Mitochondrial Potentiation Ameliorates Age-Related Heterogeneity in Hematopoietic Stem Cell Function

**Highlights**

- HSCs from old and young mice are heterogeneous for mitochondrial activity (MMP)
- MMP\textsuperscript{high} HSCs from old mice have young-like transcriptional and functional features
- MMP of HSCs can be manipulated \textit{in vivo} with consequences for function
- Mitochondrial potentiation can prevent or ameliorate onset of hematopoietic aging

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**In Brief**

HSC aging is clinically associated with risk of anemia, immune compromise, and malignancy. Mansell et al. show that mitochondrial activity is directly linked to age-related performance of HSCs and that its perturbation has direct consequences for HSC function, resulting in alleviation or prevention of hematopoietic aging.
Mitochondrial Potentiation Ameliorates Age-Related Heterogeneity in Hematopoietic Stem Cell Function

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SUMMARY

Aging is associated with reduced fitness and increased myeloid bias of the hematopoietic stem cell (HSC) compartment, causing increased risk of immune compromise, anemia, and malignancy. We show that mitochondrial membrane potential (MMP) can be used to prospectively isolate chronologically old HSCs with transcriptional features and functional attributes characteristic of young HSCs, including a high rate of transcription and balanced lineage-affiliated programs. Strikingly, MMP is a stronger determinant of the quantitative and qualitative transcriptional state of HSCs than chronological age, and transcriptional consequences of manipulation of MMP in HSCs within their native niche suggest a causal relationship. Accordingly, we show that pharmacological enhancement of MMP in old HSCs in vivo increases engraftment potential upon transplantation and reverses myeloid-biased peripheral blood output at steady state. Our results demonstrate that MMP is a source of heterogeneity in old HSCs, and its pharmacological manipulation can alter transcriptional programs with beneficial consequences for function.

INTRODUCTION

Hematopoietic stem cells (HSCs) are a rare population of bone marrow (BM) resident cells that sit at the apex of the hematopoietic hierarchy and are capable of self-renewal and lifelong replenishment of all blood lineages (Orkin and Zon, 2008). HSCs are functionally defined by their capacity to reconstitute long-term multi-lineage hematopoiesis of a recipient (Till and McCulloch, 1961; Szilvassy et al., 1990). Murine HSCs defined "phenotypically" on the basis of cell-surface markers can now be purified, such that 40% of single isolated cells produce long-term multi-lineage reconstitution (Oguro et al., 2013). However, even the most phenotypically homogeneous HSCs may be heterogeneous in respect of their metabolic, epigenetic, transcriptional, and, therefore, behavioral state. With respect to metabolic state, several studies have linked differences in mitochondrial activity or content to differences in HSC state and fate, including cell-cycle status, lineage potential, and engraftment upon transplantation (Luchsinger et al., 2016; Ito et al., 2016; Liang et al., 2020; Umemoto et al., 2018). Traditionally, such studies reported that HSCs have low mitochondrial membrane potential (MMP) and low mitochondrial mass, and these attributes are supportive of stem cell function (Simsek et al., 2010; Rimmelé et al., 2015; Sukumar et al., 2016; Vannini et al., 2016). Recently, however, a number of studies have shown that HSCs have high mitochondrial mass and MMP compared to committed BM populations, and higher mass and potential equates to greater stem cell function (de Almeida et al., 2017; Bonora et al., 2018; Morganti et al., 2019a; Takihara et al., 2019). The contradictory nature of these results may in part relate to how mitochondrial mass and MMP are measured. Mitochondrial mass and potential can be simply assessed using a range of mitochondrial targeted or cationic fluorescent dyes. However, the cellular expulsion of these dyes through xenobiotic efflux pumps may confound analysis, and "true" readings may be obtained only under conditions where these pumps are blocked by inhibitors such as verapamil (VP). This becomes particularly important in the mitochondrial analysis of HSCs and progenitors that express such efflux pumps to a much higher degree than mature BM populations (Chaudhary and Roninson, 1991; Norddahl et al., 2011; de Almeida et al., 2017; Morganti et al., 2019b).

Conventionally, MMP, which represents the electrical potential and proton gradient across the inner mitochondrial membrane, was thought to simply reflect electrical potential generated through oxidative reactions linked to ATP synthesis. However, HSCs reside in low-oxygen niches and rely primarily on anaerobic glycolysis for energy production during quiescence and self-renewal (Ito and Suda, 2014; Kohli and Passegué, 2016).
Recent studies have focused on aspects of mitochondrial regulation of stem cells beyond ATP production; mitochondria are signaling organelles involved in calcium homeostasis, lysosomal trafficking, inflammation, cell death, and survival signaling, as well as biosynthetic organelles essential for heme, amino acid, and nucleotide metabolism and epigenetic markers (Snoeck, 2017; Zorova et al., 2018; Luchsinger et al., 2019; Ito et al., 2019; Umemoto et al., 2018). The extent to which these different processes are represented by measurements of MMP in stem cells is not fully understood. Furthermore, studies in cell lines have shown that the rate at which cells transcribe RNA is in part determined by mitochondrial content and membrane potential (das Neves et al., 2010). Interestingly, in contrast to many of the aforementioned roles of mitochondria, the transcriptional rate of HSCs and its relation to mitochondrial activity have not yet been explored.

The role that aging plays in stem cell function is an emerging research topic of interest, in part because of a global demographic shift toward an older population. Aging is accompanied by steady functional decline of a variety of organs and tissues (et al., 2000; Liang et al., 2005; Norddahl et al., 2011; Dykstra et al., 2014). Upon aging of the hematopoietic system, the phenotypic HSC pool expands but collectively displays reduced repopulation capacity, increased clonality, myeloid-biased blood output, and lymphoid and erythroid deficiency (Sudo et al., 2000; Liang et al., 2005; Norddahl et al., 2011; Dykstra et al., 2011; Grover et al., 2016). As a consequence, many hematological disorders such as anemia, adaptive immune compromise, and malignancy are strongly age-associated (Pang et al., 2011).

Conceptually, aging can be considered in two distinct ways: chronological age and physiological age. Chronological age reflects the actual age of the organism, and consequently, all stem cells within an organism will share the same chronological age. Physiological age reflects an age-linked performance characteristic. Cells of the same chronological age may thus map to different physiological ages. This raises the possibility that stem cells in aged mice may be heterogeneous with regard to their physiological age with, for example, an age-related characteristic such as myeloid bias being distributed in a heterocellular fashion in old mice (Beerman et al., 2010; Wahlestedt et al., 2017; Montecino-Rodriguez et al., 2019; Gulati et al., 2019).

Since it has been shown that myeloid bias and reduced regenerative capacity of old HSCs are maintained upon transplantation into young mice, it has been suggested that functional alteration with age predominantly results from cell-autologous changes (Rossi et al., 2005). A number of studies have identified cell-intrinsic mechanisms underlying HSC aging, including DNA damage, cellular senescence, increased reactive oxygen species (ROS) production, and mitochondrial dysfunction (de Haan and Lazare, 2018).

We herein explore the possibility that MMP can be used to identify physiologically young HSCs in chronologically aged mice. We test if the transcriptional and functional states of young and old HSCs correlate directly with MMP and, consequently, if manipulation of MMP in vivo may impact on age-related performance. We show that despite the decrease in MMP of aging HSCs at the population level, a fraction of HSCs exist in old mice that are similar in MMP to the bulk of HSCs from young mice, thereby identifying MMP as a source of heterogeneity in chronologically old HSCs. Furthermore, we show that MMP directly determines both the rate of transcription and the nature of gene expression of HSCs in their native niche. Finally, we show that MMP of HSCs from old mice can be pharmacologically enhanced in vivo with direct consequences for HSC function, as indicated by increased engraftment potential and balanced lineage output upon transplantation. In a steady-state setting, we show that mitochondrial-targeted treatment of aged mice causes reversal of myeloid-biased peripheral blood output and rescue of lympho-erythroid output, highlighting the translational potential of our findings.

