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Quiescent hematopoietic stem cells accumulate DNA damage during aging that is repaired upon entry into cell cycle

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Summary

Hematopoietic stem cells (HSCs) maintain homeostasis and regenerate the blood system throughout life. It has been postulated that HSCs may be uniquely capable of preserving their genomic integrity to ensure lifelong function. To directly test this, we quantified DNA damage in HSCs and downstream progenitors from young and old mice revealing that strand breaks significantly accrue in HSCs during aging. DNA damage accumulation in HSCs was associated with broad attenuation of DNA repair and response pathways that was dependent upon HSC quiescence. Accordingly, cycling fetal HSCs and adult HSCs driven into cycle up-regulated these pathways leading to repair of strand breaks. Our results demonstrate that HSCs are not comprehensively geno-protected during aging. Rather, HSC quiescence and concomitant

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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 and the cell-cycle assay. J.S. and M.A.I. generated microarray data. J.S. performed microarray data analysis and statistical tests. I.B. and D.J.R. wrote manuscript and I.B. and J.S. generated figures. All authors edited manuscript.

Conflict of Interest

I.L.W. is a member of the scientific advisory board of StemCells. All of other authors declare no conflict of interest.

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attenuation of DNA repair and response pathways underlies DNA damage accumulation in HSCs during aging. These results provide a potential mechanism through which pre-malignant mutations accrue in HSCs.

Introduction

Aging of 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 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; Rube 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 HSC, and which progress eventually 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-protective and geno-protective mechanisms 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 ABC transporters including ABCG2 confer xenobiotic efflux activity on HSCs (Krishnamurthy et al., 2004; Zhou et al., 2002; Zhou et al., 2001). HSCs also maintain low levels of reactive oxygen species (ROS) due to the combined action of their low metabolic activity, their reliance on glycolytic metabolism, together 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., 2008; 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 (Allsopp 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 immuno-histochemical evidence of γH2AX accumulation, an indicator of DNA damage response, in HSCs isolated from old mice (Rossi et al., 2007a) and aged

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humans (Rube et al., 2011). It has been proposed that diminished DNA repair capacity may underlie this age-associated DNA damage accrual, (Chambers et al., 2007; Rube 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 amongst 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 HSC 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 DNA damage response and repair pathways are broadly attenuated in quiescent but not cycling HSCs. We show that HSCs stimulated to enter cell cycle up-regulate multiple DNA response and repair pathways and concomitantly repair accumulated DNA damage. Taken together, 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

Immuno-staining 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; Rube et al., 2011). However, it remains possible that the observed γH2AX foci in aged stem cells may mark cellular processes distinct from DNA damage. We therefore sought to directly evaluate and quantify DNA damage in HSCs and progenitors during aging. To this end we used alkaline comet assays (Figure S1) (Olive and Banath, 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 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 compared to young HSCs (Figure 1B-C). These data were verified in six independent experiments in which a cumulative total of 4940 young and 3186 old HSCs with the immuno-phenotype of $LSKCD34^{-}Flk2^{-}$ were scored in a blinded fashion. In two additional experiments, we assayed comets of over two thousand young and old HSCs purified using an alternative cell surface marker combination for HSCs-$LKSCD150^{+}CD48^{-}$ (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, with 33% showing evidence of single or double strand breaks, defined here as having greater than 10% DNA in their comet tails (Figure 1C, S1). By contrast, ~70% of old HSCs scored as damaged (Figure 1C). Tail DNA analysis further revealed that whereas 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

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in HSCs purified from old (42%) compared 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 compared to their downstream progenitor progeny from young and old mice. Purified multi-potent (MPP^{Flk2-} and MPP^{Flk2+}) and oligo-potent (GMP and CLP) progenitors, together with HSCs, were analyzed in single cell comet assays. Analysis of the Olive tail moment of each downstream progenitor populations compared to HSCs isolated from young mice showed no significant differences (Figure 2A-B), although CLP displayed slightly elevated Olive tail moments, possibly due to immunoglobulin heavy chain DJ rearrangements actively occurring in this population. Consistent with this, the percent tail DNA of the young progenitor populations and HSCs was comparable, with the majority of scored cells (>70% in all populations) having 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 compared to all of the downstream progenitors, either by Olive tail moment (Figure 2E) or percent tail DNA (Figure 2F, S2A). We further 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 had observed previously (Figure 1B), HSCs from old mice consistently showed a significant increase of DNA breaks compared to young (Figure S2B-C). Interestingly, despite the fact that all progenitor populations from old mice showed significantly reduced levels of DNA damage in comparisons to aged HSCs (Figure 2E and S2A), the majority of these aged progenitors nonetheless showed evidence of significantly greater levels of DNA breaks than their young counterparts (Figure S2B-C). Taken together, 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 if old, damaged HSCs could resolve accrued DNA damage, we FACS sorted young and old HSCs and assayed for strand breaks by comet assays either immediately after purification (steady state) or 24-hours after culturing them in a cytokine-rich media which stimulates the quiescent cells into cycle. As we previously observed (Figure 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-hours post culture stimulation showed very similar comet profiles, with the old cells showing significantly reduced numbers of damaged cells compared 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 (Harrison and Lerner, 1991). Animals were dosed two times at three-week intervals, and three weeks after the final 5-FU injection, the animals were sacrificed and 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

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HSCs derived from mice that received PBS injections (Figure 3B). It is possible, however, 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 twelve months post transplant. 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 non-damaged status, and further 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 compared to the young with a marked myeloid bias lineage output (Figure S3) 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 that demonstrate reduced amount 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 post-stimulation. 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 clone sorted HSCs from young (318 cells) and old (337 cells) mice and scored their viability 24 hours post-plating (Table S2). These experiments revealed that all HSCs survived the first 24 hours in culture regardless of age (Figure 4A). This data supports the idea that old HSCs repair accrued strand breaks upon entry into cycle, and do not undergo cell death during the first 24 hours post stimulation.

