Published in final edited form as:

Stem Cells. 2013 March ; 31(3): 511–525. doi:10.1002/stem.1261.

Cell autonomous and non-autonomous mechanisms drive hematopoietic stem/progenitor cell loss in the absence of DNA repair

JoonSeok Cho^{1,2,*}, SungHo Kook^{1,2,*}, Andria Rasile Robinson^{1,3}, Laura J. Niedernhofer^{1,4,¶}, and Byeong-Chel Lee^{1,2,¶¶}

¹University of Pittsburgh Cancer Institute, 5117 Centre Ave, Hillman Cancer Center, Pittsburgh, PA 15213 USA

²Department of Medicine, University of Pittsburgh, 1218 Scaife Hall, 3550 Terrace Street, Pittsburgh, PA, 15216

³Department of Human Genetics, University of Pittsburgh Graduate School of Public Health, 130 DeSoto Street, Pittsburgh, PA 15261 USA

⁴Department of Microbiology and Molecular Genetics, University of Pittsburgh, 523 Bridgeside Point II, 450 Technology Drive, Pittsburgh, PA 15219 USA

Abstract

Daily cells incur tens of thousands of DNA lesions caused by endogenous processes. Due to their long-lived nature, adult stem cells may be particularly susceptible to the negative impact of this constant genotoxic stress. Indeed, in murine models of DNA repair deficiencies, there is accumulation of DNA damage in hematopoietic stem cells and premature loss of function. Herein, we demonstrate that mice expressing reduced levels of ERCC1-XPF DNA repair endonuclease (*Ercc1*–/Δ mice) spontaneously display a progressive decline in the number and function of hematopoietic stem/progenitor cells (HSPCs). This was accompanied by increased cell death, expression of senescence markers, reactive oxygen species and DNA damage in HSPC populations, illustrating cell autonomous mechanisms that contribute to loss of function. In addition, the bone marrow microenvironment of *Ercc1*–/Δ mice was not permissive to engraftment of transplanted normal stem cells. Bones from *Ercc1*–/Δ mice displayed excessive osteoclastic activity, which alters the microenvironment in a way that is unfavorable to HSPC maintenance. This was accompanied by increased proinflammatory cytokines in the bone marrow of *Ercc1*–/Δ mice. These data provide novel evidence that spontaneous, endogenous DNA damage, if not repaired, promotes progressive attrition of adult stem cells via both cell autonomous and non-autonomous mechanisms.

¶¶ Address correspondence to: Byeong-Chel Lee, Hillman Cancer Center, 2.32d, 5117 Centre Avenue, Pittsburgh, PA 15213, Phone: 412-623-2285, leeb4@upmc.edu.

*These authors contributed equally to this work.

¶Co-corresponding author

Author Contributions: JoonSeok Cho: Collection and/or assembly of data, Data analysis and interpretation; SungHo Kook: Collection and/or assembly of data, Data analysis and interpretation; Andria Rasile Robinson: Collection and/or assembly of data, Data analysis and interpretation; Laura J. Niedernhofer: Data analysis and interpretation, Conception and design, Manuscript writing, Final approval of manuscript provision; Byeong-Chel Lee: Data analysis and interpretation, Conception and design, Manuscript writing, Final approval of manuscript provision

INTRODUCTION

With aging, there is a decline in functional hematopoietic stem cells (HSCs) ¹. As a consequence, HSCs from old donors have impaired differentiation capacity and impaired ability to regenerate the hematopoietic system of lethally irradiated hosts ². This loss of regenerative capacity in the hematopoietic compartment has been attributed to intrinsic (cell autonomous) changes ³, suggesting that time-dependent accumulation of damaged organelles or macromolecules drives age-related decline in HSC function.

Multiple human diseases caused by inherited defects in DNA repair display accelerated aging of the hematopoietic system ⁴, strongly suggesting that DNA damage is one type of cellular damage that contributes to aging-related loss of HSC function. All cells in the body are continuously challenged by DNA damage as a consequence of the instability of DNA in an aqueous, oxidative environment and vulnerability to reactive electrophiles ⁵. DNA damage is repaired via numerous complex pathways. Given that tens of thousands of lesions that occur per nuclear genome per day, DNA repair may be inadequate to remove all of the damage over the lifetime of mammals. Long-lived cells such as adult stem cells may be particularly prone to accumulation of unrepaired DNA damage ³.

In mouse models of many genome instability disorders, there is spontaneous and progressive loss of HSC function ^{6–7}. This includes mice with defects in transcription-coupled nucleotide excision repair, increased mutations in the mitochondrial genome, telomere maintenance and non-homologous end-joining of double-strand breaks. In other models, there is a loss of progenitor and HSC number. This includes mice with mutations in the translesion polymerase pol μ or regulatory factors in the Fanconi anemia pathway of DNA interstrand crosslink repair ^{8–9}. However, the mechanism by which endogenous DNA damage affects HSC function is still not sufficiently understood, in particular in the absence of confounding factors such as impaired transcription or immunodeficiency. To address this gap in knowledge, we carefully examined the hematopoietic system of a DNA-repair deficient model, *Ercc1*^{-/ Δ} mice without the introduction of exogenous genotoxic stress.

Ercc1^{-/ Δ} mice express ~5% of the normal level of ERCC1-XPF DNA repair endonuclease, which is involved in nucleotide excision repair, DNA interstrand crosslink repair ^{10–11}, and the repair of double-strand breaks with 3' overhangs ¹². *Ercc1*^{-/ Δ} mice are healthy into adulthood (8 wks) then begin to show numerous progressive symptoms associated with old age ¹³. By 21 weeks of age, *Ercc1*^{-/ Δ} mice exhibit symptoms, as well as functional, histopathological, ultrastructural and gene expression changes that significantly correlate with those observed in 2–3 year-old mice, relative to young mice ^{13–16}. Therefore, *Ercc1*^{-/ Δ} mice offer a unique model in which to investigate the impact of endogenous DNA damage on HSPC function that is pertinent to a human progeroid syndrome with strong correlations to normal aging. Herein, we establish that there is spontaneous and rapid loss of hematopoietic stem/progenitor cell number and function in *Ercc1*^{-/ Δ} mice and provide evidence that this is due not only to changes in damaged HSPCs but also their niche.

RESULTS

A hypomorphic mutation in *Ercc1* leads to progressive attrition of HSPC function

DNA repair-deficient *Ercc1*^{-/ Δ} mice have normal peripheral blood counts in young adulthood (7–9 weeks). By 21–24 weeks of age, the mutant animals had lymphopenia, indicative of hematopoietic dysfunction (Supplementary figure 1). Accordingly, *Ercc1*^{-/ Δ} mice exhibit a slight but significant reduction (1.5-fold, $p=0.0007$) in bone marrow cellularity at 7 weeks of age compared to littermate controls (Figure 1A). The reduction was more pronounced in 21 week-old *Ercc1*^{-/ Δ} mice (4.4-fold, $p<0.0001$). These data

demonstrate that *Ercc1*^{-/Δ} mice spontaneously develop progressive loss of hematopoiesis in the first four to five months of life.

