germ cell loss occurred prior to meiotic entry and there was no evidence that Ercc1 was essential for meiotic crossing over. An increased level of DNA strand breaks and oxidative DNA damage was found in *Ercc1*-deficient testis and increased apoptosis was noted in male germ cells. We conclude that the repair functions of Ercc1 are required in both male and female germ cells at all stages of their maturation. The role of endogenous oxidative DNA damage and the reason for the sensitivity of the germ cells to *Ercc1* deficiency are discussed.

Key words: Meiosis, Nucleotide excision repair, Oxidative DNA damage, Recombination, Spermatozoa, Xeroderma pigmentosum

INTRODUCTION

The nucleotide excision repair (NER) pathway (reviewed by Wood, 1996) has evolved to deal with UV-induced DNA lesions, but also protects against a broad range of endogenously generated bulky DNA adducts. The NER pathway is defective in patients with the human inherited disease xeroderma pigmentosum (XP), which is characterised by UV hypersensitivity, pigmentation abnormalities and a thousand-fold increased risk of skin cancer (Friedberg et al., 1995). XP patients also have a significantly increased incidence of internal tumours and, in some cases, neurological abnormalities, reflecting the importance of NER in the repair of endogenous, as well as UV-induced DNA damage. Ercc1 is essential for NER where it acts in a complex with Xpf (Ercc4) to make the incision 5′ to the lesion site. Although *Ercc1*, in common with other NER genes, is expressed in all cell types, its particular importance in a developmental context is suggested by the observation that it is preferentially expressed in both neural and haematopoietic stem cells (Geschwind et al., 2001). We began to study the role of Ercc1 by inactivating the mouse *Ercc1* gene (McWhir et al., 1993). *Ercc1*-deficient mice were not a model for XP: animals were severely runted and died before weaning from liver failure with a striking premature polyploidy in hepatocytes that was more reminiscent of a premature ageing than an NER deficiency disease (McWhir et al., 1993; Nuñez et al., 2000).

Unlike other NER proteins Ercc1 and Xpf are also involved in homologous recombination, double-strand break repair and the repair of interstrand cross-links. Their role in recombination was deduced originally from studies on the *Saccharomyces cerevisiae* homologues, RAD10 (Ercc1) and RAD1 (Xpf) (Schiestl and Prakash, 1990). In mammalian cells Ercc1 is required for the correct processing of heteroduplex intermediates formed during homologous recombination (Adair et al., 2000; Sargent et al., 2000) and also acts to remove protruding single-stranded ends in the single-stranded annealing pathway for homologous recombination and double-strand break repair (Davies et al., 1995; Fishman-Lobell and Haber, 1992). In addition, *Ercc1*- or *Xpf*-deficient mammalian cells are characteristically hypersensitive to interstrand cross-linking agents. In a reaction distinct from NER, the Ercc1/Xpf

complex has the ability to cut adjacent to such cross-links (Kuraoka et al., 2000a). The key role of the Ercc1/Xpf complex in all these repair pathways is the ability to cleave single-stranded 3′ tails projecting from DNA duplexes.

Ercc1 was the first NER gene to be inactivated in the mouse (McWhir et al., 1993). Subsequently *Xpa* (de Vries et al., 1995; Nakane et al., 1995) and *Xpc* (Sands et al., 1995) knockouts have proved to be good models for XP and this has led to the suggestion that it is the recombination, rather than the NER deficit that is the key to the *Ercc1* knockout phenotype (Weeda et al., 1997). A number of different repair genes are highly expressed in testis [e.g. *Polb* (Alcivar et al., 1992); *Lig3* (Chen et al., 1995); *Xrcc1* (Walter et al., 1996)] and as part of our investigation of the importance of the recombination repair functions of Ercc1 we decided to study spermatogenesis and oogenesis in *Ercc1*-deficient mice. Functional homologous recombination pathways are essential for the successful completion of meiosis. In yeast, unresolved double-strand breaks are thought to trigger a checkpoint leading to pachytene arrest and, in the mouse, the apoptotic elimination of spermatocytes with synaptic errors occurs via a p53-independent pathway (Odorisio et al., 1998). Knockouts for a number of mismatch repair genes have been shown to lead to a specific failure of spermatogenesis at the pachytene stage, consistent with the requirement for mismatch repair to process heteroduplex recombination intermediates [*Pms2* (Baker et al., 1950; *Mlh1* (Edelmann et al., 1996); *Msh5* (de Vries et al., 1999); *Msh4* (Kneitz et al., 2000)]. If *Ercc1* were essential at the same stage, a similar phenotype would be anticipated. Support for the notion that *Ercc1* may be required for meiosis comes from the *Drosophila melanogaster* homologue of *Xpf*, *mei-9*, where mutation causes reduced meiotic recombination and increased non-disjunction as well as defective NER (Sekelsky et al., 1995).

Within the testis several germ cell stages, including pachytene spermatocytes, have been shown to produce high levels of reactive oxygen species (Fisher and Aitken, 1997), which induce a variety of DNA lesions, with one of the most abundant being 7,8-dihydro-8-oxoguanine (8-oxoG) (for a review, see Lindahl, 1993). This lesion is strongly mutagenic and also acts as a block to transcription by RNA polymerase II (Le Page et al., 2000a). A complex anti-oxidant defence system has been described in the rat testis (Bauche et al., 1994). Traditionally base excision repair (BER) was considered to have the key role in removing 8-oxoG and 8-oxoG DNA glycosylase (Ogg1) is highly expressed in the testis (Rosenquist et al., 1997). However, the discovery of transcription-coupled repair of 8-oxoG and the observation that this process continues to operate in *Ogg1*-null cells (Le Page et al., 2000b) has, belatedly, led to the recognition that NER may also have an important role to play in the repair of 8-oxoG.

Ercc1 knockout mice die before the first wave of spermatogenesis has been completed in control littermates, so in this study we have also used animals where the *Ercc1*-deficient liver phenotype has been corrected by an *Ercc1* transgene under the control of a liver-specific promoter (Selfridge et al., 2001). The increased lifespan of these animals means that the consequences of *Ercc1* deficiency can now be studied in other tissues. Here we show that *Ercc1* is essential for normal spermatogenesis and oogenesis, but that the premeiotic lesions and DNA damage observed are consistent with a general role for the repair functions of *Ercc1* throughout gametogenesis rather than a specific requirement at meiotic crossing over.

MATERIALS AND METHODS

Ercc1-deficient mice

The production, maintenance and genotyping of *Ercc1*-deficient mice (McWhir et al., 1993) and *Ercc1*-deficient mice with an *Ercc1* transgene under the control of the transthyretin gene promoter (Selfridge et al., 2001), have been described previously. RNA and protein were extracted from mouse tissues and analysed for *Ercc1* expression by northern and western blotting as described previously (Selfridge et al., 2001).