To further investigate the clonal potential of young and old HSCs, we continued to assay viability and cell division kinetics over 6 days of culturing (Figure 4A-C), and then scored their colony forming potential on day 12 (Figure 4D-E). Over the 12 day course of the experiment, each of the 318 young HSCs assayed survived (Figure 4A-B), underwent division by 48 hours (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 hours in culture either prior to cell division (1/337) or after giving rise to one or more daughter cells (7/337) (Figure 4A-B). 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 ($p<0.001$ Figure 4D-E).

Taken together 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.

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Attenuated expression of DNA damage repair and response genes in HSCs

Our observations of significant DNA break accrual in old HSCs compared to young HSCs or aged downstream progenitor cells raised the possibility that DNA damage response 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, multi-potent progenitors (MPP^{Flk2-} and MPP^{Flk2+}), and downstream myeloid and lymphoid progenitors (GMP, CMP, MEP, CLP, BLP, and Pre-ProB) from young and old mice, and analyzed the expression of 190 genes involved in DNA damage response and repair. These included all genes associated with DNA damage response and checkpoint (DDRC), nucleotide excision repair (NER), mismatch repair (MMR), base excision repair (BER), homologous recombination (HR), and non-homologous end joining (NHEJ) that were represented on the arrays (Figure 5A). Strikingly, this analysis revealed that the vast majority of genes examined were significantly up-regulated in progenitors downstream of HSCs (FDR < 0.05, Fold change > 1.5) with many key regulators from different pathways showing highly elevated expression in all progenitors downstream of HSCs independent of age (Figure 5A-B). This highly skewed pattern of expression was very specific to these pathways as global analysis of genes up- or down-regulated in comparisons of HSCs versus their downstream progeny generally showed comparable numbers of genes significantly up- or down-regulated 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, *Wrn,* which encodes the Werner syndrome homolog, showed an opposite pattern where the majority of progenitors from young mice showed significantly decreased expression compared 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 DNA damage response and repair pathways between young and old HSCs (Figure S4A-B). 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).

We next sought to determine if the differences we observed in individual genes led to significant differential regulation of the DNA repair and response pathways as a whole. Compared to HSCs, we found that most pathways were significantly up-regulated ($p<0.001$) in the majority of downstream progenitors populations examined, and further that attenuation of these pathways in HSCs was largely age-independent (Figure 5C). Of note, the up-regulation of genes involved in HR and DNA damage response and checkpoints were significantly over-represented (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

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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 (Mohrin et al., 2010). Analysis of down-regulation of these pathways in progenitors compared to HSCs also demonstrated significance in a few instances, yet this significance was invariably due to an under-representation of downregulation of these genes in progenitors (Odds ratio <1) in contrast to the significant up-regulation of these pathways that was consistently due to an over-representation of genes with significantly increased expression (Figure 5C).

It has previously been suggested that the expression of certain DNA repair genes are down-regulated in HSCs during aging and that this may contribute to aging-associated damage accrual (Chambers et al., 2007; Rube 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 DNA damage response 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 down-regulated in aged HSCs.

Taken together, 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.

DNA damage response and repair pathways are attenuated in quiescent but not cycling HSCs

HSCs reside largely in a state of dormancy, with >90% in the quiescent G_0 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., 2008; 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 oligo-potent progenitors (Passegue 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 (E14.5), 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 down-regulated in adult versus fetal HSCs (Figure 6A-B). Consistent with this, statistical analysis at the pathway levels also showed significant down-regulation 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

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young or old HSCs showed comparable numbers of genes up- and down-regulated (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, cycling adult HSCs would be expected to up-regulate these pathways. To examine this we analyzed cell cycle status (Ki-67 and PI) of steady state adult HSCs in conjunction with immuno-staining for Rad51. These experiments showed that Rad51 protein expression in young and old HSCs was mainly restricted to cells in the $S/G_2/M$ 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 up-regulation of DNA damage response and repair pathways as they exit G_0 and progressed through cell cycle. To test this we sorted HSCs from young and old mice and cultured them for 3, 6, 12 or 24 hours in cytokine-rich media, followed by transcriptional profiling at each timepoint. As expected, HSCs stimulated into cycle quickly down-regulated *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 up-regulate their DNA damage repair and response pathways when in cycle, many of the 190 genes showed robust up-regulation starting at 12 hours and further increasing by 24-hours post-stimulation (Figure 7A-B) with most pathways being significantly up-regulated by 24 hours (Figure 7C). Interestingly, we also observed that many of the genes we examined exhibited reduced expression at the early time points post-stimulation, which led to many of the pathways displaying significant down-regulation at 3 hours, 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 DNA damage response and checkpoint genes that included *Cdkn1a*, *Gadd45α*, *Gadd45α, Gadd45β*, *Gadd45γ*, *Trex1/ Atrip*, *Plk3*, and *Crebbp* with expression of these genes spiking at 3 hours post-stimulation, followed by a return to steady state levels by 12 to 24 hours (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).

