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Reduced hematopoietic reserves in DNA interstrand crosslink repair-deficient *Ercc1*$^{-/-}$ mice

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The ERCC1-XPF heterodimer is a structure-specific endonuclease involved in both nucleotide excision repair and interstrand crosslink repair. Mice carrying a genetic defect in *Ercc1* display symptoms suggestive of a progressive, segmental progeria, indicating that disruption of one or both of these DNA damage repair pathways accelerates aging. In the hematopoietic system, there are defined age-associated changes for which the cause is unknown. To determine if DNA repair is critical to prolonged hematopoietic function, hematopoiesis in *Ercc1*$^{-/-}$ mice was compared to that in young and old wild-type mice. *Ercc1*$^{-/-}$ mice (3-week-old) exhibited multilineage cytopenia and fatty replacement of bone marrow, similar to old wild-type mice. In addition, the proliferative reserves of hematopoietic progenitors and stress erythropoiesis were significantly reduced in *Ercc1*$^{-/-}$ mice compared to age-matched controls. These features were not seen in nucleotide excision repair-deficient *Xpa*$^{-/-}$ mice, but are characteristic of Fanconi anemia, a human cancer syndrome caused by defects in interstrand crosslink repair. These data support the hypothesis that spontaneous interstrand crosslink damage contributes to the functional decline of the hematopoietic system associated with aging.

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Introduction

The hematopoietic system possesses a robust proliferative capacity that provides 10^{12} blood cells per day in an adult human (Effros and Globerson, 2002), as well as a large reserve that can be mobilized following acute challenges including blood loss or systemic infection. However, the hematopoietic stem cells that give rise to all blood cell lineages are limited in their replicative and repopulating abilities (Van Zant and Liang, 2003) and the hematopoietic system functionally declines with age. This is evidenced by the mild cytopenia, particularly anemia, found in 13% of otherwise healthy individuals over 70 years of age (Salive *et al*, 1992) and by a reduced capacity for bursts of red blood cell (RBC) production following hypoxia (Udupa and Lipschitz, 1984b) and neutrophil production after endotoxin challenge (Timaffy, 1962). Age-associated hematopoietic deficits result in decreased physical performance (Penninx *et al*, 2003) and mortality (Wilkinson and Warren, 2003). The cause(s) of this functional decline is unknown but may include decreased hematopoietic stem cell reserves, imbalance in the cytokines required for effective blood cell production, alterations in the hematopoietic microenvironment that reduce output and/or cell mobilization to the periphery. Recently, the reported increase in genomic instability in the peripheral blood of aged individuals has suggested that an accumulation of genetic defects in hematopoietic progenitors may be involved (Ben Yehuda *et al*, 2000).

ERCC1-XPF is a protein heterodimer that is essential for the multistep, 'cut and patch' nucleotide excision repair (NER) pathway that eliminates a wide range of helix-distorting DNA lesions induced by UV irradiation and numerous chemicals (Biggerstaff *et al*, 1993; Sijbers *et al*, 1996). Together the proteins act as a structure-specific endonuclease, which makes an incision 5′ of DNA lesions to allow removal of the damaged patch of DNA (Mu *et al*, 1995; de Laat *et al*, 1998). The ERCC1-XPF protein complex has functions outside of NER, for instance it is essential for DNA interstrand crosslink (ICL) repair (Niedernhofer *et al*, 2004). Mutations in *Ercc1* have not been described in man, but *Ercc1*$^{-/-}$ mice are viable. However, their phenotype is severe and quite distinct from other NER-deficient mice (McWhir *et al*, 1993; Weeda *et al*, 1997). Whereas most NER-deficient mice are phenotypically normal, albeit prone to UV- and chemical-induced cancer (de Vries *et al*, 1995), *Ercc1*$^{-/-}$ mice develop normally but quickly display severe wasting that culminates in death in the fourth week of life. Many of the features of the *Ercc1*$^{-/-}$ mice are reminiscent of mammalian aging, including ataxia, kyphosis, osteopenia, weight loss, skin atrophy, sarcopenia and hepatocellular polyploidization (Hasty *et al*, 2003). This led to the designation of the *Ercc1*$^{-/-}$ phenotype as a highly accelerated and segmental progeria (Weeda *et al*, 1997).

We utilized *Ercc1*$^{-/-}$ mice to examine the effects of loss of DNA repair pathways on degeneration of the hematopoietic system. We demonstrate here for the first time that both basal hematopoiesis and reserve capacity under stress are severely reduced in *Ercc1*$^{-/-}$ mice, in a manner consistent with that found in normal aging. This supports the validity of the *Ercc1*$^{-/-}$ mouse as a progeroid model of hematopoietic aging. Furthermore, we show that progenitor activity is only mildly affected in the absence of NER alone, and *Ercc1*$^{-/-}$ progenitors are hypersensitive to exogenous ICL

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damage, suggesting that the loss of ICL repair may play an important role in spontaneous tissue exhaustion in these mice. In support of this, the results with the *Ercc1*$^{-/-}$ mice create a striking parallel with the deficits exhibited by the human ICL-sensitive inherited syndrome Fanconi anemia. These data support a significant role for ICL repair in the maintenance of hematopoietic function.

Results

Ercc1$^{-/-}$ mice have multilineage peripheral blood cytopenia

To determine if the hematopoietic system was affected in the DNA repair-deficient *Ercc1*$^{-/-}$ mice, we analyzed the peripheral blood of 3-week-old, prematurely aged *Ercc1*$^{-/-}$ mice ($n = 34$) and their wild-type (wt) littermates (Table I). The mutant mice had significantly decreased white blood cell and platelet counts compared to wt mice. White blood cell counts were not affected in 13- to 15-day (d)-old *Ercc1*$^{-/-}$ mice compared to littermates, demonstrating that this hematopoietic deficit was not due to a developmental defect, but rather declined over the lifetime of the mice. Platelet levels were suppressed in the *Ercc1*$^{-/-}$ mice as early as 15 d of age (Table I). Peripheral RBCs and hemoglobin levels were not significantly affected in the *Ercc1*$^{-/-}$ mice, although there was a trend toward decreased erythropoiesis. Reticulocyte counts were also within normal ranges in knockout mice, suggesting that RBC lifespan in these mice was unaffected (data not shown).

Systemic, progressive hematopoietic deficits in *Ercc1*$^{-/-}$ mice

Histological examination of the femoral bone marrow (BM) space of 3-week-old *Ercc1*$^{-/-}$ mice revealed striking hypocellularity compared to age-matched wt mice ($n = 3$; Figure 1A). The BM space exhibited substantial adipose transformation and closely resembled bone sections from aged wt mice. Bone architecture in the *Ercc1*$^{-/-}$ mice was normal, including osteoclast-mediated lacunae formation and the endosteal osteoblast lining of these spaces that normally accompanies hematopoietic colonization, suggesting a hematopoietic-specific defect. Sections from 15-d-old *Ercc1*$^{-/-}$ revealed slightly reduced cellularity and early fatty changes compared to aged-matched animals. The BM of 1-d-old mutant and that of wt mice were indistinguishable. This demonstrates progressive BM degeneration in *Ercc1*$^{-/-}$ mice.