RESULTS

Mitochondrial Activity Is a Source of Heterogeneity in Old and Young HSCs

We compared the mitochondrial activity of old and young HSCs and assessed the extent to which age-related changes in mitochondrial parameters apply to all or only a fraction of HSCs within an organism. Staining for mitochondrial mass (mitotracker green [MTG]) or membrane potential (Tetramethylrhodamine methyl ester [TMRM], DilC1(5), Tetramethylrhodamine ethyl ester [TMRE], and JC-1) in the presence of VP was combined with a panel of cell-surface markers to discriminate between stem, progenitor, and mature compartments in the BM of young and old mice (Figure 1A). In line with published literature, we observed a significant increase in the frequency of immunophenotypic HSCs with age, at the expense of the hematopoietic progenitor cell compartment-1 (HPC1) population (Figure 1A).

We first validated the effect of VP on mitochondrial dye retention in HSCs versus bulk BM cells; the impact of VP has been reported for MTG (Norddahl et al., 2011; de Almeida et al., 2017) and recently for TMRM (Bonora et al., 2018) but not for DilC1(5) and TMRE. In absence of VP, primitive progenitor cells (Lin− Sca1+ cKit+; LSK) and HSCs in particular appear to have lower MMP than committed BM populations (Lin+) in both young and old animals (Figure S1A). In contrast to a recent report (Liang et al., 2020), we show that inclusion of VP significantly increases staining intensity of all mitochondrial mass and MMP dyes in HSCs, without affecting total BM staining (Figures 1B, S1B, and S1C); thus, we have used VP throughout our study. In accordance with related studies (de Almeida et al., 2017; Bonora et al., 2018; Takihara et al., 2019; Morganti et al., 2019a), our data confirm that HSCs are indeed high in mitochondrial mass as well as MMP relative to mature BM populations, a trend observed in both young and old mice (Figures 1C and S1D). We also saw a marked reduction of MMP with age in progenitors, most pronouncedly in HSCs (Figures 1D and S1E); however, this was not accompanied by any major change in mitochondrial mass as measured by MTG (Figure 1D) and quantifications of the nuclear to mitochondrial DNA ratio (data not shown), indicating a per-mitochondrial mass decrease in activity in old HSCs.

Assessment of the distribution of MMP values showed that both young and old HSCs are heterogeneous with respect to MMP, and the range of MMP values covered by young and old HSCs is broadly similar (Figure 1E). The difference in average MMP value seen at the population level results from a distribution...
Figure 1. Mitochondrial Activity Is a Source of Heterogeneity in Old and Young HSCs

(A) Left: gating strategy of HSPCs based on signaling lymphocyte activation molecule (SLAM) expression in the lineage (Mac1, Gr1, B220, CD3e, Ter119) Sca1+ c-Kit+ fraction (LSK). Right: frequencies of phenotypic HSCs (CD150+CD48−/C0−/LSK), multipotent progenitors (MPP, CD150−/C0−/CD48−/LSK), and more restricted progenitor populations HPC1 (CD150−/CD48−/LSK) and HPC2 (CD150−/CD48+LSK) in old and young mice (n = 15).

(B) Representative FACS plots of MMP in HSCs (blue) and Lin+ (gray) cells, as measured by TMRM in absence (top) or presence (bottom) of VP.

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shift of old HSCs toward the lower end of this range, while young HSCs center toward the higher end. We categorized HSCs as MMPhigh or MMPlow by splitting this range of values into two components centered around the average median staining intensity (MFI) of young and old HSCs combined, while allowing for a gap between high and low that spans at least 15% of each sample. Based on this, the majority of HSCs in young mice are classified as MMPhigh. In contrast, only 10%–15% of HSCs from old mice are classified as MMPhigh, with the MMPlow fraction representing the majority of HSCs (Figure 1E).

Next, we confirmed that the cells we operationally defined as MMPhigh by dye staining express high levels of key mitochondrial genes including Cox8a, Pmpca, and Mff (Figures 1F and 1S1F), validating our assessment of MMP status.

In addition, we explored how MMP relates to other metabolic parameters. Our data reveal that MMP of HSCs positively correlates with their intracellular ATP content (Figure 1G) and inversely correlates with mitochondrial superoxide generation (Figure S1G) and intracellular ROS (Figure S1H) in both young and old mice. This is of particular interest because low ROS levels are known to support long-term HSC function (Ito et al., 2006; Jang and Sharkis, 2007; Le et al., 2016).

In sum, HSCs are heterogeneous for MMP, and a population of MMPhigh stem cells exists in the old mice that is similar in MMP to the bulk of young HSCs. This raises the possibility that their similarity may extend beyond MMP to a shared cell state and, by extension, to similarity in function.

Mitochondrial Membrane Potential Determines Transcriptional Rate of HSCs

We first assessed cell state through quantitative and qualitative analyses of transcriptional state. We have previously shown in cell lines that MMP contributes to heterogeneity in global RNA transcription rate, generating cell-to-cell variability in otherwise seemingly homogeneous populations (das Neves et al., 2010; Guantes et al., 2015). Here, we explored this relationship in HSCs in the context of their native BM niche using mitochondrial membrane potential. The global rate of transcription of HSCs in their native niche is both heterogeneous and dependent on mitochondrial activity.

We measured transcription rate in vivo, using click-it chemistry (Kolb et al., 2001) to detect 5-ethylthiouridine (5-EU) incorporation into nascent RNA over a set period post-intraperitoneal injection (Figure 2A). Remarkably, in young mice, hematopoietic stem and progenitor cells (HSPCs) and HSCs in particular are transcribing at a significantly faster rate than MMPhigh sorted cells in both young and old mice, validating this relationship. In contrast, expression of RNA Pol I transcription termination factor 1 (Ttf1) does not appear to correlate with MMP.

In vivo treatment with the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP) resulted in a reduction of MMP (Figure S2F) concomitant with a significant decrease in transcription rate of BM cells including HSCs (Figures 2I and S2G). To address whether transcription rate is directly dependent on MMP, we compared the effect of CCCP with that of conventional RNA Pol inhibitors (Figure 2H). The magnitude of transcription rate decrease resulting from mitochondrial uncoupling is similar to that produced by broad RNA Pol inhibitor Actinomycin-D and comparable concentrations of the RNA-Pol-II-specific inhibitors 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) and Flavopiridol (Figure 2I). Furthermore, gene expression data of HSPCs isolated from CCCP-treated animals reveal specific downregulation of RNA Pol II subunits alongside downregulation of mitochondrial genes (Figure S2H). Our data are consistent with the notion that the global rate of transcription of HSCs in their native niche is both heterogeneous and dependent on mitochondrial activity.

Mitochondrial Activity Separates Transcriptionally Distinct Subsets of HSCs

We next assessed the qualitative transcriptional state of HSCs that differ in MMP through bulk RNA sequencing. MMPhigh and MMPlow HSCs were sorted from young and old mice as
Figure 2. Mitochondrial Membrane Potential Determines Transcriptional Rate of HSCs

(A) Schematic diagram of in vivo measurements of transcription (5-EU) and translation (O-propargyl-puromycin; OPP) rate.

(B) In vivo transcription rate measurements of BM lineages in young mice normalized to BM (n = 5).

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described in Figure 1, albeit enhancing the separation between high and low to span 30% of each sample (Figure 3A).

Using gene set enrichment analysis (GSEA), we validated that MMP\textsuperscript{high} and MMP\textsuperscript{low} HSCs appropriately reflect their mitochondrial state at the transcriptional level (Figures 3B and S3A). Additionally, old HSCs are enriched for age-associated gene expression signatures (Figure S3B, purple box), while young HSCs show higher expression of certain lymphoid lineage–age–associated genes (Figure S3B, blue box), although expression of some of these genes is also clearly influenced by MMP status (Figure S3B, black box).

Principal-component analysis (PCA) reveals that HSCs group predominantly by MMP rather than by chronological age (Figure 3C). Approximately 1000 genes are significantly differentially expressed between MMP\textsuperscript{high} and MMP\textsuperscript{low} HSCs irrespective of their donor age (Figure 3D). Closest-neighbor analysis (Figure 3E) shows that more genes are differentially expressed between MMP\textsuperscript{high} and MMP\textsuperscript{low} HSCs, irrespective of donor age, than between young and old HSCs, irrespective of their MMP. Overall, this suggests that MMP is a greater determinant of transcriptional state than chronological age, and while old HSCs as a population are broadly transcriptionally distinct from their younger counterparts, a fraction do share significant transcriptional similarity with young HSCs, and these may be identified through MMP.