Collection of tissues for histology

Testes and ovaries were immersion fixed, either in Bouins for 6 hours (ovaries) or 10 hours (testes), or in 4% neutral buffered formaldehyde (NBF) overnight at 4°C, or in Collidine overnight (testes only). Tissues fixed in Bouins or NBF were processed into paraffin wax using a standard 16 hour cycle. Testes fixed in Collidine were processed manually into Araldyte and stained with 1% Toluidine Blue containing 1% borax (BDH, Poole, Dorset, UK) at 60°C until a suitable staining intensity was obtained (Kerr et al., 1993). Sections (5 μm) of Bouins-fixed testes were stained with Haematoxylin and Eosin, or using the Apotag method (Sharpe et al., 1998). Epididymides from adult mice were minced in Biggers, Whitten and Whittingham medium (BWW) (Biggers et al., 1971) and allowed to stand for 5 minutes before recovering the supernatant, avoiding tissue debris. Cells were resuspended at approx. 3×10^6 cells/ml in BWW, aliquoted and frozen at –20°C.

Antibodies

A rabbit polyclonal antibody to mitochondrial core protein II of bovine complex III was kindly supplied by Dr Hermann Schaegger (University of Frankfurt). An affinity-purified rabbit polyclonal antibody raised against a peptide specific for the RNA binding protein Dazl (Ruggiu et al., 1997; Ruggiu et al., 2000) was a gift from Dr Nicola Reynolds (MRC Human Genetics Unit, Edinburgh). The antibody to mouse Ercc1 was raised in a rabbit against a His-tagged recombinant protein containing a central fragment (amino acids 36-175) of mouse Ercc1 (K.-T. Hsia and D. Melton, unpublished). The antibody was affinity-purified from crude serum using antigen immobilised on nitrocellulose membrane (Robinson et al., 1988). Pilot experiments with the Ercc1 antibody found that no immunopositive signal was obtained when Bouins-fixed wild-type testes were used so all further experiments were carried out using testes fixed in NBF.

Immunohistochemistry

Briefly, sections (5 μm) were mounted on slides coated with 3-aminopropyl triethoxy-silane (Sigma Chemical Co., Poole, Dorset, UK), dried overnight (50°C), dewaxed and rehydrated. Thereafter, sections were incubated with 3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase, washed once each (5 minutes) in distilled water and TBS (0.05 M Tris-HCl pH 7.4, 0.85% NaCl) and blocked for 30 minutes using normal rabbit serum diluted in TBS (1:5, NSS-TBS). Sections were incubated with primary antibody diluted in TBS (anti-Ercc1, 1 in 100; anti-Dazl, 1 in 100) overnight at 4°C. Sections were washed twice in TBS (5 minutes each), incubated for 30 minutes with biotinylated swine anti-rabbit immunoglobulin, diluted 1:500 in NSS-TBS for 30 minutes, then washed again in TBS (2 times 5 minutes). Bound antibodies were detected according to standard methods (Saunders et al., 2001). Images were captured using an Olympus Provis microscope (Olympus Optical Co., London, UK) equipped with a Kodak DCS330 camera

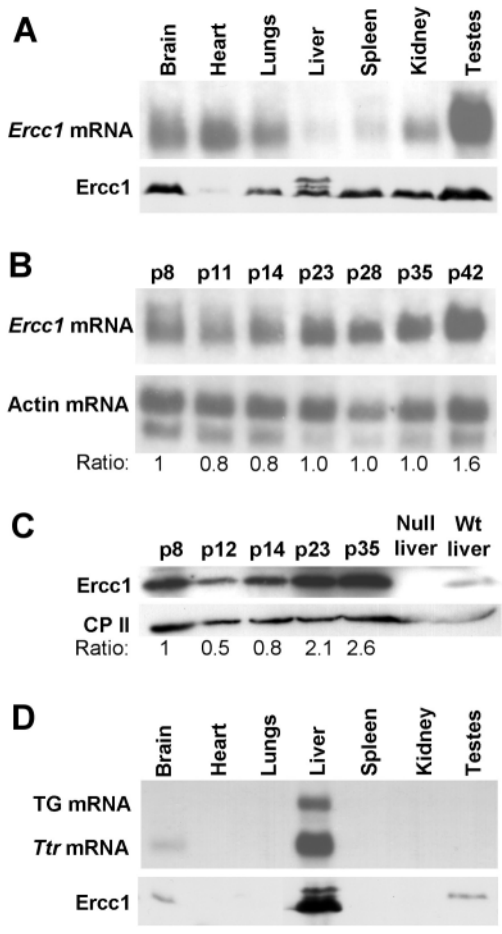
(Eastman Kodak, London, UK) and assembled on a Macintosh PowerPC computer using Photoshop 6 (Adobe).

Comet assay on sperm DNA

The alkaline single cell gel electrophoresis (Comet) assay was performed using the CometAssay™ Kit from R&D Systems (Abingdon, Oxon., UK), adapted as follows. Cells were defrosted at room temperature and 5 µl of cells at ~3×10^6/ml were mixed with 25 µl low melt agarose (37°C). This cell suspension was then dropped into one of the wells of a CometSlide (R&D Systems), immediately covered and allowed to set at 4°C. The coverslips were removed and the cells lysed in 0.75% (w/v) SDS (Sigma, Poole, Dorset, UK) and 1% (v/v) DMSO (Sigma) in lysis solution supplied with the kit for 30 minutes at 37°C. The lysis solution was replaced with alkali solution [0.3 M NaOH, 1 mM EDTA (Sigma) and 20 µg/ml Proteinase K (Amresco-Anachem Ltd., Luton, Bedfordshire, UK)] for 30 minutes at 4°C to denature the DNA. Slides were placed in a horizontal electrophoresis tank filled with alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 12.3), left for 20 minutes, electrophoresed for 10 minutes at 25 V (300 mA) and dehydrated in ice-cold methanol (100% – 5 minutes), then ethanol (100% – 5 minutes) and allowed to air-dry overnight at room temperature. The slides were then stained using 20 µl per well of ethidium bromide (15 µg/ml; Sigma) and covered. Slides were viewed using a Leitz DMRB microscope fitted with a N2.1 filter block, providing an excitation filter of 515-560 nm from a 50 W mercury lamp and a barrier filter of 580 nm. Cells were analysed using Komet 4 software (Kinetic Imaging Ltd., Liverpool, UK).

HPLC analysis of testis DNA

HPLC of DNA from fresh and frozen testis samples was performed as described (Selfridge et al., 2001).



