Quiescent Hematopoietic Stem Cells Accumulate DNA Damage during Aging that Is Repaired upon Entry into Cell Cycle

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INTRODUCTION

The hematopoietic system is associated with many changes, including diminished lymphoid potential, elevated autoimmunity, reduced regenerative potential, and onset of a spectrum of hematopoietic diseases including myelodysplastic syndrome and leukemias. Mounting evidence suggests that aging-associated changes in hematopoietic stem cells (HSCs) autonomously contribute to many of these age-related phenotypes through diverse mechanisms involving diminution of regenerative potential (Dykstra et al., 2011; Rossi et al., 2005; Sudo et al., 2000), changes in lineage potential and HSC subtype composition (Beerman et al., 2010; Challen et al., 2010; Dykstra et al., 2011; Pang et al., 2011), loss of polarity (Florian et al., 2012), alterations of the epigenetic landscape (Beerman et al., 2013; Chambers et al., 2007), and DNA damage accumulation (Rossi et al., 2007a; Rübe et al., 2011). Both myelodysplastic syndrome (Pang et al., 2013) and acute and chronic myelogenous leukemias begin with nonlethal mutations in the HSC pool, often leading to successful expansion of mutant HSC clones at the expense of normal HSCs and that eventually progress to leukemia (Corces-Zimmerman et al., 2014; Jamieson et al., 2004; Jan et al., 2012).

It has been postulated that tissue-specific stem cells, including HSCs, must possess cyto- and geno-protective mechanisms in order to ensure their long-term functional potential. Consistent with this idea, HSCs are imbued with a number of protective properties that are believed to contribute to the preservation of their activity. For example, the high levels of expression of certain ATP binding cassette (ABC) transporters, including ABCG2, confer xenobiotic efflux activity on HSCs (Krishnamurthy et al., 2004; Zhou et al., 2001, 2002). HSCs also maintain low levels of reactive oxygen species due to the combined action of their low metabolic activity and their reliance on glycolytic metabolism along with the inherent hypoxic nature of HSCs and their niche (Kocabas et al., 2012; Nombela-Arrieta et al., 2013; Parmar et al., 2007; Shyh-Chang et al., 2013; Suda et al., 2011; Takubo et al., 2010). Moreover, the dormant nature of HSCs (Cheshier et al., 1999; Foudi et al., 2009; Wilson et al., 2008), combined with the expression of telomerase in HSCs (Broccoli et al., 1995; Hiyama et al., 1995; Morrison et al., 1996), minimizes the introduction of replication-based errors and uncapping of telomeres during replication (Alsopp et al., 2003; Flores et al., 2006; Morrison et al., 1996).

In addition to these inherent cyto-protective properties, it is also clear that genome repair is important for HSC regenerative potential, as highlighted in studies using mice with engineered mutations in diverse DNA repair and response pathways that invariably show diminished HSC functional potential under conditions of stress (Cho et al., 2013; Nijnik et al., 2007; Parmar et al., 2010; Prasher et al., 2005; Rossi et al., 2007a). The aging dependent exacerbation of functional deficits in several DNA-repair-deficient mice suggested that the physiologic process of aging may be associated with progressive DNA damage accrual in...
HSCs (Nijnik et al., 2007; Rossi et al., 2007a). Indeed, this idea has been supported by immunohistochemical evidence of γH2AX accumulation, an indicator of DNA damage response (DDR), in HSCs isolated from old mice (Rossi et al., 2007a) and aged humans (Rübe et al., 2011). It has been proposed that diminished DNA repair capacity may underlie this age-associated DNA damage accrual (Chambers et al., 2007; Rübe et al., 2011), although this hypothesis has not been directly tested.

Herein, we present direct evidence of DNA damage accumulation in HSCs during aging. We report that, among diverse hematopoietic progenitor cells, age-associated DNA damage accrual measured by comet assays of DNA strand breaks is greatest within the HSC compartment. However, when HSCs are brought into cycle, the accrued damage does not result in measurable cell death, inability to produce hematopoietic colonies in vitro, or failure to reconstitute blood cells in vivo. Utilizing microarray expression analysis, we show that multiple DDR and repair pathways are broadly attenuated in quiescent, but not cycling, HSCs. We show that HSCs stimulated to enter cell-cycle upregulate multiple DNA response and repair pathways and concomitantly repair accumulated DNA damage. Altogether, our results refute the doctrine that HSCs are uniquely geno-protected during aging and instead demonstrates that stem cell quiescence attenuates DNA repair and response pathways in HSCs leading to DNA damage accumulation in the hematopoietic stem cell compartment during aging.

RESULTS

DNA Damage Accumulates in HSCs during Aging

Immunostaining of γH2AX in human and murine hematopoietic progenitors has provided indirect evidence that DNA strand breaks may accrue in HSCs during aging (Rossi et al., 2007a; Rübe et al., 2011). However, it remains possible that the observed γH2AX foci in aged stem cells may mark cellular processes distinct from DNA damage. Therefore, we sought to directly evaluate and quantify DNA damage in HSCs and progenitors during aging. To this end, we used alkaline comet assays (Figure S1 available online) (Olive and Banáth, 2006; Olive et al., 2001; Singh et al., 1988) to measure single- and double-strand breaks in stringently purified HSCs from young (3–4 months) and old (24–26 months) mice (Figure 1A). HSCs