We next explored the underlying basis of transcriptional similarity of cells sharing the same MMP. GSEA (Figures 3F and S3C) reveals an age-related signature associated with low MMP, exemplified by upregulation of inflammatory signaling and apoptotic pathways and downregulation of DNA repair pathways (Nijnik et al., 2007; Rossi et al., 2007; Kirschner et al., 2017). Critically, upregulation of inflammation and downregulation of DNA repair are drivers of oncogenesis, relevant perhaps to the strong age dependency of leukemia (Akunuru and Geiger, 2016). GSEA also shows that MMP\textsuperscript{high} HSCs are enriched for pathways linked to transcriptional rate, including purine and pyrimidine metabolism, RNA turnover, and spliceosome pathways.

Furthermore, GSEA comparing our raw dataset to published lineage signatures (Novershtern et al., 2011) reveals that MMP\textsuperscript{high} HSCs are enriched for granulocyte and monocyte progenitor (GMP) signatures and downregulation of erythroid, early erythroid, Pro-B, and early T cell programs, compared to MMP\textsuperscript{high} HSCs (Figures 3F and S3C). For a more global assessment of lineage in relation to MMP, we used CellRadar, which compares selected gene sets to published lineage-associated genes. These data show that MMP\textsuperscript{high} HSCs are primarily enriched for granulocyte, monocyte, and long-term HSC (LT-HSC) signatures (Figure 3G) in a manner nearly identical to CellRadar analyses of genes upregulated upon HSC aging (Figure 3H). Genes upregulated in MMP\textsuperscript{high} HSCs, in contrast, reveal a more diverse lineage pattern covering numerous lymphoid, erythroid, and mixed-lineage precursor populations (Figure 3G) in a manner more closely resembling CellRadar analyses of genes upregulated in young HSCs (Figure S3D).

Finally, we tested the connection between MMP and lineage using a literature-derived candidate gene approach (Rossi et al., 2005; Challen et al., 2010); age-associated genes with implications for myeloid or platelet bias are nearly exclusively upregulated in MMP\textsuperscript{low} HSCs, whereas genes involved in lymphoid and erythroid priming are found to be upregulated in MMP\textsuperscript{high} HSCs (Figures 3D and S3E).

We conclude from these data that MMP separates HSCs with quantitatively and qualitatively distinct transcriptional states, irrespective of chronological age. Furthermore, sorting cells based on low and high MMP segregates the transcriptional changes that are characteristic of old and young HSCs, respectively.

In Vivo Manipulation of MMP Alters the Transcriptional States of HSCs

To see if MMP is a functional determinant of transcriptional state, we next experimentally manipulated MMP levels of HSCs in vivo (Figure 4A). We both uncoupled the mitochondrial respiratory chain by injection of CCCP (Figure 2H) and enhanced MMP using mitoquinol (Mito-Q), a mitochondrial-targeted coenzyme-Q10 (Murphy and Smith, 2007), which successfully increased MMP of old HSCs in vitro (Figure S4A). We show that successive Mito-Q injection significantly enhanced the MMP of old HSCs in their native niche (Figures 4B and S4B), increased their global rate of transcription (Figures 4B and S4C), and decreased their levels of intracellular ROS (Figure S4D).

Mito-Q treatment of old mice caused a significant frequency shift from HSCs to the HPC1 fraction, thereby partially restoring the skewed distribution of HSPCs (Figure 4C) normally seen in aged animals (see Figure 1A). CD150 expression has been used to distinguish between predominantly lymphoid-biased (Ly; CD150\textsuperscript{low}) and myeloid-biased (My; CD150\textsuperscript{high}) HSCs, the latter becoming dominant with age (Beerman et al., 2010; Figures 4C and 4D). Interestingly, short-term Mito-Q treatment causes a significant increase in Ly-HSCs at the expense of My-HSCs (Figures 4D) through selective cycling of Ly-HSCs (Figures 4E and S4E). These changes are in line with our observation that Ly-HSCs have higher MMP than My-HSCs (Figure S4F). Collectively these results show that a 5-day treatment of old mice with Mito-Q causes phenotypic changes in HSCs that are

(C) In vivo protein translation rate measurements of BM lineages in young mice normalized to BM (n = 4).
(D) Comparison of transcription rate between young (blue) and old (purple) BM lineages, normalized to BM (n = 5).
(E) Representative FACS plot of transcription rate of young (blue) and old (purple) HSCs (left) including analysis of the percentage distribution (right) of young and old HSCs that fall into the transcription rate\textsuperscript{fast} (orange) and transcription rate\textsuperscript{slow} (gray) categories (n = 5).
(F) Transcription rate analysis in the 30% MMP\textsuperscript{low}– and MMP\textsuperscript{high}–sorted HSCs from young or old (18–24 months) mice (n = 9 [Y], n = 5 [O]). Ratio paired t test.
(G) mRNA expression of RNA Pol II subunits Poir2a, Poir2e, and elongation factor El2, as well as RNA Pol I transcription termination factor 1 (Ttf1) in young and old LSK cells sorted by MMP, normalized to expression of B2m (n = 3).
(H) Schematic diagram of experimental procedure.
(I) Transcription rate in HSCs after in vivo treatment with mitochondrial uncoupler CCCP compared to RNA Pol inhibitors Actinomycin-D, DRB, and Flavopiridol at three concentrations. (n = 1 per concentration).

Results are represented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
suggestive of a change in functional state, with the possible caveat that Mito-Q treatment may affect cell-surface marker expression.

We therefore assessed whether increasing the MMP of old HSCs toward values observed in young HSCs would increase their transcriptional similarity to young HSCs and vice versa. RNA sequencing was performed on old HSCs isolated from old untreated animals (O), old Mito-Q-treated animals (O+MQ), young untreated animals (Y), and young CCCP-treated animals (Y-CCCP) (Figure 4A).

Interestingly, PCA reveals that old HSCs isolated from Mito-Q-treated animals show greater transcriptional similarity to young HSCs than to old HSCs from untreated animals (Figure S4G). We next grouped samples into “higher MMP,” consisting of young untreated HSCs and old HSCs with enhanced MMP through Mito-Q treatment, and “lower MMP,” consisting of old untreated HSCs and young HSCs after mitochondrial uncoupling through CCCP (Figure 4F). We then identified commonly upregulated and downregulated genes in higher MMP compared to lower MMP HSCs, irrespective of donor age (Figure 4G). In parallel, we compared all old samples to all young samples, irrespective of treatment. More genes are differentially expressed between higher MMP and lower MMP samples, irrespective of donor age, than between old and young samples, irrespective of treatment (Figure 4F). While the transcriptional state change effected by manipulation of MMP is not complete, our data suggest that the MMP of HSCs, whether naturally occurring or achieved through in vivo pharmacological manipulation, is a bigger determinant of transcriptional state than is the age of the donor mice.

We next explored the nature of genes that are commonly up- or downregulated in both old Mito-Q-treated and young untreated HSCs to assess if the transcriptional state change in response to Mito-Q is suggestive of a functional state shift. The differentially expressed genes (DEGs) that are significantly upregulated in higher MMP samples irrespective of chronological age comprise many genes that are critical for HSC function and self-renewal, including many transcription factors (TFs) (Figure 4H). Furthermore, Gene Ontology analysis reveals that the top upregulated pathways that are shared between young untreated and old Mito-Q-treated HSCs are highly representative of increased transcriptional rate and also include upregulation of DNA repair pathways (Figure 4H). We functionally validated the increased DNA repair capacity of old HSCs after Mito-Q treatment through measurements of DNA double-strand breaks (marked by y-H2AX) after 2 Gy of irradiation. We show that HSCs, but not bulk BM, from old Mito-Q-treated mice have significantly reduced levels of DNA damage after irradiation compared to HSCs from old untreated mice, despite no difference in y-H2AX levels at steady state (Figure S4H). Similar results are obtained upon short-term ex vivo treatment with Mito-Q of HSCs that were exposed to irradiation in vivo (Figure S4I).

Furthermore, CellRadar indicates that these DEGs are relevant to lineage (Figure 4I): higher MMP correlates with increased lymphoid programming, reflected in signatures for lymphoid-primed multipotent progenitors (LMPPs), early T cell progenitors (ETPs), natural killer cells (NKs), and both pre- and pro-B cell lineages. These signatures are absent in the CellRadar profiles of both old untreated and Mito-Qsorted HSCs (Figures 4I and 3H). The CellRadar plot based on genes that are upregulated in old Mito-Q-treated versus old untreated HSCs is nearly identical in shape to the CellRadar plot based on genes that are upregulated in young compared to young uncoupled HSCs, suggesting that this lineage signature is specific to elevated MMP of HSCs from both young and old animals.

