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The Epigenetic Basis of Hematopoietic Stem Cell Aging

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Abstract

Highly proliferative tissues such as the gut, skin and bone marrow lose millions of cells each day to normal attrition and challenge from different biological adversities. To achieve a lifespan beyond the longevity of individual cell types, tissue-specific stem cells sustain these tissues throughout the life of a human. For example, the lifespan of erythrocytes is about 100 days and adults make about two million new erythrocytes every second. A small pool of hematopoietic stem cells (HSCs) in the bone marrow is responsible for the lifetime maintenance of these populations. However, there are changes that occur within the HSC pool during aging. Biologically, these changes manifest as blunted immune responses, decreased bone marrow cellularity, and increased risk of myeloid diseases. Understanding the molecular mechanisms underlying dysfunction of aging HSCs is an important focus of biomedical research. With advances in modern health care, the average age of the general population is ever increasing. If molecular or pharmacological interventions could be discovered that rejuvenate aging HSCs, it could reduce the burden of age related immune system compromise as well as open up new opportunities for treatment of hematological disorders with regenerative therapy.

Functional and Molecular Manifestations of HSC Aging

Hematopoietic stem cells are the quiescent, multipotent precursors of the hematopoietic system that reside in specialized niches in the bone marrow and regenerate the hematopoietic system for the life of the organism. HSCs possess two unique functional properties that distinguish them from other cells of the hematopoietic system – the ability to self-renew and generate daughter HSCs which maintain the HSC pool over time, and the capacity for multilineage differentiation to generate all the effector cells of the blood and bone marrow. Over time, the aging process exerts fundamental changes in both of these

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processes in HSCs. Compared to their young counterparts, old HSCs are characterized by diminished capacity for self-renewal, and a bias in their lineage output towards myeloid lineages[1–6].

The diminished self-renewal capacity of old HSCs seems to be partly linked to their replicative history, as HSCs which have undergone more cell divisions (either biologically induced by age or artificially induced by experimentation) have diminished potential in transplantation assays[7]. But exactly how self-renewal capacity is eroded over time at the molecular level remains to be determined. The observation that old HSCs are more biased towards myeloid cell output can at least be partly explained by the changing dynamics of the stem cell pool over time. Many groups have now experimentally validated that different subtypes of HSCs exist in the bone marrow with inherent biases in lineage output[8–13]. Aging does not necessarily change the lineage output of individual HSCs, but changes the composition of the HSC pool over time. While lineage-balanced (HSCs that generate blood cell types in the range of that found in an unmanipulated mouse) and lymphoid-biased (generate proportionally more B- and T-cells than myeloid cells) predominate in young bone marrow, the composition of the compartment shifts during age to become dominated by myeloid-biased (or more accurately termed lymphoid-deficient) HSCs[2,3,14]. Given the observation that these differentiation biases are stable over time, this suggests that at least certain aspects of their behavior may be epigenetically pre-programming.

Epigenetic Modifications Guide HSC Fate Decisions

The term epigenetics refers to changes in gene expression that does not involve changes to the underlying DNA sequence: a change in phenotype without a change in genotype. Epigenetic modifications act like a blueprint to maintain cell identity by informing each specific cell type which genes should be expressed and to what level. Unlike the underlying genetic sequence, epigenetic modifications are malleable and can be altered in response to signals from the environment. Drifting of the epigenetic landscape over time may be responsible for much of the age-related dysfunction of old HSCs.

Epigenetic Modifications to Cytosine Nucleotides

While the protein-coding information in the genome is encoded by the sequence of the four nucleotides, additional epigenetic information can be encoded by chemical modifications to the cytosine base. One such modification occurs when the carbon 5 hydrogen of the cytosine base is replaced by a methyl group, forming 5-methylcytosine (5-mC). 5-mC encodes the same nucleotide sequence as unmodified cytosine and does not affect Watson-Crick base pairing. 5-mC almost always occurs at cytosines in CpG dinucleotides and in mammals, 60–90% of all CpGs are methylated[15,16]. Normal biological functions of DNA methylation include X-chromosome inactivation, silencing of repetitive elements, and maintenance of parental imprinting patterns[17]. The pattern of 5-mC distribution can influence binding of regulatory proteins to target sequences in the genome and impact subsequent expression levels of downstream genes. This function of DNA methylation can help orchestrate physiological processes such as stem cell differentiation, and pathologically contribute to oncogenesis.

