HemaSphere

EUROPEAN HEMATOLOGY ASSOCIATION EHA

Review Article
Open Access

Unraveling Heterogeneity in the Aging Hematopoietic Stem Cell Compartment: An Insight From Single-cell Approaches

Fei Yang[1,2], Craig Nourse[1,2], G. Vignir Helgason[1], Kristina Kirschner[1,2]

Correspondence: Kristina Kirschner (kristina.kirschner@glasgow.ac.uk).

ABSTRACT

Specific cell types and, therefore, organs respond differently during aging. This is also true for the hematopoietic system, where it has been demonstrated that hematopoietic stem cells alter a variety of features, such as their metabolism, and accumulate DNA damage, which can lead to clonal outgrowth over time. In addition, profound changes in the bone marrow microenvironment upon aging lead to senescence in certain cell types such as mesenchymal stem cells and result in increased inflammation. This heterogeneity makes it difficult to pinpoint the molecular drivers of organismal aging gained from bulk approaches, such as RNA sequencing. A better understanding of the heterogeneity underlying the aging process in the hematopoietic compartment is, therefore, needed. With the advances of single-cell technologies in recent years, it is now possible to address fundamental questions of aging. In this review, we discuss how single-cell approaches can and indeed are already being used to understand changes observed during aging in the hematopoietic compartment. We will touch on established and novel methods for flow cytometric detection, single-cell culture approaches, and single-cell omics.

INTRODUCTION

With the world's population of individuals over 60 years increasing from 12% to 22% between 2015 and 2050 (https://www.who.int/news-room/fact-sheets/detail/ageing-and-health), the need to study and understand the many and heterogenous aging-related changes in the body, including the hematopoietic stem cell compartment, has become a priority. Hematopoiesis itself is a complex and hierarchical system that generates ~10^{12} functionally diverse cells each day.[1] Hematopoiesis can function with high fidelity for many decades but is inevitably challenged by aging and the time-dependent accumulation of somatic variation.[2] Over a lifetime, human cells continually acquire mutations, some of which may alter the complex homeostasis of cell division and lead to the subsequent expansion of somatic clones that alter the hematopoietic compartment.[2] Such expansions, termed clonal hematopoiesis of indeterminate potential (CHIP), are frequent in the hematopoietic system and become detectable as we age.[3]

Changes in the hematopoietic system have been well documented during aging, both in individual cell types as well as the aging microenvironment. Age is the single biggest factor underlying the onset of many hematological malignancies.[4] Increased myeloid cell production alongside increased phenotypic hematopoietic stem and progenitor cell (HSPC) numbers, accumulation of DNA damage and clonal hematopoiesis in the hematopoietic stem cell (HSC) compartment that occurs with increasing age predisposes individuals to myeloid malignancies, although the relationship between HSC heterogeneity with age and transformation to leukemic stem cells is still unclear.[5] Additional changes that have been reported to occur in HSPCs are a decrease in the activity of autophagy and alterations in cellular metabolism.[6] In addition, age-related changes in the bone marrow niche can exacerbate aging features in the hematopoietic compartment, leading to a proinflammatory microenvironment[7,8] (Figure 1).

In this review, we will discuss recent technical advances to allow for the interrogation of heterogeneity caused by aging of the hematopoietic compartment using single-cell approaches.

FLOW CYTOMETRIC APPROACHES TO STUDY HETEROGENEITY OF THE AGING HEMATOPOIETIC COMPARTMENT

Fluorescence-activated cell sorting enables the isolation and purification of single cells, which greatly facilitates their mechanistic study. Flow cytometry enables the quantification of the expression of cell surface and cell-intrinsic markers and is widely used in defining a specific HSC group in either young or old HSPCs (Figure 2). Flow cytometric approaches define HSPC populations phenotypically, for example, by the use of cell surface markers, which often leads to a mixed population of

[1]School of Cancer Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, United Kingdom
[2]Cancer Research UK Beatson Institute, Glasgow, United Kingdom
FY and CN have contributed equally to this work.
Copyright © 2023 the Author(s). Published by Wolters Kluwer Health, Inc. on behalf of the European Hematology Association. This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial License 4.0 (CCBY-NC), where it is permissible to download, share, remix, transform, and buildup the work provided it is properly cited. The work cannot be used commercially without permission from the journal.
HemaSphere (2023) 7:6(e895).
http://dx.doi.org/10.1097/HS9.0000000000000895.
Received: December 16, 2022 / Accepted: April 18, 2023