In mice, the primary site of hematopoiesis migrates during development, transitioning perinatally from the fetal liver to the spleen and then to the BM by 2 weeks of age. To examine the possibility that hematopoietic colonization of the BM is merely delayed in *Ercc1*$^{-/-}$ mice, spleen and liver were also analyzed. The ratio of splenic red pulp to lymphocytic white pulp was lower in both *Ercc1*$^{-/-}$ and aged wt mice compared to young wt mice (Figure 1B). While significant hematopoiesis was observed in the splenic red pulp of young wt mice, the red pulp of young *Ercc1*$^{-/-}$ and aged wt mice was predominantly filled with mature RBCs. Furthermore, there was evidence of accumulation of splenic hemosiderin with increasing age of the *Ercc1*$^{-/-}$ mice, suggesting high turnover of erythrocytes. In line with the severely decreased platelet levels observed in the peripheral blood of *Ercc1*$^{-/-}$ mice, both BM and splenic sections from these mice showed reduced megakaryopoiesis, even compared to the normal, aged animals. No significant liver hematopoiesis was observed in any mice, regardless of age or genotype (Figure 1C). The decreased BM hematopoiesis observed in the 3-week-old *Ercc1*$^{-/-}$ mice does not therefore reflect delayed hematopoietic migration to the BM, but rather recapitulates many of the physiological features found in 2-year-old wt mice.

Livers from both the *Ercc1*$^{-/-}$ and the 2-year-old wt mice ($n = 3$ of each genotype) contained increased numbers of polyploid cells with dramatically enlarged nuclei and prominent nuclear inclusions (Figure 1C) as previously reported (Weeda *et al*, 1997). Both features are hallmarks of liver aging (Epstein, 1967). There was also a pronounced infiltration of mature lymphocytes, monocytes and granulocytes in the livers of all *Ercc1*$^{-/-}$ and aged wt mice and this was not observed in young wt mice. This infiltrate is also seen in another DNA repair-deficient, progeroid mouse model expressing a mutant form of the double-strand break (DSB) end-joining repair protein Ku86 (Vogel *et al*, 1999). Together these observations suggest profoundly accelerated tissue damage in *Ercc1*$^{-/-}$ mice and emphasize the parallels between ERCC1-deficient progeria and natural aging.

Loss of Ercc1 decreases immature BM progenitor proliferative potential

Studies of aged humans and mice demonstrate age-related hematopoietic changes affecting multiple cell lineages, suggesting failure in a multipotent hematopoietic progenitor compartment, although this remains controversial (reviewed in Van Zant and Liang, 2003). In addition to the overall hypocellularity observed in *Ercc1*$^{-/-}$ BM, the number of low-density progenitor cells was specifically decreased twofold compared to wt controls (Figure 2A). This cellular fraction is enriched for both lineage-committed and multipotent progenitor activity. To determine if the multipotent early progenitors were specifically affected, we plated low-density progenitors in a cytokine cocktail that stimulates the specific expansion of immature cells (Engelhardt *et al*, 1997;

Table I Peripheral blood parameters of wt and *Ercc1*$^{-/-}$ mice at 2 and 3 weeks of age

Cohort (n)	Age (weeks)	WBC	Plt	RBC	HCT	Hb	MCHC
wt (10)	2	5.7±1.5	1051±112	6.9±0.3	0.34±0.02	6.7±0.4	20.0±0.4
Ercc1$^{-/-}$ (9)	2	4.4±3.3	517±211**	6.1±1.3	0.33±0.02	6.9±1.3	21.0±1.0
wt (27)	3	5.4±0.5	795±44	6.6±0.2	0.32±0.01	6.8±0.2	20.9±0.1
Ercc1$^{-/-}$ (34)	3	3.9±0.5*	614±28**	6.4±0.2	0.32±0.01	6.6±0.2	21.3±0.2

WBC, white blood cells ($\times 10^9$/l); Plt, platelets ($\times 10^9$/l); RBC, red blood cells ($\times 10^{12}$/l); HCT, hematocrit; Hb, hemoglobin (arbitrary units); MCHC, mean corpuscular hemoglobin concentration (arbitrary units).
Significant difference between *Ercc1*$^{-/-}$ mice and wt mice as determined by Student's t-test, *$P<0.05$, **$P<0.005$.

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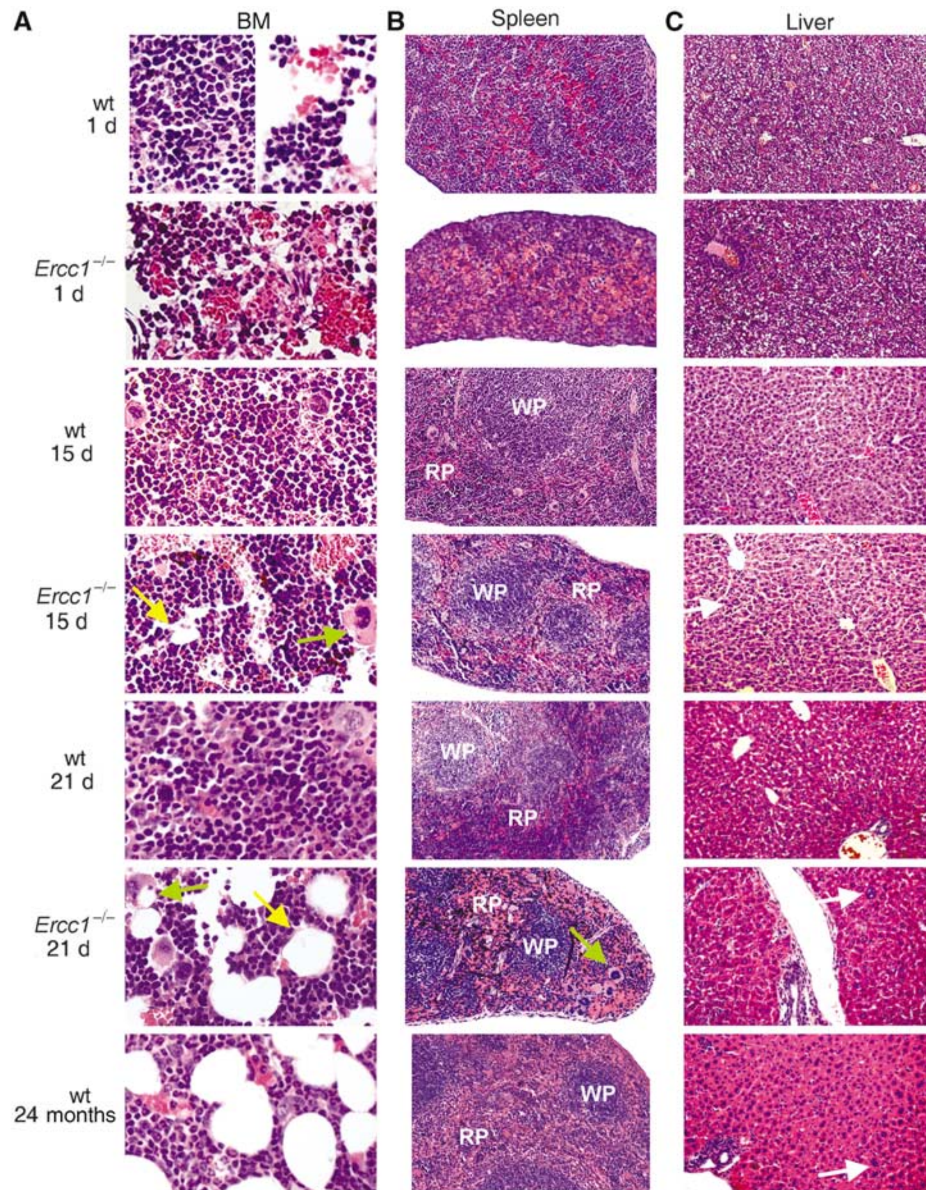