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DNA methylation is established by the DNA methyltransferase enzymes, of which there are three catalytically-active members in mammals. The principle function of Dnmt1 is to reestablish existing DNA methylation patterns during cellular replication by recognizing hemimethylated DNA and copying pre-existing DNA methylation marks from the parental strand to the daughter strand[18]. Dnmt1 has specific and important functions in HSCs as genetic inactivation leads to nearly immediate and complete loss of HSCs *in vivo*[19]. Moreover, HSCs from mice with reduced Dnmt1 activity become restricted to myeloerythroid differentiation due to both impaired silencing of key lineage determinant genes such as Gata1, Id2 and CEBP/α, and an inability to prime master lymphoid regulators such as Ebf1, Pax5 and Il7rα[20]. DNMT1 is upregulated in some acute myeloid leukemia (AML) patients, and inhibition of Dnmt1 impairs leukemogenesis and inhibits leukemia stem cell function in a mouse model of MLL-AF9 driven AML[21]. Dnmt3a and Dnmt3b act as *de novo* DNA methyltransferase enzymes which coordinate the establishment of new DNA methylation patterns during development and stem cell differentiation[22]. Dnmt3a regulates HSC fate decisions as evidenced by conditional inactivation of Dnmt3a, which skews HSC divisions towards self-renewal divisions at the expense of differentiation. This fate bias of Dnmt3a-null HSCs leads to an accumulation of mutant HSCs in the bone marrow and a relative dearth of their differentiated progeny in the blood[23]. Over time, the aberrant hematopoiesis resulting from loss of Dnmt3a leads to bone marrow failure and mice succumb to anemia and cytopenias[24,25]. While deletion of Dnmt3b in adult HSCs seems to be dispensable for HSC function, conditional inactivation of Dnmt3b in a Dnmt3a-null HSC background exacerbates the self-renewal phenotype suggesting Dnmt3b collaborates with Dnmt3a to confer differentiation potential[26]. Underscoring the importance of these enzymes in hematopoiesis, DNMT3A is one of the most recurrently mutated genes in myeloid malignancies such as AML[27,28] and myelodysplastic syndromes (MDS)[29]. Conversely, DNMT3B is almost never mutated in these diseases, but may also have tumor suppressor function[30,31].

HSC aging is associated with changes to the DNA methylome. Old HSCs are characterized by a gradual increase in global DNA methylation levels[32], a hypermethylation phenotype that contrasts with aging in post-mitotic somatic cells which show global hypomethylation. The mechanism for HSC hypermethylation during aging has not been defined. As a group, the genes encoding DNA methyltransferase enzymes decrease in expression in old HSCs compared to young[32], although this does not account for the expression and function of specific isoforms of Dnmt3a and Dnmt3b. A substantial proportion of differentially methylated regions (DMRs) that change with HSC aging are associated with lineage determinant factors. DMRs that increase in 5-mC content can be localized to genomic loci associated with lymphoid and erythroid lineages[33], cell types that decline during aging. This would be consistent with the dogma of DNA methylation acting predominantly as a repressive epigenetic mark. The alterations in DNA methylation at genomic loci in aging HSCs does not correlate with accompanying changes in expression levels of downstream genes. This lack of correlation between DNA methylation and gene expression suggests that alterations in DNA methylation do not necessarily produce functional consequences for the HSCs themselves, but serves as a heritable readout of their lineage potential which ultimately manifests in their downstream progeny.