Taken together these data indicate that DNA repair and response pathways are broadly attenuated in quiescent but not cycling HSCs. They further suggest that HSCs exit from $G_{0,}$ 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 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 HSC 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

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(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; Till and Mc, 1961), and examination of γH2AX in HSCs (Rossi et al., 2007a; Rube et al., 2011). Moreover, increasing evidence that mutation accrual in the HSC compartment underlies numerous age-associated hematopoietic malignancies, including 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; Rube 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 down-regulation of the NER pathway in aged HSCs. It seems unlikely however that the observed accrual of strand breaks in old HSCs could be attributable to diminished expression of this pathway. It nonetheless remains possible and perhaps likely that down-regulation 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 compared 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 up-regulation in downstream progenitors, regardless of age. We did note however 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 we observed in multiple independent experiments. We speculate that the transcriptional activity of NHEJ in HSCs may prime HSCs for repair once 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 G_0 and entry into cycle led to global up-regulation 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., 2008; Wilson et al., 2008). Interestingly, exit from G_0 was associated with robust induction of canonical DNA damage response genes, including *Cdkn1a*, *Gadd45α, Gadd45β, Gadd45γ*, and *Rb1* shortly after

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entry into cycle. These data suggest the existence of a post-G_0 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 a MDS 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; Weissman, 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- and 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 of age and old mice, obtained from the National Institute of Aging (NIA, Bethesda, MD), 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 using the following cell surface phenotypes:

HSC: Lin(Mac1, Gr1, Ter119, B220, Il7rα, CD3, CD4, CD8)$^-$c-Kit$^+$Sca-1$^+$CD34-Flk2$^-$ or when noted Lin(Mac1, Gr1, Ter119, B220, Il7rα, CD3, CD4, CD8)$^-$c-Kit$^+$ Sca-1$^+$CD150$^+$CD48$^-$ ***MPP^{Flk2-}***: Lin(Mac1, Gr1, Ter119, B220, Il7rα, CD3, CD4, CD8)$^-$c-Kit$^+$Sca1$^+$CD34$^+$Flk2$^-$, ***MPP^{Flk2+}***: Lin(Mac1, Gr1, Ter119, B220, Il7rα, CD3, CD4, CD8)$^-$ c-Kit$^+$Sca-1$^+$CD34$^+$Flk2$^+$, ***GMP***: Lin(Mac1, Gr1, Ter119, B220, Il7rα, CD3, CD4, CD8)$^-$c-Kit$^+$Sca1$^-$CD34$^+$FcγR$^+$, ***CLP***: Lin(Mac1, Gr1, Ter119, CD3, CD4, CD8)$^-$CD19$^-$CD11c$^-$B220$^-$CD27$^+$c-Kit^{mid}Flk2$^+$IL7Rα$^+$Ly6d$^-$ ***CMP***: Lin(Mac1, Gr1, Ter119, B220, Il7rα, CD3, CD4, CD8)$^-$c-Kit$^+$Sca-1$^-$CD34$^+$FcγR^{low}, ***MEP***: Lin(Mac1, Gr1, Ter119, B220, Il7rα, CD3, CD4, CD8)$^-$c-Kit$^+$Sca-1$^-$CD34$^-$FcγR$^-$, ***BLP***: Lin(Mac1, Gr1, Ter119, CD3, CD4, CD8)$^-$CD19$^-$CD11c$^-$ B220$^-$CD27$^+$c-Kit^{mid}Flk2$^+$IL7Rα$^+$Ly6d$^+$, ***PreProB***: Lin(Mac1, Gr1, Ter119, CD3, CD4, CD8)$^-$ CD19$^-$CD11c$^-$B220$^+$CD27$^+$c-Kit^{mid}Flk2$^+$IL7Rα$^+$Ly6d$^+$. All cells were sorted on a FACSAria II (Becton Dickinson) and

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propidium iodide was used to exclude dead cells. FACS data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Comet Assays

Alkaline comet assays were performed using Trevigen CometAssay® kits and slides according to the manufacturer's protocol. Briefly, purified cells were embedded in Comet LMAgarose and transferred onto Trevigen HTCometSlides. For each experiment, all cells used in comparison analyses were assayed on a single high throughput slide, such that all of the following steps were consistent for those comparisons. The immobilized cells were lysed overnight and treated with freshly made alkaline unwinding solution followed by electrophoresis in alkaline conditions using the Trevigen's CometAssay® Electrophoresis System. Cells were stained with Sybr® Green or Sybr® Gold and imaged. Analysis was performed on blinded files using CometScore (TriTek Corp) where values for percent tail DNA and Olive moment were generated. Control HSCs received 2Gy of gamma irradiation. Statistical significance was calculated in Prism (GraphPad) utilizing the Mann-Whitney test for comparisons of two populations (young to old HSC) and One-way ANOVA for HSCs to multiple downstream progenitor populations.