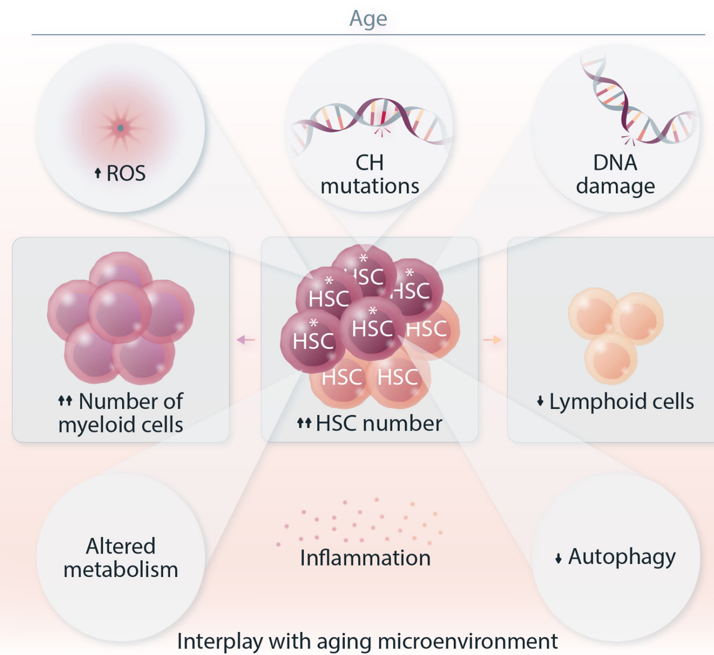

Figure 1. Aging-related changes in the hematopoietic compartment. With aging, phenotypic hematopoietic stem cells (HSCs) increase in numbers alongside an increase in myeloid cells and a decrease in lymphoid cells. Aged HSCs accumulate DNA damage which can lead to clonal hematopoiesis (CH) mutations over time. Other features of aging HSCs include an increase in reactive oxygen species (ROS), alterations in their metabolism and decreased levels of autophagy. The interplay with the aging microenvironment leads to local inflammation.

HSPCs, some of which might have reduced HSC function. These cell surface markers are often complemented with transplantations into mice, the gold standard assay to identify long-term repopulating HSCs (LT-HSCs).

Murine HSC isolation strategies upon aging

Murine HSCs were initially isolated using Lineage (Lin)$^-$Sca1$^+$c-Kit$^+$ (LSK) as conventional markers both in young and aged mice.[9,10] All 4 types of multipotent progenitors (MPPs) are included in the LSK population. Subsequently, different separation strategies for purer HSC populations evolved in different labs over time. For example, CD11b$^-$CD4$^-$CD90^{low}Lin$^-$Sca1$^+$ can be used to gate functional LT-HSCs in the bone marrow of young mice.[11] To obtain purer LT-HSC populations, CD150$^+$CD244$^-$CD48$^-$, also known as SLAM markers, were used in murine bone marrow. Those cell surface proteins faithfully mark young and old stem cells phenotypically.[12] Another commonly used combination of markers to enrich for LT-HSCs is known as the 34F markers consisting of LSK$^+$CD34$^-$FLT3.[13] A variety of other markers have also been reported to enrich functional murine LT-HSCs, such as LSK markers alongside Hoechst dye efflux, which can additionally distinguish between myeloid- and lymphoid-biased HSCs and is stable with aging.[14] Other groups defined murine LT-HSCs as LSK CD90^{low}, fetal liver kinase2$^-$ (FLK2), which is now usually designated as Fms-related receptor tyrosine kinase 3 (FLT3).[15] This variety in markers used to identify LT-HSCs already points to heterogeneity in the LT-HSC compartment and makes direct comparison of studies difficult.