Previous studies indicate that with aging alterations in hematopoiesis are caused by a loss of HSPC function¹⁷. We first assessed hematopoietic progenitor cell (HPC) function by performing the colony-forming assay. Bone marrow cells from 7 week-old *Ercc1*^{-/Δ} mice had a significantly reduced ability to form CFU-GM and pre-B colonies compared to control littermates (Figure 1B, left panels). A more pronounced reduction in clonogenic capacity was seen in bone marrow cells from 21 week-old *Ercc1*^{-/Δ} mice (Figure 1B, right panels). In particular, formation of pre-B colonies was almost completely absent in 21 week-old *Ercc1*^{-/Δ} mice (Figure 1B, lower right panel). These data demonstrate a progressive decline in proliferative capacity of HPCs isolated from progeroid *Ercc1*^{-/Δ} mice, similar to what was previously observed in the more severe *Ercc1*^{-/-} knock-out mice¹⁸.

The effect of *Ercc1* mutation on hematopoietic lineage determination was examined using CD11b and B220 as markers of myeloid and lymphoid lineages, respectively. The percent of CD11b⁺ and B220⁺ cells in the peripheral blood of *Ercc1*^{-/Δ} and normal littermates were similar at 7 weeks of age (Figure 1C, upper panel). However, the lineage differentiation of *Ercc1*^{-/Δ} mice was significantly skewed away from lymphoid lineages in favor of myeloid lineages by 21 weeks of age (Figure 1C, lower panel). The skewed myeloid differentiation and reduced pre-B colony formation in *Ercc1*^{-/Δ} mice mimic changes that occur in rodents with aging¹⁹⁻²¹.

To further assess HSPC function, bone marrow cells from *Ercc1*^{-/Δ} mice and control littermates were transplanted into lethally irradiated recipient animals. Survival of the recipient animals was measured as an index of the capacity of the donor cells to repopulate the recipients' hematopoietic system. As expected, bone marrow cells from 7 and 21 week-old control wild-type littermates were able to fully rescue the lethally irradiated recipients (Figure 2A, open circles). Bone marrow cells from 7 week-old *Ercc1*^{-/Δ} mice were sufficient to provide radioprotection (or short-term repopulating cells) (Figure 2A, upper panel, closed diamonds): recipient survival was 100% until 8 weeks post-transplantation. However, their ability to support long-term survival of lethally irradiated recipients was partially impaired (60% recipient survival at >12 weeks post-transplantation). Of note, as the *Ercc1*^{-/Δ} mice reached an age of 21 weeks, the radioprotective capacity of their bone marrow cells was substantially decreased (only 20% of the recipients were alive 5 weeks post-transplant) (Figure 2A, lower panel, closed diamonds). Furthermore, the bone marrow cells from 21 week-old *Ercc1*^{-/Δ} mice showed complete loss of long-term repopulating capacity and failed to rescue any of lethally irradiated recipient animals beyond 7 weeks post-transplant. These results indicate that while *Ercc1*^{-/Δ} mice have functional HSPCs at 7 weeks of age, they are lost or become exhausted by the time *Ercc1*^{-/Δ} mice are 21 weeks-old.

Competitive repopulation assays were also conducted. Bone marrow cells from *Ercc1*^{-/Δ} mice or control littermates (CD45, 1/2) were mixed in equal proportion with WT competitor cells (CD45.1) and then transplanted into lethally irradiated recipient animals (CD45.2). Competitor bone marrow cells (CD45.1) were obtained from the first-generation cross of C57BL/6 (CD45.1) and FVB (CD45.1) mice to minimize potential skewing due to variation in genetic background. Bone marrow cells from control littermates were able to compete equally with the competitor cells at 4, 8, (data not shown) and 12 weeks (competitor, 42.94%; wild-type littermate, 49.65%) and > 1 year post-transplant (competitor, 44.94%; wild-type littermate, 47.95%) (Figure 2B), indicating that there is no competitive advantage or disadvantage resulting from histocompatibility mismatches. In contrast, *Ercc1*^{-/Δ} bone marrow cells were almost completely outcompeted by the competitor cells at all time points

analyzed post-transplantation (Figure 2B). Collectively these data support that there is a spontaneous and progressive loss of functional HSPCs in *Ercc1*^{-/-} mice.

A hypomorphic mutation in *Ercc1* leads to progressive attrition of HSPC number

We next used multicolor flow cytometry to measure the number and frequency of stem and progenitor populations in the bone marrow compartment of *Ercc1*^{-/-} mice. The gating strategy used to define various stem/progenitor cell populations is shown in Figure 2C. Lineage⁻Sca-1⁺c-kit⁺ (LSK) cells are composed of hematopoietic stem cells and a heterogeneous mix of multipotent/committed progenitors. LSK cells can be further subdivided into more primitive stem cell populations based on the expression of CD150, CD48 and CD34²²⁻²³ (Figure 2C). Meanwhile, lineage⁻Sca-1⁻IL-7R⁻c-kit⁺ cells can be further divided on the basis of the expression of CD34 and Fc receptor (FcR), yielding common myeloid progenitors (CMP; CD34⁺, FcR(low/-)), granulocyte-monocyte progenitor cells (GMP; CD34⁺, FcR⁺) and megakaryocyte-erythroid progenitor cells (MEP; CD34⁻, FcR(low/-))²⁴. The absolute number of each type of cell in the bone marrow compartment of *Ercc1*^{-/-} mice and littermate controls at two ages were plotted in Figure 2D. A moderate decrease in the absolute number of LSK (~2 fold, p=0.013) was observed in 7 week-old *Ercc1*^{-/-} mice compared to control littermates (Figure 2D, upper panel). SLAM LSK cells (CD150⁺, CD48⁻, LSK), which are currently considered to be the most primitive HSCs²³, were also modestly reduced (~3 fold, p=0.014) in 7 week-old *Ercc1*^{-/-} mice compared to control littermates (Figure 2D, upper panel). Bone marrow cells from 7 week-old *Ercc1*^{-/-} mice displayed a modest but significant reduction in the number of HPCs, such as common lymphoid progenitor cells (CLP; defined as Lin⁻, IL-7Rα⁺, c-Kit⁺, Sca-1⁺) (p=0.009)²⁵ and CMP cells (p=0.04) but not GMP or MEP cells (Figure 2D, upper panel).

While HPCs in 21 week-old *Ercc1*^{-/-} mice were not markedly altered compared to 7 week-old *Ercc1*^{-/-} mice, there was a substantial further reduction in CD34-LSK (~28 fold, p=0.021) and SLAM LSK cells (~30 fold, p=0.023) (Figure 2C-D), both of which are highly enriched for long term repopulating HSPCs. These results demonstrate a time-dependent attrition of *Ercc1*^{-/-} HSPCs, in particular the more primitive stem/progenitors cells.