RESULTS

Ercc1 expression in testis

Northern analysis of RNA extracted from adult mice revealed that *Ercc1* mRNA was expressed in a wide range of tissues, but was most abundant in the testis (Fig. 1A). The high level of *Ercc1* expression in the testis was confirmed by a western blot, using an antibody raised against recombinant mouse Ercc1, although the difference between testis and other tissues was less pronounced at the protein level (Fig. 1A). The significance of the multiple Ercc1 protein species in the liver is discussed elsewhere (Selfridge et al., 2001). We have found that our standard loading controls for blots that involve reprobing for housekeeping genes, such as *Gapdh* or actin, are inappropriate when different tissues are being compared. For this reason densitometric analysis of the level of ethidium bromide staining of northern gels and Coomassie blue staining of duplicate western gels was used to confirm that equal loadings were achieved (data not shown).

In mice, the first wave of spermatogenesis occurs synchronously during testis maturation with spermatocytes at the leptotene stage of prophase I detectable on about day 10 (Bellve et al., 1977). To see if the high levels of *Ercc1* expression were associated with meiotic crossing over, RNA and protein samples from testes of wild-type mice (p8-42) were subjected to northern (Fig. 1B) and western analysis (Fig. 1C). The levels of *Ercc1* mRNA increased with age, but when the filter was reprobed for actin mRNA, the *Ercc1*/actin ratio determined by phosphorimagery was essentially constant from p8-35 and only increased slightly at p42. The levels of Ercc1 protein were more variable, perhaps because they were determined by densitometry rather than phosphorimagery. The Ercc1/mitochondrial core protein II ratios shown in Fig. 1C are the means of two separate

Fig. 1. *Ercc1* expression in mouse testis. (A) Pattern of *Ercc1* expression in mouse tissues. Upper panel: total RNA (30 µg) from a range of tissues was analysed by northern blotting using an *Ercc1* cDNA probe [Probe a in Selfridge et al. (Selfridge et al., 2001)]. Lower panel: total protein (80 µg) was analysed by western blotting using an antibody raised against a fragment of mouse Ercc1. (B) Developmental pattern of *Ercc1* mRNA in testis. Total RNA (30 µg) extracted from testes of mice (ranging from 8-42 days post partum) was analysed by northern blotting using an *Ercc1* cDNA probe. The filter was then reprobed for actin mRNA using a mouse α-actin cDNA probe (Minty et al., 1981). The *Ercc1*/actin mRNA ratio was determined by phosphorimagery and is expressed relative to the p8 sample. (C) Developmental pattern of Ercc1 protein in testis. Total protein (80 µg) extracted from testes of mice (ranging from 8-35 days post partum) was analysed by western blotting using the antibody against Ercc1. Wild type (Wt) and *Ercc1* null liver samples were used to demonstrate the specificity of the antibody. The filter was then reprobed with an antibody against mitochondrial core protein II (CP II). The Ercc1/CP II protein ratio was determined by densitometry and is expressed relative to the p8 sample. The ratios shown are the means of two separate determinations on two independent samples at each age. (D) Analysis of *Ercc1* transgene expression. Upper panel: total RNA (30 µg) extracted from a range of tissues from transgene-positive *Ercc1*-deficient mice was analysed by northern blotting using a probe from the 5′ end of the mouse transthyretin (*Ttr*) gene [Probe b in Selfridge et al. (Selfridge et al., 2001)]. mRNA from the endogenous *Ttr* gene and from the transgene (TG) is indicated. Lower panel: total protein (80 µg) was analysed by western blotting using the antibody against Ercc1.

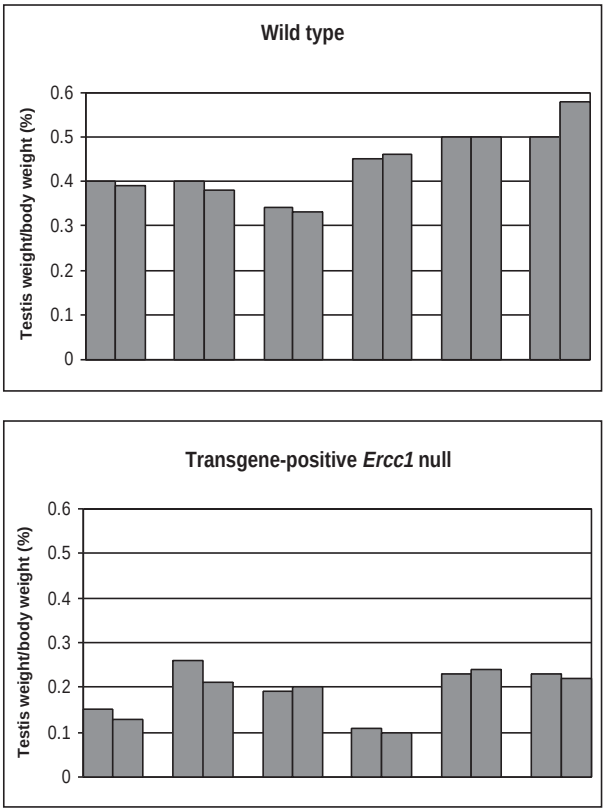


Fig. 2. Testis weights. Testis weights from 6-week old wild-type and transgene-positive *Ercc1* nulls are expressed as a percentage of body weight. 6 animals of each genotype were used. The weights of individual testes from the same animal are displayed together.

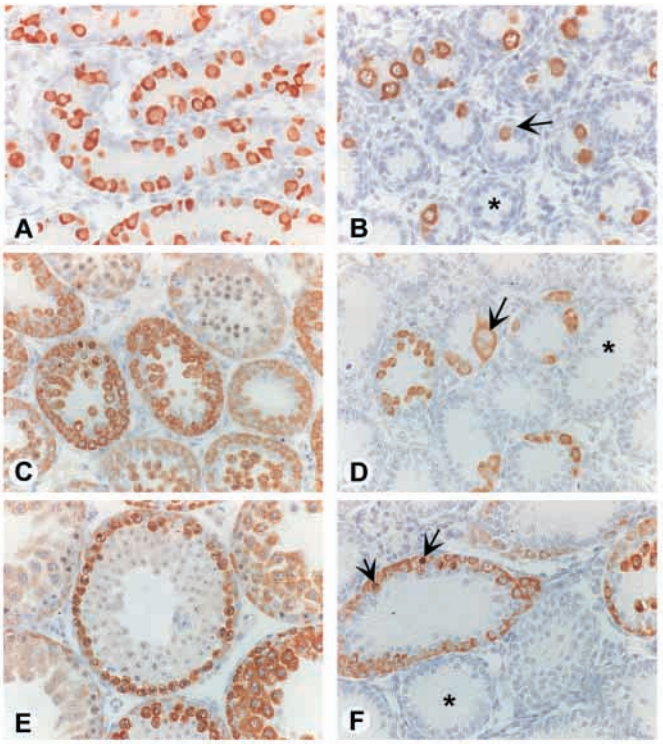


Fig. 3. Testes from control and *Ercc1*-null mice immunostained for the germ cell-specific marker Dazl. (A) 3-day wild type, (B) 3-day *Ercc1* null, (C) 12-day wild type, (D) 12-day *Ercc1* null, (E) 22-day wild type, (F) 22-day *Ercc1* null. The number of germ cells in the *Ercc1* nulls was reduced compared with wild-type littermates at all ages examined. The number of germ cells in individual tubules in the nulls was highly variable even within the same testis. Tubules devoid of germ cells (*) and germ cells with abnormal morphology (arrows) in the nulls were seen at all ages.

determinations on two independent samples. Ercc1 protein was also present from the earliest stages examined with an increase in the p23 and p35 samples. This increase could reflect expression in round spermatids detected by immunohistochemistry (see later section on *Ercc1* expression in male germ cells), as they would be present at these times. However, there was no suggestion at the mRNA or protein level that *Ercc1* expression was linked with the first wave of meiotic crossing over.