Several groups reported changes in the number of aged HSCPs in mice. Dykstra et al[16] reported an increase in LSK cells in the bone marrow of old mice with a simultaneous increase in purer populations marked by CD48$^-$CD34$^-$EPCR$^+$CD150$^+$. The frequency of those cells was highly variable in individual mice during aging, again pointing to heterogeneity on a mouse-to-mouse basis. Overall, these CD48$^-$CD34$^-$EPCR$^+$CD150$^+$ cells displayed a myeloid bias, reduced self-renewal activity, and a reduction in homing capacity to the bone marrow. Another study confirmed both the age-associated accumulation of HSCs and an increase in myeloid-biased HSCs in mice. When isolating LT-HSCs by using LSK$^+$FLT3$^-$CD34$^-$ markers, myeloid-biased HSCs could be distinguished by high expression levels of SLAMF1/CD150, with lower levels of SLAM1 marking balanced HSCs.[17] Several groups have since confirmed an accumulation of myeloid-dominant HSCs in the aged HSC compartment at the expense of lymphoid-dominant HSCs in mice.[14,18] Lymphoid-deficient HSCs were also found to be significantly expanded in mice with increasing age, leading to a myeloid bias, whereas balanced HSCs only show a minor increase in frequency as determined by flow cytometry using CD45$^+$EPCR$^+$CD48$^-$CD150$^+$ (ESLAM) markers.[19] This myeloid bias partially stems from a relative defect in the frequency of common lymphoid progenitors (CLPs), produced by lymphoid deficient so-called αHSCs with all other groups of progenitor cells not changing when comparing young and aged HSCs outputs in mice. This is possibly due to the persistence of ßHSCs, which give rise to a balanced output as defined by granulocytes/monocytes to B-cell and T-cell ratios upon transplantation.[19,20] In addition, cell examination in old mice revealed loss of lymphoid priming alongside a decreased differentiation potential of lymphoid-primed MPPs (MMP4), suggesting alterations of MPP populations rather than changes in HSC subtypes as effectors of the aging-related impairment of lymphoid cell production.[21]

Commonly used cell surface markers defining myeloid-biased HSCs are LSK CD150^{hi}CD41^{+}[22] or LSK CD48$^+$CD150$^+$CD34$^+$FLk2$^+$Neogen (NEO1)$^+$ with HoxB5^{+}[23] with balanced HSCs being defined by NEO1$^-$.[23] *Neo1* is a gene encoding a cell surface protein that is a member of the immunoglobulin superfamily. It has been identified on murine LT-HSCs.[24] Shin et al[23] further gated LSK CD150$^+$CD34$^-$ HSCs and found c-Kit^{low} cells to lead to a balanced output with c-Kit^{high} HSCs giving rise to long-term lympho-myeloid grafts, displaying an intrinsic megakaryocytic lineage bias. Gekas and Graf[22] used LSK CD34$^-$FLT3$^-$ or LSK CD48$^-$CD150$^+$ as HSC markers from which they could then identify CD41$^+$ as marking