Figure 1 Progressive systemic hematopoietic deficits in *Ercc1*$^{-/-}$ mice. (**A**) Hematoxylin- and eosin-stained histologic sections of the femoral BM space from *Ercc1*$^{-/-}$ mice and wt littermates at 1, 15 and 21 d of age, as well as from an aged, 2-year-old wt mouse. At 1 d of age, the BM of *Ercc1*$^{-/-}$ and that of wt mice show equivalent cellularity and areas of erythropoiesis. By 15 d of age, the BM of *Ercc1*$^{-/-}$ mice shows early signs of fatty replacement (yellow arrow) and accumulation of large debris-laden macrophages (green arrow), which progresses by 21 d of age. Adipose deposition in the BM is a hallmark of aged mice. (**B**) Splenic sections of the same *Ercc1*$^{-/-}$ and wt mice. Early postnatal splenic development is unchanged in the *Ercc1*$^{-/-}$ background. By 15 d of age, there is evidence of replacement of the hematopoietic red pulp (RP; white pulp, WP) by mature erythrocytes in the mutant mice. This becomes exaggerated by 21 d of age in the *Ercc1*$^{-/-}$ mice and is also seen in the aged wt mouse spleen. Hemosiderin deposition is apparent in the spleens of 15- and 21-d-old *Ercc1*$^{-/-}$ mice (black deposits), indicating increased erythrocyte death. (**C**) Liver sections of the same mice. Sections from 1- and 15-d-old wt and *Ercc1*$^{-/-}$ mice are indistinguishable, showing an equivalent amount of residual hematopoiesis. Thus, *Ercc1*$^{-/-}$ mice do not have a developmental delay of hematopoiesis. Polyploid nuclei in hepatocytes (white arrows) and perivascular lymphocytic infiltrates are evident in the 21 d *Ercc1*$^{-/-}$ mouse and aged mouse livers.

Ladd *et al*, 1997). Over 8 d in culture, wt progenitors expanded an average of 65-fold ($\pm$8), while *Ercc1*$^{-/-}$ cells achieved only a 12-fold ($\pm$1.6) expansion during the same time period (Figure 2B).

The high level of oxygen present in normal tissue culture conditions causes oxidative damage, which may account for both the decreased proliferation rate and early senescence of murine fibroblasts compared to human fibroblasts (Parrinello *et al*, 2003). However, when total BM was plated in the cytokine conditions utilized above, the proliferation rate of progenitors grown in low-oxygen conditions (3% versus the normal 20%) was not altered during the first 6 d of culture, regardless of genotype (Figure 2C). These data support a cell-intrinsic proliferative defect in the early progenitor compartment of *Ercc1*$^{-/-}$ mice that is independent of *in vitro* culture conditions.

Decreased lineage-committed progenitors in Ercc1$^{-/-}$ *mice*

To determine if the hematopoietic defect extended to lineage-committed progenitors, we assayed the ability of progenitor cells to proliferate and produce differentiated cells in

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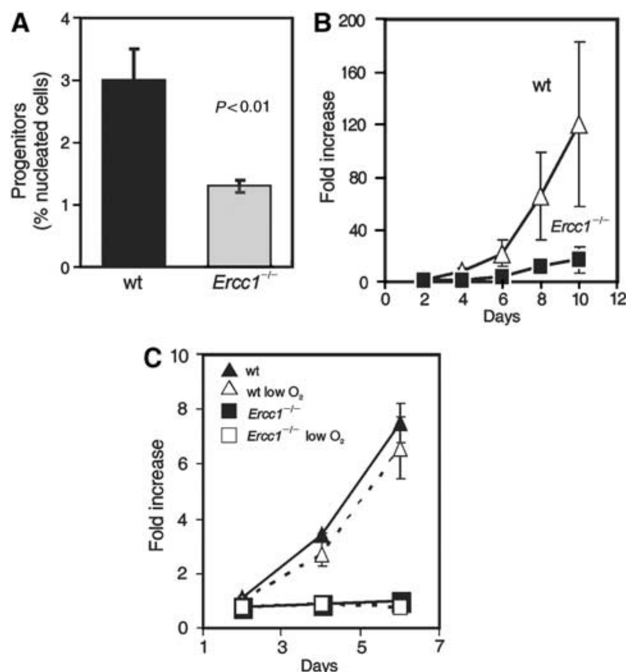

Figure 2 Decreased hematopoietic progenitors in *Ercc1*$^{-/-}$ mice. (**A**) Progenitors were isolated from 3-week-old *Ercc1*$^{-/-}$ mice and wt littermates and directly counted. The mean (±s.e.m.) for five pools of mice (2–4 mice per pool) is plotted. (**B**) Progenitor cells were cultured in a cytokine-rich environment to stimulate maximal proliferation. Every 2 d, cells were counted and the results plotted as fold increase from day 0. Each point represents the mean (±s.e.m.) of at least three samples. (**C**) Total BM was isolated from 3-week-old mice and plated with lineage-specific cytokines under 20 or 3% (low) oxygen. Progenitor numbers are expressed as fold increase from day 1. Each point represents the mean (±s.e.m.) of three mice.

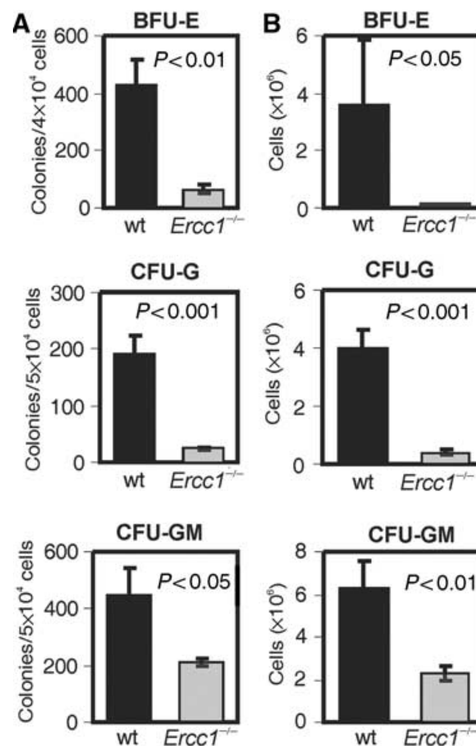

Figure 3 Lineage-committed progenitor numbers are decreased in *Ercc1*$^{-/-}$ BM. (**A**) Hematopoietic progenitors were isolated from 3-week-old mice. The indicated numbers of cells (*y*-axis) were seeded in methylcellulose with lineage-specific cytokines and grown for 6 d prior to colony counting. Bar graphs represent the mean (±s.e.m.) of at least four independent cell pools (2–4 mice per pool). (**B**) Outgrowth of cells from lineage-committed progenitors. Following colony counting, cultures were harvested and total cell numbers determined.

response to various cytokines *in vitro*. The number of lineage-committed progenitors capable of forming colonies was dramatically decreased across all lineages examined in *Ercc1*$^{-/-}$ BM (Figure 3A). The severity of this decrease varied by lineage, with both the erythroid lineage (BFU-E) and granulocytic lineage progenitors (CFU-G) being decreased approximately seven-fold, while the bipotent myeloid lineage progenitors (CFU-GM) demonstrated a two-fold decline in the absence of ERCC1. The proliferative capacity of those *Ercc1*$^{-/-}$ progenitors that did form colonies was also decreased, again most severely in the BFU-E and CFU-G compartments, producing far fewer terminally differentiated cells during 6 d of *in vitro* culture (Figure 3B). All appropriate cell types were present in each cytokine condition, demonstrating that terminal differentiation of particular lineages was not blocked (data not shown). Together, these data suggest a severe decrease in the proliferative potential of both *Ercc1*$^{-/-}$ early and lineage-committed progenitors that could account for the cytopenia observed in these mice at 3 weeks of age.