Our data thus suggest that changing MMP acts directly on the level of stem cells to change their qualitative and quantitative transcriptional state, as well as the balance between lymphoid- and myeloid-biased stem cells. The nature of change in old HSCs upon Mito-Q treatment reveals greater transcriptional similarity with young HSCs that extends to pathways highly relevant to stem cell fitness and lineage output, thus suggesting that this transcriptional state change might be accompanied by a functional state change.

HSCs from Mito-Q-Treated Old Mice Display Superior Engraftment and Lineage Kinetics upon Transplantation

To assess the extent to which HSC function is intrinsically altered upon Mito-Q treatment, we performed competitive transplantation assays. Three hundred HSCs isolated from old Mito-Q-treated mice (O+MQ, according to Figure 4A) or from old untreated mice (O) were competitively transplanted against 100 HSCs from young mice (Y) together with 200,000 BM support cells into lethally irradiated mice. Since recipient mice were never exposed to Mito-Q, any difference in readout would result from intrinsically sustained changes in old HSCs that were treated in vivo prior to isolation and transplantation. Peripheral blood (PB) engraftment was monitored until the 16-week endpoint, when BM engraftment was also analyzed (Figure 5A). First, we assessed if the MMP of HSCs that are transplanted influences the MMP of their progeny. We observed that young donor HSCs reconstitute BM, which, 16 weeks after...
Figure 4. In Vivo Manipulation of MMP Alters the Transcriptional States of HSCs

(A) Strategy of in vivo modulation of MMP in old (purple) and young (blue) mice using Mito-Q or CCCP.

(B) Representative FACS plots of MMP (left) and transcription rate (right) in old HSCs after Mito-Q (lilac) compared to old control HSCs (purple).

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transplantation, shows a similar trend in MMP values across lineages as observed in young mice at steady state (Figures 5B and 1C). Old donor HSCs reconstitute lineages that are lower in MMP than lineages derived from young donor HSCs (Figures 5B and 5A). Interestingly, BM reconstituted by old Mito-Q-treated donor HSCs has significantly higher MMP than BM derived from old untreated donor HSCs and is similar in MMP to BM reconstituted by young HSCs (Figures 5B and 5C). Moreover, both old Mito-Q-treated and young HSCs reconstitute a stem cell pool that is higher in MMP compared to the HSC pool derived from old untreated donors (Figures 5C and 5B).

Importantly, analysis of the kinetics of reconstitution revealed that old HSCs isolated from Mito-Q-treated mice showed significantly superior PB engraftment compared to old untreated HSCs (Figure 5D). Old HSCs from Mito-Q-treated mice also show improved lineage kinetics, producing significantly more B cells than old HSCs from untreated mice, together with a quicker reduction of myeloid-biased output (Figure 5D). BM engraftment 16 weeks after transplantation reflects the blood observations (Figures 5E and 5F), with a nearly 2-fold-greater engraftment from HSCs derived from Mito-Q-treated old mice compared to untreated old mice (Figure 5E). Further analysis revealed that O+MØ HSCs perform equally well as their competitors, consisting of both young HSCs and support BM, whereas old untreated HSCs are significantly outcompeted by their competitors (Figures 5E and 5C).

Thus, the transcriptional state change in old HSCs in response to mitochondrial potentiation is accompanied by increased engraftment potential, improved B cell output, and reduced myeloid bias and suggests that the effect of short-term in vivo Mito-Q treatment on HSCs is cell intrinsic and, intriguingly, can be maintained upon stem cell transplantation with long-term consequences for function.

Mito-Q Treatment Can Both Revert and Prevent Onset of the Hematopoietic Aging Phenotype

Since stem cell behavior in a homeostatic setting may be distinct from that seen in a transplant (Busch and Rodewald, 2016; Rodríguez-Fraticelli et al., 2018; Säven et al., 2018), we next tested the activity of Mito-Q on hematopoiesis at steady state by asking whether it could revert or prevent the onset of hematopoietic aging, thereby assessing its translational potential in the amelioration or prevention of age-related blood disorders.

We measured PB parameters before, during, and after 5 days of treatment of old mice with Mito-Q (Figure 6A) using correction of myeloid bias and rescue of lymphoid output as a measure of functional improvement. Because the trajectory of aging varies from mouse to mouse, resulting in a cohort of 18-month-old mice with different age-related phenotypes, we individually monitored mice receiving Mito-Q. Strikingly, every old Mito-Q-treated animal shows reduction in myeloid output between the start and end of treatment, in the magnitude of 2% to 46% (Figure 6B). This is accompanied by an increased output of B cells of up to 34% seen in all but one individual (Figure 6C). Qualitative changes in B cells were also evident, as shown by a proportionate increase in Immunoglobulin-M expressing (IgM+) B cells (Figures 6D and 5B). Upon Mito-Q treatment, myeloid and B cell output have significantly changed compared to old untreated mice to such an extent that they are no longer significantly different from young mice (Figure 6E). Mito-Q treatment also caused a significant increase in T cells (Figure 6B). Importantly, the proportional changes in lymphoid versus myeloid output occurred without changing total blood counts (Figure 6F). Of note, treatment of young mice with Mito-Q did not alter PB parameters (data not shown).

We extended these findings in a smaller cohort of young and old mice treated with or without Mito-Q in their drinking water for a period of 10 weeks to assess the utility of Mito-Q delivered in a manner more compatible with any potential therapeutic use (Figure 6C). Adding Mito-Q to the drinking water of old mice produced similar changes in myeloid, lymphoid, and erythroid kinetics (Figures 6D–6F) to direct Mito-Q injection. Supplementing the drinking water of young mice for 10 weeks did not significantly affect blood and BM parameters.

These results further raise the possibility that earlier intervention might prevent or attenuate the onset of an aging phenotype. To test this, we supplemented the drinking water of 14-month-old middle-aged (MA) mice without (O) or with Mito-Q (OMQ), monitoring the ratio of myeloid to B cells of individual mice over 5 months (Figure 6G). Mice that had Mito-Q-supplemented water have a similar ratio of myeloid to B cells in the PB as MA mice at the start of the experiment, whereas naturally aged mice developed significant myeloid bias (Figures 6H and 5G). Accordingly, 19-month-old mice that aged with Mito-Q water have significantly more B cells and fewer myeloid cells than naturally aged mice (Figure 6H). Again, these changes occurred without affecting total white blood cell counts (Figure 6H). Critically, Mito-Q treatment also prevented onset of anemia that occurred with natural aging, as evidenced by the significant increase in red blood cell count, hematocrit, and hematocrit of mice that received Mito-Q water.
Figure 5. HSCs from Mito-Q-Treated Old Mice Display Superior Engraftment and Lineage Kinetics upon Transplantation

(A) Transplantation setup.

(B) FACS analysis of MMP (TMRM+VP) in BM lineages retrieved 16 weeks after transplantation, separated based on donor origin. Left: derived from old untreated donor HSCs (n = 10); middle: derived from old Mito-Q-treated donor HSCs (n = 9); right: young HSC donor derived (n = 21).

(C) Comparison of MMP in BM (left) or HSCs (right) 16 weeks after transplantation, reconstituted by old untreated donor HSCs (purple, n = 10), old HSCs from Mito-Q-treated donors (lilac, n = 9), or young donor HSCs (blue, n = 21).

(D) Graphs showing % Engraftment, % B cells, and % Myeloid over weeks post Tx (wks post Tx).

(E) Graphs showing % of BM engraftment over 16 weeks post Tx.

(F) Graphs showing Myeloid (% BM) and p220+ (% BM) over 16 weeks post Tx.

(legend continued on next page)
for 5 months (Figure 6J). Mirroring the PB observations, BMs of old mice that aged with Mito-Q-supplemented water have a significantly smaller proportion of myeloid cells and a significantly greater proportion of B cells, including the IgM B220+ compartment consisting of pre- and pro-B cells, compared to naturally aged mice (Figure 6J), as well as higher levels of mature erythroid progenitors (Figures 6K and S6I). Additionally, there is a less-exaggerated expansion of phenotypic HSPCs including HSCs when mice age with Mito-Q-supplemented drinking water (Figure S6J).