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Cytosine hydroxymethylation (5-hmC) is another important epigenetic modification to the cytosine base. Similar to 5-mC, hydroxymethylation replaces the hydrogen atom at carbon 5 of cytosine with a hydroxymethyl group. 5-hmC is produced by oxidation of 5-mC by the ten-eleven translocation (TET) family of enzymes, and is thought to be an initial step in a potential demethylation pathway[34,35]. However, the observations that (1) 5-hmC is stable, (2) can be found in relative abundance in certain cell types, and (3) can influence gene expression suggests 5-hmC is a unique commodity and an important part of the regulatory epigenome. While 5-hmC is rare in most somatic cells (<0.2% of all CpGs), it is relatively enriched in pluripotent embryonic stem (ES) cells, and in other adult somatic stem cells[34,36,37]. Mouse mutant models and patient genome sequencing studies reinforce important roles for the Tet enzymes (Tet1, Tet2, Tet3) in hematopoiesis. Along with DNMT3A, TET2 is one of the most recurrently mutated genes in myeloid malignancies[28,38–40]. Murine Tet2-null HSCs have increased self-renewal and competitive repopulation capacity compared to wild-type HSCs and increased myeloid cell output[41–43]. Over the long-term, Tet2-null mice develop a chronic myelomonocytic leukemia (CMML)-like disease, but with long latency and variable penetrance[41]. However, Tet2-null mice acutely depleted for Tet3 develop a fully-penetrant and highly aggressive myeloid leukemia within a few weeks[44]. While Tet1 is rarely mutated in human hematopoietic pathologies, conditional deletion in mouse HSCs increases B-cell output and leads to development of B-cell malignancies[45]. This presents an interesting dichotomy for the epigenetic regulation of B-cell potential in HSCs as Dnmt1 seems necessary for B-lineage priming, while Tet1 may restrict B-cell output.

Corresponding with the global DNA hypermethylation of aging HSCs, there is a concomitant reduction in 5-hmC in old HSCs compared to young[32]. While all three Tet enzymes are expressed in HSCs, their levels decrease in abundance with age. The role of 5-hmC in gene regulation is still controversial. Although the presence of 5-mC at gene promoters and enhancers is generally associated with gene repression, accumulation of 5-hmC is associated with active genes[46–48]. While technical advances have facilitated mapping of 5-mC and 5-hmC patterns at unprecedented resolution and permitted correlative observation, our ability to assign functional significance of the modification at specific CpG residues has remained limited. Technological advances such as adaptation of CRISPR/Cas9 systems for site-specific epigenome editing should help address these questions[49,50]. As abnormal DNA methylation and hydroxymethylation patterns are widely reported in a variety of human blood disorders, assigning functional significance to the placement of these marks is an important priority for biomedical research.

Post-Translational Modifications to Histone Tails

Each human cell has about six feet of linear DNA which is compacted into higher-order structures in the nucleus by wrapping around histone proteins. Covalent post-translational modifications (including methylation, phosphorylation, acetylation, ubiquitylation and sumoylation) to specific amino acid residues of histone tails are another major mechanism of epigenetic regulation. These histone modifications can impact gene expression by altering chromatin structure to make it more/less accessible to transcription factors and the transcriptional machinery, or by recruiting “readers” of the histone code.

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Global profiling of histone modifications is performed by chromatin immunoprecipitation (ChIP) followed by next-generation sequencing (ChIP-seq). Although emerging technologies have reduced the scale of material required for such assays[51,52], the large cell numbers required to generate sufficient input DNA for ChIP-seq coupled with the limited numbers of long-term HSCs that can be obtained per animal have hindered genome-wide profiling of chromatin marks in HSCs. As such, current genome-wide studies have focused on histone modifications with well-characterized epigenetic roles. Trimethylation of lysine 4 on histone 3 (H3K4me3) is a chromatin landmark that is present at the transcription start sites of protein-coding genes that are either actively transcribed or primed to do so. H3K4me3 protects promoters from DNA methylation, and helps keep the chromatin in an open state for access of the transcriptional machinery. In mammals, at least six genes (predominantly MLL family members) encode for histone methyltransferases that catalyze H3K4me3. Old HSCs exhibit a modest increase in the total number H3K4me3 peaks compared to their young counterparts[32]. But more interestingly, the breadth of these peaks at existing marks expands considerably[32]. The spreading of H3K4me3 from existing H3K4me3 peaks could be an important contributing factor to the dysfunction of aging HSCs as they are enriched for genes associated with HSC self-renewal and loss of differentiation capacity. There is a strong correlation between altered H3K4me3 levels and transcriptional activity as some of the genes that increase in expression level most significantly with age (e.g. Selp, Nupr1, Sdpr, Plscr2, HoxB cluster genes) are those which gain the most H3K4me3[32].