Suboptimal stress erythropoiesis is associated with Ercc1$^{-/-}$ *erythroid progenitor senescence*

The liver is the primary site of hematopoiesis during late embryogenesis in both mice and man. RBC production during this period is 3–5 times higher than that found during basal adult erythropoiesis (reviewed in Palis *et al*, 1999) and therefore represents a period of greatly increased hematopoietic demand. Deficient fetal erythropoiesis translates to a lack of optimal adult stress erythropoiesis capacity in mice (Masuoka and Townes, 2002). Although basal hematocrit and RBC levels were not significantly affected in 3-week-old *Ercc1*$^{-/-}$ mice (Table I), the proliferative defect observed in erythroid lineage progenitors (Figure 3) suggested that the hyperproliferation required under stress conditions could be more severely affected.

The number of fetal liver hematopoietic progenitors was decreased by two-fold in the *Ercc1*$^{-/-}$ mice (Figure 4A). BFU-E progenitor numbers were also specifically decreased (data not shown). Fetal liver erythroid progenitors can be cultured with differentiation-promoting cytokines *in vitro*, and under these conditions cells synchronously arrest, accumulate hemoglobin, reduce in size and extrude their nuclei to become mature erythrocytes (von Lindern *et al*, 2001). *Ercc1*$^{-/-}$ fetal liver erythroid progenitors differentiated normally (Figure 4B) and quantification of hemoglobin accumulation showed neither a delay nor a decrease (data not shown). The observed defects in fetal liver hematopoiesis do not stem, therefore, from a block in terminal differentiation capacity, in agreement with the normal differentiation observed in BM BFU-E colonies from 3-week-old *Ercc1*$^{-/-}$ mice.

Fetal liver erythroid progenitors can also be induced to expand *in vitro* in response to a proliferative cytokine cocktail. Maximal proliferation in this system requires glucocorticoid stimulation, as does stress erythropoiesis *in vivo*. Therefore, growth under these conditions is considered an *in vitro* model of stress erythropoiesis (Bauer *et al*, 1999;

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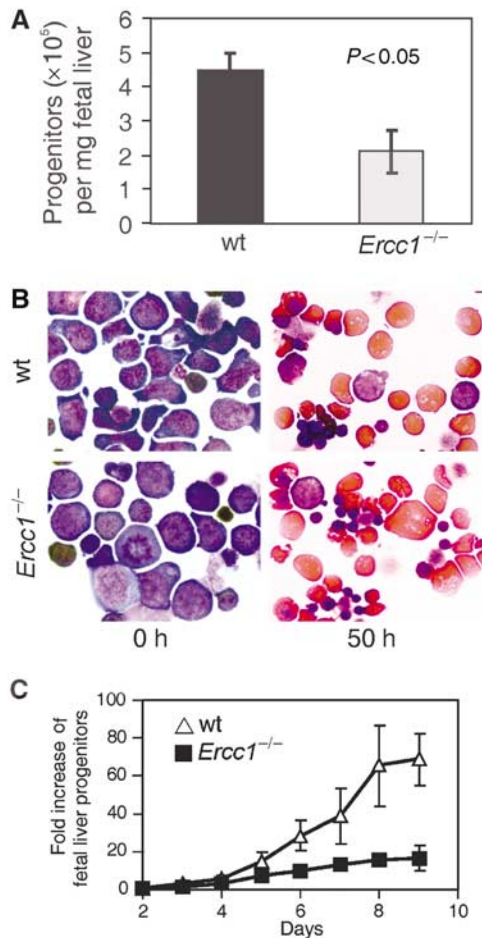

Figure 4 Fetal liver progenitor number, but not differentiation capacity, is decreased in *Ercc1*$^{-/-}$ mice. (**A**) Fetal liver hematopoietic progenitors were isolated from E12.5 embryos, counted, corrected for liver weight and plotted ($\pm$s.e.m.; $n\geqslant4$ mice). (**B**) Erythroid progenitors were plated with cytokines promoting differentiation. Neutral benzidine staining of cytospins demonstrates normal hemoglobin accumulation and cell size reduction in the *Ercc1*$^{-/-}$ samples. (**C**) Progenitors isolated from fetal livers of *Ercc1*$^{-/-}$ (■) and wt (Δ) mice were plated in a cytokine-rich environment promoting maximal expansion of erythroid progenitors. Cells were counted daily and the results plotted as the fold increase from day 0.

von Lindern *et al*, 2001). In the presence of glucocorticoids, *Ercc1*$^{-/-}$ progenitor cells expanded four-fold less than did wt cells (Figure 4C) with an average doubling time that was 16 h longer than that calculated for wt cells under identical conditions (36 h$\pm$1.4 versus 20.6 h$\pm$1.3 respectively, $P<0.005$). To determine if this decreased proliferation was the result of accelerated or increased differentiation, we analyzed proliferating progenitor cultures for the presence of terminally differentiated erythrocytes. There was a low level of spontaneous differentiation under proliferative conditions, as previously reported (von Lindern *et al*, 2001), which remained constant during the exponential growth period. This was not altered in *Ercc1*$^{-/-}$ cells (data not shown).

To determine the cause of the diminished proliferative potential in *Ercc1*$^{-/-}$ progenitors, we measured the frequency of apoptosis and cellular senescence. The viability of *Ercc1*$^{-/-}$ progenitors, determined by Trypan blue exclusion, remained high throughout the early culture period of fetal liver progenitors (Figure 5A). This was supported by the observation that there was no significant difference in the cell cycle profile of *Ercc1*$^{-/-}$ and wt BM progenitor cells (data not shown), including the fraction of sub-G1 cells representing apoptotic/necrotic cells (Figure 5B). This corroborated the low level of cell death found previously in *Ercc1*^{mutant} mice (Weeda *et al*, 1997). Direct detection of apoptotic cells using the TUNEL assay also did not reveal a difference in the number of apoptotic cells between *Ercc1*$^{-/-}$ and wt BM progenitors for the length of the culture (Figure 5C). We conclude that cell death is not the predominant factor affecting proliferation in *Ercc1*$^{-/-}$ progenitors.

It was previously reported that both *Ercc1*$^{-/-}$ mouse embryo fibroblasts and liver cells appear to undergo a premature senescence, withdrawing from the cell cycle and exhibiting a flattened morphology (McWhir *et al*, 1993; Weeda *et al*, 1997). Senescing cells of multiple types (Dimri *et al*, 1995), including BM hematopoietic progenitors (Meng *et al*, 2003), accumulate senescence-associated β-galactosidase (SA β-gal) in their cytoplasm and this activity is widely accepted as a marker of cellular senescence. The fraction of SA β-gal-positive cells increased in both wt and *Ercc1*$^{-/-}$ BM progenitor cultures with increasing time *ex vivo* (Figure 5D). However, the fraction of senescence cells rose much more sharply in the *Ercc1*$^{-/-}$ cells, ranging from two- to –five-fold above the levels in wt cultures.