Figure 1. DNA Damage Accumulates in HSCs during Aging

(A) Representative alkaline comets of young and old HSCs. (B and C) Olive tail moment (B) and percent of DNA in tail (C) of 710 HSCs from young mice, 447 HSCs from old mice, and 77 HSCs dosed with 2 Gy of irradiation. ***p < 0.001. Error bars represent SEM. See also Figure S1 and Table S1.
subjected to gamma irradiation were assayed in parallel as a positive control. Analysis of the two most reliable DNA damage measurements, Olive moment and percent tail DNA (Kumaravel and Jha, 2006), showed significantly elevated levels of DNA damage in HSCs purified from old mice in comparison to young HSCs (Figures 1B and 1C). These data were verified in six independent experiments in which a cumulative total of 4,940 young and 3,186 old HSCs with the immunophenotype of LSKCD34+/C0Flk2+ were scored in a blinded fashion. In two additional experiments, we assayed comets of over 2,000 young and old HSCs purified with an alternative cell-surface marker combination for HSCs: LSKCD150+/CD48+ (Kiel et al., 2005). In all experiments, a highly significant increase in DNA breaks was observed in old HSCs (Table S1). Interestingly, we consistently observed that young HSCs are not impervious to DNA damage, and 33% showed evidence of single- or double-strand breaks, defined here as having greater than 10% DNA in their comet tails (Figures 1C and S1). In contrast, ~70% of old HSCs scored as damaged (Figure 1C). Furthermore, tail DNA analysis revealed that, although young and old HSCs showed comparable frequencies of cells presenting evidence of modest DNA damage (classified as 10%–30% tail DNA), cells showing evidence of significant DNA damage (>30% tail DNA) were much more prevalent in HSCs purified from old (42%) in comparison to young (9%) mice (Figure 1C). Nonetheless, the fact that over 30% of old HSCs showed no evidence of strand breaks indicates that the HSC population as a whole does not comprehensively accrue strand breaks during aging.

To address the specificity of aging-associated damage in hematopoietic progenitor cells, we analyzed strand breaks in HSCs in comparison to their downstream progenitor progeny from young and old mice. Purified multipotent (MPP(+Flk2+) and MPP(-Flk2-)) and oligopotent progenitors (granulocyte-macrophage progenitor [GMP] and common lymphoid progenitor [CLP]), along with HSCs, were analyzed in single-cell comet assays. Analysis of the Olive tail moment of each downstream progenitor populations in comparison to HSCs isolated from young mice showed no significant differences (Figures 2A and 2B), although CLPs displayed slightly elevated Olive tail moments, possibly because of immunoglobulin heavy chain diversity and joining gene segment rearrangements actively occurring in this population. Consistent with this, the percent tail DNA of the young progenitor populations and HSCs was comparable, and the majority of scored cells (~70% in all populations) had no measurable DNA damage (<10% tail DNA; Figure 2C). In contrast, analysis of these same stem and progenitor populations from old mice (Figure 2D) demonstrated that the amount of damage accrued in the HSC compartment was significantly greater in comparison to all of the downstream progenitors by either Olive tail moment (Figure 2E) or percent tail DNA (Figures 2F and S2A). Furthermore, we analyzed the Olive tail moment of the stem and progenitor populations by comparing cells isolated from young to those purified from old mice. As we observed previously (Figure 1B), HSCs from old mice consistently showed a significant increase of DNA breaks in comparison to young (Figures S2B and S2C). Interestingly, despite the fact that all progenitor populations from old mice showed significantly reduced levels of DNA damage in comparison to aged HSCs (Figures 2E and S2A), the majority of these aged progenitors nonetheless showed evidence of significantly greater levels of DNA breaks than their young counterparts (Figures S2B and S2C). Altogether, these experiments show that HSCs and their progenitors accumulate DNA breaks during aging, yet the greatest amount of damage accrual is found within the stem cell compartment.

Old HSCs Repair DNA Damage upon Entering Cell Cycle

To establish whether old damaged HSCs could resolve accrued DNA damage, we used fluorescence-activated cell sorting (FACS) to purify young and old HSCs and assayed them for strand breaks by comet assays either immediately after purification (steady state) or 24 hr after culturing them in a cytokine-rich media, which stimulates the quiescent cells into cycle. As we previously observed (Figures 1, 2, and S2), HSCs at steady state from both young and old mice presented evidence of DNA breaks with significantly more damage observed in the old HSCs (Figure 3A). However, young and old HSCs assayed 24 hr postculture stimulation showed very similar comet profiles, and the old cells showed significantly reduced numbers of damaged cells in comparison to steady state (Figure 3A). To examine how old damaged HSCs would respond to induced cycling in vivo, we injected aged animals with 5-fluorouracil (5-FU), an agent that kills cycling cells (Van Zant, 1984) and drives the quiescent HSCs into cycle (Harison and Lerner, 1991). Animals were dosed two times at 3-week intervals, and, 3 weeks after the final 5-FU injection, the animals were sacrificed, HSCs were purified, and alkaline comet assays performed. These experiments revealed that old HSCs driven into cycle by this treatment showed a significant decrease in the levels of DNA damage in comparison to aged control HSCs derived from mice that received PBS injections (Figure 3B). However, it is possible that the 5-FU may differentially affect damaged HSCs, so we performed an additional in vivo experiment whereby we competitively transplanted 100 HSCs purified from either young or old mice into lethally irradiated recipients and performed comet assays 12 months posttransplant. This analysis showed no significant differences in the DNA damage burden of HSCs derived from either the young or old donor HSCs (Figure 3C). This suggests that HSCs driven into cycle by transplantation repaired their accumulated DNA damage, resetting both the young and old HSCs to a nondamaged status, and that both young and old HSCs acquired similar levels of DNA damage over the time course of the experiment. Nonetheless, examination of donor-derived reconstitution from old HSCs was significantly lower in comparison to the young HSCs with a marked myeloid bias lineage output (Figure S3), which was consistent with previous reports (Beerman et al., 2013; Cho et al., 2008; Rossi et al., 2005; Sudo et al., 2000; Wang et al., 2012).