Ercc1 was absent from the testes and all other tissues of *Ercc1*-deficient mice examined (data not shown). The *Ercc1* transgene used to correct the liver phenotype is under the control of the transthyretin (*Ttr*) gene promoter (Selfridge et al., 2001). The endogenous *Ttr* gene is expressed strongly in the liver, but also in the choroid plexus of the brain. Northern analysis of transgene-containing *Ercc1* nulls with a *Ttr* probe revealed the liver-specific pattern of transgene expression, with no transgene transcripts detectable in other tissues (Fig. 1D). However, low levels of Ercc1 protein were detected in the testes and brain of some transgene-containing *Ercc1* nulls analysed, including the one shown in Fig. 1D. The level of Ercc1 protein in the testes of these animals was variable, but was always <10% of the wild-type level.

Infertility in *Ercc1*-deficient mice

Ercc1 nulls die by 3 weeks of age, but it was possible to assess the fertility of transgene-positive *Ercc1* nulls, which live for up to 12 weeks (Selfridge et al., 2001). Although copulation plugs were observed, no pregnancies were ever detected from matings between male or female transgene-positive *Ercc1* nulls and wild-type partners and we conclude that transgene-positive *Ercc1* nulls of both sexes are infertile. Individual testis weights, expressed as a percentage of body weight, from 3- and 6-week transgene-positive *Ercc1* nulls were compared with wild-type littermates (see Fig. 2 for 6-week data). This correction was made because body weights of transgene-positive *Ercc1* nulls are, on average, only 60% of wild type (Selfridge et al., 2001). Even correcting for body weight, mean testis weights from transgene-positive *Ercc1* nulls were only 50% of controls (3-week: wild type 0.27±0.07(s.d.)%, transgene-positive *Ercc1* null 0.16±0.03%, $P=2.8\times10^{-5}$ by Student's *t*-test. 6-week: wild type 0.44±0.08%, transgene-positive *Ercc1* null 0.19±0.05%, $P=4.9\times10^{-9}$).

Morphological appearance of testes from *Ercc1*-null mice

The germ cell complement of testes from wild-type and *Ercc1* nulls were examined using immunohistochemistry for the germ cell-specific protein Dazl (Ruggiu et al., 1997). On days 3, 7 (not shown), 10 (not shown), 12 and 22, mutant animals contained less germ cells within the seminiferous epithelium than their wild-type littermates (Fig. 3, compare A, C, and E, with B, D, and F, respectively). The number of germ cells in individual

tubules in the nulls was highly variable, even within the same testis, when compared to the uniform appearance of tubules from control littermates. Tubules devoid of germ cells (Sertoli cell only, SCO; Fig. 3 asterisk) and germ cells with abnormal morphology (arrows) were seen at all ages in the *Ercc1*-null testes. In 22-day old wild-type mice the seminiferous epithelium was well developed (Fig. 3E), a lumen had formed and, in agreement with published data (Bellve et al., 1977), the most advanced germ cell stage was an early round spermatid. In age-matched *Ercc1*-null littermates (Fig. 3F) germ cells were limited to spermatogonia and a very few pachytene spermatocytes while the majority of tubules appeared to be SCO. Formation of tubule lumens was either incomplete, or absent and the Sertoli cell cytoplasm occupied the centre of the tubules.

Morphological appearance of testes from transgene-positive *Ercc1*-null mice

The introduction of the *Ercc1* transgene onto the null background resulted in partial restoration of the early wave of spermatogenesis and germ cells up to and including pachytene spermatocytes were observed in 3-week-old animals (Fig. 4B, labelled P). Although some tubules appeared to be SCO (asterisks), tubule lumens had begun to form. At 6-7 weeks of age the testes of wild-type mice contained a full complement of germ cells with spermatozoa ready for release into the tubule lumens (Fig. 4C, labelled s). In age-matched transgene-positive *Ercc1* nulls the testicular phenotype was variable, both between animals and within the testes of individual males. Although, in every case the number of germ cells within the seminiferous tubules was substantially reduced (Fig. 4D) and SCO tubules were observed (asterisk), all stages of germ cell development from spermatocytes (arrowheads) to mature elongated spermatids (§) were detected. In tubules containing a substantial population of germ cells 'gaps' within the epithelium (arrows) were often observed. Critically, the loss of germ cells within any individual tubule did not appear to be confined to a single stage of development and the net result was a more disordered arrangement of germ cells than seen in normal spermatogenesis (Oakberg, 1956).

The reduction in the diameter of the seminiferous tubules (around 50%) and in the height of the seminiferous epithelium in transgene-positive *Ercc1* nulls compared with controls was most evident when tissue embedded in Araldyte was examined (Fig. 4E compared with F, double-headed arrows). Toluidine Blue staining revealed occasional lipid droplets close to the basement membrane (Fig. 4E, arrows) of 10-week controls. In the 10-week transgene-positive *Ercc1* nulls (Fig. 4F) there was an accumulation of numerous lipid droplets in the cytoplasm of Sertoli cells (arrows) and interstitial Leydig cells (L). The lipid in the Sertoli cells is consistent with accumulation of waste products following phagocytosis of germ cell remnants.