Cell culture conditions including oxidative stress can induce senescence *in vitro* (reviewed in Sherr and DePinho, 2000; Parrinello *et al*, 2003). However, incubation of *Ercc1*$^{-/-}$ progenitors at physiological oxygen tension (3%), rather than 20% typical of tissue culture conditions, had no effect (Figure 5D). To determine if cellular senescence was a late event or intrinsic to *Ercc1* deficiency, we also examined progenitors isolated from fetal livers ($n=7$ mice of each genotype). While SA β-gal-positive cells were rare in fetal material, a significantly larger fraction of cells were SA β-gal positive in *Ercc1*$^{-/-}$ livers, reaching approximately 2% of the total progenitor population (Figure 5E). Thus, even progenitors isolated from young, asymptomatic *Ercc1*$^{-/-}$ animals are prone to senesce. In summary, these data suggest that hematopoietic progenitor senescence occurs with greater frequency in the absence of ERCC1. This increased senescence causes a drop in hematopoietic proliferative reserve capacity and a suboptimal response to stress conditions *in vivo* and *in vitro*.

Progenitor number and function are unaffected by NER loss alone, but are dramatically decreased following ICL induction

ERCC1 is involved in two distinct DNA repair pathways: NER, which removes bulky lesions restricted to one DNA strand, and ICL repair, which processes highly cytotoxic double-strand lesions. Having demonstrated the sensitivity of hematopoietic progenitor cells to premature senescence in the absence of ERCC1, we now sought to determine the contribution of each of these types of DNA lesions to this progeroid phenotype in *Ercc1*$^{-/-}$ mice.

To determine if loss of NER alone causes hematopoietic progenitor failure similar to that observed in *Ercc1*$^{-/-}$ mice, we isolated BM cells from the femurs of young mice in which *Xpa* (xeroderma pigmentosum complementation group A)

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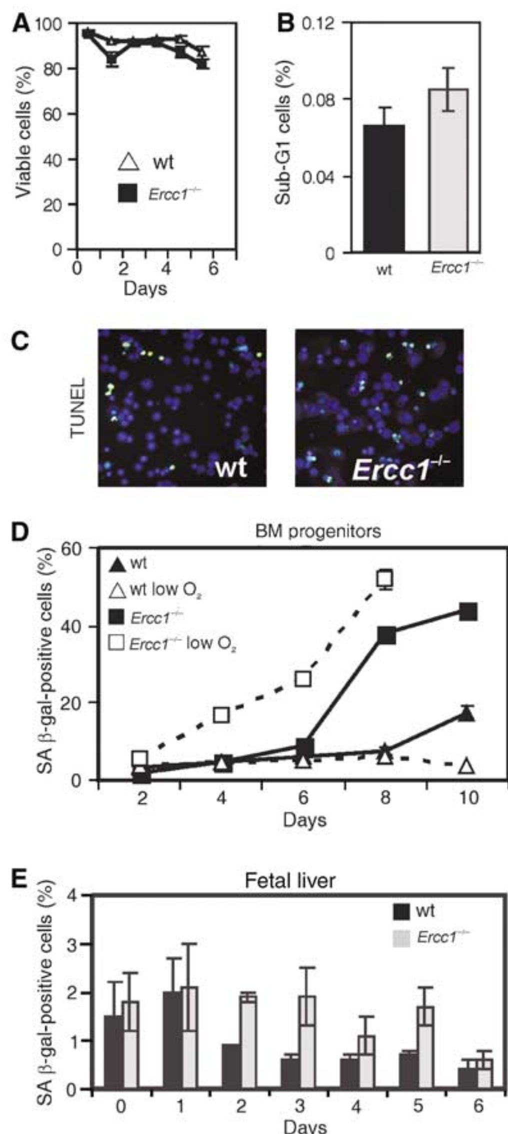

Figure 5 Decreased reserve capacity in the *Ercc1*$^{-/-}$ fetal liver erythroid progenitor pool is associated with progenitor senescence. (**A**) Fetal liver progenitor cultures were stained with the vital dye Trypan blue to determine viability at multiple time points after the initiation of cultures. A minimum of 200 cells were analyzed at each time point and the fraction of viable cells plotted. (**B**) BM progenitors were isolated from 21-d-old *Ercc1*$^{-/-}$ mice and wt littermates (three pools of two animals each), stained with propidium iodide to determine DNA content and analyzed by flow cytometry. Cells with less than 2*N* DNA content were gated and the percent calculated from the total number of viable cells. (**C**) TUNEL assay on fetal liver progenitors. Cells were isolated and cultured under growth-promoting conditions. Replica platings were harvested and analyzed for apoptosis every 24 h after isolation for a total of 8 d. No difference in the fraction of apoptotic cells between *Ercc1*$^{-/-}$ and wt cultures was detected. An example image from day 5 is depicted. (**D**) BM progenitors were isolated from femurs of 3-week-old *Ercc1*$^{-/-}$ mice and wt littermates ($n=3$, each genotype) and cultured for 10 d under growth-promoting conditions at either 20 or 3% (low) O_2. A replicate of the culture was fixed every 2 d and stained for SA β-gal. Positively staining cells were counted and plotted as a percentage (mean±s.e.m.). (**E**) Fetal liver progenitors were isolated from three mice of each genotype and cultured as described in (A), then washed, fixed and stained for SA β-gal. The percentage of positively staining, senescent cells is plotted (mean±s.e.m.).

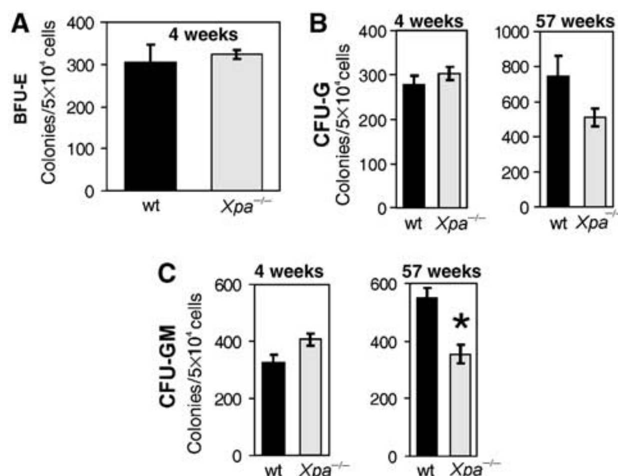

Figure 6 Normal progenitor pools in NER-deficient *Xpa*$^{-/-}$ mice. Hematopoietic progenitor cells were isolated from young (4 weeks) and old (56–58 weeks) wt (black bars) and *Xpa*$^{-/-}$ (gray bars) mice and plated in CFU-G or CFU-GM (or in the case of young mice BFU-E) stimulating cytokine conditions. The colony numbers were counted on the 6th d of culture. The mean (±s.e.m.) of three cell pools (two mice per pool) is shown for the young mice and three individual animals for the aged mice. The only significant difference between wt and *Xpa*$^{-/-}$ hematopoietic progenitor activity was detected in the aged mice and was restricted to the granulocyte–macrophage lineage (*$P<0.05$).

was genetically deleted (*Xpa*$^{-/-}$ mice; de Vries *et al*, 1995). Cells from these mice are completely deficient in NER activity but retain ICL repair capacity. There were no differences in CFU-G, CFU-GM or BFU-E numbers in 4-week-old *Xpa*$^{-/-}$ mice compared to littermate controls (Figure 6) and colony size remained within the normal range (data not shown). Both the previous characterization of these mice (reviewed in Wijnhoven and van Steeg, 2003) and our own observations revealed no differences in peripheral blood values or BM cell counts between newly weaned *Xpa*$^{-/-}$ mice and wt littermate controls. However, CFU-GM (but not CFU-G) colony number was significantly reduced in 1-year-old *Xpa*$^{-/-}$ mice, indicating that an absence of NER does affect hematopoietic progenitors, but only later in life and in a lineage-restricted manner. These data demonstrate that loss of NER alone is not sufficient to cause the decrease in BM progenitor activity observed in *Ercc1*$^{-/-}$ mice.