The combined results of these experiments demonstrating reduced amounts of DNA damage in aged cells stimulated to cycle could be explained by two possibilities: (1) DNA breaks accrued at steady state were repaired upon entry into cycle or (2) damaged HSCs underwent apoptosis and were not scored poststimulation. To discriminate between these possibilities, we quantified the rate of apoptotic attrition of young and old HSCs at a single-cell level after stimulation into cycle. To this end, we cloned sorted HSCs from young (318 cells) and old (337 cells) mice and scored their viability 24 hr poststimulating (Table S2). These experiments revealed that all HSCs survived the first 24 hr in culture regardless of age (Figure 4A). These data support...
Figure 2. Age-Associated DNA Damage Accrual Is Greatest in the HSC Compartment

(A) Representative alkaline comets of HSCs, multipotent progenitors (MPP^{Flk2-} and MPP^{Flk2+}), and oligopotent progenitors (GMP and CLP) isolated from young mice.

(B and C) Olive tail moment (B) and percent of DNA in tail (C) of HSCs (n = 1620), MPP^{Flk2-} (n = 714), MPP^{Flk2+} (n = 324), GMP (n = 333), and CLP (n = 713) from young mice. HSCs (n = 292) that received 2 Gy of irradiation were also scored.

(D) Representative alkaline comets of HSCs, multipotent progenitors (MPP^{Flk2-} and MPP^{Flk2+}), and oligopotent progenitors (GMP and CLP) isolated from old mice.

(E and F) Olive tail moment (E) and percent of DNA in tail (F) of HSCs (n = 424), MPP^{Flk2-} (n = 578), MPP^{Flk2+} (n = 479), GMP (n = 309), and CLP (n = 503) from old mice. The same irradiated controls (292 HSCs with 2 Gy) are shown, given that all samples were arrayed on one slide. ***p < 0.001.

Error bars represent SEM. See also Figures S1 and S2.
Comparison to young HSCs or aged downstream progenitor cell populations revealed that old HSCs exhibit significant DNA break accrual, even after strand break repair, indicating that these cells repair accrued strand breaks upon entry into cycle and do not undergo cell death during the first 24 hr poststimulation. To further investigate the clonal potential of young and old HSCs, we continued to assay viability and cell division kinetics over 6 days of culture (Figures 4A–4C) and then scored their colony-forming potential on day 12 (Figures 4D and 4E). Over the 12-day course of the experiment, each of the 318 young HSCs assayed survived (Figures 4A and 4B) and underwent division by 48 hr (Figure 4C), with the majority of single HSCs giving rise to colonies of >30 cells by day 6 (Figure 4B) and large colonies by day 12 (Figure 4D). Old HSCs exhibited a similar cell division kinetic (Figure 4C), with the majority of clones giving rise to colonies of >30 cells by day 6 (Figure 4B). However, in contrast to the young HSCs, we observed a small number of old clones that died after 48 hr in culture either prior to colony division (1 of 337) or after giving rise to one or more daughter cells (7 of 337; Figures 4A and 4B). We also observed a single aged HSC that did not divide over the first 6 days of stimulation, suggesting that this cell was growth arrested and possibly senescent. Moreover, whereas the cell types present in the colonies at day 12 were comparable, the old HSCs generally gave rise to smaller colonies than the young HSCs (p < 0.001; Figures 4D and 4E).

Altogether, these results indicate that aged HSCs repair accrued DNA strand breaks upon entry into cycle; however, even after strand break repair, aged HSCs displayed diminished proliferative potential.

**Attenuated Expression of DNA Damage Repair and Response Genes in HSCs**

Our observations of significant DNA break accrual in old HSCs in comparison to young HSCs or aged downstream progenitor cells raised the possibility that DDR and repair may be differentially regulated in quiescent HSCs and their downstream progenitors. To explore this possibility at a global level, we generated transcriptome-wide expression profiles of HSCs, multipotent progenitors (MPPFlk2+ and MPPFlk2−), and downstream myeloid and lymphoid progenitors [CMP, common myeloid progenitor [CMP], megakaryocyte-erythroid progenitor [MEP], CLP, B cell-biased lymphoid progenitor [BLP], and pre-ProB] from young and old mice and analyzed the expression of 190 genes involved in DDR and repair. These included all genes associated with DDR and checkpoint, nucleotide excision repair (NER), mismatch repair, base excision repair, homologous recombination (HR), and nonhomologous end-joining (NHEJ) that were represented on the arrays (Figure 5A). Strikingly, this analysis revealed that the vast majority of genes examined were significantly upregulated in progenitors downstream of HSCs (false discovery rate [FDR] < 0.05, fold change > 1.5), and many key regulators from different pathways showed highly elevated expression in all progenitors downstream of HSCs independent of age (Figures 5A and 5B). This highly skewed pattern of expression was very specific to these pathways as global analysis of genes up- or downregulated in comparisons of HSCs versus their downstream progeny generally showed comparable numbers of genes significantly up- or downregulated in each comparison, which was independent of age (Figure 5B).