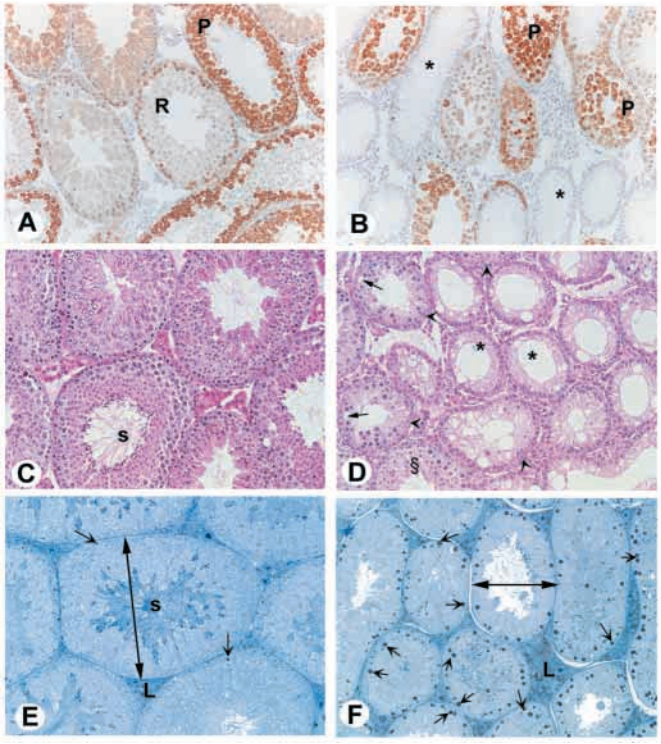


Fig. 4. Testes from control and transgene-positive *Ercc1*-deficient mice. (A) 3-week wild type, immunostained for Dazl, containing a normal complement of germ cells including pachytene spermatocytes (P) and round spermatids (R). (B) 3-week transgene-positive *Ercc1* null, immunostained for Dazl; note that germ cells are present up to and including pachytene spermatocytes (P), but many tubules are SCO (Sertoli cell only, *). Although germ cell numbers are reduced compared with wild type (A) they are substantially increased compared to age-matched null animals (Fig. 3F). (C) 6-week wild type, Haematoxylin and Eosin stained; full spermatogenesis is present with germ cells at different stages of development arranged in characteristic associations (stages), mature spermatozoa (s) are seen in the centre of the tubule at stage VIII. (D) 7-week transgene-positive *Ercc1* null, Haematoxylin and Eosin stained; note that the germ cell complement is variable with some SCO tubules (*), whilst in others, although significant germ cell loss has occurred (arrows point to gaps within the epithelium), germ cells including spermatocytes (arrowheads) and elongate spermatids (§) are present. (E) 10-week wild-type, plastic section; note very occasional small lipid droplets close to the basement membrane (arrows). (F) 10-week transgene-positive *Ercc1* null, plastic section; note reduced diameter of the seminiferous tubules due to reduced germ cell numbers (double-headed arrow) compared with age-matched wild type (E), and accumulation of numerous lipid droplets within the Sertoli cells (arrows). Positive lipid staining was noted in interstitial Leydig cells in both wild-type and *Ercc1* null testes (L).

Apoptosis within testes of *Ercc1*-deficient mice

Germ cells with damaged DNA or aberrant meioses are eliminated by apoptosis (for a review, see Baarends et al., 2001). Testicular sections were stained using the Apotag method that has been used previously on fixed testis sections (Sharpe et al., 1998). In 7-week wild-type mice immunopositive germ cells were detected at stages XI and XII (Fig. 5A, arrows). These cells were infrequent and located close to the basement membrane, consistent with previous reports that most cells undergoing apoptosis in the normal testis are mitotically active A type spermatogonia (Krishnamurthy et al., 1998). In age-matched transgene-positive *Ercc1* null littermates, although considerably fewer germ cells were present than in controls, the immunopositive staining was sometimes associated with groups of germ cells (Fig. 5B). We did not detect elevated levels of apoptotic cells in *Ercc1* nulls between days 7 and 22, consistent with rapid elimination of cells containing abnormal DNA (not shown).

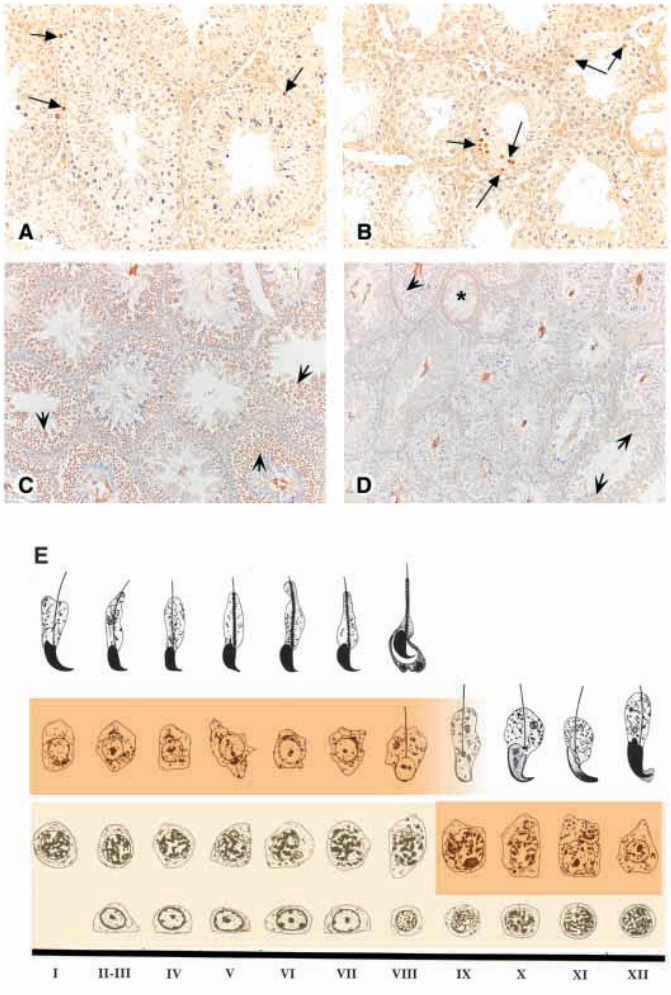


Fig. 5. (A,B) Apoptosis in *Ercc1*-deficient testes and (B,C) pattern of expression of the Ercc1 protein. (A) 7-week wild type and (B) 7-week transgene-positive *Ercc1* deficient testes. Note that apoptosis, visualised by the Apotag assay, was detected in only a few cells in each sample. Apoptotic cells in the wild type (arrows) were stage dependent (stages I and XII), whereas in *Ercc1*-deficient testis some apoptotic cells were observed at all ages and often occurred in clusters (arrows in B). (C) 9-week wild-type and (D) 9-week transgene-positive *Ercc1*-deficient testes. Non-specific staining of sperm tails was seen in both samples. Weak immunopositive staining was detected in cell nuclei of Leydig and Sertoli cells from wild-type testis. Examination of specific staining of wild-type germ cells (C) revealed that some immunopositive reaction was present in pre-meiotic germ cells and that the most intense immunopositive reaction was localised to late pachytene spermatocytes (stages IX-XI) and round spermatids (stages I-VII, arrowheads). Very faint specific nuclear staining was detected in a few round spermatids from transgene-positive *Ercc1*-deficient testis (arrowheads in D) (*, SCO). (E) A summary diagram based on the stages of the spermatogenic cycle (adapted from Oakberg, 1956) showing germ-cell-specific staining for Ercc1. As germ cells proceed through meiosis from pre-meiotic spermatogonia (bottom left) to mature spermatozoa (top right) they are arranged vertically in the seminiferous tubules in characteristic associations (numbered stages) as indicated. The intensity of the immunopositive staining observed is indicated by the intensity of shading: the heaviest shading denotes the intense immunopositive reaction from late pachytene spermatocytes (stages IX-XI) to round spermatids (stages I-VII); the lighter shading denotes the faint staining in pre-meiotic germ cells through to mid pachytene spermatocytes.