To determine if ICL damage induces hematopoietic progenitor failure, we treated BM progenitors from 3-week-old *Ercc1*$^{-/-}$ or wt littermates with the ICL-inducing agent mitomycin C (MMC). Figure 7 demonstrates a decrease in progenitor capacity with increasing concentrations of MMC in both wt and *Ercc1*$^{-/-}$ progenitors. *Ercc1*$^{-/-}$ BM progenitor cells were dramatically more sensitive to MMC than wt cells, regardless of the age of the animal from which the cells were derived. In contrast, the sensitivity of NER-deficient *Xpa*$^{-/-}$ progenitors was indistinguishable from wt cells. Thus, the exquisite sensitivity of the *Ercc1*$^{-/-}$ cells to ICL-inducing drugs is a unique property of the progeroid animal-derived cells. We were unable to detect a decrease in ICL repair in BM progenitors isolated from 18 month-old wt mice, indicating that if DNA repair capacity would decline with age, it is a late event.

Analysis of CFU-GM colonies revealed striking similarities between wt cells treated with high concentrations of MMC

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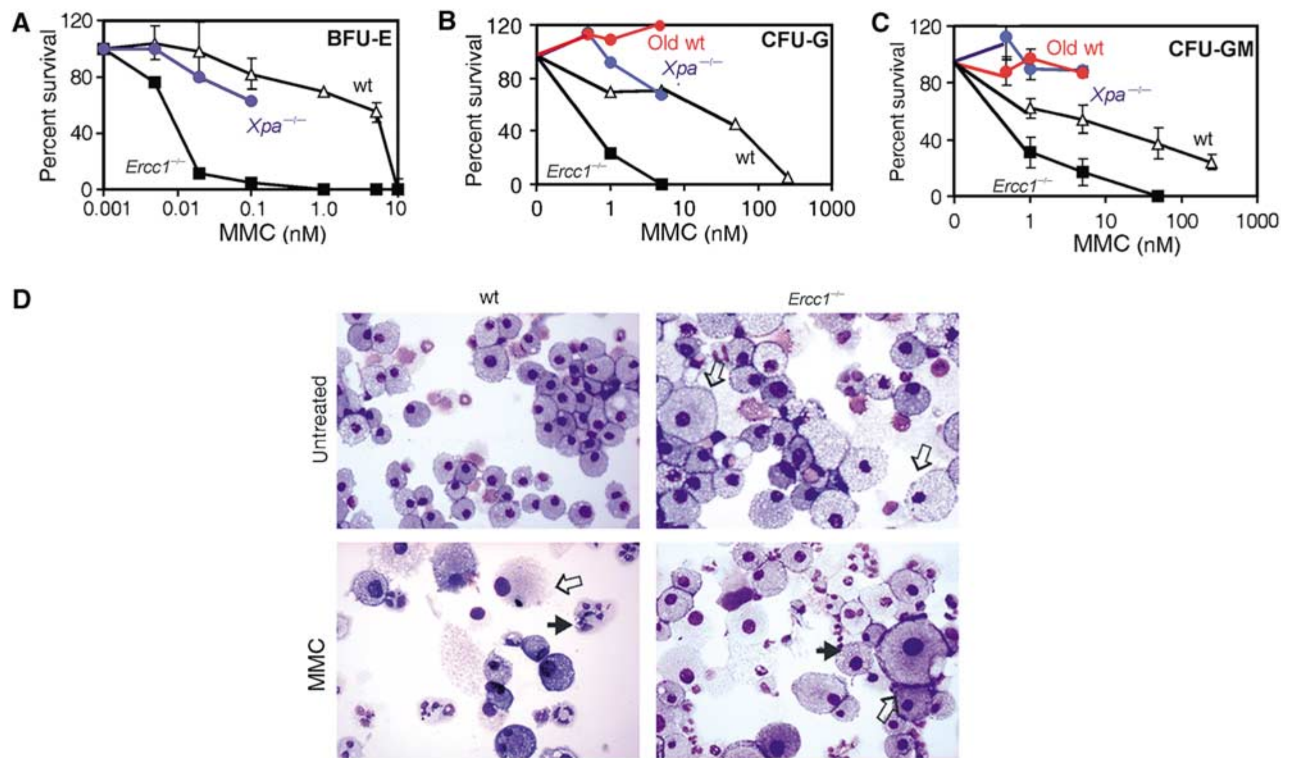

Figure 7 Hypersensitivity of *Ercc1*$^{-/-}$ progenitors to ICL damage. Progenitor cells from 3-week-old wt (Δ), *Ercc1*$^{-/-}$ (■) or *Xpa*$^{-/-}$ (blue ●) mice and 78-week-old wt (red ●) mice were assayed for BFU-E (**A**), CFU-G (**B**) and CFU-GM (**C**) activity in the presence of increasing concentrations of the crosslinking agent MMC. Colonies were counted after 6 d in culture. Each data point represents the mean colony number (±s.e.m. from at least three cell pools) as a percentage of the colony number detected in untreated samples. (**D**) Cytology of wt and *Ercc1*$^{-/-}$ CFU-GM cultures before and after exposure to 50 or 1 nM MMC, respectively. Filled arrows indicate nuclear fragmentation; open arrows indicate enlarged macrophages.

and untreated *Ercc1*$^{-/-}$ cells (Figure 7D). Untreated *Ercc1*$^{-/-}$ cultures exhibited abundant, dramatically enlarged macrophages, as well as cells with fragmented nuclei. These abnormalities, while rare in untreated wt cultures, became prevalent upon MMC treatment, suggesting that these changes may be cellular responses to and/or consequences of ICL damage. Treatment of wt cells with an ICL-inducing agent therefore recapitulates the cytological profile and progenitor capacity loss found in *Ercc1*$^{-/-}$ mice, supporting the hypothesis that the phenotype of the *Ercc1*$^{-/-}$ mice is due to a failure to repair spontaneous DNA ICLs.

Discussion

Hematopoietic changes in progeroid Ercc1$^{-/-}$ *mice mimic normal human aging*

Age-associated changes in the human hematopoietic system include mild cytopenia, in particular anemia (Salive *et al*, 1992), as well as a reduced capacity for bursts of RBC production following hypoxia (Udupa and Lipschitz, 1984b) and neutrophil production after endotoxin challenge (Timaffy, 1962). These deficits impact physical performance and survival (Penninx *et al*, 2003; Wilkinson and Warren, 2003). We investigated the hematopoietic system of DNA repair-deficient *Ercc1*$^{-/-}$ mice and discovered striking parallels with human aging.

Ercc1$^{-/-}$ mice (3-week-old) are mildly cytopenic, and time-course analysis indicates that this cytopenia is progressive over their lifespan (Table I). The prognostic value of mild peripheral cytopenia in elderly patients is not well established. However, we demonstrate here that the mild cytopenia in *Ercc1*$^{-/-}$ mice is a direct reflection of dramatically diminished numbers of BM hematopoietic progenitors (see below), which leads to suboptimal stress hematopoiesis. Thus, our results support recent data indicating that aged individuals with low–normal peripheral blood counts are at elevated risk of morbidity and mortality during periods of acute hematopoietic stress including blood loss or systemic infection (Wilkinson and Warren, 2003).