The effect of H3K27me3 is unambiguously and strongly repressive[53,54]. H3K27me3-associated domains congregate in silenced foci that inhibit transcription by preventing access to RNA polymerase II and other trans-factors[55,56]. However, this repressive state is both temporally and spatially dynamic. Accordingly, the PRC2 proteins and appropriate regulation of H3K27me3 have been shown to be necessary for differentiation and maintenance of cell type identity in organisms across eukaryotes[57,58], and dysregulation of H3K27me3 is implicated in the genesis and progression of cancer[59,60]. Consistent with the importance of this epigenetic mark in stem cell function, depletion of each of the core PRC2 components (Ezh2, Suz12, Eed) in HSCs severely compromises hematopoiesis. Loss of Eed and Suz12 in HSCs leads to a significant reduction in the number of phenotypically-defined HSCs, compromised function in competitive repopulation assays, and reductions in global H3K27me3[61–63]. While genetic inactivation of Ezh2 has little effect on HSC homeostasis, this may be complicated by compensation by Ezh1[61]. However, over-expression of Ezh2 preserves HSC self-renewal potential over serial transplantation[64], and inhibition of both Ezh1 and Ezh2 is catastrophic for HSCs[61], showing the importance of this PRC2 component in hematopoiesis. As HSCs age, there appears to be essentially no change in the distribution and total number of H3K27me3 peaks. But the breadth of the peaks increases by ~29%, and the H3K27me3 signal intensity at transcription start sites increases by ~50%[32]. This pattern may suggest a gradual evolution of this mark over time in HSCs to lock down epigenetic repression of non-HSC genes, or Darwinian selection for HSCs with broader and stronger H3K27me3 peaks during aging.

In pluripotent ES cells and multipotent adult somatic stem cells, many promoters of crucial developmental genes are marked by both the activating H3K4me3 and repressive

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H3K27me3 histone modifications. Such loci are termed bivalent domains, and are thought to represent a gene poised status ready for rapid activation/repression depending upon the fate choice during lineage commitment[57]. Accordingly, in the hematopoietic system the frequency of bivalent promoters is highest in HSCs which progressively declines during differentiation as these domains are resolved in lineage-restricted cells[65]. Genes with a bivalent promoter status are generally not expressed or lowly expressed because of the presence of the repressive H3K27me3 mark. In young HSCs, a large number of H3K27me3-marked loci are also enriched for the H3K4me3 histone modification, and the total number of bivalent domains is comparable to the number found in ES cells (~2000)[32]. The bivalent domains present in HSCs include many master transcription factors involved in HSC fate determination such as C/EBPα, Ebf1, Pax5, Meis1, Gata2 and Gata3. Over one-third of the bivalent genes are glycoproteins involved in key HSC signaling pathways including Wnt, Hedgehog, BMPs and TGFβ[32]. As HSCs age, there is a net increase in the number of bivalent domains, which occurs due to gains of both H3K4me3 and H3K27me3 (entirely new bivalent domains), or H3K27me3 only at promoters with existing H3K4me3[32]. However, the mechanisms of this cannot be explained by changing expression levels of the writers and erasers of these modifications, and the functional consequences for HSCs remains to be determined.

Are Epigenome Changes Intrinsic to Each HSC Over Time or Reflect the Reduced Clonal Diversity of the Aging HSC Pool?

HSC aging can be characterized in terms of the underlying epigenetic changes. However, because of technical limitations it remains to be determined if these epigenetic changes represent inherent changes to aging HSCs, or reflect changes in the composition of the HSC pool over time. The aging pool of HSCs in the bone marrow becomes enriched for myeloid-biased HSCs with the highest self-renewal potential[2,3]. Because all of the assays used to define epigenome features require pools of HSCs, the observed changes in aging epigenetic features may represent different proportions of lineage-biased HSCs in the young/old HSC pools (Figure 1). For a hypothetical example, if Flt3 is marked by H3K27me3 in myeloid-biased HSCs, but less in lymphoid-biased HSCs, then it would appear that H3K27me3 increases at Flt3 as HSCs age. The observation that Flt3 H3K27me3 increases with age, however, could be alternatively explained by the skewing in ratio of myeloid-biased to lymphoid-biased HSCs in the assay sample as lymphoid-biased HSCs may represent up to 50% of the young HSC pool, but less than 10% of the old HSC pool. Even if strict sorting gates are used for flow cytometric purification of HSCs from young and old mice, currently there is little way to avoid this as the trade-off for including more stringent gates for HSC subtypes (e.g. CD150^{high}, CD229, Hoechst low) would mean a final yield of HSCs typically insufficient for the assay.