Ercc1 expression in male germ cells

Northern analysis on extracts from whole testes had not indicated a clear association between *Ercc1* expression and a particular stage in spermatogenesis. To investigate *Ercc1* expression in individual germ cells, immunohistochemistry was undertaken on testis sections from 9-week-old animals using an affinity-purified Ercc1-specific antiserum. In wild-type males Ercc1 immunopositive staining was barely detectable in Sertoli and Leydig cells and, while some immunopositive reaction was present in all germ cells, the most intense immunoreaction was in germ cells from late pachytene spermatocytes (stage IX) to round spermatids (stage VIII) (Fig. 5C). These data, summarised on a diagram of the stages of the spermatogenic cycle in Fig. 5E, are consistent with the increased levels of Ercc1 protein detected in p23 and p35 testes by western blotting (Fig. 1C). Some non-specific staining of sperm tails was seen in all samples and, in age-matched transgene-positive *Ercc1*-null littermates very faint immunopositive staining was observed in some germ cells (Fig. 5D, arrows), consistent with the low levels of transgene-encoded *Ercc1* expression detected on western blots (Fig. 1D). No immunopositive staining was seen in 3-week-old *Ercc1* nulls (data not shown).

Analysis of spermatozoa from *Ercc1*-deficient mice

Consistent with the deficit in spermatogenesis observed, the number of sperm recovered from the epididymides of 7-week-old transgene-positive *Ercc1* nulls was very limited. While sperm from age-matched controls (Fig. 6A) were morphologically normal, those from transgene-positive *Ercc1*-null littermates (Fig. 6C) showed a range of head (§) and tail (*) malformations.

Alkaline comet analysis was undertaken to assess the level of DNA damage (single-and double-strand breaks and alkali-labile sites) in sperm from the different *Ercc1* genotypes. Transgene-positive *Ercc1* nulls (Fig. 6D) consistently showed much larger comet tails than wild-type littermates (Fig. 6B). Statistical analysis of the comet data (summarised in Fig. 6E) confirmed that the increase in the median percentage of DNA in comet tails in transgene-positive *Ercc1* nulls compared to wild type was statistically significant ($P<0.05$ by Mann-Whitney U test). Sperm comet tails in *Ercc1* heterozygous males were intermediate between the other two genotypes, presumably reflecting a 50% reduction in Ercc1 levels. Thus, the limited number of sperm produced in transgene-positive *Ercc1* null animals contained DNA with high levels of damage.

Oxidative DNA damage in *Ercc1*-deficient testis

The level of oxidative DNA damage in testis DNA was assessed by measuring the commonest oxidised base, 8-oxoG, by HPLC with electrochemical detection. Levels were calculated as moles of 8-oxoG/10^5 moles of deoxyguanosine. The mean value obtained for 6- to 10-week-old control testis was 0.16±0.05 (n=7). The value for transgene-positive *Ercc1*-null littermates was 0.54±0.30 (n=4). The 3-fold increase in oxidative DNA damage levels in *Ercc1*-deficient testis was statistically significant (P=0.008 by Student's t-test).

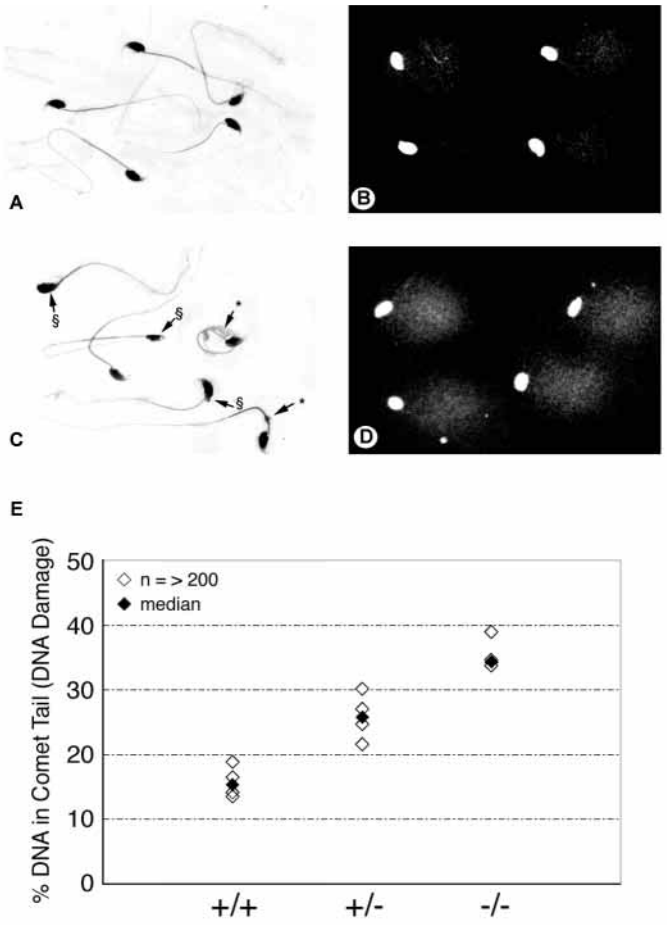


Fig. 6. Morphology and comet assay on sperm isolated from 7-week wild-type and *Ercc1*-deficient mice. Morphology of (A) wild-type and (C) transgene-positive *Ercc1*-deficient sperm, note abnormal shapes of sperm heads (§) and presence of tail abnormalities (*). Comet assay of (B) wild-type and (D) transgene-positive *Ercc1*-deficient sperm. Note increased size of comet tails compared with B. (E) Summary of comet assays. At least 200 sperm from four animals of each genotype were scored. The median value for the percentage of the DNA in the comet tail is shown for each sample, along with the median value (black diamond) for each genotype (+/+, wild type; +/–, *Ercc1* heterozygote; –/–, transgene-positive *Ercc1* deficient). DNA damage was significantly higher (P<0.05 by Mann-Whitney U test) in transgene-positive *Ercc1*-deficient than wild-type sperm.