The prevalence of anemia increases with age and at least 20% of age-associated anemia have no identifiable cause beyond aging itself (Rothstein, 2003). Surprisingly, the RBC counts of progeroid *Ercc1*$^{-/-}$ mice were not significantly reduced (Table I). This may be attributable to the fact that the lifespan of *Ercc1*$^{-/-}$ mice is shorter than the 45 d circulatory lifespan of mouse RBCs. It is possible that if the mice survived longer, anemia would become critical. In support of this, a patient with dramatically reduced levels of ERCC1-XPF as a consequence of a mutation in *XPF* had a severe progeria that included anemia (LJ Niedernhofer and JHJ Hoeijmakers, unpublished data).

Despite the lack of peripheral anemia, there are several lines of evidence suggesting a severe, underlying erythropoietic deficit in *Ercc1*$^{-/-}$ mice. First, BM erythroid progenitor activity was the most severely impaired of all hematopoietic lineages in colony forming assays (Figure 3). Second, erythropoiesis was reduced in *Ercc1*$^{-/-}$ mice during fetal development and under *in vitro* conditions mimicking stress erythropoiesis (Figure 4; Masuoka and Townes, 2002). This mirrors the reduced progenitor expansion and RBC

production seen in aged mice under hypoxic stress conditions (Udupa and Lipschitz, 1984a). In both aged and *Ercc1*$^{-/-}$ mice, erythroid differentiation, measured by hemoglobin accumulation per cell, was normal. Rather, erythropoietic deficits were due to decreased progenitor proliferation (Figure 4C; Udupa and Lipschitz, 1984b). Thus, the reduced stress erythropoiesis found in *Ercc1*$^{-/-}$ mice recapitulates that seen in aged wt mice and the decline of BM hematopoietic reserves that cause inadequate acute response in humans (Globerson, 2001). These data extend the range of tissues for which premature signs of aging are detected as a result of ERCC1-XPF deficiency (McWhir *et al*, 1993; Weeda *et al*, 1997). Furthermore, these data promote the *Ercc1*$^{-/-}$ mice as a valid model for studying degeneration of the hematopoietic system in aging.

The decreased proliferative potential in Ercc1$^{-/-}$ *mice is caused by progenitor senescence*

The mechanism(s) underlying the age-associated functional decline in hematopoietic progenitors remains controversial but may include increases in both cellular senescence and apoptosis (reviewed in Van Zant and Liang, 2003). The reduced capacity of *Ercc1*$^{-/-}$ hematopoietic progenitor cells to proliferate is not due to increased cell death (Figure 5). Similarly, no increased apoptosis is detected in the liver, kidney or spleen of *Ercc1*$^{-/-}$ mice compared to wt littermates (Weeda *et al*, 1997). Rather, in the absence of ERCC1, a significant fraction of hematopoietic progenitors senesce, accumulating SA β-gal (Figures 2 and 5). These data suggest that premature senescence is the primary mechanism underlying progenitor proliferative defects. Importantly, DNA damage promotes senescence of mouse fibroblasts, supporting the significance of senescence in DNA repair-deficient *Ercc1*$^{-/-}$ cells (Parrinello *et al*, 2003).

Hematopoietic deficits in Ercc1$^{-/-}$ *mice are linked to the loss of ICL repair and mimic Fanconi anemia*

The ERCC1-XPF endonuclease is required for NER of large bulky DNA lesions and a distinct DNA repair pathway for ICLs (Niedernhofer *et al*, 2004). Deficiency in NER alone is not sufficient to explain the phenotype of the *Ercc1*$^{-/-}$ mice, including their hematopoietic deficits (Figure 6; de Vries *et al*, 1995; Weeda *et al*, 1997). Genetic disruption of *Xpa* in the mouse results in a complete loss of NER. However, unlike the *Ercc1*$^{-/-}$ mice, the hematopoietic system of young *Xpa*$^{-/-}$ mice is normal as determined by peripheral blood cell counts and lineage-committed progenitor numbers in the BM. However, 1-year-old *Xpa*$^{-/-}$ mice display a reduction in granulocyte–macrophage-committed progenitors. This relatively mild phenotype associated with NER deficiency is further supported by normal peripheral blood counts observed in human xeroderma pigmentosum patients (Polani, 1979). Thus, an inability to excise bulky DNA adducts via NER is detrimental to the hematopoietic system, but is insufficient to explain the dramatic deficits observed in *Ercc1*$^{-/-}$ mice.

Several lines of evidence support a link between the loss of ICL repair in *Ercc1*$^{-/-}$ mice and the hematopoietic phenotype. First, *Ercc1*$^{-/-}$ primary hematopoietic progenitor cells are very sensitive to the ICL-inducing agent MMC (Figure 7; Murray *et al*, 2002). Second, ICL damage leads to the formation of DSBs (Bredberg *et al*, 1982), and in the absence of ERCC1-XPF, these ICL-induced DSBs are not repaired (Niedernhofer *et al*, 2004). Senescent cells and fragmented nuclei are common in untreated *Ercc1*$^{-/-}$ hematopoietic progenitor cultures and induced in wt cells by the crosslinking agent MMC (Figure 7). Thus, ICLs are capable of inducing the cellular changes observed in *Ercc1*$^{-/-}$ hematopoietic cells. Importantly, DSBs are known to accumulate in senescent human cells and in the nuclei of aged mouse tissues (Sedelnikova *et al*, 2004), indicating that ICL repair intermediates could contribute to the pathophysiology of aging. Third, numerous other proteins implicated in ICL repair, when mutated in humans or mice, impact the hematopoietic system including FancC, FancG, Rev3 and Rad50 (Hadjur *et al*, 2001; Bender *et al*, 2002; Koomen *et al*, 2002). Interestingly, peripheral blood mononuclear cells from elderly patients exhibit decreased ICL repair following cisplatin treatment (McHugh *et al*, 2001), suggesting that a decrease in ICL repair mechanisms may also be involved in natural aging.

The progeroid hematopoietic phenotype of the *Ercc1*$^{-/-}$ mice is spontaneous. Thus, for ICLs to play a causative role, there must be an endogenous source of crosslink damage. Spontaneous ICLs have not yet been detected *in vivo*, most likely because the number of lesions required to elicit a cytotoxic response is well below the limit of detection (Dronkert and Kanaar, 2001). However, endogenous compounds, which are chemically capable of forming DNA ICLs under physiological conditions, are known (Kasai *et al*, 1998; Niedernhofer *et al*, 2003). The majority of these compounds are by-products of lipid peroxidation, which is triggered when oxygen free radicals attack polyunsaturated membrane fatty acids. Importantly, elevated levels of lipid peroxidation and reactive oxygen species promote DNA damage and accelerate aging (Finkel and Holbrook, 2000; Barja, 2002; Pamplona *et al*, 2002).