In sum, Mito-Q supplementation of MA or old mice results, respectively, in the attenuation or partial reversal of an aging phenotype, highlighting the translational potential of our findings.

**DISCUSSION**

Herein, we have shown that (1) MMP is a source of heterogeneity of old HSCs, with a fraction of old HSCs having similarly high MMP as the bulk of young HSCs; (2) MMP is a bigger determinant of the quantitative and qualitative transcriptional state of HSCs than chronological age; (3) MMP of HSCs can be altered in vivo; (4) enhancement of MMP changes the transcriptional landscape of old HSCs, including rescue of lympho-erythroid programs and DNA repair pathways; (5) these transcriptional changes read out as functional improvement in stem cell transplantation assays; and (6) Mito-Q can ameliorate or prevent onset of an aging phenotype.

Age-related changes in hematopoiesis include expansion of phenotypic HSCs, reduced engraftment, and myeloid skewing. While mitochondrial dysfunction has been implicated in aging of the blood system (de Haan and Lazare, 2018), the extent to which age-related phenotypes of HSCs directly result from changes in mitochondrial biology is not fully understood. One study demonstrated that a mouse model of mitochondrial DNA mutagenesis can manifest some aspects of chronological stem cell aging (lineage skewing), but not all (molecular and phenotypic changes) (Norddahl et al., 2011). Of note, MMP was not affected in this mouse model. Our demonstration that Mito-Q treatment causes reversal or stabilization of age-related parameters of the PB and BM addresses this issue directly, and several lines of evidence suggest that these functional changes may be attributed to altering MMP at the level of HSCs. First, short-term Mito-Q treatment directly changes both quantitative and qualitative transcriptional programs at the level of stem cells, including a shift in lineage-affiliated programs, consistent with the re-balancing of lineage output observed in treated mice. Second, Mito-Q treatment corrects the excess representation of phenotypic HSC seen in aged mice and results in selective cycling of lymphoid-biased HSCs at the expense of myeloid-biased HSCs. Lastly, HSCs isolated from Mito-Q-treated old mice are functionally superior to HSCs isolated from untreated old mice upon transplantation into untreated animals, both in respect to total engraftment and lineage distribution of reconstitution. These data highlight that HSCs are intrinsically changed upon short-term Mito-Q treatment with long-term functional consequences. This aside, we do not exclude the possibility that there may be additional and potentially beneficial effects of Mito-Q downstream of stem cells (e.g., stemming from the increased HPC1 fraction harboring LMPPs) when mice receive Mito-Q.

Germane to the issue of chronological versus physiological aging, we conclude that HSCs age in a heterocellular manner, leading to functional heterogeneity in the aged stem cell pool, which may both be dynamic and, importantly, perturbable. Strikingly, we show that HSCs transcribe RNA quickly, relative to both mature BM and uncommitted progenitor populations. In line with prior cell line studies (das Neves et al., 2010), we show that the transcriptional rate of HSCs correlates with their MMP, and both are significantly decreased in aging HSCs. Remarkably, the rate of transcription changes when MMP is perturbed, suggesting a cause-and-effect relationship. Why a high rate of transcription might be beneficial to stem cells is less clear, but we are intrigued by the possibility that a high transcriptional rate might in some way facilitate high stem cell fate plasticity. In any event, the extent to which transcription rate is or is not related to qualitatively different patterns in gene expression merits further investigation. Interestingly, the high transcriptional rate is not reflected in the rate of protein synthesis. On the contrary, HSPCs are characterized by a significantly lower rate of translation compared to more mature populations, thus suggesting an uncoupling of transcription from protein translation in HSCs at steady state. Such a phenomenon has recently been described in neuronal stem cells (Baser et al., 2019), raising the possibility that this might apply to other multipotent and potentially pluripotent stem cells.

Finally, our experiments that show that age-related parameters in the blood can be reversed under homeostatic conditions, or indeed prevented by the simple addition of Mito-Q to the drinking water, suggest that Mito-Q could be used in a setting where it might offer clinical benefit. Indeed, Mito-Q has been used in clinical trials of neurodegenerative diseases such as Alzheimer’s disease, multiple sclerosis and Parkinson’s disease, liver pathologies, and kidney and cardiovascular disease, and registered trials in the recruitment phase also involve the musculoskeletal system, platelet function, and asthma (Jin et al., 2014; Kumar and Singh, 2015; Rossman et al., 2018; Ribeiro et al., 2018). With respect to the blood system, we speculate that possible situations in which mitochondrial potentiation may be worth considering include autologous stem cell transplantation in elderly patients receiving high-dose chemotherapy and anemia of the elderly. Furthermore, MMP\textsuperscript{low} HSCs that become abundant with age are characterized by myeloid skewing, upregulation of ROS and inflammatory pathways, and downregulation of DNA repair programs, all of which are predisposing factors to clonal diseases such as myelodysplastic syndrome (MDS) that primarily...
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affect patients of advanced age. Given the fact that Mito-Q at least partly resolves these age-related features at the stem cell level, we speculate that it may have a role in the prevention of age-related clonal diseases such as MDS.

Limitations of the Study
As mentioned earlier, analysis of MMP in HSCs is confounded by expulsion of mitochondrial dyes unless cellular efflux pumps are inhibited by VP, potentially accounting for conflicting published reports. We, like other recent studies (de Almeida et al., 2017; Takihara et al., 2019; Morganti et al., 2019a), observe that the MMP\textsuperscript{high} fraction is enriched for stem cell potential. Our transcriptional analyses confirm that the cells we prospectively isolate as MMP\textsuperscript{high} in the presence of VP are enriched for appropriate signatures of mitochondrial activity. While VP per se and its blocking of efflux pumps may interfere with the steady-state biology of HSCs, these transcriptional assays were conducted at very short time frames, during which no evidence of toxicity or change in cell viability was observed. However, VP’s toxicity does interfere with functional studies of HSCs in longer-term studies, and thus we could not directly compare the function of MMP\textsuperscript{high} and MMP\textsuperscript{low} HSCs. Furthermore, mitochondrial dyes themselves can inhibit aspects of mitochondrial function (Perry et al., 2011), and this level of interference may be greater in cells that retain more of the dye. We considered MitoDendra2 reporter mice as an alternative to MMP dyes, but MitoDendra2 is a marker of mitochondrial content and is consequently less useful in aging where MMP is decreased but mass is stable (Figure 1). We have shown here that MMP positively correlates with ATP content and DNA repair capacity of HSCs and negatively correlates with their ROS levels. It will be interesting to understand how MMP relates more broadly to the mitochondrial and metabolic biology of HSCs during aging, particularly in the context of their hypoxic in vivo niches; however, a current limitation is the small number of HSCs, which at the moment precludes some of these analyses.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.stem.2020.09.018.

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Figure 6. Mito-Q Treatment Can Both Revert and Prevent Onset of the Hematopoietic Aging Phenotype
(A) Schematic diagram of treatment regimen.
(B) Percentage of myeloid (Mac1/Gr1\textsuperscript{+}) cells in PB of individually measured old mice before (pre), in the middle (mid), and at the end of treatment (see Figure 5A) with the range and average (box) percentage change between pre- and end-treatment values (n = 15).
(C) As in (B), but for percentage of B cells (B220\textsuperscript{+}) (n = 15).
(D) Representative histogram of IgM expression in B cells from old (O, purple), young (Y, blue), and old Mito-Q-treated (O+MQ, lilac) mice.
(E) Distribution of myeloid and B cells in PB of old mice (purple) compared to O+MQ mice at the end of a 5-day treatment (lilac) compared to young (blue) mice (n = 15–19).
(F) Sysmex analysis of total white blood cell counts (WBC) and the percentage (Lym\%) and absolute number (Lym#) of lymphocytes in old mice at the end of Mito-Q treatment (lilac) compared to young (blue) mice (n = 10–15).
(G) Experimental overview.
(H) Left: ratio of myeloid to B cells in MA mice (green) and aged mice kept on normal drinking water (O, purple) compared to mice aged with Mito-Q water (O+MQ, lilac). Right: comparison of PB myeloid, B, and T cells between naturally aged (O, purple) or mice aged with Mito-Q water (O+MQ, lilac) at the endpoint (n = 15, MA; n = 5; O; n = 10 OMQ).
(I) Sysmex whole blood analysis of erythroid parameters of naturally aged (O) or Mito-Q-supplemented (O+MQ) mice (n = 5; O; n = 10 OMQ)
(J) BM analysis of myeloid (M), B cells, and the collective of pre-/pro-B cells (IgM\textsuperscript{+} B220\textsuperscript{+}) of naturally aged (O) or Mito-Q-supplemented (O+MQ) mice (n = 5; O; n = 10 OMQ).
(K) Representative FACS plots of erythroid progenitor populations, gated on Lin\textsuperscript{-} cells (B220\textsuperscript{-} Mac1\textsuperscript{-} Gr1\textsuperscript{-} CD3\textsuperscript{+}) in the BM of naturally aged (O) or Mito-Q-supplemented (O+MQ) mice (see also Figure S6).