A hypomorphic mutation in *Ercc1* increases the turnover of HSPCs

Most HSPCs reside in a non-cycling quiescent state, but prolonged regenerative demands could draw a greater fraction of HSPCs into active cell cycling, leading to premature loss of this stem progenitor cell population³. Given the more dramatic decline in the number of primitive HSPCs than intermediate progenitors in *Ercc1*^{-/-} mice (Figure 2C and 2D), we asked whether there is increased turnover of *Ercc1*^{-/-} HSPCs between 7 and 21 weeks of age. We tested this by measuring the cell cycle profile of LSK cells isolated from 14 to 16 week-old mice. The proportion of LSK cells in the quiescent state (G0) was significantly reduced in *Ercc1*^{-/-} animals compared to control littermates (from 81% to 58%; Figure 3). This coincided with an increase in LSK cells in the G1 (from 10% to 23%) and S/G2/M phase (from 9% to 19%). Interestingly, the active cycling of *Ercc1*^{-/-} LSK cells coincided with the period of time in which there was a significant decrease in HSPC pool size (Figure 2), suggesting that *Ercc1*^{-/-} HSPCs may undergo cell death and/or senescence rather than self-renewal.

A hypomorphic mutation in *Ercc1* leads to cell death and premature senescence of HSPCs

To determine if the attrition of HSPCs is due to cell death, LSK cells isolated from 16 week-old *Ercc1*^{-/-} mice and control littermates were stained with Annexin V and PI. *Ercc1*^{-/-} mice showed only a modest increase in the percent of early apoptotic cells (Annexin V⁺, PI⁻ cells; 2% vs. 0.8%). The percent of late apoptotic (Annexin V⁺, PI⁺) and dead cells

(Annexin V⁻, PI⁺) was significantly increased in LSK cells from *Ercc1*⁻/ Δ mice compared to normal littermates (Figure 4A).

We next determined the extent of senescence in *Ercc1*⁻/ Δ HSPCs by measuring senescence-associated beta-galactosidase (SA- β -gal) activity. SA- β -gal was not significantly increased in LSK cells from *Ercc1*⁻/ Δ mice at 7 weeks of age (Figure 4B). In contrast, LSK cells from 21 week-old *Ercc1*⁻/ Δ mice displayed a dramatic increase in SA- β -gal activity compared to age-matched WT counterparts (Figure 4B, 2.5-fold, $p=0.001$). This was accompanied by a significant up-regulation of *p16*^{INK4a} (Figure 4C). At 7 weeks of age, the *p16*^{INK4a} level in *Ercc1*⁻/ Δ LSK cells was approximately 6-fold higher than control littermates ($p=0.02$). A more marked increase in the level of *p16*^{INK4a} was detected in *Ercc1*⁻/ Δ LSK cells from 21 week-old mice (20-fold increase, $p=0.03$).

A hypomorphic mutation in *Ercc1* leads to increased ROS and DNA damage in HSPCs

Cells from aged organisms have increased oxidative damage, which arises from endogenously produced reactive oxygen species (ROS) ³. ROS can drive cell senescence and/or death ²⁶. In addition, ROS can drive HSPCs into the cell cycle, compromising their quiescent state ²⁷. We, therefore, examined whether ROS is increased in LSK cells from *Ercc1*⁻/ Δ mice compared to normal controls. Indeed, we observed a trend of increasing mitochondrial superoxide anion in LSK cells from 7 week-old *Ercc1*⁻/ Δ mice compared to littermate controls (Figure 5A). LSK cells from 21 week-old *Ercc1*⁻/ Δ mice exhibited a more pronounced increase in the level of mitochondrial ROS relative to littermate controls.

We next measured γ H2AX, a marker of DNA strand breaks, in HSPCs from 16- and 21-week-old mice. A modestly higher proportion of LSK cells were positive for γ H2AX in 16 week old *Ercc1*⁻/ Δ mice compared with their normal littermate counterparts (8.7% vs. 2.7%) (Figure 5B, top histograms). However, by 21 weeks of age, there was a much more dramatic increase in the fraction of LSK cells staining positively for γ H2AX in *Ercc1*⁻/ Δ mice compared to control littermates (24.5% vs. 4.3%) (Figure 5B, lower histograms). Importantly, the accumulation of γ H2AX was observed predominantly in LSK cells but not in other subpopulations of HSPCs such as Lin⁻Sca-1⁻c-kit⁺ cells (myeloid progenitors). We were unable to analyze levels of γ -H2AX in SLAM LSK or CD34-LSK cells due to their very low frequency of this population of cells in 21 week-old *Ercc1*⁻/ Δ mice (see Figure 2). Collectively, these data support the conclusion that *Ercc1*⁻/ Δ mice display an age-dependent increase in oxidative stress and spontaneous accumulation of DNA damage preferentially in stem/progenitor cell population.

A hypomorphic mutation in *Ercc1* also leads to loss of HSPCs via non-cell-autonomous mechanisms

While the results shown above indicate cell autonomous defects within the *Ercc1*⁻/ Δ HSPCs, we asked whether the fate of *Ercc1*⁻/ Δ HSPCs is also influenced by their microenvironment, i.e., via a non-cell-autonomous manner. For this purpose, transplantation experiments were performed with or without conditioning regimen, as illustrated in Figure 6A. When normal donor cells (CD45.1) were transplanted into unconditioned WT recipients (CD45.1/2), donor-derived cells (CD45.1) made a modest contribution to the recipient's peripheral blood cells (13.6%) 5 weeks post-transplant, but were barely detectable (~0.05%) in the peripheral blood 9 weeks post-transplant and thereafter (Figure 6B, upper). In contrast, normal donor cells (CD45.1) showed a higher contribution in unconditioned *Ercc1*⁻/ Δ recipients at 5 weeks post-transplant (~46%) (Figure 6B, upper). However, the contribution of donor cells to *Ercc1*⁻/ Δ recipient's hematopoietic system was drastically reduced thereafter and donor cell chimerism was almost undetectable (<1%) at 9 weeks post-transplantation (Figure 6B, upper). Because stable engraftment is dependent upon

successful lodging and expansion of donor HSPCs in the recipient's bone marrow, we monitored the frequency of donor cells in the recipient's bone marrow. Donor cells were barely detectable in the bone marrow of WT recipient mice (<0.01%) (Figure 6B, lower left). This is expected since the stem cell niches in WT mice have already been occupied by the endogenous HSPCs. A higher percentage of donor cells (0.32%) were detected in the bone marrow of *Ercc1*^{-/-} recipients. However, amongst the donor-derived cells in the bone marrow of *Ercc1*^{-/-} recipients, donor-derived LSK cells were virtually undetectable (<0.1%) (Figure 6B, lower right), suggesting the possibility that the microenvironment of *Ercc1*^{-/-} mice is not permissive for stable and productive engraftment of transplanted donor HSPCs.