In general, most of the genes analyzed showed consistent expression patterns regardless of age. However, there were a small number of genes whose expression was significantly divergent between young and old HSCs compared to progenitors. Most striking was Trp53 (which encodes p53) that showed statistically significant increased expression downstream of HSCs in young mice, whereas aged progenitors had significantly diminished expression downstream of HSCs (Figure 5A). In contrast,
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Quiescence Underlies Damage Accrual in Aged HSCs

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Wrn, which encodes the Werner syndrome homolog, showed an opposite pattern wherein the majority of progenitors from young mice showed significantly decreased expression in comparison to HSCs, whereas most progenitors from old mice exhibited significantly increased expression. To discriminate between the possibilities that these observations were due to age-associated changes in the HSC compartment or the progenitor cells, we directly compared the expression profiles of the 190 genes involved in DDR and repair pathways between young and old HSCs (Figures S4A and S4B). Although we found a number of age-regulated genes, which included Trp53 and Wrn, the majority of genes involved in DNA damage repair and response were not significantly age regulated in HSCs (Figure S4A).

Next, we sought to determine whether the differences we observed in individual genes led to significant differential regulation of the DNA repair and response pathways as a whole. In comparison to HSCs, we found that most pathways were significantly upregulated (p < 0.001) in the majority of downstream progenitor populations examined and that attenuation of these pathways in HSCs was largely age independent (Figure 5C). Notably, the upregulation of genes involved in HR and DDR and checkpoints were significantly overrepresented (odds ratio > 1, p < 0.001) in all of the progenitor populations downstream of HSCs. The only pathway that was not significantly differentially regulated in HSCs and progenitor cells was NHEJ, which remained mostly unchanged between HSCs and downstream progenitors in both young and old mice, consistent with evidence suggesting that NHEJ is transcriptionally active in HSCs (Moirin et al., 2010). Analysis of downregulation of these pathways in progenitors in comparison to HSCs also demonstrated significance in a few instances, but this significance was invariably due to an underrepresentation of downregulation of these genes in progenitors (odds ratio < 1) in contrast to the significant upregulation of these pathways, which was consistently due to an overrepresentation of genes with significantly increased expression (Figure 5C).

It has previously been suggested that the expression of certain DNA repair genes are downregulated in HSCs during aging and that this may contribute to aging-associated damage accrual (Chambers et al., 2007; Rübe et al., 2011). To examine this broadly across all DNA damage repair and response pathways, we compared the expression of these pathways in young and old HSCs. Although several genes from diverse pathways displayed significant expression changes with age, analysis of the pathways showed that all but one of the DDR and repair pathways were not significantly different between young and aged HSCs (Figure S4B). The sole exception was the NER pathway (p < 0.01), which was significantly downregulated in aged HSCs.

Altogether, these results demonstrate that HSCs exhibit attenuated expression of most DNA damage repair and response pathways in comparison to their downstream progenitors that is largely age-independent. Moreover, HSC aging itself is not associated with significant differences in the regulation of the majority of these pathways.

**DDR and Repair Pathways Are Attenuated in Quiescent, but Not Cycling, HSCs**

HSCs reside largely in a state of dormancy, >90% of them residing in the quiescent G0 phase of the cell cycle in both young and old mice (Figure S5) (Rossi et al., 2007b; Sudo et al., 2000), entering into cycle infrequently throughout the adult lifespan (Cheshier et al., 1999; Foudi et al., 2009; Wilson et al., 2008). In contrast, downstream progenitors are more actively cycling with a progressive increase in the steady-state cycling rate from MPPs (Figure S5) to oligopotent progenitors (Passegué et al., 2005). As many DNA damage repair pathways are known to be tightly coordinated with phases of the cell cycle, we hypothesized that the broad attenuation of these pathways we observed in HSCs may be due to their quiescent state. To test this, we generated transcriptome-wide expression profiles of HSCs isolated from the fetal liver (FL-HSCs) at embryonic day 14.5, a developmental time at which HSCs are known to be highly cycling (Bowie et al., 2006; Morrison et al., 1995; Nygren et al., 2006; Pietras et al., 2011), and analyzed the 190 DNA damage repair and response genes in comparison to both young and old adult HSCs (Figure 6A). Of the differentially expressed genes, the vast majority were downregulated in adult versus fetal HSCs (Figures 6A and 6B). Consistent with this, statistical analysis at the pathway levels also showed significant downregulation for the majority of the pathways for FL-HSC comparisons to both young and old HSCs (Figure 6C). As we had observed in our HSC to progenitor comparisons, this skewed expression pattern was very specific to these genes as global analysis of all genes significantly differentially regulated between FL-HSC and young or old HSCs showed comparable numbers of genes up- and downregulated (Figure 6B).

The observed expression differences between FL and adult HSCs could be explained by either intrinsic differences in HSCs at these defined stages of ontogeny or could reflect different cell cycle status of these cells. If the latter were true, then cycling adult HSCs would be expected to upregulate these pathways. To examine this, we analyzed cell-cycle status (Ki-67 and propidium iodide) of steady-state adult HSCs in conjunction with immunostaining for Rad51. These experiments showed that Rad51 protein expression in young and old HSCs was mainly restricted to cells in the G0/M phase of the cell cycle, which, at steady state, represents only a very small fraction of HSCs (<2%; Figure S5). We hypothesized that adult quiescent HSCs driven into cycle would lead to upregulation of DDR and repair pathways as they exit G0 and progressed through cell cycle.