Results are represented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
AUTHOR CONTRIBUTIONS

E.M. and T.E. conceived the experiments, E.M. and V.S. performed the experiments, and E.D. and J.B. performed the RNA-sequencing. C.J. and S.S. performed the bioinformatics analysis. K.M. and J.L. provided significant feedback on the project. E.M. and T.E. wrote the manuscript. V.S., J.B., E.D., K.M., and J.L. proofread and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR Methods

### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| PE/Cy5 anti-mouse TER-119 | BioLegend | Cat#116210; RRID: AB_313711 |
| PE/Cy5 anti-mouse Ly-6G/Ly-6C (Gr-1); RB6-8C5 | BioLegend | Cat#108410; RRID: AB_313375 |
| PE/Cy5 anti-mouse/human CD45R/B220; RA3-6B2 | BioLegend | Cat#103210; RRID: AB_312995 |
| PE/Cy5 anti-mouse CD3e; 145-2C11 | BioLegend | Cat#100310; RRID: AB_312675 |
| PE/Cy5 anti-mouse/human CD11b; M1/70 | BioLegend | Cat#101210; RRID: AB_312793 |
| APC-eFluor 780 anti-mouse CD117 (c-Kit); 2B8 | Invitrogen (eBioscience) | Cat#47-1171-82; RRID: AB_1272177 |
| Brilliant Violet 421 anti-mouse Ly-6A/E (Sca-1); D7 | BioLegend | Cat#108128; RRID: AB_2563064 |
| PE/Cy7 anti-mouse CD48; HM48-1 | BioLegend | Cat#103424; RRID: AB_2075049 |
| Brilliant Violet 605 anti-mouse CD150 (SLAM); TC15-12F12.2 | BioLegend | Cat#115927; RRID: AB_11204248 |
| Brilliant Violet 605 anti-mouse CD45.1; A20 | BioLegend | Cat#110738; RRID: AB_2562565 |
| PE anti-mouse CD45.2; 104 | BioLegend | Cat#109807; RRID: AB_313444 |
| PE anti-mouse IgM; RMM-1 | BioLegend | Cat#406508; RRID: AB_315058 |
| PE anti-mouse CD45.1; A20 | BioLegend | Cat#110708; RRID: AB_313497 |
| PE anti-mouse Ly-6A/E (Sca-1); E13-161.7 | BioLegend | Cat#122508; RRID: AB_756193 |
| APC anti-mouse CD3e; 145-2C11 | BioLegend | Cat#100312; RRID: AB_312677 |
| APC anti-mouse/human CD45R/B220; RA3-6B2 | BioLegend | Cat#103212; RRID: AB_312997 |
| APC anti-mouse CD48; HM48-1 | BioLegend | Cat#103412; RRID: AB_571997 |
| APC anti-mouse CD45.2; 104 | BioLegend | Cat#109814; RRID: AB_389211 |
| APC anti-mouse Ly-6G/Ly-6C (Gr-1); RB6-8C5 | BioLegend | Cat#108412; RRID: AB_313377 |
| APC anti-mouse/human CD11b; M1/70 | BioLegend | Cat#101212; RRID: AB_312795 |
| APC anti-mouse CD117 (c-Kit); 2B8 | BioLegend | Cat#105812; RRID: AB_313221 |
| APC/Cyanine7 anti-mouse CD45.2; 104 | BioLegend | Cat#109824; RRID: AB_830789 |
| FITC hamster anti-mouse CD3e; 145-2C11 | BD Biosciences | Cat#553062; RRID: AB_394595 |
| FITC rat anti-mouse CD4; H129.19 | BD Biosciences | Cat#553651; RRID: AB_394971 |
| FITC anti-mouse CD48; HM48-1 | BioLegend | Cat#103403; RRID: AB_313018 |
| FITC anti-mouse CD45.1; A20 | BioLegend | Cat#110706; RRID: AB_313495 |
| FITC anti-mouse CD71; RI7217 | BioLegend | Cat#113806; RRID: AB_313567 |
| PerCP rat anti-mouse CD8a; 53-6.7 | BD Biosciences | Cat#553036; RRID: AB_394573 |
| PE/Cy7 anti-mouse TER119 | eBioscience | Cat#25-5921-82; RRID: AB_469661 |
| PE/Cy7 anti-mouse CD150; TC15-12F12.2 | BioLegend | Cat#115914; RRID: AB_439797 |
| PE/Cy7 anti-mouse CD45.1; A20 | BioLegend | Cat#110730; RRID: AB_1134168 |
| BV421 Rat Anti-Mouse CD135; A2F10.1 | BD Biosciences | Cat#652898; RRID: AB_273787 |
| PE anti-mouse CD127 (IL-7Rα); A7R34 | BioLegend | Cat#135010; RRID: AB_1937251 |
| Zombie Red Fixable Viability dye | BioLegend | Cat#423109 |
| FITC anti-H2A.X Phospho (Ser139); 2F3 | BioLegend | Cat#613404; RRID: AB_528919 |
| Chemicals, Peptides, and Recombinant Proteins | STEMCELL technologies | Cat#07800 |
| Ammonium Chloride Solution | ThermoFischer | Cat#D1306 |
| 4',6-diamidino-2-phenylindole (DAPI) | ThermoFischer | Cat#E10345 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| o-propargyl puromycin (OPP) | Jena Bioscience | Cat#Nu-931-5 |
| Mitoquinol (for injection) | Cayman Chemicals | Cat# 89950 |
| Mitoquonol (for drinking water supplementation) | MitoQ Limited | gifted |
| CCCP | Sigma Aldrich | Cat# C2759 |
| 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) | Sigma Aldrich | Cat# D1916 |
| Actinomycin-D | Sigma Aldrich | Cat# A1410 |
| Flavopiridol | Sigma Aldrich | Cat# F3055 |
| Verapamil | Sigma Aldrich | Cat# V4629 |
| Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM) | ThermoFischer | Cat# I34361 |
| Tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE) | ThermoFischer | Cat# T669 |
| 1,1',3,3',3''-hexamethylylindocarboxyanine iodide (DiIC1(5)) | ThermoFischer | Cat# M34151 |
| Mitotracker Green | ThermoFischer | Cat# M7514 |
| JC-1 | ThermoFischer | Cat# M34142 |

Critical Commercial Assays

| FITC anti-mouse Ki-67 kit | BD PharMingen | Cat#556026 |
| CD117 Microbeads, mouse | Miltenyi Biotec | Cat#130-091-224 |
| Click-IT RNA Alexa Fluor 488 Imaging Kit | ThermoFischer | Cat#C10329 |
| Click-IT Plus OPP Alexa Fluor 488 Protein Synthesis Assay Kit | ThermoFischer | Cat#C10456 |
| DCFDA/H2DCFDA- Cellular ROS Assay Kit | Abcam | Cat#fab113851 |
| Luminescent ATP Detection Assay Kit | Abcam | Cat#fab113849 |
| MitoSOX Red Mitochondrial Superoxide Indicator | N/A | Cat#M36008 |
| RNeasy Micro Kit | QIAGEN | Cat#74004 |
| Agencourt AMPureXP magnetic beads | Beckman Coulter | Cat#A63881 |
| Bioanalyser high sensitivity DNA chip | Agilent | Cat#5067-4626 |
| Bioanalyser RNA 6000 Picco chip | Agilent | Cat#5067-1513 |
| Qubit High Sensitivity DNA kit | ThermoFisher | Cat#Q32851 |
| Nextera XT DNA Preparation Kit | Illumina | Cat# FC-131-1096 |
| NextSeq500/550 High Output Kit v2.5 | Illumina | Cat#20024908 |

Deposited Data

| RNA seq #1 (Figures 3 and S3) | This paper | GEO: GSE156805 |
| RNA seq #2 (Figures 4 and S4) | This paper | GEO: GSE156807 |

Experimental Models: Organisms/Strains

| C57BL/6 JRj mice | Janvier | RRID: MGI:2670020 |
| C57BL/6 NRj mice | Janvier | RRID: MGI:6236253 |
| C57BL/6 x B6.SJL mice | In house | N/A |
| B6.SJL | In house | N/A |

Oligonucleotides

See Table S1

Software and Algorithms

| GSEA | Broad Institute | https://www.gsea-msigdb.org/gsea/index.jsp |
| CellRadar | Dhapola et al., personal communication | https://karlsson.github.io/cellradar/ |
| Flowjo v10 | BD | https://www.flowjo.com |

(Continued on next page)
RESOURCES AVAILABILITY

Lead Contact
Further information and requests for resources or reagents should be directed to and will be fulfilled by the Lead Contact, Tariq Enver (t.enver@ucl.ac.uk).