To rule out the possibility that the poor engraftment in unconditioned wild-type and *Ercc1*^{-/-} recipients was due to differences in genetic backgrounds between donor and recipient mice, we tested the compatibility of the strains under conditions of lethal irradiation. The majority of peripheral blood cells in conditioned (10 Gy) control littermate recipients were of donor origin (Supplementary figure 2, upper panel). Furthermore, there was robust engraftment in the bone marrow as evidenced by a donor-dominated (>93%) chimerism and the presence of LSK cells in the bone marrow of conditioned control littermate recipients (Supplementary figure 2, lower panel). Transplantation into lethally conditioned *Ercc1*^{-/-} recipients is not technically feasible, since *Ercc1*^{-/-} mice are hypersensitive to radiation¹². Therefore, in subsequent experiments, the *Ercc1*^{-/-} mice were conditioned with 3 Gy total body irradiation. At 3Gy, *Ercc1*^{-/-} recipient mice could survive up to 7–9 weeks after transplantation. At 9 weeks post-transplantation, *Ercc1*^{-/-} mice had a significantly reduced number of LSK cell in their bone marrow as compared with similarly conditioned wild-type recipients (Figure 6C). These results reinforce the view that the microenvironment of *Ercc1*^{-/-} mice does not provide an optimal milieu for HSPCs.

Osteoblasts provide the niche necessary to retain HSCs in a quiescent state^{28–29}. An imbalance of osteoblast/osteoclast activity is detrimental to long-term HSC maintenance^{30–31}. To determine whether the observed non-autonomous defects are associated with a shift in the balance of osteoclast and osteoblast activity, bone/bone marrow sections were stained for tartrate resistant acid phosphatase (TRAP), a marker of osteoclast activity. TRAP positive cells were barely detectable in the bone marrow of normal mice at 7 and 21 weeks of age (Figure 6D, left panel). In contrast, 21 week-old *Ercc1*^{-/-} mice demonstrated a considerably higher level of TRAP staining (Figure 6D, lower right panel).

Interestingly, the serum from *Ercc1*^{-/-} mice was more potent in promoting osteoclast formation than serum from littermate controls (Figure 6F, p=0.001), suggesting that humoral factors in *Ercc1*^{-/-} mice may play a role in shifting the balance towards to osteoclast formation. *Ercc1*^{-/-} mice displayed an increased level of several proinflammatory cytokines in their serum (Figure 6E). TNF- α and IL-1 α were the most upregulated (>2 fold, p<0.05). Addition of antibodies against TNF- α and IL-1 α to the serum of *Ercc1*^{-/-} mice attenuated osteoclast formation in vitro (Figure 6F) demonstrating a causative role for these cytokines in promoting osteoclastogenesis. Of note, the TNF- α neutralizing antibody had a more potent inhibitory effect on osteoclast formation than the IL-1 α antibody. However, the combination was additive. Since the serum level of the cytokines may not accurately reflect the characteristic of bone marrow microenvironment, we further measured cytokine expression in the bone marrow itself. In bone marrow supernatant, TNF- α and IL-1 α were higher in *Ercc1*^{-/-} mice than in littermate controls (Figure 6G). To determine the source of the cytokine production in the bone marrow of *Ercc1*^{-/-} mice, TNF- α and IL-1 α expression were analyzed in various lineages of bone marrow cells. Flow cytometric analysis revealed that TNF- α and IL-1 α were predominantly expressed by CD4 and CD8

expressing cells in *Ercc1*^{-/-} bone marrow (Figure 6G). In assays done with other cell lineage markers (e.g. B220+, CD11b+, Gr-1+, etc.) we found no significant difference.

RANKL, the primary mediator of osteoclast formation, was also expressed at high levels in the bone marrow of *Ercc1*^{-/-} mice (Figure 6H). Interestingly, the increased expression of RANKL was observed in *Ercc1*^{-/-} mice at 7 weeks of age, which was before the appearance of TRAP positive cells. These findings support the observation that the bone marrow of *Ercc1*^{-/-} mice is not conducive to HSPC engraftment and maintenance.

DISCUSSION

As humans age, there is loss of bone marrow cellularity, increased cell turnover and cell death in the hematopoietic compartment, and myeloid skewing³². These characteristics indicate a loss of regenerative capacity in the hematopoietic system with aging. Many genetic diseases caused by mutations in genome maintenance mechanisms show involvement of the hematopoietic system³³, leading to the hypothesis that DNA damage contributes to aging-related loss of HSC function. In support of this, *Xpd*^{TTD} mice defective in nucleotide excision repair and transcription, *Ku80*^{-/-} mice missing non-homologous end-joining of double-strand breaks, G3 *mTR*^{-/-} mice missing telomerase and *poly*^{Exo} mice missing the exonuclease of the mitochondrial polymerase γ , show accelerated loss of HSC function, but not number⁴. This led to the view that HSCs accumulate DNA damage, which directly affects HSC function^{1, 34-35}. In contrast, there is attrition of HSCs in *polu*^{-/-} mice defective in DNA damage tolerance, as well as *Atm*^{-/-} and *p53*^{+/m} mice with altered DNA damage response^{4, 8}. *Lig4*^{Y288C} mice, defective in non-homologous end-joining of double-strand breaks, spontaneously develop bone marrow failure and lose HSCs as a consequence of increased HSC turnover⁷.

Ercc1^{-/-} mice are immunocompetent, have normal telomere lengths, transcription and DNA damage response³⁶⁻³⁷, allowing us to focus on the impact of unrepaired endogenous DNA damage on the hematopoietic system. *Ercc1*^{-/-} mice have early onset and progressive loss of HSPC function and number (Figure 1 and 2). This provides evidence that endogenous DNA damage, if not repaired, can drive premature exhaustion of HSPCs.

Several factors associated with HSPC dysfunction were discovered to be perturbed in LSK cells from *Ercc1*^{-/-} mice (Figure. 4-5). γ H2AX is elevated in LSK cells from 21 week old *Ercc1*^{-/-} mice, but not younger animals or progenitor cells, indicative of age-dependent DNA damage accumulation in the HSPC subsets. ROS is elevated in HSPCs of progeroid *Ercc1*^{-/-} mice compared to littermate controls. Deletion of the DNA damage signaling proteins ATM or MDM2, or mutation of the telomerase protein DKC1 also leads to increased ROS in HSCs and stem cell depletion³⁸⁻⁴⁰.

Expression of the *p16*^{INK4a} is associated closely with cellular senescence⁴¹. *p16*^{INK4a} expression was significantly elevated in *Ercc1*^{-/-} LSK cells, which has been shown to attenuates HSC self-renewal⁴².

DNA damage is thought to drive aging-related loss of HSC function via a cell autonomous mechanism^{1, 3, 43}, which is consistent with all of the above. However, in other tissue-specific, adult stem cell populations, the niche is also critical for maintaining a reservoir of stem cells and tissue regenerative capacity⁴⁴. Thus we also examined non-cell autonomous mechanisms for hematopoietic failure in the *Ercc1*^{-/-} mice. Normal HSPCs were not able to establish sustainable, productive engraftment in *Ercc1*^{-/-} mice, indicating that defects in the microenvironment contribute to the loss of HSPC number and function. This was supported by the observations that osteoclastic activity was significantly increased in *Ercc1*^{-/-} mice compared to wild-type littermates as were pro-inflammatory cytokines in the

serum of *Ercc1*^{-/-} mice that drive osteoclastogenesis. Deletion of the cell cycle regulator RB leads to osteoclast-mediated destruction of the osteoblastic niche, displacement of the HSCs, and myeloid expansion⁴⁵, which are similar to changes observed in *Ercc1*^{-/-} mice.