**Figure 4. Clonal Analysis of Single HSCs from Young and Old Mice**

(A) Individual HSC clones scored daily for 6 days from three young mice and three old mice. Numbers of cells scored daily are presented in a color scale from white (n = 1 cell) to red (n > 32 cells). Each clone was then cultured an additional 6 days and scored for types of cells generated from each clone.

(B) Summary of clones derived from 318 young and 337 old single HSCs after 6 days in culture. Each clone was assayed at time points 0.5, 1, 2, 3, 4, 5, and 6 days, and the composite data are presented.

(C) Cell division kinetics of young and old HSCs.

(D) Overall colony size at day 12 of clones derived from single young or old HSCs.

(E) Colony composition of colonies generated from single HSCs isolated from young and old mice. \*\*p < 0.001.
Figure 5. Attenuation of DDR and Repair Pathways in HSCs in Comparison to Downstream Progenitor Populations

(A) Fold-change comparisons of genes involved in DDR and checkpoints (DDRC), nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), nonhomologous end-joining (NHEJ), and homologous recombination (HR) in progenitor populations in comparison to their age-matched HSCs. Each column represents an individual replicate, and the log2 fold change in comparison to the average expression of the HSCs is shown. Significant expression changes defined as >1.5 fold and p < 0.05, are designated with a bold black border.

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To test this, we sorted HSCs from young and old mice and cultured them for 3, 6, 12, or 24 hr in cytokine-rich media, followed by transcriptional profiling at each time point. As expected, HSCs stimulated into cycle quickly downregulated Cdkn1c, which encodes the Cdk inhibitor p57 responsible for maintaining HSC quiescence (Matsumoto et al., 2011; Zou et al., 2011) (Figure 7A). Consistent with the idea that HSCs broadly upregulate their DNA damage repair and response

See also Figure S4.

Figure 6. Attenuation of DDR and Repair Genes in Quiescent HSCs

(A) Fold-change comparisons between genes involved in DDR and repair in fetal liver HSCs in comparison to adult young or old HSCs. Each column represents an individual replicate, and the log2 fold change in comparison to the average expression of the fetal liver HSCs is shown. Significant expression changes, defined as >1.5 fold and p < 0.05, are designated with a bold black border.

(B) Frequency of genes that show significant upregulation (red), significant downregulation (blue), or no significant change (gray) in each pathway for comparisons between fetal liver HSCs and either young or old HSCs. The global frequencies of the total number of genes and those with significant differential regulation (up or downregulated) out of the total 17,872 genes examined on the arrays are also included.

(C) Analysis of the changes of the overall pathways involved with DDR and repair in young and old HSCs in comparison to cycling fetal liver HSCs. p values are presented by a color scale, and odds ratios < 1 are indicated with a hash through the box.

See also Figure S5.
pathways when in cycle, many of the 190 genes showed robust upregulation starting at 12 hr and increasing by 24 hr post-stimulation (Figures 7A and 7B), and most pathways are significantly upregulated by 24 hr (Figure 7C). Interestingly, we also observed that many of the genes we examined exhibited reduced expression at the early time points poststimulation,
which led to many of the pathways displaying significant down-regulation at 3 hr, with the old cells showing attenuation of almost all DNA response and repair pathways. This early decrease in expression of these pathways was accompanied by robust induction of a subset of DDR and checkpoint genes that included Cdkn1a, Gadd45α, Gadd45β, Gadd45γ, Trex1/Atip, Pik3, and Crebbp, and expression of these genes spiked at 3 hr poststimulation, followed by a return to steady state levels by 12 to 24 hr (Figure 7D). Though this pattern was evident in both young and old HSCs, in almost all cases, we observed a greater induction of these genes in aged HSCs (Figure 7D).

Altogether, these data indicate that DNA repair and response pathways are broadly attenuated in quiescent, but not cycling, HSCs. Furthermore, they suggest that HSCs exit from G0 and progression into the cell cycle leads to induction of DNA damage repair and response genes. The broad attenuation of DNA damage repair and response pathways in quiescent HSCs most likely underlies the accrual of DNA damage during aging.

**DISCUSSION**

The lifelong potential of HSCs has led to the supposition that these cells must be imbued with a unique ability to preserve their genomic integrity. Indeed, many properties associated with HSCs, such as high ABC transporter activity, low metabolic activity, hypoxic environment, and long periods of dormancy, are potential means through which HSCs could maintain genomic fidelity to preserve function. However, evidence that HSCs may not be impervious to DNA damage accrual has been implicated in studies utilizing genetic models (Cho et al., 2013; Nijnik et al., 2007; Parmar et al., 2010; Prasher et al., 2005; Rossi et al., 2007a; Rudolph et al., 1999), their comparable radiation sensitivity to other cells that undergo mitotic death (Domen et al., 1998; Tili and McCulloch, 1961), and examination of γH2AX in HSCs (Rossi et al., 2007a; Rübe et al., 2011).

Moreover, increasing evidence that mutation accrual in the HSC compartment underlies numerous age-associated hematopoietic malignancies, including acute myeloid leukemia (AML) (Corces-Zimmerman et al., 2014; Jamieson et al., 2004; Jan and Majeti, 2013; Pang et al., 2013), is also inconsistent with the idea of a privileged, exquisitely geno-protected HSC compartment.