Materials Availability
This study did not generate new materials or reagents.

Data and Code Availability
RNA-seq data are available at NCBI GEO with the following reference series: GSE156808 and accession numbers: GSE156805 and GSE156807.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
Young adult (8-16 weeks) and Aged (18-24 months) C56Bl/6Jrj mice (CD45.2), as well as young (8-16 weeks) C56Bl/6Njrj mice (CD45.2) were purchased from Janvier Labs. Aged mice were analyzed at 18-20 months of age, unless indicated otherwise. B6.SJL (CD45.1) mice and C56Bl/6 x B6.SJL mice (CD45.1/CD45.2) used for transplantation experiments were generated in house. Mice were housed in plastic cages in a controlled environment with 12-hour light-dark cycles and chow and water were provided ad libitum. Experiments and animal care were performed in accordance with the Lund University Animal Ethical Committee.

METHOD DETAILS

Peripheral Blood analysis
PB was collected from the tail vein into EDTA-coated microvette tubes (Sarstedt Cat# 20.1341.100) and placed on a roller to prevent coagulation. Whole blood count analysis was performed using an automated hematology analyzer (Sysmex KX-21N). For FACS analysis, PB was lysed in ammonium chloride solution (STEMCELL technologies) for 10 min at room temperature and washed twice prior to staining for cell surface markers for 45 min at 4°C in the dark. Cells were washed and stained for cell surface markers for 45 min at 4°C in the dark. Cells were washed in FACS buffer (PBS+2% FBS) and filtered prior to flow cytometric analysis.

Bone Marrow analysis
Mice were euthanized by spinal dislocation followed by the dissection of the spinal bones and both right and left femurs, tibias and iliacs. Bones were crushed using a pestle and mortar and bone marrow cells were collected in 20 mL ice-cold FACS buffer (PBS+2% FBS), filtered and washed (350xg, 5 min). BM cells were either c-kit enriched using CD117 beads and magnetic separation (MACS Miltenyi Biotec, according to manufacturer’s instructions) when progenitor populations were analyzed, or simply lysed for red blood cells (ammonium chloride solution, STEMCELL technologies) for 10 min at room temperature, when mature populations were analyzed. Cells were washed and stained for cell surface markers for 45 min at 4°C in the dark. Cells were washed in FACS buffer and filtered prior to FACS sorting or flow cytometric analysis.

FACS sorting and analysis
For HSC sorting, bone marrow was c-Kit enriched and stained for HSC-SLAM cell surface markers (Lineage (B220, CD3ε, Ter119, Mac1, Gr1), cKit (CD117), Sca1, CD48, CD150, see table for antibody specification), washed, filtered and kept on ice as single cell
After cell surface staining, cells were washed and incubated for 20-30 min at 37°C. Mitochondrial membrane potential and mass were measured using the DCFDA/H2DCFDA kit (Abcam, Cat#ab113851) according to manufacturer’s instructions. Cells were washed in 1X buffer and analyzed by Flow Cytometry. Verapamil inclusion in this staining resulted in significantly increased levels of dye fluorescence across bone marrow lineages including HSCs (data not shown) and therefore verapamil was not included in this staining. Dye sensitivity at this concentration was validated by measuring dye fluorescence after tert-butyl hydroperoxide (TBHP) exposure which readily increases DCFDA signal.

Mitochondrial superoxide was measured by FACS following cell surface staining using MitoSOX Red Mitochondrial Superoxide Indicator (ThermoFisher, Cat#ab113808). Cells were stained with MitoSOX (5µM) for 30 min at 37°C and analyzed by Flow Cytometry. Dye sensitivity of all dyes at the used concentrations was validated by measuring dye fluorescence after tert-butyl hydroperoxide (TBHP) exposure which readily increases MitoSOX signal.

Global transcription rate was measured in vivo through Click-iT detection of incorporation of labeled nucleotide over a specific period of time (Jao and Salic, 2008). In brief, mice were intraperitoneally injected with 0.5 mg alkylene-labeled 5-ethyluridine (5-EU; ThermoFisher, Cat# E10345) which readily incorporates into nascent RNA. Titration of 5-EU was done to ensure signal saturation in all BM lineages at the injected dose (Figure S2A). Mice were sacrificed one hour later after which BM was harvested and stained for cell surface markers. Cells were fixed and permeabilised (Cytofix/Cytoperm, BD, Cat# 554714) for 20 min at 4°C washed in 1X buffer and analyzed by Flow Cytometry. Verapamil inclusion in this staining resulted in significantly increased levels of dye fluorescence across bone marrow lineages including HSCs (data not shown) and therefore verapamil was included in this staining. Dye sensitivity at this concentration was validated by measuring dye fluorescence after tert-butyl hydroperoxide (TBHP) exposure which readily increases MitoSOX signal.

Intracellular ROS levels were measured by FACS following cell surface staining, using the DCFDA/H2DCFDA kit (Abcam, Cat#ab113851) according to manufacturer’s instructions. Cells were stained with 20µM DCFDA in 1X buffer for 30 min at 37°C, washed in 1X buffer and analyzed by Flow Cytometry. Verapamil inclusion in this staining resulted in significantly reduced levels of dye fluorescence across bone marrow lineages including HSCs (data not shown) and therefore verapamil was not included in this staining. Dye sensitivity at this concentration was validated by measuring dye fluorescence after tert-butyl hydroperoxide (TBHP) exposure which readily increases DCFDA signal.

Intracellular mitochondrial superoxide was measured by FACS following cell surface staining using MitoSOX Red Mitochondrial Superoxide Indicator (ThermoFisher, Cat#ab113808). Cells were stained with MitoSOX (5µM) for 30 min at 37°C and analyzed by Flow Cytometry. Dye sensitivity of all dyes at the used concentrations was validated by measuring dye fluorescence after tert-butyl hydroperoxide (TBHP) exposure which readily increases MitoSOX signal.

The transcription/translation rate analysis

To circumvent the incompatibility of the Click-iT protocol which requires fixation and permeabilisation with the staining for MTG, TMRM or DiIC1(5) whose signal is not retained upon fixation, a two-step protocol was used. Cells were FACS sorted based on 30% highest or lowest MMP after in vivo incubation with labeled 5-EU. MMPhigh or MMPlow sorted cells were then fixed, permeabilised, followed by a Click-iT reaction to label the incorporated 5-EU and re-analyzed by FACS to measure transcriptional rate in relation to MMP.
lution) for 60 min at 4 °C, washed (500xg for 5 min) and stained with FITC-Ki-67 (1:100 concentration in wash/perm solution) and stained for 60 min at 4 °C, and washed. Cell concentration was kept below 2x10^6/ml. DAPI was added at a final concentration of 1µg/ml (or 5x10^-13 g/cell); any sub-dilution was made in H2O. Cells were analyzed by FACS (LSRII; BD), on low flow rate ensuring that acquisition rate always remained below 1000 events/s.