Defective homing of *Ercc1*^{-/-} HSPC via downregulation of homing-associated molecules could be another potential explanation for the hematopoietic deficiencies observed in *Ercc1*^{-/-} mice. Unexpectedly, we found that the HSPCs from 15- and 21-weeks old *Ercc1*^{-/-} mice express significantly high levels of CXCR4 (Supplementary figure 3), which is one of the critical mediators of HSC homing⁴⁶. This suggests that the dysfunction of *Ercc1*^{-/-} HSPCs is not likely mediated by down-regulating the expression of CXCR4. Because the majority of intravenously injected cells are trapped in the lung and liver, with very limited number of *Ercc1*^{-/-} HSPCs, it was technically difficult to quantify homing of transplanted *Ercc1*^{-/-} HSPCs.

FoxO^{-/-}, *Bmi-1*^{-/-} and *Lig*^{4Y288C} mice, which exhibit premature aging-like phenotypes, also undergo significant attrition in HSC numbers^{7, 47–48}. In contrast, it was reported that the number of HSCs increases in normal aged B6 mice, although there is considerable strain to strain variation⁴⁹.

In summary, analysis of the hematopoietic system of *Ercc1*^{-/-} mice provides evidence that endogenous DNA damage promotes the loss of HSPCs through both cell autonomous and non-autonomous mechanisms. The heretofore unappreciated contribution of cell non-autonomous mechanisms of HSPC attrition could have important implications for elderly patients and patients with genome instability disorders requiring bone marrow transplantation.

Material and Methods

Mice

Ercc1^{-/-} mice were generated by mating of heterozygous mice in two different inbred backgrounds to create isogenic F1 hybrids: *Ercc1*^{+/-} FVB/n×*Ercc1*^{+/-} C57Bl/6. Genomic DNA was isolated from a 1 mm ear plug of 10–14 day-old mice using a NucleoSpin® 96 Tissue DNA extraction system (Macherey-Nagel, Inc.). Genotyping of the *Ercc1* null allele was done by PCR co-amplification of the 3' end of exon 7 from the wild-type (wt) allele and the neomycin resistance marker cloned into exon 7 of the targeted allele using primers specific for exon 7, neo^r and intron 7 (5'-AGCCGACCTCCTTATGGAAA, 5'-TCGCCCTTCTTGACGAGTTCT and 5'-ACAGATGCTGAGGGCAGACT, respectively). Wt (0.25-kb) and mutant (0.4-kb) fragments were separated by electrophoresis on a 2% agarose gel. The hypomorphic allele of *Ercc1* (Δ) was amplified by adding a fourth primer (5'-CTAGGTGGCAGCAGGTCATC) to amplify the neomycin cassette in the mutant allele (0.5-kb). Wild-type or heterozygous littermates were retained as aging-matched controls. All animal experiments were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Colony Forming Cell (CFC) Assays

For the CFU-GM assay, bone marrow cells (1×10⁴/dish) were seeded into 35 mm dishes in MethoCult® GF (Stem Cell Technologies Inc.). For the pre-B assay, bone marrow cells (1×10⁵/dish) were plated in MethoCult® M3630 (StemCell Technologies Inc.). All Colony Forming Cell Assays were performed in triplicate. The colonies were counted on day 7 for pre-B and day 12 for CFU-GM colonies.

Quantitative Real-Time PCR

Total RNA from LSK cells was isolated using RNeasy Micro kit (Qiagen). Reverse transcription of total RNA was performed using QuantiTech reverse transcription kit (Qiagen). Primers for *p16^{INK4a}* was purchased from Applied Biosystems.

Flow cytometry analysis

LSK cells were defined by staining with a lineage cocktail, Sca-1 and c-kit antibodies. LSK cells were further defined by staining with anti-CD34, CD48 and CD150 antibodies as previously described⁵⁰. Cell sorting was performed by Moflow (DakoCytomation). The SA- β -Gal activity was assessed using C₁₂FDG (Molecular Probes). The level of mitochondrial superoxide anion within LSK cells was assessed using MitoSOX™ (Invitrogen). Cell death was determined by Annexin V and PI staining. Intracellular cytokine levels were measured by flow cytometry. Briefly, bone marrow cells were stained antibodies to lineage surface markers. Cells were then fixed, permeabilized and stained with anti-TNF- α and IL-1 α antibodies according to the manufacturer's instructions (e-BioScience). Cell populations were gated (e.g. CD4+, CD8+ and double positive cells) and the fraction of cells expressing TNF- α and IL-1 α were measured. γ -H2AX was assessed by anti-phospho-histone H2AX (Ser139) antibody (Cell Signaling) using the Cytofix/Cytoperm plus Solution Kit (BD Bioscience).

Cell cycle analysis

Cell cycle analysis was performed by staining the cells with FITC conjugated Ki-67 antibody (BD Pharmingen). Briefly, LSK cells were fixed and permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) followed by staining with Ki-67-FITC (BD Biosciences) and DAPI (Invitrogen).

Transplantation

To evaluate short- and long-term reconstituting activity of donor cells, recipient mice were lethally irradiated (Shepherd Mark I 68 irradiator, ¹³⁷Cs γ source) 8 to 24 hours prior to transplantation. Bone marrow cells from 7 and 21 week-old *Ercc*^{-/ Δ} mice or their control littermates were transplanted by tail vein injection. For the competitive repopulation assay, test cells (CD45.1/2) from *Ercc*^{-/ Δ} mice and control littermates were transplanted along with an equal number of competitor cells (CD45.1) into lethally irradiated recipient mice (CD45.2). Competitor cells, like the test cells were obtained from F1 mice (FVB/B6). To examine the fate of transplanted cells in non-ablated hosts, 5 $\times$ 10⁶ normal WT bone marrow cells (B6, CD45.1) were transplanted into non-irradiated recipient mice (CD45.1/2). Peripheral blood and bone marrow cells were collected from recipient mice at various times post-transplant, and the ratio of CD45.1/CD45.1.2 was determined by flow cytometry.

TRAP staining and Immunohistochemistry

The femurs dissected from 7 and 21 weeks-old *Ercc*^{-/ Δ} mice and wild-type littermates were fixed in 4% paraformaldehyde solution in phosphate buffered saline (PBS, pH 7.2) for 2 days and then decalcified in 10% EDTA (pH 7.5) for 10 days. After decalcification, they were embedded in paraffin and cut longitudinally to 5 μ m thickness. For identification of osteoclasts, the sections were deparaffinized, dehydrated, and stained using TRAP staining kit (B-Bridge International, Inc.) according to the manufacturer's instructions. For immunohistochemical staining of RANKL, bone marrow sections were immunolabeled overnight with goat polyclonal antibody against mouse RANKL (Santa Cruz Biotechnology, 1:50) at 4°C. Subsequently, the bone sections were incubated with biotinylated anti-goat antibody (Vector Laboratories) and DAB substrate (Vector Laboratories) according to the manufacturer's instructions.