To truly resolve the epigenetic changes intrinsic to aging HSCs, single cell resolution is required for epigenome profiling. Methods are currently being adapted for transcriptional profiling of single cells, but such methods still need to be developed for profiling epigenetic marks. However, recent technological advances in ChIP-seq adaptations have reduced the input material to a point (~10,000 cells) that is reasonably achievable for HSC subtype

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purification[51,52]. Single cell epigenome profiling may one day be possible with the rate of innovation of next-generation sequencing approaches. Coupling such technology with new strains of fluorescent reporter mice[66–68] which isolate more pure populations of functional long-term HSCs should provide the power to answer these important questions.

Is Aging a Byproduct of HSC Epigenome Changes Over Time?

If epigenetic changes are mechanistically shown to be a driver of the aging HSC phenotype, then this raises the possibility that resetting the epigenome to a more youthful state may help restore the functional potential of old HSCs. This premise of this concept has been demonstrated at least indirectly by reprogramming studies. Using transcription-based methods to first reprogram young or old hematopoietic progenitors to induced pluripotent stem (iPS) cells, re-differentiation of these lines back into blood lineages does not show a functional deficit in the potential of re-derived old cells[69]. This suggests that resetting the old epigenome can overcome the functional deficits of HSC aging. Although global changes to the epigenome occur during aging, it is more likely that the functional decline in aging HSCs is due to specific changes to key regulatory loci. Examining the molecular changes associated with other approaches of rejuvenating old HSCs such as transcription factor-based reprogramming to induced HSCs (iHSCs)[70,71], calorie restriction[72], or exposure to a young circulatory system through parabiosis[73] may identify epigenetic differences at critical loci which enforce the aging phenotype. Reversal of these specific modifications may provide a mechanism to restore self-renewal and lymphoid differentiation potential to old HSCs. Current epigenetic therapies (some of which are used clinically for treatment of hematopoietic malignancies) cause global changes to the epigenome. To more efficaciously reactivate the potential of old HSCs, it is more likely necessary to revert the epigenetic changes at specific age-associated regulatory loci without inducing non-specific global changes. Perhaps most promising for this purpose are modifications to the CRISPR/Cas9 system whereby a catalytically inactive Cas9 (lacks endonuclease activity but can still be recruited by guide RNAs) is fused to the functional domains of epigenetic modifiers (such as DNMT3A for targeted DNA methylation)[49,50]. Such technologies should provide powerful tools for the study of HSC potential and stem cell biology in general.

Epigenetic Dysfunction as a Precursor to Hematologic Disease in Elderly Individuals

Aging remains the number one risk factor for cancer. The hematopoietic system is no different as age is associated with increased risk of blood cancers (especially malignancies of the myeloid lineage), and abnormal distribution of epigenetic marks is a feature of aging-associated hematopoietic malignancies. One of the most interesting findings from the genomics revolution is the observation that cancer-associated genetic variants can be detected in the blood in a significant proportion of asymptomatic elderly individuals[74–76]. This phenomenon is known as clonal hematopoiesis, disproportionate blood cell production from a single HSC clone. Studies to date have shown that the presence of such a clone increases an individual's risk of all-cause mortality, but only marginally increases the hazard ratio for developing a hematopoietic malignancy[75]. That is, the presence of one of these

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cancer-associated variants does not mean an individual is destined to develop AML or MDS (although this may just be a matter of limited lifespan at age of detection). More studies are needed to determine what is different between individuals with clonal hematopoiesis who eventually develop a hematopoietic malignancy compared to those who go on to live relatively healthy lives.

