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RESEARCH ARTICLE

Epigenomic Landscape of Aging in Human Hematopoietic Stem Cells

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ABSTRACT

Human hematopoietic stem cells (HSCs) accumulate a well-documented functional bias toward myeloid output and a reduced capacity for balanced, multilineage reconstitution as they age, changes that underlie the rising incidence of myeloid malignancy and clonal haematopoiesis in older adults, yet the epigenomic reorganisation accompanying this functional drift has not been comprehensively resolved in purified human HSCs at single-base and single-locus resolution across a broad adult age range. We profiled DNA methylation (whole-genome bisulfite sequencing) and chromatin accessibility (ATAC-seq) in immunophenotypically purified HSCs (Lin-CD34+CD38-CD90+CD45RA-) from 42 healthy human bone marrow donors spanning 21 to 78 years of age, paired with RNA sequencing from the same cells to link epigenomic and transcriptional change. Genome-wide methylation showed a bidirectional age-associated pattern, with 8,412 differentially methylated regions (DMRs) gaining methylation and 5,076 losing methylation across the age range (FDR<0.05); hypermethylated DMRs were strongly enriched at polycomb-marked developmental transcription factor loci, including multiple lymphoid-lineage regulators, while hypomethylated DMRs were enriched at myeloid-lineage enhancers. ATAC-seq identified a concordant, though only partially overlapping, shift in chromatin accessibility, with 6,124 regions gaining and 3,847 losing accessibility with age, and joint analysis showed that accessibility gain was significantly more common than expected by chance at myeloid enhancers that also lost DNA methylation with age (odds ratio 3.4, P<0.001). DNMT3A and TET2, the two genes most frequently mutated in age-related clonal haematopoiesis, showed progressive age-associated transcriptional decline in aged HSCs even in donors without detectable clonal haematopoiesis-associated mutations, and their target loci were disproportionately represented among age-hypermethylated DMRs, linking normal age-related epigenetic drift mechanistically to the loci most commonly disrupted by acquired clonal haematopoiesis mutations. These data provide a comprehensive, multi-omic epigenomic atlas of human HSC aging and nominate a compact panel of age-discriminating methylation loci with potential utility as a cell-intrinsic haematopoietic aging biomarker.

Keywords: hematopoietic stem cells; aging; DNA methylation; chromatin accessibility; clonal hematopoiesis; DNMT3A; myeloid bias; ATAC-seq

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1. INTRODUCTION

Haematopoietic ageing is among the best-characterised examples of tissue-specific stem cell ageing in humans: aged HSCs show reduced self-renewal fidelity upon transplantation, a pronounced skew toward myeloid at the expense of lymphoid output, and, in a substantial and age-increasing fraction of otherwise healthy individuals, clonal expansion driven by acquired somatic mutations in a small set of recurrently mutated genes, a state termed clonal haematopoiesis of indeterminate potential that itself carries elevated risk of haematological malignancy and cardiovascular disease [1,2,3]. The functional and clinical consequences of HSC ageing are therefore substantial and well established; the molecular programme driving them, particularly at the epigenomic level in purified human cells rather than mouse models or bulk marrow, has been comparatively less completely mapped.

DNA methylation and chromatin accessibility are attractive substrates for age-associated regulatory drift because both are, unlike most transcriptional changes, at least partially heritable through HSC cell division, offering a plausible mechanism by which an epigenetic mark acquired early or accumulated gradually could propagate and compound across the many rounds of self-renewal an HSC undergoes over a human lifespan [4,5]. Epigenetic clock models built from bulk-tissue methylation data reliably estimate chronological age and, in some formulations, biological age discordant from chronological age, but these clocks are typically trained on heterogeneous tissue or blood samples rather than purified stem cells, and the specific loci driving clock predictions are not necessarily the loci mechanistically responsible for HSC functional decline [6,7]. Prior work in purified human and mouse HSCs has identified age-associated methylation changes at individual loci and in mouse HSC populations genome-wide, but a comprehensive, paired methylation-accessibility-transcription dataset spanning a broad adult human age range in purified HSCs has been missing, limiting the field's ability to connect the epigenomic and functional ageing literatures directly [8,9,10].