In vivo stimulation of HSCs

Aged mice received either two doses of 5-Fluorouricil (150 mg/kg) or PBS by intra-peritoneal injection. The mice were given three weeks 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 (LSKCD34$^-$Flk2$^-$) were purified from either young or old donor (CD45.2) mice, and competitively transplanted against 200,000 whole bone marrow cells (CD45.1) into lethally irradiated young recipient mice (CD45.1). Twelve months post-transplant, donor derived (CD45.2) HSCs were purified and assayed for DNA damage.

Microarray expression analysis

Genome-wide gene expression analysis was performed using the Affymetrix GeneChip Mouse Genome 430 2.0 Array platform. RNA was isolated using TRIzol (Life Technologies) and purified RNA was amplified, labeled, hybridized, and scanned according to Affymetrix's specifications at Stanford Protein and Nucleic Acid Facility. Raw microarray data were submitted to Gene Expression Commons (https://gexc.stanford.edu) (Seita et al., 2012) where data normalization was computed against the Common Reference, which is large collection (n=11,939) of publically available microarray data from NIH GEO. Meta-analysis of the Common Reference also provides the dynamic range of each probeset on the array, and in situations where there are multiple probesets for the same gene, the probeset with widest dynamic range was used for analysis. The Affymetrix Mouse Genome 430 2.0 Array includes 45,101 probesets, of which 17,872 annotated genes are measurable.

Heatmaps representing fold-change of gene expression were generated in Gene Expression Commons. Pair-wise statistical comparisons at the gene level between cell types was computed utilizing Significance Analysis of Microarray (SAM) (Tusher et al., 2001)

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provided in R. Genes with the criteria FDR < 0.05 and Fold-change > 1.5 were scored as significant genes, and results were merged into Gene Expression Commons.

Pathway-level statistical comparison was performed by Fisher's exact test in Gene Expression Commons in which the frequency of significantly up-regulated or down-regulated genes was compared between genes in the pathway and in all of the other annotated genes on the microarray.

The raw data has been deposited in GEO public repository (GSE55525). All normalized expression intensities, results of statistical comparisons, and heatmap representations are available at Gene Expression Commons as HSC and Progenitors Young vs Old (https://gexc.stanford.edu/model/detail/786), FL-Young-Old HSCs (https://gexc.stanford.edu/model/detail/775), and HSCs in vitro Stimulation (https://gexc.stanford.edu/model/detail/313).

Clonal *ex vivo* stimulation assay

Using single-cell mode of the BD FACSAria, cells were individually sorted into a single well of a 96-well round-bottom plate. For each experiment, 120 wells were used, with three replicates of each age group. Each well contained DMEM/F-12 medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Hyclone/Thermo Scientific, Waltham, MA), 1× penicillin/streptomycin, 2mM GlutaMax, 50µM 2-mercaptoethanol (Gibco/Invitrogen, Carlsbad, CA), and the following cytokines: 10 ng/ml mouse SCF, 10 ng/ml mouse TPO, 10 ng/ml mouse Flt3l, 10 ng/ml mouse IL-3, 1 U/ml mouse EPO, 10 ng/ml mouse GM-CSF (all purchased from PeproTech, Rocky Hill, NJ). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. At 12, 24, 48, 72, 96, 120, and 144 hours of culture, cell numbers in each well were counted under microscope. 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-Gruenwald-Giemsa staining. The same culture conditions were used for short-term *ex vivo* stimulation of pooled cells (Figure 3A and Figure 7).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- HSCs are not uniquely geno-protected and accumulate DNA damage ith age
- DNA damage response (DDR) and repair pathways are differentially regulated in HSCs
- Attenuated damage repair and response in HSCs is quiescence, but not age, dependent
- HSCs G_0 exit leads to upregulation of DDR and repair pathways and repair of damage