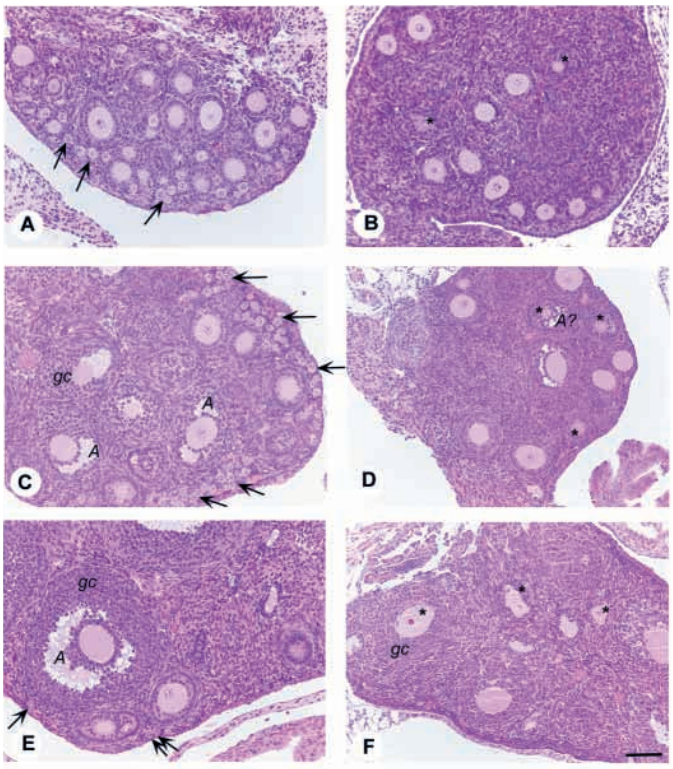


Fig. 7. Ovaries from control and *Ercc1*-deficient mice. (A) 8-day wild type, (B) 8-day *Ercc1* null, (C) 14-day wild type, (D) 14-day *Ercc1* null, (E) 6-week wild type, (F) 6-week transgene-positive *Ercc1* null mice. Note that numerous primary follicles (arrows) are present in the wild-type samples (A,C,E), but appear to be absent from the ovaries in both types of *Ercc1*-deficient sample (B,D,F). Organisation of granulosa cells (gc) around oocytes occurred in both wild-type and *Ercc1*-deficient ovaries. By day 14 some follicles had matured sufficiently to form an antrum (A) in both wild-type and *Ercc1*-null ovaries (C,D). The development of the most mature follicles appeared less advanced in the null animals on day 14 and in the adult transgene-positive females compared with their wild-type littermates. Oocytes with an abnormal appearance were frequently observed in both types of *Ercc1*-deficient ovary (*). Scale bar, 100 μm.

Morphological appearance of ovaries from *Ercc1* null females

Ovaries were analysed from both *Ercc1* nulls (days 8, 10, 14) and transgene-positive *Ercc1* nulls (day 16 and adult). All transgene-positive *Ercc1* null adult females were infertile and no fully mature antral follicles were detected in any of the ovaries examined (e.g. Fig. 7F). Female germ cells enter meiotic prophase during fetal life (Hartung and Stahl, 1977) and, after birth, the oocyte remains arrested in diplotene (the so-called dictyate stage) during an extended growth phase within the follicle whilst it matures in size. At all ages examined, in both types of *Ercc1*-deficient animal, the number of oocytes was reduced although some variation in numbers was observed between animals and between ovaries from the same animal. Although some large oocytes, enclosed in follicles with multiple layers of granulosa cells, were seen even in null females, oocytes often appeared to be in the process of degeneration (asterisks in Fig. 7B,D,F). Thus, contrary to the situation in spermatogenesis, oogenesis was similarly affected in *Ercc1* null and transgene-positive *Ercc1* nulls. The most striking difference between ovaries from immature *Ercc1*-null and transgene-positive *Ercc1*-null females and their wild-type littermates was the absence of primary follicles from the periphery of the ovary.

DISCUSSION

In this study we investigated the hypothesis that the recombination repair functions of Ercc1 were essential for meiotic crossing over. Instead, we found a general requirement for Ercc1 throughout spermatogenesis and oogenesis. Two types of mice were used: *Ercc1* knockouts (McWhir et al., 1993) and *Ercc1* knockouts where the liver phenotype has been

corrected by an *Ercc1* transgene under the control of a liver-specific promoter (Selfridge et al., 2001).

We have shown that *Ercc1*, like its partner *Xpf* (Shannon et al., 1999), is expressed at the highest level in the testis. This alone does not imply a key role for the recombination repair functions of Ercc1/Xpf, because many other DNA repair proteins are also highly expressed in testis. In particular, the expression of four genes involved in NER only is also elevated in the testis [*Xpb* (Weeda et al., 1991); *Xpc* (Li et al., 1996); *Mhr23a* and *b* (*Rad23a* and *b*) (van der Spek et al., 1996)]. Meiotic crossing over during the first synchronous wave of spermatogenesis coincides with a sharp increase in the expression of some mismatch repair genes, such as *Mlh1*, which are essential for the repair of heteroduplex intermediates formed during homologous recombination (Edelmann et al., 1996). In contrast similar levels of *Ercc1* expression were detected in testes of all ages examined. This and a previous study (Shannon et al., 1999) are in agreement that *Ercc1/Xpf* do not show a mismatch repair protein-like surge in expression coincident with the first wave of meiotic crossing over.

The highest levels of immunostaining for Ercc1 were seen in meiotic cells from late pachytene in prophase I, through the second meiotic division, to round spermatids. These results are consistent with the western blot data where an increase in the total amount of Ercc1 protein was noted in samples from day 23 onwards at a time when pachytene spermatocytes and round spermatids are the most abundant cell types within the testis. Earlier germ cell stages contained less intense immunostaining. Crossing over initiates with the appearance of double-strand breaks in leptotene and is completed by late pachytene (Mahadevaiah et al., 2001). Thus, the stages with the highest levels of Ercc1 protein follow rather than precede meiotic crossing over, again providing no support for Ercc1 having a critical role in meiotic crossing over.

In *Ercc1*-null males germ cell depletion was observed in testes on days 3 and 7 at a time when they are mitotically active, but prior to entry into meiotic prophase, which occurs on and after day 8 (Bellve et al., 1977). Germ cell loss was highly variable between individual testes, even those from the same animal and, even in the absence of Ercc1, a very few germ cells in day 22 males were observed to have the appearance of pachytene spermatocytes. Strikingly, the low level of transgene-derived *Ercc1* expression was sufficient to markedly increase the numbers of germ cells and allowed some to complete meiosis and mature into spermatozoa.

Ercc1-deficient female mice were also infertile, but the oogenesis defect was less severe and, even in the complete absence of Ercc1, some germ cells were able to complete meiotic prophase, arrest in dictyate, produce signals to the granulosa cells to initiate organisation of follicles and grow. Unlike the situation in males, the same phenotype, namely reduced numbers of primary follicles that have completed prophase I and degenerating maturing oocytes, was seen in both *Ercc1* nulls and transgene-positive *Ercc1* nulls, suggesting that there is no ectopic *Ercc1* transgene expression in the ovary.