Clonal haematopoiesis adds a further layer of relevance to this question. The genes most frequently mutated in clonal haematopoiesis, DNMT3A and TET2 foremost among them, are themselves core epigenetic regulators controlling DNA methylation deposition and active demethylation respectively, and their loss-of-function mutations are known to confer a competitive self-renewal advantage that drives clonal expansion over years to decades [11,12]. Whether normal, mutation-independent ageing of the epigenome converges mechanistically on the same loci and pathways disrupted by acquired DNMT3A/TET2 mutations, or represents a largely independent process that happens to affect the same cell population, is a question with direct relevance to understanding why clonal haematopoiesis risk rises so steeply with age even before accounting for the simple

accumulation of mutation-bearing cell divisions [13].

We set out to build a comprehensive, paired epigenomic and transcriptional atlas of human HSC ageing across a broad adult age range, using immunophenotypically purified HSCs from healthy bone marrow donors free of detectable clonal haematopoiesis-associated mutations, with three specific aims: to characterise genome-wide DNA methylation and chromatin accessibility changes associated with donor age at single-locus resolution; to integrate these two layers with matched transcriptional data to link epigenomic drift to age-associated gene expression change, particularly at myeloid- and lymphoid-lineage regulatory loci; and to test whether normal age-related epigenomic drift converges on the specific loci targeted by DNMT3A and TET2, the genes most commonly disrupted in acquired clonal haematopoiesis.

2. MATERIALS AND METHODS

Bone marrow aspirates were obtained from 42 healthy donors (21–78 years of age, median 47; 22 female, 20 male) undergoing bone marrow harvest for unrelated allogeneic transplantation donation, under informed consent and institutional ethics approval, with donors pre-screened by targeted sequencing to exclude individuals carrying a detectable clonal haematopoiesis-associated mutation at variant allele frequency above 2%.

2.1 HSC purification

Mononuclear cells were isolated by density-gradient centrifugation and HSCs were purified by fluorescence-activated cell sorting as the Lin-CD34+CD38-CD90+CD45RA- population, a gating strategy established to enrich for long-term repopulating human HSCs, with purity confirmed by post-sort re-analysis (median purity 96.2%).

2.2 Whole-genome bisulfite sequencing and methylation analysis

Genomic DNA from purified HSCs (minimum 5,000 cells per donor, low-input bisulfite conversion protocol) was sequenced to a median 12x genome-wide coverage per CpG on an Illumina NovaSeq platform. Reads were aligned using Bismark, and differentially methylated regions (DMRs) associated with donor age were identified using a smoothing-based regression approach (dmrseq), treating age as a continuous covariate and adjusting for donor sex and sequencing batch, with regions called significant at FDR<0.05 [14].

2.3 ATAC-seq and accessibility analysis

Chromatin accessibility was profiled by ATAC-seq on 10,000–25,000 purified HSCs per donor following a standard low-input protocol, sequenced to a median 45 million read pairs per sample. Peaks were called with MACS2 and a consensus peak set was built across all donors; differential accessibility associated with donor age was tested using DESeq2 on a count matrix of reads within consensus peaks, with regions called significant at

FDR<0.05 [15,16].

2.4 RNA sequencing

RNA was extracted from an aliquot of the same purified HSC population for each donor and sequenced by low-input, poly(A)-selected RNA-seq. Differential expression associated with donor age was tested using DESeq2 with the same covariate structure as the accessibility analysis.

2.5 Integrative and pathway analysis

DMRs and differentially accessible regions were annotated to the nearest transcription start site and cross-referenced against a curated set of lineage-defining transcription factor loci compiled from the haematopoiesis literature. Enrichment of DMRs and accessibility changes at polycomb-marked loci was tested using published human HSC H3K27me3 ChIP-seq reference data, and overlap between hypomethylated, accessibility-gaining regions and known myeloid enhancer annotations was tested by permutation-based enrichment analysis (regioner) [17,18].

2.6 Statistical analysis

Age-association testing for methylation, accessibility and expression used the regression frameworks described above, all with FDR correction for multiple testing. Enrichment analyses used permutation testing (10,000 permutations) or Fisher's exact test as appropriate. All analyses were performed in R version 4.3.