In opposition to the concept that HSCs are entirely geno-protected during aging, we observed significant and consistent evidence of DNA strand breaks in HSCs isolated from old mice. A possible explanation for this could be that DNA repair and/or responses may be differentially regulated in HSCs during aging, as has previously been suggested (Chambers et al., 2007; Rübe et al., 2011). However, our global examination of DNA repair and response genes and pathways revealed minimal differences during aging in HSCs. The sole exception to this was the downregulation of the NER pathway in aged HSCs. However, it seems unlikely that the observed accrual of strand breaks in old HSCs could be attributable to diminished expression of this pathway. Nonetheless, it remains possible, and perhaps likely, that downregulation of NER might lead to accumulation of bulky DNA adducts in HSCs (that we did not evaluate in this study) that could affect function during aging. Consistent with this, we have previously observed an aging-dependent decline in HSC activity in a murine model of NER deficiency (Rossi et al., 2007a).

In further refutation of the concept that HSCs possess inherent mechanisms that uniquely protect their genome, examination of DNA damage accrual in multiple early progenitor cell compartments in comparison to HSCs revealed that the greatest amount of age-associated damage was concentrated in HSCs. Global examination of DNA repair and response pathways in these progenitors revealed that, in contrast to the minimal differences observed between young and old HSCs, the majority of these pathways showed robust and highly significant upregulation in downstream progenitors regardless of age. However, we did note that NHEJ appeared to be transcriptionally active in both young and old HSCs, which appears inconsistent with the significant accrual of strand breaks in old HSCs that we observed in multiple independent experiments. We speculate that the transcriptional activity of NHEJ in HSCs may prime HSCs for repair upon the cells enter cycle in a manner similar to the transcriptional priming of lineage potential observed in HSCs (Kirstetter et al., 2006; Mead et al., 2013; Miyamoto et al., 2002). Consistent with this, we observed robust repair of accrued strand breaks in old HSCs upon stimulation and entry into cell cycle both ex vivo and in vivo.

Exit from G0 and entry into cycle led to global upregulation of essentially all DNA repair and response pathways. This raises the possibility that attenuation of these pathways in quiescent HSCs may more broadly contribute to the accrual of diverse genomic lesions not assayed in this study, particularly given the low turnover rate of HSCs and their prolonged periods of dormancy (Cheshier et al., 1999; Foudi et al., 2009; Wilson et al., 2008). Interestingly, exit from G0 was associated with robust induction of canonical DDR genes, including Cdkn1a, Gadd45α, Gadd45β, Gadd45γ, and Rb1, shortly after entry into cycle. These data suggest the existence of a post-G0 DNA-damage-induced checkpoint in HSCs that we postulate may be in place to ensure that damage accrued during periods of dormancy is repaired prior to either differentiation or self-renewal divisions. Though our data clearly indicates that old HSCs can repair strand breaks upon entry into cycle, we did not address the fidelity of repair or examine other types of DNA damage in this study, which could also contribute to the diminished functional potential observed with HSC aging. Moreover, it seems likely that the attenuation of DNA repair and response pathways we observe in quiescent HSCs could lead to age-associated mutation accrual beyond strand breaks. Indeed, the idea that HSCs serve as the primary reservoir for mutation accrual underlying the development of diverse age-associated hematopoietic diseases such as myelodysplastic syndrome and AML is now widely accepted (Jan et al., 2012; Krivtsov et al., 2013; Rossi et al., 2007a; Shlush et al., 2014; Taussig et al., 2005; Tehranchi et al., 2010; Weisman, 2005; Will et al., 2012). Our data demonstrating that HSC quiescence and concomitant attenuation of DNA repair and response pathways provides a mechanism through which such premalignant mutations in HSCs may accrue. It should be stated that other adult stem cell tissue systems, such as brain, gastrointestinal tract, skin, etc., will very likely follow the same energy-saving paradigm, accumulate DNA damage in quiescent stem cells, and repair these insults when it is necessary to enter cell cycle.
EXPERIMENTAL PROCEDURES

Mice
All mice used were C57BL/6 males. Young mice were 3–4 months old, and old mice, obtained from the National Institute on Aging, were 24–26 months old. All mice were maintained according to protocols approved by Harvard Medical School Animal Facility or Stanford University’s Administrative Panel on Laboratory Animal Care, and all procedures were performed with consent from the local ethics committees.