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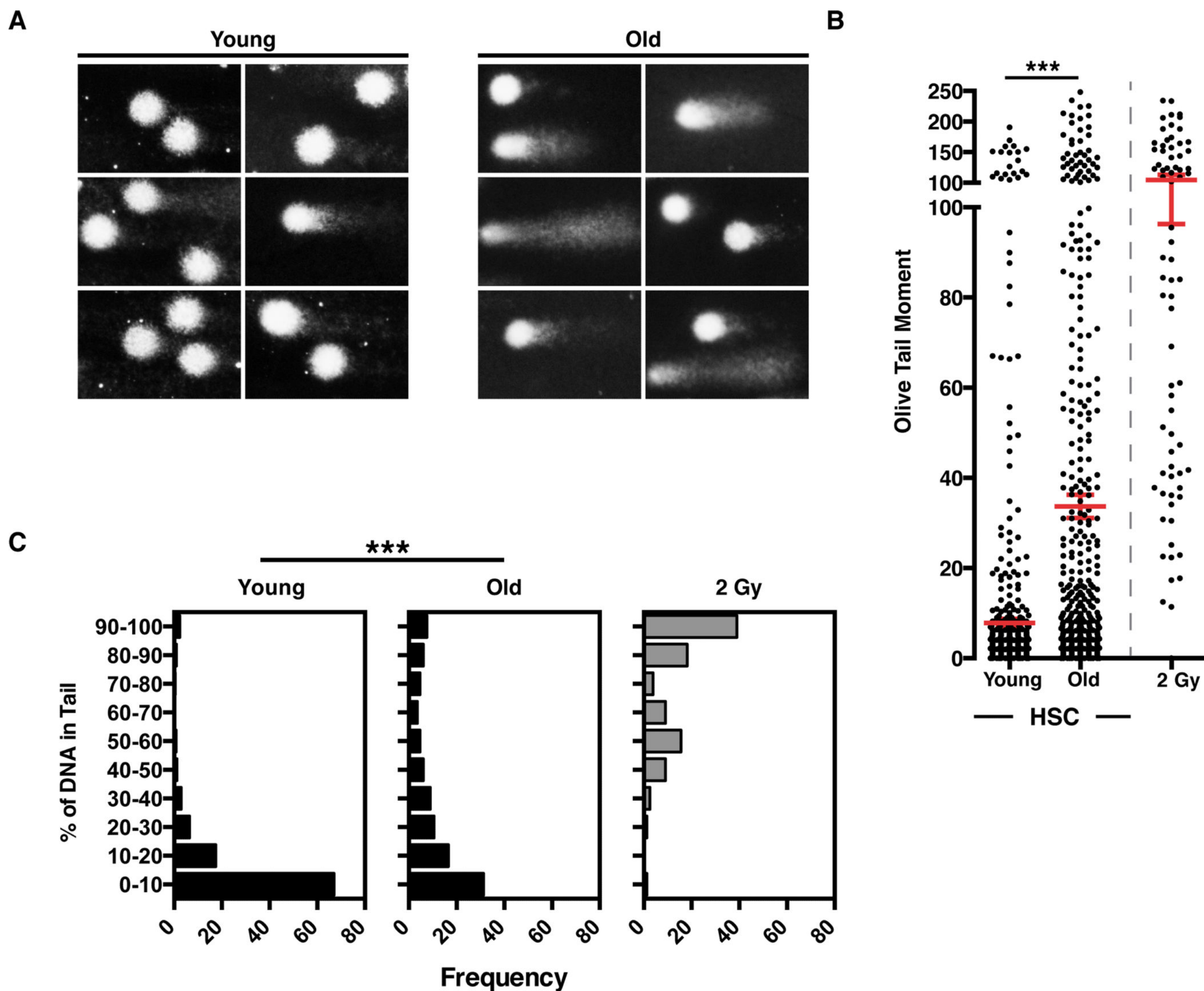

Figure 1.

DNA damage accumulates in HSCs during aging. (A) Representative alkaline comets of young and old HSCs. (B-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 2Gy IR. *** p<0.001 (see also Figure S1 and Table S1)

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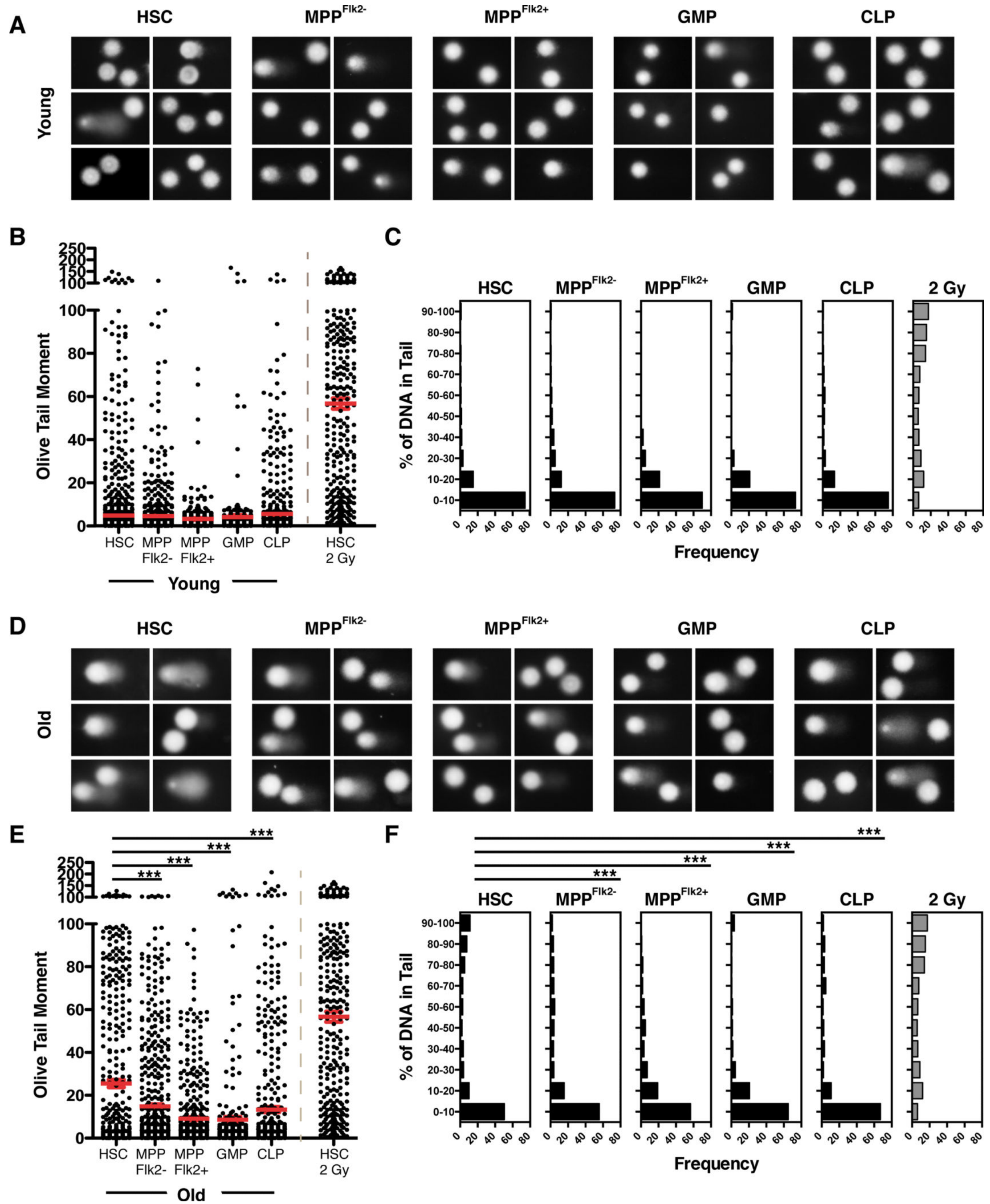