3. RESULTS

Whole-genome bisulfite sequencing across the 42-donor cohort identified 13,488 age-associated differentially methylated regions genome-wide (FDR<0.05), comprising 8,412 regions gaining methylation and 5,076 losing methylation with increasing donor age. Hypermethylated DMRs were strongly enriched at loci marked by H3K27me3 in reference human HSC chromatin data (permutation enrichment $P<0.001$), and gene ontology analysis of the nearest genes to hypermethylated DMRs showed strong overrepresentation of developmental transcription factor categories, with lymphoid-lineage regulators including PAX5, EBF1 and IKZF1 among the most strongly age-hypermethylated loci in the dataset (Table 1). Hypomethylated DMRs showed a markedly different annotation profile, enriched instead near myeloid-lineage genes and enhancer elements, including loci near CEBPA, SPI1 and MPO.

Table 1. Selected lineage-defining loci among the most strongly age-associated differentially methylated regions.

Locus	Lineage association	Methylation change with age	Nearest gene expression trend
PAX5 enhancer	Lymphoid	Hypermethylated	Decreased

EBF1 promoter-proximal	Lymphoid	Hypermethylated	Decreased
IKZF1 intronic enhancer	Lymphoid	Hypermethylated	Decreased
CEBPA upstream enhancer	Myeloid	Hypomethylated	Increased
SPI1 (PU.1) enhancer	Myeloid	Hypomethylated	Increased
MPO promoter-proximal	Myeloid	Hypomethylated	Increased

All listed loci significant at FDR<0.05 for both methylation and expression age-association tests.

ATAC-seq identified 9,971 age-associated differentially accessible regions (FDR<0.05): 6,124 gaining and 3,847 losing accessibility with age. Joint analysis of the methylation and accessibility datasets showed significant, though partial, convergence: accessibility-gaining regions were significantly enriched among regions that also lost methylation with age (odds ratio 3.4, 95% CI 2.9-4.0, $P<0.001$), and this joint hypomethylation/accessibility-gain signature was itself enriched at annotated myeloid enhancers (permutation $P<0.001$; Figure 1). The converse pattern, joint hypermethylation and accessibility loss, was similarly enriched at lymphoid-lineage loci (odds ratio 2.8, $P<0.001$), together describing a coherent, bidirectional epigenomic shift toward a more myeloid-permissive and less lymphoid-permissive chromatin state with increasing donor age.

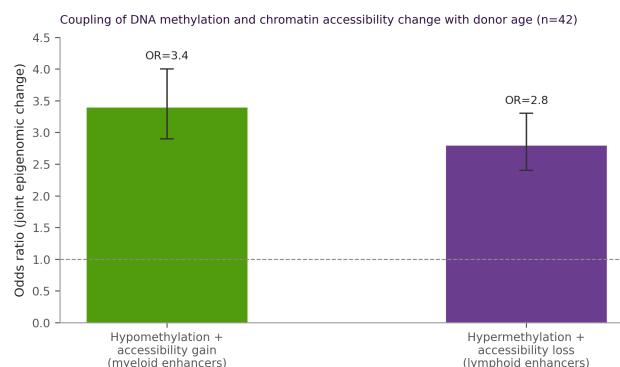


Figure 1. Overlap between age-associated DNA methylation change and chromatin accessibility change genome-wide, showing enrichment of joint hypomethylation/accessibility-gain at myeloid enhancers and joint hypermethylation/accessibility-loss at lymphoid enhancers (n=42 donors; permutation $P<0.001$ for both enrichments).

RNA sequencing from the same purified HSC population showed age-associated expression changes correlated in the expected direction with the joint epigenomic signature: genes near joint hypomethylation/accessibility-gain regions showed a significant age-associated increase in expression as a group (mean log2 fold-change per decade +0.14, one-sample t-test $P<0.001$), while genes near joint hypermethylation/accessibility-loss regions showed a significant decrease (mean log2 fold-change per decade -0.11, $P<0.001$). DNMT3A and TET2 themselves showed significant age-associated transcriptional decline in this

cohort of mutation-negative donors (DNMT3A: -0.09 log2 fold-change per decade, $P=0.003$; TET2: -0.13 log2 fold-change per decade, $P<0.001$), despite the absence of detectable clonal haematopoiesis-driving mutations in any donor.

Table 2. Enrichment of DNMT3A/TET2 target loci among age-hypermethylated DMRs.