Purification of Cells
Adult bone marrow cells were extracted by crushing the bones of donor mice. Cells were stained and sorted with the following cell-surface phenotypes: HSCs, Lin(Mac1, Gr1, Ter119, CD3, CD4, CD8) c-Kit+Sca-1− CD34− Flk2−, or when noted, Lin(Mac1, Gr1, Ter119, B220, Il7r, CD3, CD4, CD8) c-Kit+Sca-1−CD150−CD48−; MPP+, Lin(Mac1, Gr1, Ter119, B220, Il7r, CD3, CD4, CD8) c-Kit+Sca-1−CD34−Flk2−; CD19+ CD34− Lin(Mac1, Gr1, Ter119, B220, Il7r, CD3, CD4, CD8) c-Kit+Sca-1−CD34−Flk2−; CD34+, CD19− Ly6d+/C0 CD45.2+ c-Kit+Sca-1−CD34−Flk2−; MPP+, Lin(Mac1, Gr1, Ter119, B220, CD19+ CD34− Lin(Mac1, Gr1, Ter119, B220, CD19+ CD34− Flk2−; SLAM, Lin(Mac1, Gr1, Ter119, B220, CD19+ CD34− Lin(Mac1, Gr1, Ter119, B220, CD19+ CD34− Flk2−; B220+, CD4−); MEP, Lin(Mac1, Gr1, Ter119, B220, CD19+ CD34− Lin(Mac1, Gr1, Ter119, B220, CD19+ CD34− Flk2−; Sca-1+ c-KitFlk2−; MPP+, Lin(Mac1, Gr1, Ter119, B220, CD19+ CD34− Lin(Mac1, Gr1, Ter119, B220, CD19+ CD34− Flk2−; IL-7r−; Lin(Mac1, Gr1, Ter119, B220, CD19+ CD34− Lin(Mac1, Gr1, Ter119, B220, CD19+ CD34− Flk2−; CD19+ CD34− Lin(Mac1, Gr1, Ter119, B220, CD19+ CD34− Flk2−; c-Kit+Sca-1−CD34−FcγR− inactive) were purified from either young or old donor (CD45.2+) mice and competitively transplanted against 200,000 whole-bone-marrow cells derived (CD45.2) HSCs were purified and assayed for DNA damage 12 months after transplantation.

In Vivo Stimulation of HSCs
Aged mice received either two doses of 5-FU (150 mg/kg) or PBS by intraperitoneal injection. The mice were given 3-week intervals between injections, and bone marrow was harvested three weeks after the last injection for isolation of HSCs used in comet assays. In the transplant setting, 100 HSCs were assayed as perProtocols. All normalized expression intensities, results of statistical comparisons, and heat map representations are available at Gene Expression Commons as HSCs and progenitors young versus old (https://gexc.stanford.edu/model/detail/786), FL-young-old HSCs (https://gexc.stanford.edu/model/detail/773), and HSCs in vitro stimulation (https://gexc.stanford.edu/model/detail/313).

Clonal Ex Vivo Stimulation Assay
Using the single-cell mode of the FACSAria, we sorted cells individually into a single well of a 96-well round-bottom plate. For each experiment, 120 wells were used with three replications of each age group. Each well contained Dulbecco’s modified Eagle’s medium and F-12 medium (Gibco and Invitrogen) supplemented with 10% fetal calf serum (Hyclone and Thermo Scientific), 1x penicillin/streptomycin, 2 mM GlutaMAX, 50 μM 2-mercaptoethanol (Gibco and Invitrogen), and the following cytokines: 10 ng/ml mouse stem cell factor, 10 ng/ml mouse thrombopoietin, 10 ng/ml mouse Flt3l, 10 ng/ml mouse IL-3, 1 U/ml mouse erythropoietin, 10 ng/ml mouse granulocyte macrophage colony-stimulating factor (all purchased from PeproTech). The cells were incubated at 37°C in a humidified atmosphere with 5% CO2. After 12 days of culture, the size of each colony was measured under a microscope, and colonies were subjected to cytospin and characterized morphologically and cytochemically by May-Grunwald-Giemsa staining. The same culture conditions were used for short-term ex vivo stimulation of pooled cells (Figures 3A and 7).

ACCESSION NUMBERS
Raw data were deposited to the NCBI GEO under accession number GSE55525.

SUPPLEMENTAL INFORMATION
Supplemental Information contains Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2014.04.016.

AUTHOR CONTRIBUTIONS
I.B., J.S., I.L.W., and D.J.R designed the study. I.B. and J.S. performed cell sorting. I.B. performed the comet assays and analysis and the in vivo assays. J.S. performed the clonal in vitro assay, the cell-cycle assay, microarray data analysis, and statistical tests. J.S. and M.A.I. generated microarray data. I.B. and D.J.R. wrote the manuscript, and I.B. and J.S. generated figures. All authors edited the manuscript. Co-first authorship of I.B. and J.S. is equal, and this citation will be listed with the alternative author orders in the author curriculum vitae and bibliographies.

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Quiescence Underlies Damage Accrual in Aged HSCs

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REFERENCES


Quiescence Underlies Damage Accrual in Aged HSCs


Figure S1:

0 - 10%

10 - 20%

20 - 30%

30 - 40%

40 - 50%

50 - 60%

60 - 70%

70 - 80%

80 - 90%

90 - 100%
Figure S2:

A

B

Olive Tail Moment

HSC MPP MPP GMP CLP 2 Gy IR

Young

Old

HSC MPP MPP GMP CLP 2 Gy IR

Young Old Young Old Young Old Young Old Young Old 2 Gy HSC

Olive Tail Moment

HSC MPP MPP GMP CLP 2 Gy IR

Young

Old

HSC MPP MPP GMP CLP 2 Gy HSC

Olive Tail Moment

Young Old Young Old Young Old Young Old Young Old 2 Gy HSC
Figure S3:

A

B

Total Reconstitution

**

Young Donor

Old Donor

% of Donor

Young Old

***

Mac1+ B220+ CD3+

% of Donor

Young Donor

Old Donor

**

***
Figure S4:

DNA damage response and checkpoint

A

Significant by SAM (FDR < 0.05)

Fold Change vs. Young HSC [log2]

Young

Old

Young

Old

Young

Old

NHEJ

BER

MMR

HR

Young

Old

NHEJ

BER

MMR

HR

B

Up-regulated from Young

Down-regulated from Young

DDRC

NER

BER

MMR

NHEJ

HR

P value

n.s.