Figure 2.

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-C) Olive Tail Moment (B) and percent of DNA in tail (C) of HSC (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 2Gy of irradiation were also scored. (D) Representative alkaline comets of HSCs, multipotent progenitors (MPP^{Flk2-} and MPP^{Flk2+}) and oligopotent progenitors (GMP and

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CLP) isolated from old mice. (E-F) Olive Tail Moment (E) and percent of DNA in tail (F) of HSC (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 2Gy) are shown, as all samples were arrayed on one slide. *** $p<0.001$ (see also Figure S1, S2)

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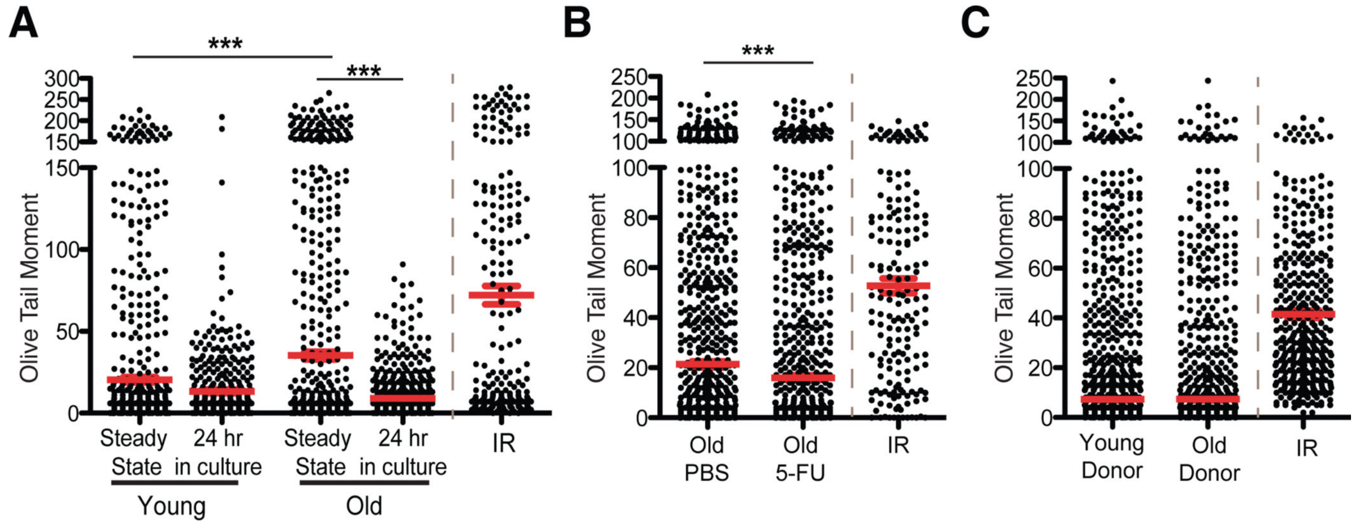

Figure 3.

HSCs recognize and repair DNA damage upon stimulation into cell cycle regardless of age. (A) Olive Tail Moment of HSCs isolated from young and old mice at steady state (n=749 and 694 respectively) or after 24 hours in culture (Young 24 hour (n=385) Old 24 hour (n=649)). 294 irradiated HSCs were used as a positive control. (B) Olive Tail Moment of aged HSCs after receiving two doses of PBS (n= 1107) or 5-FU (n=1195) and irradiated control cells (n=176) (C) Olive Tail Moment of donor derived HSCs from either young donor HSC (n=2310) or aged HSC (n=1746) 12 months post competitive transplant and irradiated control cells (n=379) *** $p<0.001$ (see also Figure S3)