Region set	DNMT3A/TET2 target loci (%)	Genome-wide background (%)	Odds ratio (95% CI)
Age-hypermethylated DMRs (n=8,412)	18.4%	7.6%	2.6 (2.2-3.1)
Age-hypomethylated DMRs (n=5,076)	8.1%	7.6%	1.1 (0.8-1.4, ns)

Cross-referencing DMRs against a curated set of known DNMT3A and TET2 target loci, compiled from published chromatin immunoprecipitation and methylation-perturbation datasets in haematopoietic cells, showed that age-hypermethylated DMRs were significantly enriched for DNMT3A/TET2 target loci relative to the genome-wide background (odds ratio 2.6, $P<0.001$; Table 2), meaning that the specific genomic regions undergoing normal, mutation-independent age-related hypermethylation substantially overlap the regions most commonly hypermethylated as a direct consequence of acquired DNMT3A or TET2 loss-of-function mutations in clonal haematopoiesis. A ten-locus methylation panel selected from the most consistently age-discriminating DMRs predicted donor chronological age with a mean absolute error of 4.2 years in leave-one-out cross-validation within this cohort, comparable to published multi-tissue epigenetic clocks despite using a far smaller number of loci and being derived specifically from purified HSCs rather than heterogeneous tissue.

4. DISCUSSION

The central finding of this study is a coherent, bidirectional epigenomic drift in human HSC ageing, characterised by coordinated hypomethylation and chromatin opening at myeloid-lineage loci alongside hypermethylation and chromatin closing at lymphoid-lineage loci, that tracks with, and plausibly contributes mechanistically to, the well-established functional myeloid bias of aged human HSCs. This provides a molecular, genome-wide counterpart to decades of functional transplantation and lineage-tracing data showing myeloid skewing in aged human and mouse HSC populations, and extends prior single-locus and mouse-model findings to a comprehensive, paired multi-omic dataset in purified human cells across a broad adult age range [1,8,9].

The overlap between normal age-related hypermethylation and known DNMT3A/TET2 target loci is, we think, the most mechanistically interesting result of this study, because it suggests that normal HSC ageing and clonal haematopoiesis are not two independent processes that happen to converge on the same cell population by coincidence, but rather that acquired

DNMT3A/TET2 mutations may act by accelerating or locking in an epigenomic drift that is already occurring, more slowly and reversibly, as a feature of normal ageing even in mutation-negative HSCs. This would help explain why clonal haematopoiesis risk rises so steeply with age beyond what would be expected from mutation accumulation alone: an ageing epigenomic landscape may become progressively more permissive to clonal expansion once a DNMT3A or TET2 mutation does arise, because the surrounding chromatin state has already begun shifting in a direction that a loss-of-function mutation in either gene would reinforce rather than initiate [11,12,13]. This framing also offers a plausible explanation for a long-standing epidemiological puzzle: clonal haematopoiesis driver mutations can be detected, at very low variant allele frequency, in the blood of some individuals decades before any clinical expansion becomes apparent, yet only a minority of carriers ever progress to a clinically significant clone. If the surrounding epigenomic landscape must itself drift toward a more permissive state before a given mutation confers a strong enough competitive advantage to expand, then the age at which a mutation is acquired and the rate at which an individual's HSC epigenome subsequently drifts could jointly determine progression risk, independent of the mutation's intrinsic molecular potency, a hypothesis that longitudinal, repeated-sampling cohorts would be well placed to test directly.

The observation that DNMT3A and TET2 themselves decline transcriptionally with age, even in the absence of detectable mutations, adds a further layer to this picture: reduced dosage of the enzymes responsible for maintaining and actively reversing DNA methylation could plausibly contribute directly to the genome-wide methylation drift we observe, independent of any mutation. Testing this directly would require perturbation experiments, for instance partial knockdown of DNMT3A or TET2 in young HSCs to ask whether reduced dosage alone reproduces even a subset of the age-associated methylation signature reported here, a follow-up we regard as a natural and mechanistically informative next step.