**DNA damage analysis**

DNA double strand breaks were measured using staining for γ-H2AX (phosphorylation of Ser139 of H2A histone family member X which occurs upon DNA double strand breaks) followed by FACS analysis, either at steady state or after irradiation. Mice received a 2Gy single dose of irradiation and the femur and tibia were harvested 30 min, 1 hr, 2 hr or 4 hr later. We first validated that γ-H2AX staining in young HSCs peaked at 30 min-1 hr after a single dose of 2Gy irradiation and recovers to pre-irradiation levels around 4 hr post-irradiation (data not shown), and therefore γ-H2AX was subsequently measured at 30 min-1 hr after irradiation. For γ-H2AX detection, BM cells were stained using conventional SLAM markers (LSK CD48 CD150+) before they were fixed using the BD Fix/Perm kit (Cytofix/Cytoperm, BD, Cat# 554714) for 30 min. Cells were subsequently permeabilised using the BD Fix/Perm solution + 0.25% of Triton X-100 for 30 min followed by incubation with γ-H2AX antibody (Abcam, 5uL per 100ul, according to manufacturer’s instructions) for 30 min. Cells were then stained with Zombie Red Fixable Viability dye (Biolegend, according to manufacturer’s instructions) to allow for dead cell exclusion. Samples were analyzed and data collected using LSRII flow cytometer.

**Chemical treatment of mice**

Mitoquinol was either administered through intra-peritoneal injection (2 mg/kg body weight, Cayman Cat# 89950) or administered orally through drinking water supplementation (350 mM (kindly provided by MitoQ Limited) dissolved in sucrose-supplemented water (5g/L) which was given to both the MitoQ and the control group.

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 0.3-1.0 mg/kg bw, Sigma), Actinomycin-D (0.1-1.0 mg/kg bw, Flavopiridol (0.5-5.0 mg/kg bw) and 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB; 1-10 mg/kg bw) were administered through single intraperitoneal injection. All chemicals were administered in 200uL PBS (with < 10% DMSO when initial stock solutions were prepared in DMSO).

**Mito-Q treatment in vitro**

Short-term (2 hr) in vitro treatment with Mito-Q was performed to directly assess the impact of Mito-Q on MMP and DNA damage repair in HSCs from old mice.

For assessment of DNA damage repair, bone marrow cells were harvested 1 hr after mice were exposed to 2Gy of irradiation. cKit-enriched cells were then seeded in 48-well plates at a concentration of 5*10^5 cells/well in 150 uL of StemSpan SFEM (STEMCELL technologies) supplemented with 1% Penicillin/Streptomycin (HyClone), with or without Mito-Q at 0.5, 1, 2, 4 or 8 uM concentrations, for a 2 hr incubation at 37 °C. Cells were then washed, stained for cell surface antibodies followed by staining DNA damage marker γ-H2AX.

A similar protocol was used for assessment of MMP after short-term (2hr) in vitro treatment with Mito-Q, albeit cells were harvested from old mice at steady state in absence of irradiation insult.

**RT-PCR gene expression analysis**

For real-time quantitative polymerase chain reaction (RT-qPCR), cells were sorted into 350uL RLT lysis buffer (QIAGEN) and total RNA was isolated (RNaseasy Micro, QIAGEN) according to manufacturer’s instructions. cDNA was prepared through reversed transcription (Superscript III First Strand Synthesis kit, Invitrogen) using a T100 thermal cycler (BioRad). qRT-PCR analysis was performed using Taqman gene expression mastermix (ThermoFisher Cat#4369016) and primers (Taqman real-time PCR primers (FAM), see resource table for primer specifications) on a 7900HT Fast Real-Time PCR system (iScience). Gene expression was normalized to B2m expression (Matsuzaki et al., 2015).

**RNA sequencing (low cell number bulk)**

HSCs (1000 per sample) were sorted directly into 800 µL TRIzol Reagent® (ThermoFisher, Cat#15596018) and frozen at –80 °C. Total RNA was extracted from the aqueous phase after addition of 200 µL chloroform and precipitated by mixing an equal volume of isopropanol supplemented with 5 µg of linear polyacrylamide (Sigma). The RNA pellet was washed twice with fresh 80% ethanol and resuspended in 5 µL RNase-free water. 1 µL of RNA was quantified on an Agilent Bioanalyser RNA 6000 Pico chip. 100pg of RNA were used to generate cDNA using a modified Smart-seq2 protocol (Picelli et al., 2014). cDNA was amplified using SeqAmp Polymerase (Takara) and 16 cycles at thermal conditions recommended by the manufacturer. The resulting cDNA libraries were purified with Agencourt AMPureXP magnetic beads (0.8 to 1 beads to cDNA ratio), washed twice with fresh 80% ethanol and eluted in 15 µL EB buffer. 1 µL cDNA was quality checked on an Agilent Bioanalyzer high sensitivity DNA chip and quantified using Qubit High Sensitivity DNA kit (Thermo Fisher, Cat# Q32851). Sequencing libraries were produced from the cDNA libraries using Nextera XT DNA Preparation Kit (Illumina) following a modified version of the manufacturer’s protocol with 20-fold volume reduction (2.5 µL final volume). 50pg of cDNA were tagmented followed by 13 cycles of amplification. Equimolar amounts of each sequencing library were ascertainment and pooled using the Echo 525 liquid handler (Labcyte). Pool was purified with 0.8x Agencourt AMPureXP magnetic beads, eluted in 25 µL EB buffer, quality checked on an Agilent Bioanalyzer high sensitivity DNA chip and quantified using Qubit High...
Sensitivity DNA kit. Pool was diluted to a final concentration of 2pM and sequenced on the Illumina NextSeq 500 platform using NextSeq500/550 High Output Kit v2.5 (2x76 paired end cycles).

**Differential Gene Expression Analysis**

Raw reads were quality controlled and trimmed using Trim Galore ([https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/](https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)) prior to mapping using STAR v2.7 against the mouse GRCm38.95 assembly. Gene counts were produced using QoRTs (26187896) after which duplicates were managed using Markduplicates from Picard v4.0.1.1 ([https://broadinstitute.github.io/picard/](https://broadinstitute.github.io/picard/)) and DupRadar (27769170). Samples were clustered using the hclust function for R using correlation distance, and differentially expressed genes were identified using DESeq2 (25516281).

**Gene Set Enrichment Analysis** was performed ([Subramanian et al., 2005](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2652088/)), computing overlap with our complete raw dataset and MSigDB gene sets provided by the software (Hallmark and KEGG), as well as published gene sets: MitoCarta2.0 (Broad Institute) and Novershtern et al. (2011).

**Gene ontology analysis** was performed by computing overlap between our differentially expressed gene sets and known gene ontology signatures using DAVID Bioinformatics Resources v6.8 (Laboratory of Human Retrovirology and Immunoinformatics (LHRI)).

**CellRadar**

Visualization of the differentially expressed gene was performed using software CellRadar ([https://karlsson.github.io/cellradar/](https://karlsson.github.io/cellradar/)); developed by the G. Karlsson lab, Lund University, (Parashar Dhapola et al., personal communication) based on normalized gene expression data from normal human hematopoiesis (HemaExplorer) from the Bloodspot database ([http://servers.binf.ku.dk/bloodspot/](http://servers.binf.ku.dk/bloodspot/)).

**Transplantation assays**

Recipient mice were lethally irradiated (900cGY) 6-12 hr prior to transplantation. Isolated HSCs from young (100 HSCs) and old mice (300 HSCs), together with 200,000 whole BM support cells were transplanted in 350uL FACS buffer (PBS+2%FBS) through intravenous tail vein injection. Drinking water of recipient mice was supplemented with ciprofloxacin (125mg/L, HEXAL) for 3 weeks to prevent infection during this period of myelo-ablation. Peripheral blood was analyzed for donor engraftment and lineage kinetics at weeks 3, 4, 6, 10 and 16 after transplantation. Bone marrow engraftment, lineage kinetics and MMP status were analyzed at the 16-week endpoint.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was performed in Graphpad Prism 7. Results are represented as mean ± SEM. The number of experimental subjects per experiment is given (n = ) and these are generated from 2, 3 or 4 independently repeated experiments unless otherwise stated. When comparing more than 2 groups, ANOVA was performed with post hoc Tukey correction for multiple comparisons. When ANOVA showed significant difference (p < 0.05), multiple comparison analysis was performed using unpaired Student’s t test, unless otherwise stated. When only 2 groups were compared, an unpaired Student’s t test was used, unless otherwise stated. Statistical significance is indicated in figures as follows: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. In bulk RNA-sequencing analysis, differentially expressed genes are defined as having a greater than 2-fold change in expression and a false discovery rate below 5% (Figures 3 and S3) or below 10% (Figures 4 and S4).