In all studies of clonal hematopoiesis across aging humans, the two most commonly variant genes are DNMT3A and TET2[74–76]. Given that murine models of Dnmt3a and Tet2 mutation enhance HSC self-renewal, perhaps this means clonal hematopoiesis is a necessary evil to compensate for the diminished potential of aging HSCs. In other words, DNMT3A and TET2 variants empower old human HSCs with enough self-renewal to extend their lifespan, at the expense of increased risk of malignant transformation. We speculate that this situation may have arisen as a compensatory mechanism over the last two centuries as the rate of stem cell evolution has not kept pace with the extension of human lifespan due to advances in health care technology. Up until relatively recently in human history, the HSC pool was only expected to sustain the blood and bone marrow of an individual for 50 years or so. Over thousands of year, HSCs had evolved to do this very well for this time period. However, average human life expectancy has more than doubled in the last 200 years which has not afforded stem cell evolution the time to adjust to this extended output requirement. One way for HSCs to extend their self-renewal potential beyond their normal lifespan is to select for variants that enhance their self-renewal potential such as those found in DNMT3A and TET2. Thus, there is much to be learned about the relationship between clonal hematopoiesis and risk for hematopoietic transformation, weighed against the overall lifespan of an individual.

Concluding Remarks

Though technological advances have allowed unprecedented access to the HSC epigenome over the last few years, there is still much to be learned about the relationships between different epigenetic marks at distinct regions in the genome and their influence on target gene expression and cellular behavior. Although HSC aging is characterized by changes underlying epigenetic changes, it remains to be determined if the changes in the epigenome over time a cause or a consequence of HSC aging. However, as epigenetic modifications are dynamically malleable, further insights into these processes can pave the way for manipulation of the epigenome to change cell fate, and perhaps restore functional potential to aging HSCs.

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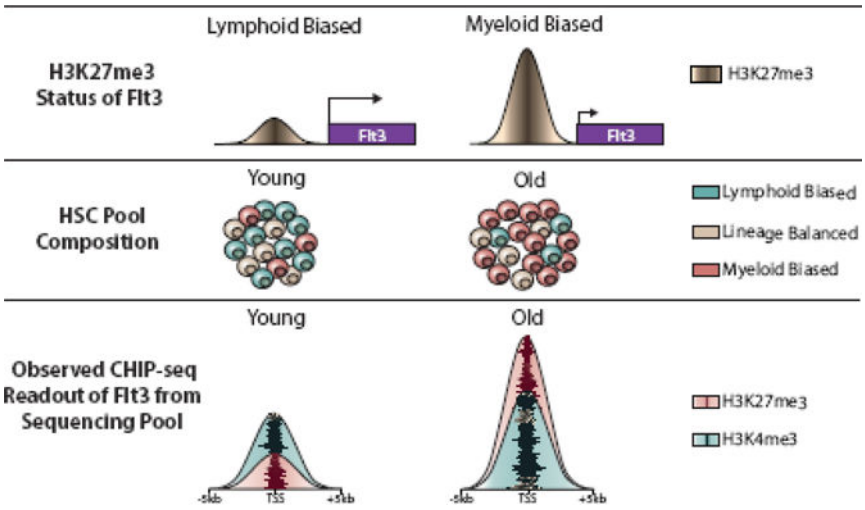

Figure 1.

The influence of the composition of the HSC pool in epigenetic comparisons of young and old HSCs. ChIP-seq studies are performed using pools of HSCs, and the outcome can be influenced by variability within distinct HSC subtypes. Using H3K27me3 at the Flt3 gene as an example, there is a low level in lymphoid-biased HSCs and the gene is expressed, but significant H3K27me3 in myeloid-biased HSCs where the gene is repressed. ChIP-seq for H3K27me3 in a pool of young HSCs would show low H3K27me3 at Flt3 because this is the predominant HSC subtype at this developmental stage. The same assay from a pool of old HSCs would produce an observed increase in H3K27me3 at Flt3, but this could be largely due to the over-representation of myeloid-biased HSCs in the pool at this age. Thus, these epigenetic differences over age produced from analyzing pools of cells may in fact reflect the diversity of the cellular composition rather than inherent changes to the HSCs themselves.