Studies in *S. cerevisiae* have indicated that mismatch repair is essential to repair mismatches and small loops generated in heteroduplex DNA during meiotic crossing over (reviewed by Kirkpatrick, 1999). The repair of large loops also involves RAD1/RAD10, presumably because of its ability to cleave 3′ single-stranded tails projecting from DNA duplexes. Our studies indicate that this role for Ercc1 is not essential for meiotic crossing over in mouse gametogenesis. Indeed, a second large loop repair pathway independent of RAD1, RAD10 and MSH2 has been identified in *S. cerevisiae* (reviewed by Kirkpatrick, 1999). The observed defects must be due instead to a more general requirement for the recombination repair and/or NER functions of Ercc1. A meiotic checkpoint that detects spermatocytes with unsynapsed chromosomes and eliminates them by p53-independent apoptosis has been described (Odorisio et al., 1998). However, the quality control system for sperm production operates at other levels too: a sizeable fraction of germ cells (mainly diploid spermatogonia) die and are removed during normal spermatogenesis (reviewed by Braun, 1998). The p53-dependent apoptotic response to ionising radiation found in mitotic cells also operates in this tissue (Odorisio et al., 1998). This classical DNA damage response would be expected to be particularly important in such a rapidly dividing tissue. The disorganised appearance and frequent gaps in *Ercc1*-deficient seminiferous tubules indicate the excessive elimination of, presumably DNA-damaged, germ cells. Clustered apoptoses, affecting a range of germ cell stages, were observed in *Ercc1*-deficient testis whereas only rare apoptoses affecting single germ cells were seen in control sections. Given the, presumably sporadic, nature of the endogenous DNA damage occurring in the testis and the transient nature of apoptosing cells, we did not expect to see the very high levels of apoptosis observed after a defined cytotoxic insult, such as treatment with methoxyacetic acid (Krishnamurthy et al., 1998). An accumulation of cytoplasmic lipid droplets in Sertoli cells is often associated with germ cell degeneration (Paniagua et al., 1987). In the present study the accumulation observed in 10-week transgene-positive *Ercc1* nulls provides testimony to the excessive germ cell elimination and phagocytosis that has occurred following germ cell death.

The limited number of sperm that were produced by transgene-positive *Ercc1*-deficient mice had a high frequency of malformations and the animals were infertile. Spermatogenesis is unaffected (at least as judged by normal fertility) in *Xpa* (de Vries et al., 1995; Nakane et al., 1995) and *Xpc* (Sands et al., 1995) knockout mice and, in man, males with XP are also fertile (Kraemer, 1993). This suggests that infertility in *Ercc1*-deficient male mice results from the lack of an additional Ercc1 function, rather than from an NER deficit.

Sperm from transgene-positive *Ercc1*-deficient mice had significantly higher levels of DNA strand breaks than control samples. This could be due to the lack of the recombination repair or NER functions of Ercc1. In vitro an NER complex will form and 3′ incision by Xpg can take place in the absence of Ercc1/Xpf (Evans et al., 1997). Strand breaks would not be predicted from the failure of Ercc1/Xpf to cut adjacent to an interstrand crosslink in the recombination repair model favoured by Kuraoka et al. (Kuraoka et al., 2000a). However, in both of these cases strand breaks could accumulate at replication forks stalled at unrepaired lesions. The failure of Ercc1 to act in the single-stranded annealing pathway for double-strand break repair and homologous recombination would lead directly to the accumulation of strand breaks because the remaining single-strand tails would prevent ligation. Comet assays on sperm from *Xpa* and *Xpc* knockout mice could help to resolve the relative contributions of the

recombination repair and NER deficits to the accumulation of strand breaks in *Ercc1*-deficient mice.

Ercc1, in common with other NER genes, is expressed in all tissues examined. This is consistent with a role for NER in the repair of endogenous, predominantly oxidative DNA damage in internal tissues as well as the key role of repairing UV-induced DNA damage in the skin (reviewed by Lindahl, 1993). Traditionally BER was considered to have the key role in removing the commonest oxidised base, 8-oxoG, which is strongly mutagenic and also acts as a block to transcription by RNA polymerase II (Le Page et al., 2000a). However, the discovery of transcription coupled repair of 8-oxoG and the observation that this process continues to operate in *Ogg1* null cells (Le Page et al., 2000b) has, belatedly, led to the recognition that NER may also have an important role to play in the repair of 8-oxoG (Kuraoka et al., 2000b; Le Page et al., 2000b). Three-fold higher levels of 8-oxoG were found in DNA extracted from *Ercc1*-deficient testis than from control littermates, demonstrating that Ercc1 is important for the repair of 8-oxoG, particularly considering that the levels of 8-oxoG in the livers of *Ogg1*-null mice were only 1.7-fold higher than controls (Klungland et al., 1999). Most likely the increase in 8-oxoG results from the lack of the NER function of Ercc1. The role of NER in the repair of 8-oxoG and the significance of this damage to the phenotype in *Ercc1*-deficient testis could be determined by measuring 8-oxoG levels in *Xpa* and *Xpc* knockout testis.

We believe that the consequences of *Ercc1* deficiency for gametogenesis can be explained by a general requirement for Ercc1 to repair DNA damage in all dividing cells, rather than a specific role in meoisis, and by the stochastic nature of DNA damage. The more severe consequences of *Ercc1* deficiency for spermatogenesis than oogenesis reflect differences between the processes themselves. Primordial germ cells migrate from the hind gut to the gonad between day 8.5-10.5 p.c. During this time they are actively proliferating in both sexes, so that starting from about 100 cells, more than 1000 are present on day 10.5 p.c. (Godin et al., 1990). Female primordial germ cells enter meiosis about day 12.5-13.5 p.c. and meiosis continues during foetal development until it becomes arrested at diplotene stage, on or about 5 days of age (p.p.). Male germ cells continue to divide mitotically until day 16 p.c. and then become arrested in G_1 (Vergouwen et al., 1991) until they resume mitotic activity after birth. Spermatogonial cells undergo up to 6 further mitotic divisions before they enter meiosis (de Rooij, 2001). Thus, in both processes, mitotic expansion precedes entry into meiosis, but the expansion is much more extensive in the male. The premeiotic loss of germ cells with damaged DNA is greater in the male because rapidly cycling cells are particularly susceptible to the deleterious effects of DNA damage on DNA replication. DNA damage is a continuous, random process and, in both sexes, some undamaged germ cells, or cells with non-lethal levels of damage, survive the mitotic expansion and enter meiosis before succumbing to damage acquired during the meiotic stages. In transgene-positive *Ercc1*-deficient males small amounts of transgene-derived Ercc1 are sufficient to partially rescue the phenotype resulting in complete spermatogenesis in some tubules and production of mature sperm, albeit abnormal and with elevated levels of DNA damage.

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