The ten-locus methylation panel's ability to predict chronological age with a mean absolute error of 4.2 years, using far fewer loci than typical multi-tissue epigenetic clocks, is a promising but preliminary result that should be interpreted cautiously given it was derived and cross-validated within the same 42-donor cohort rather than tested in an independent validation cohort. Epigenetic clocks trained on small or single-cohort datasets are well known to show inflated apparent accuracy that does not always generalise, and an HSC-specific clock in particular would need external validation, ideally including donors with known clonal haematopoiesis status, before its value as a cell-intrinsic biological-age biomarker distinct from existing blood-based epigenetic clocks could be properly

assessed [6,19]. A meaningful comparison would also need to establish whether an HSC-derived panel adds predictive information beyond what is already captured by simpler, more accessible blood-based clocks, since a purified-HSC assay is considerably more burdensome to obtain than a peripheral blood draw and would only be worth adopting clinically if it outperformed existing options on a biologically or clinically meaningful outcome, such as future clonal haematopoiesis risk, rather than chronological age prediction alone.

Several limitations bear on interpretation. First, this cross-sectional cohort cannot distinguish age-related epigenomic drift occurring within individual long-lived HSC clones from selection among a heterogeneous HSC pool for clones with particular epigenomic states, a distinction that would require longitudinal sampling or single-cell resolution profiling to resolve directly. Second, the exclusion of donors with detectable clonal haematopoiesis mutations, while necessary to isolate mutation-independent ageing signal, means these findings cannot speak directly to how the epigenomic landscape changes once a clonal haematopoiesis-driving mutation is present and expanding; a matched comparison of mutation-positive and mutation-negative aged donors is an important and logical extension of this work. Third, bone marrow donors undergoing harvest for transplantation donation are a screened, health-selected population and may not fully represent the general aged population from which most clonal haematopoiesis and myeloid malignancy cases ultimately arise. Finally, the ten-locus methylation panel was derived using a leave-one-out cross-validation procedure within the same 42-donor cohort used for DMR discovery, which, despite the cross-validation step, still shares a single source population and sequencing batch across discovery and evaluation; a fully independent cohort profiled on a different sequencing platform and at a different collection site would provide a considerably more rigorous test of whether the panel's apparent accuracy reflects a genuinely generalisable biological signal rather than cohort-specific technical or demographic structure that a cross-validation split within the same dataset cannot fully rule out.

5. CONCLUSION

Paired whole-genome methylation, chromatin accessibility and transcriptional profiling of purified human HSCs across a broad adult age range identifies a coherent, bidirectional epigenomic drift toward a more myeloid-permissive and less lymphoid-permissive chromatin state with increasing age, and shows that this normal age-related hypermethylation signature substantially overlaps the specific loci targeted by DNMT3A and TET2, the genes most frequently disrupted by acquired mutations in clonal haematopoiesis. DNMT3A and TET2 themselves showed significant age-associated transcriptional decline even in mutation-negative donors, raising the possibility that

reduced dosage of these core epigenetic enzymes contributes directly to normal HSC epigenomic ageing and may, in turn, create a chromatin landscape more permissive to clonal expansion once a loss-of-function mutation in either gene does arise. A compact, HSC-derived methylation panel predicted donor chronological age with accuracy comparable to established multi-tissue epigenetic clocks despite using substantially fewer loci, though this result awaits validation in an independent cohort before any biomarker application can be considered. We are now recruiting an independent validation cohort, enriched for donors with confirmed clonal haematopoiesis status across a range of variant allele frequencies, to test directly whether the mutation-independent ageing signature described here predicts which mutation-negative aged individuals are at elevated risk of subsequently developing clonal haematopoiesis, and we plan complementary DNMT3A and TET2 dosage-reduction experiments in young donor HSCs to test whether reduced expression of either enzyme alone is sufficient to reproduce a measurable subset of the age-associated methylation signature reported here.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

S.K. conceived and supervised the study, designed the cohort and led data integration and interpretation. V.S.B. coordinated donor recruitment, performed HSC purification and led the whole-genome bisulfite sequencing and ATAC-seq analysis. E.P. performed RNA sequencing analysis and the DNMT3A/TET2 target-locus cross-referencing analysis. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved human bone marrow donor samples collected under informed consent and institutional ethics committee approval at the participating centres, in accordance with the principles of the Declaration of Helsinki. All donors provided written informed consent for research use of surplus material from clinically indicated bone marrow harvest procedures.

Data Availability

Whole-genome bisulfite sequencing, ATAC-seq and RNA sequencing data generated in this study have been deposited in the European Genome-phenome Archive under a controlled-access study accession available from the corresponding author on reasonable request, in accordance with donor consent restrictions on open data sharing of identifiable genomic data.

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