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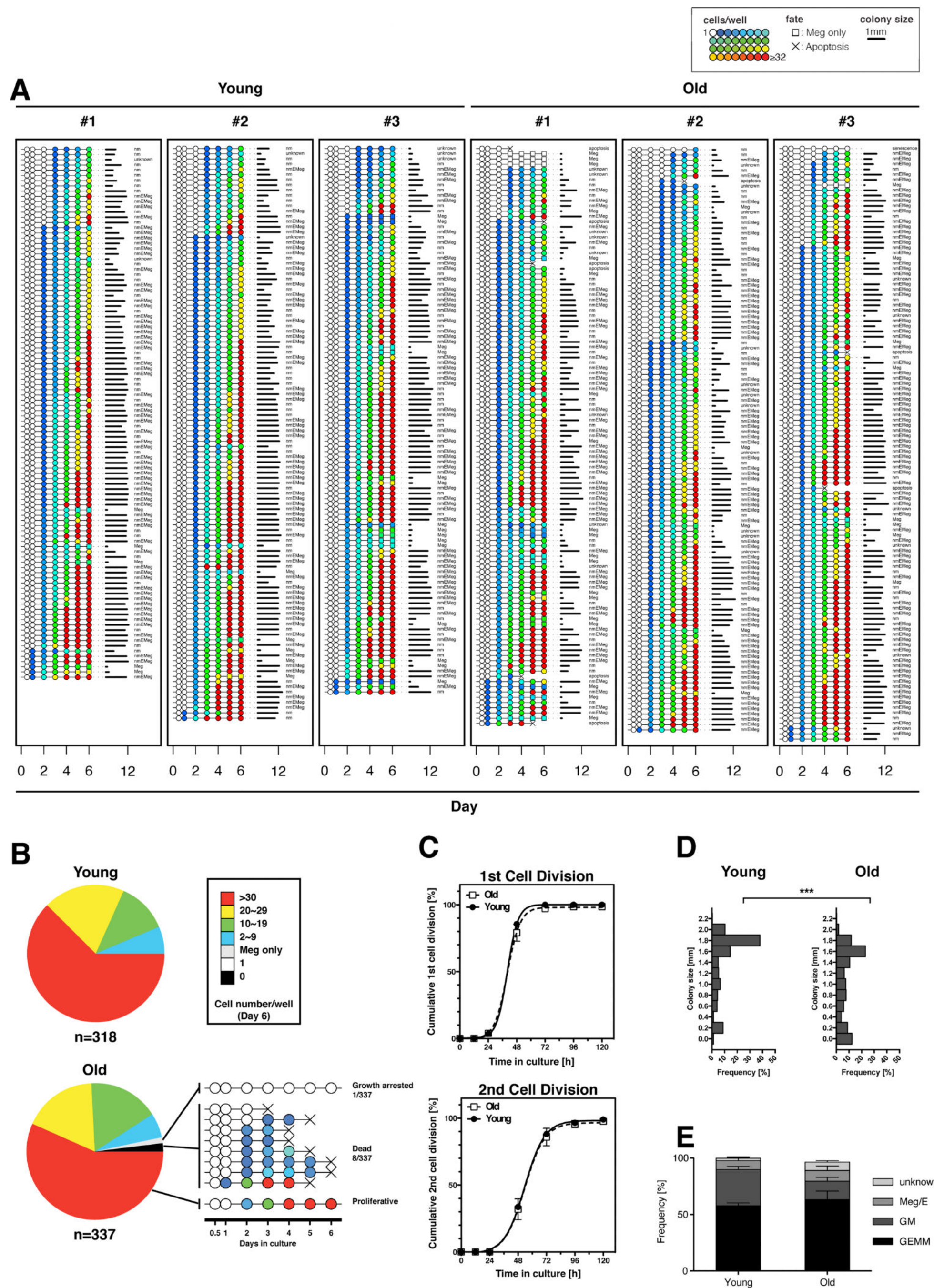

Figure 4.

Clonal analysis of single HSCs from young and old mice (A) Individual HSC clones scored daily for six days from three young mice and three old mice. Numbers of cells scored daily are presented in a color scale from white=1 cell to red>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 six days in culture. Each clone was assayed at time points 0.5, 1, 2, 3, 4, 5, and 6 days and the composite data is 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. *** $p<0.001$

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Figure 5.

Attenuation of DNA damage response and repair pathways in HSCs compared to downstream progenitor populations. (A) Fold change comparisons of genes involved in DNA damage response and checkpoints (DDRC), nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), non-homologous end joining (NHEJ), and homologous recombination (HR) in progenitor populations compared to their age-matched HSCs. Each column represents an individual replicate, and the log2 fold change compared 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 boarder. (B) Frequency of genes that show significant up-regulation (red), significant down-regulation (blue) or no significant change (grey) in each pathway for comparisons between HSCs and the indicated progenitors. The global frequencies of the total number of genes showing significant differential regulation out of the total 17,872 genes examined on the arrays is also shown (Global). (C) Statistical analysis of the significance of the changes in each of the indicated DNA damage response and repair pathways in progenitor cells compared to the HSCs from either young or old mice. p-values are presented by a color scale and odds ratios of less than one are indicated with a hash through the box. (see also Figure S4)