ELISA

The levels of cytokines were measured using Enzyme-linked immunosorbent assay (ELISA). Serum samples from 17 week-old *Ercc1*^{-/-} mice and control littermates (n=3 per group) were diluted to 1:10 in PBS. Bone marrow fluid was collected as previously described with minor modifications⁵¹. Absorbance was measured using a microplate reader set at 450 nm. Plotted is the average $\pm$ the standard error of the mean.

Osteoclast-forming assay

Bone marrow cells from 5–6 week-old mice (B6) were seeded in a 96-well plate at a density of 2×10^5 cells per well and incubated with 50 ng/ml M-CSF (eBioscience) for 3 days. Serum from 17 week-old *Ercc1*^{-/-} mice and control littermates were diluted in PBS samples and added to each well in the presence or absence of neutralizing antibodies (anti-IL-1 α or anti-TNF- α ; 1 mg/ml). The cells were incubated further for 3 days. Osteoclasts were identified by TRAP staining as describe above and counted. Plotted is the average of TRAP positive cells $\pm$ standard error of the mean

Statistical analysis

All *p* values (statistical significance) were determined by Student's *t* test, Mann-Whitney, or Wilcoxon test; * < 0.05; ** < 0.01; *** < 0.005.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Ms. Chelsea Feldman for technical assistance. This study was supported in part by research funding from Department of Defense (W81XWH-09-1-0364) to B.C. Lee and a National Institutes of Health grant (R01ES016114) to L.J. Niedernhofer. This project used the UPCI flow cytometry facility and was supported in part by award P30CA047904.

REFERENCES

1. Beerman I, Maloney WJ, Weissmann IL, et al. Stem cells and the aging hematopoietic system. *Curr Opin Immunol.* 2010; 22:500–506. [PubMed: 20650622]
2. Morrison SJ, Wandycz AM, Akashi K, et al. The aging of hematopoietic stem cells. *Nat Med.* 1996; 2:1011–1016. [PubMed: 8782459]
3. Sahin E, Depinho RA. Linking functional decline of telomeres, mitochondria and stem cells during ageing. *Nature.* 2010; 464:520–528. [PubMed: 20336134]
4. Niedernhofer LJ. DNA repair is crucial for maintaining hematopoietic stem cell function. *DNA Repair (Amst).* 2008; 7:523–529. [PubMed: 18248857]
5. Barnes DE, Lindahl T. Repair and genetic consequences of endogenous DNA base damage in mammalian cells. *Annu Rev Genet.* 2004; 38:445–476. [PubMed: 15568983]
6. Rossi DJ, Bryder D, Seita J, et al. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature.* 2007; 447:725–729. [PubMed: 17554309]
7. Nijnik A, Woodbine L, Marchetti C, et al. DNA repair is limiting for haematopoietic stem cells during ageing. *Nature.* 2007; 447:686–690. [PubMed: 17554302]
8. Lucas D, Escudero B, Ligos JM, et al. Altered hematopoiesis in mice lacking DNA polymerase mu is due to inefficient double-strand break repair. *PLoS Genet.* 2009; 5:e1000389. [PubMed: 19229323]
9. Parmar K, Kim J, Sykes SM, et al. Hematopoietic stem cell defects in mice with deficiency of *Fancd2* or *Usp1*. *Stem Cells.* 2010; 28:1186–1195. [PubMed: 20506303]

10. Niedernhofer LJ, Odijk H, Budzowska M, et al. The structure-specific endonuclease Ercc1-Xpf is required to resolve DNA interstrand cross-link-induced double-strand breaks. *Mol Cell Biol.* 2004; 24:5776–5787. [PubMed: 15199134]
11. Bhagwat N, Olsen AL, Wang AT, et al. XPF-ERCC1 participates in the Fanconi anemia pathway of cross-link repair. *Mol Cell Biol.* 2009; 29:6427–6437. [PubMed: 19805513]
12. Ahmad A, Robinson AR, Duensing A, et al. ERCC1-XPF endonuclease facilitates DNA double-strand break repair. *Mol Cell Biol.* 2008; 28:5082–5092. [PubMed: 18541667]
13. Gregg SQ, Robinson AR, Niedernhofer LJ. Physiological consequences of defects in ERCC1-XPF DNA repair endonuclease. *DNA Repair (Amst).* 2011; 10:781–791. [PubMed: 21612988]
14. Gregg SQ, Gutierrez V, Robinson AR, et al. A mouse model of accelerated liver aging caused by a defect in DNA repair. *Hepatology.* 2012; 55:609–621. [PubMed: 21953681]
15. Vo N, Seo HY, Robinson A, et al. Accelerated aging of intervertebral discs in a mouse model of progeria. *J Orthop Res.* 2010; 28:1600–1607. [PubMed: 20973062]
16. Lavasani M, Robinson AR, Lu A, et al. Muscle-derived stem/progenitor cell dysfunction limits healthspan and lifespan in a murine progeria model. *Nat Commun.* 2012; 3:608. [PubMed: 22215083]
17. Rossi DJ, Bryder D, Zahn JM, et al. Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proc Natl Acad Sci U S A.* 2005; 102:9194–9199. [PubMed: 15967997]
18. Prasher JM, Lalai AS, Heijmans-Antonissen C, et al. Reduced hematopoietic reserves in DNA interstrand crosslink repair-deficient Ercc1^{-/-} mice. *EMBO J.* 2005; 24:861–871. [PubMed: 15692571]
19. Min H, Montecino-Rodriguez E, Dorshkind K. Effects of aging on the common lymphoid progenitor to pro-B cell transition. *J Immunol.* 2006; 176:1007–1012. [PubMed: 16393987]
20. Schofield R, Dexter TM, Lord BI, et al. Comparison of haemopoiesis in young and old mice. *Mech Ageing Dev.* 1986; 34:1–12. [PubMed: 3520172]
21. Ju Z, Jiang H, Jaworski M, et al. Telomere dysfunction induces environmental alterations limiting hematopoietic stem cell function and engraftment. *Nat Med.* 2007; 13:742–747. [PubMed: 17486088]
22. Osawa M, Hanada K, Hamada H, et al. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science.* 1996; 273:242–245. [PubMed: 8662508]
23. Kiel MJ, Yilmaz OH, Iwashita T, et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell.* 2005; 121:1109–1121. [PubMed: 15989959]
24. Akashi K, Traver D, Miyamoto T, et al. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature.* 2000; 404:193–197. [PubMed: 10724173]
25. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell.* 1997; 91:661–672. [PubMed: 9393859]
26. Parrinello S, Samper E, Krtolica A, et al. Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol.* 2003; 5:741–747. [PubMed: 12855956]
27. Tothova Z, Gilliland DG. FoxO transcription factors and stem cell homeostasis: insights from the hematopoietic system. *Cell Stem Cell.* 2007; 1:140–152. [PubMed: 18371346]
28. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature.* 2003; 425:841–846. [PubMed: 14574413]
29. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature.* 2003; 425:836–841. [PubMed: 14574412]
30. Kollet O, Dar A, Shivtiel S, et al. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nat Med.* 2006; 12:657–664. [PubMed: 16715089]
31. Purton LE, Scadden DT. Osteoclasts eat stem cells out of house and home. *Nat Med.* 2006; 12:610–611. [PubMed: 16761001]
32. Ogawa T, Kitagawa M, Hirokawa K. Age-related changes of human bone marrow: a histometric estimation of proliferative cells, apoptotic cells, T cells, B cells and macrophages. *Mech Ageing Dev.* 2000; 117:57–68. [PubMed: 10958923]