< 0.05

< 0.01

< 0.001
Figure S5:

Young (3 months)

Old (26 months)
Table S1:

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<th>Exp 3: Fig S2C LSKCD34-Flk2-</th>
<th>Exp 4: Fig 5 LSKCD34-Flk2-</th>
<th>Exp 5 LSKCD34-Flk2-</th>
<th>Exp 6 LSKCD34-Flk2-</th>
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<td>447 old</td>
<td>1620 yng</td>
<td>424 old</td>
<td>725 yng</td>
<td>615 old</td>
<td>749 yng</td>
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## Table S2

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<th>Plating efficiency (%)</th>
<th>Existence of a cell/cells at 24h after sort</th>
<th>Frequency of cell loss between 12h to 24h (%)</th>
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<tr>
<td>Young #3</td>
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<td>105</td>
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<tr>
<td>Old #1</td>
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<td>111</td>
<td>92.50%</td>
<td>111</td>
<td>0.00%</td>
</tr>
<tr>
<td>Old #2</td>
<td>120</td>
<td>112</td>
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<td>Old #3</td>
<td>120</td>
<td>114</td>
<td>95.00%</td>
<td>114</td>
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Supplemental Figure Legends:

**Figure S1**: Scoring of single cells using alkaline comet assay (A) Representative images of comets scored from CometScore, binned by percent DNA in tail. TD: Percent of DNA in the tail, OM: Olive Tail Moment (related to Figure 1, 2, and 3)

**Figure S2**: DNA damage accrual is greatest in the aged HSC compartment (A) Olive Tail Moment of alkaline comets from young HSC (n=725) MPP^Flk2^- (n=716) MPP^Flk2+ (n=275) GMP (n=230) and CLP (n=949) and 215 irradiated HSCs (2 Gy) as well as comets from old HSC (615), MPP^Flk2- (n=817) MPP^Flk2+ (n=706) GMP (n=382) and CLP (n=497) and the 215 irradiated HSCs (2 Gy). (B) Comparisons of Olive Tail Moment of HSC and progenitor populations isolated from young and old mice. Tail moments presented are comparisons between data shown in Fig 2 (top) and Fig S2A (bottom). ** p<0.01 *** p<0.001 (related to Figure 1, 2, and 3)

**Figure S3**: Donor–derived analysis of the peripheral blood in competitive transplants of 100 young and old HSC (A-B) Competitive transplantation of HSCs from young and old (n=5,4 recipients respectively) showing total reconstitution (A), and contribution to B cells (B220+), myeloid cells (Mac1+) and T cells (CD3+) (B) nine months post transplant. ** p<0.01 *** p<0.001 Error bars represent SEM (related to Figure 3)

**Figure S4**: Expression of genes involved in DNA damage response and repair pathways during aging (A) Fold change comparisons between genes involved in DDR and repair in old HSCs compared to young HSCs. Each column represents an individual replicate and the log2 fold change compared to the average expression of the young HSCs. Significant expression changes, defined as >1.5 fold and p < 0.05, are designated with a black box. (B) Analysis of the changes of the overall pathways involved with DNA damage response and repair in old HSCs compared to young HSCs. p-values are presented by a color scale. (C) Cell cycle and Rad51 expression analysis of young and old HSCs. Cells in each stage of the cell cycle (colored gate) were gated on and then assayed for Rad51 expression (histogram color matches previous gate). (related to Figure 5)

**Figure S5**: Rad51 and cell cycle analysis. (A) Purified HSC, MPP^Flk2-, and MPP^Flk2+ cells were isolated from young and aged mice and stained for KI-67, PI, and Rad51 analyzed by flow-cytometry for cell-cycle status and Rad51 protein expression simultaneously. Cells were sub-divided into G0 (green), G1 (blue), or S/G2/M (red) based on DNA content (PI) and KI-67 intensity, then Rad51 intensity at each cell-cycle status is represented by histogram as frequency in subpopulation. (related to Figure 5,6)

**Table S1**: Statistical comparisons between DNA damage of young and old HSCs (A) Statistical significance of the increased stand breaks in aged HSCs from multiple experiments comparing damage in young and old HSCs. (related to Figure 1, 2, and 3)
Table S2: Plating efficiency and cell death at 24 hours post-sort (A) Breakdown of the number of single cells sorted from the 6 experiments (3 young and 3 old) and used in the clonal ex vivo culturing experiments. 120 wells containing permissive culture media were used for each experiment. 12 hours post sort, wells were examined to determine plating efficiency. At 24 hours, the wells were re-examined to assay for cell death. (related to Figure 4)
Supplemental Methods:

**Simultaneous analysis of cell-cycle and Rad51 expression:**

Sorted cells were fixed with Fix&Perm Medium A (Caltag/Life Technologies/Thermo Fisher Scientific, MA) for 15 min, washed, then permeabilized using 90% ice-cold methanol for 30 min on ice, washed twice. Cells were then stained with Ki-67-APC (BioLegend, CA) and anti-Rad51-rabbit-monoclonal Ab (Cell Signaling Technologies, MA) for 30 min. After wash, secondary antibody, anti-rabbit-IgG-PacificBlue (Molecular Probe/Life Technologies/Thermo Fisher Scientific, MA) was added for 30 min. After wash, cell pellet was diluted with staining medium containing PI (2 ug/ml) and RNAse (100 ug/ml), then analyzed on FACS Aria II.
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