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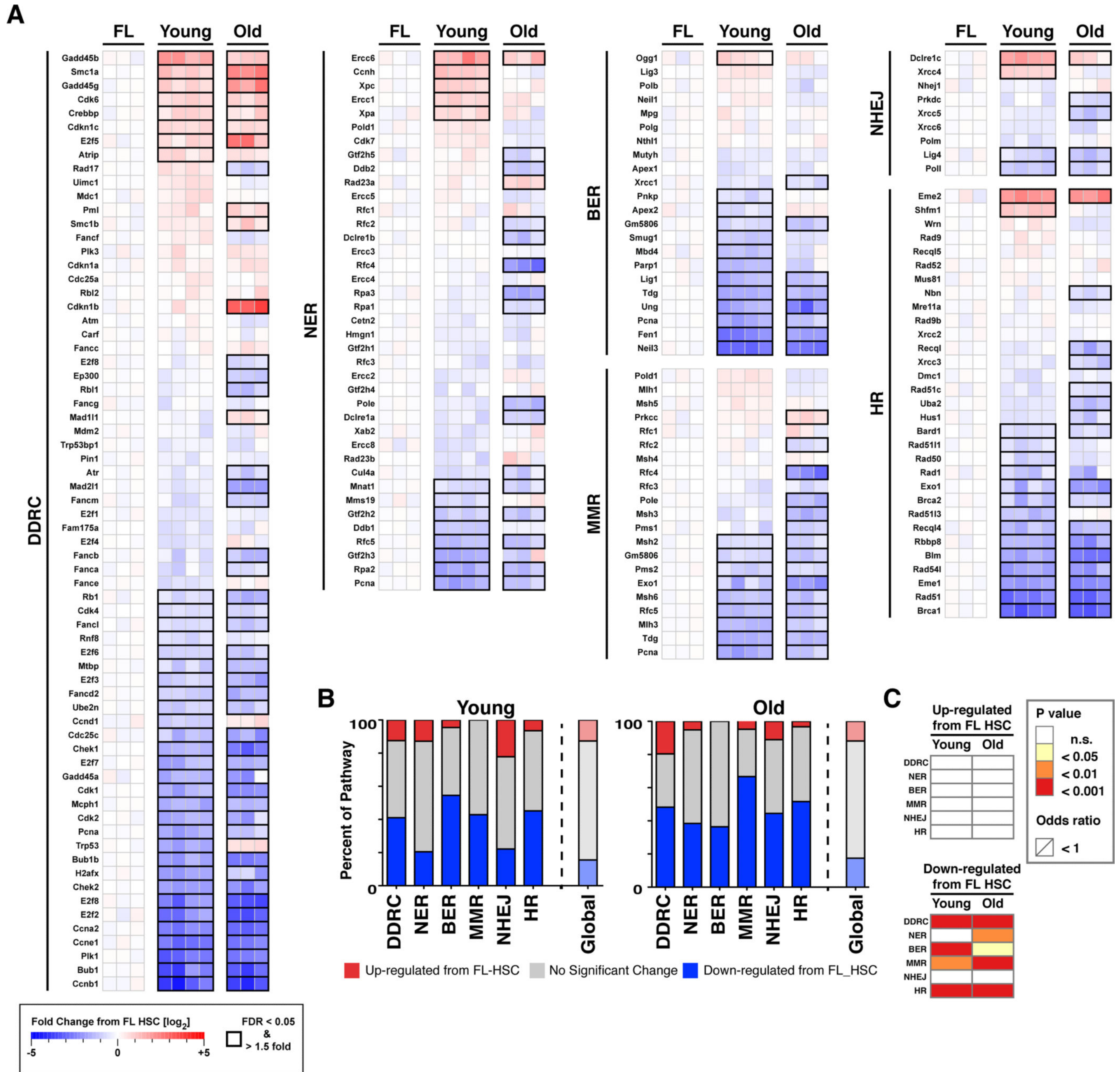

Figure 6.

Attenuation of DNA damage response and repair genes in quiescent HSCs (A) Fold change comparisons between genes involved in DDR and repair in fetal liver HSCs compared to adult HSCs young or old. Each column represents an individual replicate and the log2 fold change compared 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 boarder. (B) Frequency of genes that show significant up-regulation (red), significant down-regulation (blue) or no significant change (grey) 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 down-regulated) 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 DNA damage response and repair in young and old HSCs compared to cycling fetal liver HSCs. p-values are presented by a color scale and odds ratios of less than one are indicated with a hash through the box. (see also Figure S5)

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Figure 7.

Dynamic expression profiles of DNA damage response and repair genes in HSCs after stimulation (A) Fold change comparisons of genes involved in DDR and repair in freshly purified HSCs in comparison to HSCs stimulated into cycle at different time points (3, 6, 12 and 24 hours). Each column represents an individual replicate and the log2 fold change compared to the average expression of the age-corresponding HSCs is shown. Genes with average expression fold changes of >1.5 and p<0.05 are signified by a bold black boarder. (B) Frequency of genes that show significant up-regulation (red), significant down-

regulation (blue) or no significant change (grey) in each pathway in comparisons between HSCs at steady state or 3, 6, 12, or 24 hours post-stimulation for both young and old. (C) Analysis of significance of the changes of the overall pathways involved with DNA damage response and repair in stimulated HSCs compared to steady state HSCs from respective young or old mice. p-values are presented by a color scale and odds ratios of less than one are indicated with a hash through the box. (D) Fold-change profiles of several DNA damage response genes.

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