33. Naka K, Hirao A. Maintenance of genomic integrity in hematopoietic stem cells. *Int J Hematol*. 2011; 93:434–439. [PubMed: 21384097]
34. Rossi DJ, Bryder D, Weissman IL. Hematopoietic stem cell aging: mechanism and consequence. *Exp Gerontol*. 2007; 42:385–390. [PubMed: 17275237]
35. Sharpless NE, DePinho RA. How stem cells age and why this makes us grow old. *Nat Rev Mol Cell Biol*. 2007; 8:703–713. [PubMed: 17717515]
36. Zhu XD, Niedernhofer L, Kuster B, et al. ERCC1/XPF removes the 3' overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes. *Mol Cell*. 2003; 12:1489–1498. [PubMed: 14690602]
37. Schrader CE, Vardo J, Linehan E, et al. Deletion of the nucleotide excision repair gene *Ercc1* reduces immunoglobulin class switching and alters mutations near switch recombination junctions. *J Exp Med*. 2004; 200:321–330. [PubMed: 15280420]
38. Abbas HA, Maccio DR, Coskun S, et al. Mdm2 is required for survival of hematopoietic stem cells/progenitors via dampening of ROS-induced p53 activity. *Cell Stem Cell*. 2010; 7:606–617. [PubMed: 21040902]
39. Gu BW, Fan JM, Bessler M, et al. Accelerated hematopoietic stem cell aging in a mouse model of dyskeratosis congenita responds to antioxidant treatment. *Aging Cell*. 2011; 10:338–348. [PubMed: 21241452]
40. Ito K, Hirao A, Arai F, et al. Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature*. 2004; 431:997–1002. [PubMed: 15496926]
41. Satyanarayana A, Rudolph KL. p16 and ARF: activation of teenage proteins in old age. *J Clin Invest*. 2004; 114:1237–1240. [PubMed: 15520854]
42. Janzen V, Forkert R, Fleming HE, et al. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature*. 2006; 443:421–426. [PubMed: 16957735]
43. Rossi DJ, Jamieson CH, Weissman IL. Stems cells and the pathways to aging and cancer. *Cell*. 2008; 132:681–696. [PubMed: 18295583]
44. Silva H, Conboy IM. Aging and stem cell renewal. 2008
45. Walkley CR, Shea JM, Sims NA, et al. Rb regulates interactions between hematopoietic stem cells and their bone marrow microenvironment. *Cell*. 2007; 129:1081–1095. [PubMed: 17574022]
46. Peled A, Petit I, Kollet O, et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science*. 1999; 283:845–848. [PubMed: 9933168]
47. Tothova Z, Kollipara R, Huntly BJ, et al. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell*. 2007; 128:325–339. [PubMed: 17254970]
48. Park IK, Qian D, Kiel M, et al. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature*. 2003; 423:302–305. [PubMed: 12714971]
49. de Haan G, Van Zant G. Dynamic changes in mouse hematopoietic stem cell numbers during aging. *Blood*. 1999; 93:3294–3301. [PubMed: 10233881]
50. Cho J, Shen H, Yu H, et al. Ewing sarcoma gene *Ews* regulates hematopoietic stem cell senescence. *Blood*. 2011; 117:1156–1166. [PubMed: 21030557]
51. Benarafa C, LeCuyer TE, Baumann M, et al. SerpinB1 protects the mature neutrophil reserve in the bone marrow. *J Leukoc Biol*. 2011; 90:21–29. [PubMed: 21248149]

A.

B.

C.

7 week 21 week

Figure 1. Progressive impairment of hematopoietic progenitor cells in DNA repair-deficient *Ercc1*^{-Δ} mice

(A) Bone marrow cells were collected from the tibia and femur of *Ercc1*^{-Δ} mice and control littermates at 7 and 21 weeks of age, and the number of viable mononuclear cells was counted (Wilcoxon test, n=10 per group, p<0.0001). Asterisks (***) indicate a statistically significant difference.

(B) Bone marrow cells from mice of the indicated genotypes at 7 and 21 weeks of age were subjected to CFU-GM and pre-B colony-forming assay. The number of colonies is shown as the mean ± standard deviation (s.d.) (Wilcoxon test, n=3 individual animals per group). Asterisks (* and **) indicate a statistically significant difference.

(C) Peripheral blood cells from mice of the indicated genotypes at 7 and 21 weeks of age were stained with CD11b and B220 antibodies, to identify monocytes and B lymphocytes, respectively. Graphed are the percent of CD11b (n=5 mice, p=0.007) and B220 (n=4 mice, p=0.002) positive cells expressed as the mean ± s.d. An asterisk (** and ***) indicates a statistically significant difference.

A.

B.

C.

D.

7 week 21 week

Figure 2. Quantitative and qualitative changes in HSPCs from *Ercc1*^{-Δ} mice

(A) Bone marrow cells from mice of the indicated genotypes at 7 and 21 weeks of age were transplanted into lethally irradiated recipient animals. The ability of the donor cells to support survival of the recipient for 4–5 weeks was attributed to short-term repopulating cells (radioprotection). Survival beyond 16 weeks requires long-term repopulating cells. n represents the number of recipient animals in each group.

(B) Competitive bone marrow repopulating assay. Bone marrow cells from *Ercc1*^{-Δ} mice or their control littermates (CD45.1/2) were mixed with competitor bone marrow cells from wild-type animals (CD45.1) at a ratio of 1:1 and transplanted into lethally irradiated recipient mice (C57BL/6, CD45.2). Competitor bone marrow cells (CD45.1) were obtained

from the first-generation cross of C57BL/6 (B6.SJL-*Ptpca*^a *Pep3*^b/BoyJ, CD45.1) and FVB (CD45.1) mice, so that their genetic background was identical to that of the *Ercc1* mice but they express only the CD45.1 cell surface antigen. Peripheral blood cells from recipient animals were analyzed for CD45.1 and CD45.1.2 expression by flow cytometry every 4 weeks after transplantation. Top panels: representative flow cytometry data showing donor contributions at 12 weeks and one year post-transplant; Lower panel: a graph of the results obtained at 12 weeks and one year post-transplant. Plotted is the percent of recipient peripheral blood mononuclear cells derived from the *Ercc1*^{+/+} or *Ercc1*^{-/ Δ} mice vs. wild-type competitor-derived cells $\pm$ s.d. (n=5 recipient mice per group, p<0.0001). Asterisks (***) indicate a statistically significant difference.