The strongest evidence for a causal role of ICLs in the loss of hematopoietic progenitors is the striking parallel between the *Ercc1*$^{-/-}$ phenotype and that of Fanconi anemia, which too is linked with defective ICL repair. Fanconi anemia patients have pancytopenia, progressive BM failure and increased risk of myeloid leukemia (Wong and Buchwald, 2002). Diagnosis is based on hypersensitivity of the patient cells specifically to drugs that induce DNA ICLs. Like the *Ercc1*$^{-/-}$ mice, hematopoietic progenitors isolated from Fanconi anemia patients proliferate poorly (Martinez-Jaramillo *et al*, 2000). Also similar to *Ercc1*$^{-/-}$ mice, this defect is cell intrinsic, not caused by progenitor apoptosis and unchanged by low-oxygen culture conditions (Alter *et al*, 1991; Bagnara *et al*, 1992). Curiously, Fanconi anemia mouse models, established for three of the eight disease complementation groups, do not display hematopoietic abnormalities until challenged with MMC or increased reactive oxygen species. Under these stress conditions, the progenitors lose substantiative proliferative capacity (Wong and Buchwald, 2002). The parallels between the phenotypes of Fanconi anemia patients, *Ercc1*$^{-/-}$ mice and Fanconi mouse models challenged with ICL-inducing agents indicate that repair of ICL damage is important to the maintenance of hematopoietic reserves.

The spontaneous hematopoietic phenotype of Fanconi anemia patients is more severe than that caused by genetic disruption of this pathway in mice. This may indicate an increased burden of spontaneous DNA ICLs in human hema-

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topoietic tissue compared to mice. *Ercc1*$^{-/-}$ murine progenitors are hypersensitive to crosslink damage (50–1000 ×; Figure 7) compared to progenitors isolated from *FancA*$^{-/-}$ mice (Rio *et al*, 2002). Accordingly, *Ercc1*$^{-/-}$ mice have a more severe phenotype than *Fanc*$^{-/-}$ mice, and ERCC1 deficiency is apparently lethal in humans. Because of their hypersensitivity to crosslink damage, the *Ercc1* mutant mice therefore represent a good animal model of the human disease Fanconi anemia.

Interestingly, a recent study showed that hematopoietic stem cells in mice lacking the 'ataxia telangiectasia mutated' (*Atm*) gene are hampered in their self-renewal abilities due to activation of the p16^{INK4a}-Rb pathway. This manifests as a reduced reconstitutive capacity and progressive BM failure reminiscent of *Ercc1*$^{-/-}$ mice (Ito *et al*, 2004). Like ERCC1, ATM is involved in maintaining genomic stability, and inactivation of *Atm* results in a clinical syndrome with features of premature aging. Although the hematopoietic phenotype of *Atm*$^{-/-}$ mice is not as acute as in animals lacking Ercc1 (the hematopoietic defects in *Atm*$^{-/-}$ mice become apparent only 24 weeks after birth), it is tempting to speculate that failure to respond to the same spontaneous DNA damage causes the reduced hematopoietic activity in both mouse strains, although this needs further study.

In summary, this report demonstrates that DNA repair-deficient *Ercc1*$^{-/-}$ mice have decreased responses to hematopoietic stress and show exhaustion of hematopoietic progenitor activity, features that reflect symptoms of human aging. We propose that this phenotype is a consequence of progenitor cell depletion due to premature senescence of the hematopoietic stem cell and progenitor cell compartment, rather than apoptotic cell death and cell cycle changes in the progeny of these cells, and likely results from an accumulation of unrepaired endogenous DNA damage, specifically DNA ICLs.

The world's population is increasingly aged and therefore increasingly at risk for development of diseases of aging such as cancer. Elderly cancer patients tolerate current chemotherapeutic regimens poorly, and are often excluded from clinical trials due to comorbidities (reviewed in Rosti *et al*, 2003). The BM is a major target of most genotoxic chemotherapeutic agents and prolonged myelosuppression is one of the most common side effects of chemotherapy in the elderly (Balducci and Extermann, 1997). Therefore, there is a great need for animal models in which to test chemotherapeutic modalities in the context of an aged hematopoietic environment. Recapitulation of aged hematopoietic phenotypes in *Ercc1*$^{-/-}$ mice makes these mice not only a model of Fanconi anemia, but also a model in which to examine tissue sensitivity and rebound capacity of aged BM in response to current and novel cancer chemotherapy regimens.

Materials and methods

Mice

Ercc1$^{-/-}$ and wt littermates were bred from heterozygous crossings in a mixed (C57Bl/6:FVB/n) genetic background. Mice were killed at postnatal days 13–23 when the *Ercc1*$^{-/-}$ mice displayed symptoms of premature aging but were not moribund. *Xpa*$^{-/-}$ mice and old wt mice were C57Bl/6. Blood was obtained by post-mortem heart puncture and analyzed on an Animal Blood Counter (ABX Diagnostics). Femurs and tibiae were collected for BM isolation, or sectioning after fixation in 2% paraformaldehyde in sodium phosphate buffer, pH 7.4, 16 h, 4°C, then decalcification with 14% EDTA. Tissues were embedded in paraffin, sectioned (5 μm) and stained with hematoxylin and eosin.

BM proliferation and colony forming unit assays

Bones were cleaned and pulverized by mortar and pestle. BM cells were collected, washed, depleted of macrophages and fractionated by equilibrium density centrifugation on a Percoll gradient (Amersham Biosciences). Progenitor cells were enriched in the lightest density fraction as previously described (Sugiura *et al*, 1992). For proliferation studies, cells were plated in CellGro media (Sigma) with mIL-3 (10 ng/ml), mSCF (100 ng/ml; R&D Systems), dexamethasone (10^{-9} U/ml; Sigma), flt-3 ligand (0.052 μg/ml) and hTPO (0.01 μg/ml), and then cultivated at 5% CO_2 and 20% O_2 (or 3% where indicated) at a density of 0.8×10^6/ml. BM progenitors were harvested and then incubated in the presence of bromodeoxyuridine for 30 min prior to staining with propidium iodide for cell cycle analysis.

BM colony forming unit (CFU) assays were conducted as previously described (Hermans *et al*, 1998). For ICL induction studies, MMC was added directly to the methylcellulose mix prior to plating. Colonies containing more than 50 cells were scored 1 week after plating. For cell counts, colonies were pooled in PBS containing 20 μg/ml DNAse and rotated at room temperature for 30 min to create a single cell suspension. Statistical significance was determined using the Student's *t*-test. Cytological preparations were stained with May–Grünwald–Giemsa (Shandon Holland) and observed at ×100 magnification.

Fetal liver cell culture

Livers were removed from E12.5–13.5 embryos isolated from euthanized females. Progenitors were cultured as previously described (von Lindern *et al*, 2001). Briefly, cells were plated as single cell suspensions in Iscove's modified Dulbecco's medium + Glutamax supplemented with StemPro-34^{TM} (Invitrogen), BSA, antibiotics and 2 mM L-glutamine. Progenitors were separated from differentiated erythrocytes by harvesting the cultures, centrifuging repeatedly, then re-plating to maintain a >90% pure progenitor population. For differentiation studies, cells were washed twice with PBS and re-plated in fresh media supplemented with erythropoietin (Epo, 10 U/ml), iron-saturated human transferrin (1 mg/ml) and insulin (4×10^{-4} IE/ml). Cytological preparations were stained with histological dyes and neutral benzidine. Hemoglobin content was quantified by photometric assay (Kowenz *et al*, 1987).

For proliferation studies, Epo (0.02 U/ml), mSCF (100 ng/ml) and dexamethasone (10^{-9} U/ml) were added and cells were maintained at a density of 1.5×10^6/ml. Cells were counted on a CASY/TTC cell counter (Schärfe System) and viability determined by Trypan blue exclusion. Replica platings of the fetal liver progenitors were harvested every 24 h for 8 d and apoptosis detected by TUNEL assay using the Promega Apoptosis detection system according to the manufacturer's instructions. SA β-gal staining was performed as previously described (Dimri *et al*, 1995).

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