(C) Representative schematic diagrams of flow cytometry gating strategy to define various stem/progenitor cell populations of 21 week-old *Ercc1*^{-/ Δ} mice and their normal littermates. Bone marrow cells were stained with the antibodies indicated on each axis and analyzed by flow cytometry. Numbers represent the percent of bone marrow cells in the gated area. LSK (Lin⁻ Sca1⁺ c-Kit⁺), CD34-LSK, SLAM LSK (CD150⁺ CD48⁻ LSK), CLP (common lymphoid progenitors), CMP (common myeloid progenitors), GMP (granulocyte-monocyte progenitors) and MEP (megakaryocyte-erythroid precursor).

(D) Quantification of the absolute number of HSPCs in *Ercc1*^{-/ Δ} mice at 7 and 21 weeks of age compared to normal littermates. The data are plotted as the mean $\pm$ s.d. P values were determined by Mann-Whitney test (n=5 mice per genotype and age). Asterisk (*) indicates statistically significant differences.

Figure 3. Increased turnover of HSPCs in *Ercc1*^{-Δ} mice

HSPCs (LSK) from 15–16 week-old mice (n=7 per genotype) were pooled and analyzed for DNA content (DAPI) and intracellular Ki-67. Due to the very low number of LSK cells isolated from *Ercc1*^{-Δ} mice, bone marrow cells were pooled from 3–4 mice of each genotype. The percent of cells in each phase of the cell cycle is summarized in the accompanying graph. Data are shown as mean ± s.d. and are representative of at least two independent experiments.

A.

B.

C.

Figure 4. HSPCs from DNA repair-deficient *Ercc1*^{-/-} mice undergo premature cell death and senescence

(A) Left: LSK cells from 15–16 week -old mice of the indicated genotypes were pooled (n=3 per group) and stained with Annexin-V and PI to measure cell death. Shown are representative flow cytometry data. Numbers represent the percent of cells in each quadrant. Right: The percent of cell death was calculated on the basis of PI-positive cells. Data are shown as mean ± s.d. and are representative of at least three independent experiments.

(B) LSK cells from mice of the indicated genotypes at 7- and 21 weeks of age were stained with C₁₂ FDG to measure SA-β-gal activity. Upper: Shown are representative flow cytometry data. n represents the number of animals examined in each group. Lower: the results are expressed as the mean fluorescence intensity (MFI) of SA-β-gal staining. SA-β-gal MFI for the control littermate LSK cells was set to 1.

(C) LSK cells from mice of the indicated genotypes at 7 and 21 weeks of age were sorted and the expression of *p16*^{INK4a} was analyzed by real-time quantitative PCR. The expression in WT cells (black bar) was set as 1. The fold change in *Ercc1*^{-/-} mice was calculated using the ΔΔCt method. The expression level was normalized to the level of GAPDH.

Representative results of at least two independent experiments, each using 3 mice per group, is shown. P-values were determined by Mann-Whitney test (n=6). Asterix (*) indicate a statistically significant difference.

A.

B.

Figure 5. HSPCs from DNA repair-deficient *Ercc1*^{-/-} mice have increased oxidative stress and DNA damage

(A) LSK cells from mice of the indicated genotypes at 7- and 21 weeks of age were stained with MitoSOXTM Red to measure mitochondrial superoxide anion (ROS). Upper: Shown are representative flow cytometry data. n represents the number of animals examined in each group. Lower: the results are expressed as the mean fluorescence intensity (MFI) of MitoSox staining. MitoSox MFI for the control littermate LSK cells was set to 1.

(B) LSK cells from the indicated genotypes at 16- (n=6 per each genotype) and 21 weeks (n=9 per each genotype) of age were analyzed for endogenous expression of phosphorylated γH2AX by flow cytometry. Shown are representative flow cytometry data. The

accompanying graphs represent the percent (upper panel) and the MFI (lower panel) of γ H2AX positive LSK cells of at least two independent experiments. γ H2AX MFI for the control littermate LSK cells was set to 1

B.

Peripheral Blood

Bone Marrow

C.

D.

E.

F.

G.

H.

Figure 6. Deficiency of ERCC1 leads to changes in the HSPC niche

(A) Schematic diagram of the transplantation protocol. Normal WT bone marrow cells (5×10^6 /recipient, CD45.1) were transplanted into conditioned or non-conditioned recipient animals of the indicated genotype. As a control, normal WT recipients, which are F1 offspring of FVB X B6 (CD45.1/2), were used. Unconditioned recipient animals were 14 weeks old. Six- to 7-week-old animals were sublethally irradiated with 300 cGy.

(B) Upper: At 5 and 9 weeks post-transplant, the unconditioned recipient's peripheral blood cells were analyzed for donor contribution. The percent of donor-derived cells (CD45.1) is expressed as the mean $\pm$ s.d.; WT (*Ercc1*^{+/+}) recipients (closed diamond, n=4), *Ercc1*^{-/-} recipients (closed circle, n=3), control recipients (closed square, n=5). Lower: At 9 weeks post-transplant, unconditioned recipient's bone marrow cells were analyzed for donor contribution (CD45.1). Donor-derived cells (CD45.1) in the recipient's bone marrow (n=4) were gated and further analyzed for the percentage of LSK cells (CD45.1, LSK). Shown are representative flow cytometry plots depicting the percent of donor (CD45.1) or host (CD45.1/2) derived cells.

(C) At 9 weeks post-transplant, the conditioned (300 cGy) recipient's bone marrow cells were analyzed for donor HSPC contribution. Donor-derived cells (CD45.1) in the recipient's bone marrow (n=3/each group) were gated and analyzed for the number of LSK cells.

(D) Longitudinal sections of *Ercc1*^{+/+} and *Ercc1*^{-/-} femurs at 7 and 21 weeks of age were stained for TRAP to visualize osteoclasts and counterstained with haematoxylin. TRAP positive cells appear red/purple. Representative TRAP stain sections are shown. Scale bar = 50 μ m

(E) Cytokine levels in the sera of 17 week-old mice of each genotype (n= 3 per group) were measured by a sandwich ELISA as described in Methods. Asterisks (* and **) indicate a statistically significant difference.

(F) In vitro osteoclast differentiation was performed in the presence of M-CSF (50 ng/ml). Cells were incubated with serum from wild type (black bars) or *Ercc1*^{-/-} (white bars) mice in the presence (+) and absence (-) of anti-IL-1 α (1 μ g/ml) and/or anti-TNF- α (1 μ g/ml) antibodies as detailed in Methods. The number of TRAP+ cells was counted as described above.

(G) Upper panel: Cell-free bone marrow supernatants were recovered as previously described⁵¹. The levels of cytokines were measured as described above. Middle panel: For measurement of intracellular cytokine production, bone marrow cells were labeled for the expression of various lineage markers and for the intracellular expression of TNF- α and IL-1 α as described in Methods. Representative histograms on CD4 and CD8 gated cells are presented. Numbers represent the percentage of cells producing TNF- α and IL-1 α within

the indicated gate. Lower panel: The accompanying graphs represent mean $\pm$ s.d. from three independent experiments.

(H) Bone marrow sections were incubated with an antibody against mouse RANKL, which drives osteoclast differentiation, followed by DAPI staining. Scale bar = 50 μ m