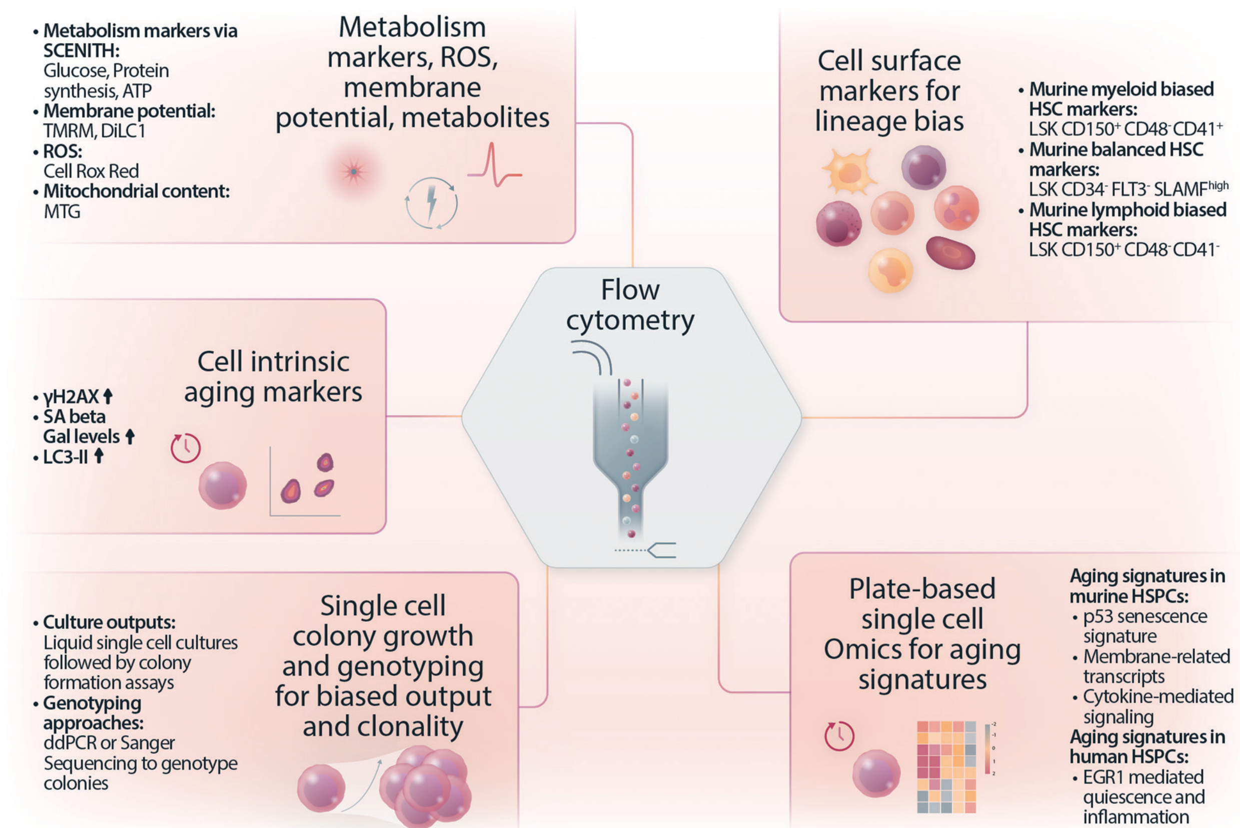

Figure 2. Multiple uses for flow cytometry in detecting heterogeneity in the aging hematopoietic compartment. Flow cytometry is a useful tool for the characterization of aging-related changes in the hematopoietic compartment. It can be used to detect the cell surface proteome and cell-intrinsic markers of aging. In addition, sorting of single HSPCs into a 96-well format allows for single-cell omics-based detection of changes in the transcriptome and enables single-cell growth kinetics to be linked with genotyping for known mutations. Finally, metabolic changes can be readily detected using flow cytometric approaches. γH2AX = g Histone H2AX; SA Beta Gal = senescence-associated beta galactosidase; HSC = hematopoietic stem cell; HSPC = hematopoietic stem and progenitor cell; LSK = Lineage–Sca1+c-Kit+; SCENITH = Single Cell ENergetIc metabolism by Profiling Translation inHibition; TMRM = tetramethylrhodamine methyl ester.

myeloid-biased LT-HSCs, which become prevalent with age. CD41⁻ HSCs in contrast yielded a relatively lymphoid-biased progeny. Therefore, different isolation protocols can be used to define myeloid- or lymphoid-biased HSCs.

A more recent study used single-cell transplantation assays in mice to assess clonal outputs with aging.[25] When transplanting LSK CD34⁻ cells from young and old mice into recipients, the authors found an increase in myeloid-restricted repopulating progenitors in old mice compared with young. This study also confirmed a myeloid bias of old LT-HSCs in primary recipients. Interestingly, this myeloid bias disappeared upon secondary transplantations, instead giving rise to a balanced clonal output.[25] These LT-HSCs were exclusively found upon aging and were termed latent-HSCs.

Alternatives to flow cytometric clonal tracing

In murine models, fluorescent markers can be introduced and tracked over time using flow cytometry. One such example stems from a mouse model containing a tetracycline-regulated (Tet-O, TetOff) HSPC-specific Histone H2B tagged to green fluorescent protein (H2B-GFP) label-retaining system.[26] These mice were generated by crossing 2 mouse strains, namely the human CD34+TetR-VP16 hybrid protein (huCD34+ -tTA) and TetO-H2B-GFP strains.[27] Labeled GFP fluorescent HSCs were followed over the adult lifespan of the mouse, and flow cytometric assessment of GFP high and low populations alongside HSC markers was performed. The study reported an increase in myeloid output, which was linked to increased divisional history, demonstrating that proliferation history and phenotypic output are closely linked. Other groups have made HSC reporter mice by using different constructs such as HoxB5-mCherry[28] or α-catulin–GFP⁺ reporters,[29] demonstrating an increase of phenotypic HSCs in old mice. HoxB5 expression was found to be restricted to LT-HSCs, and therefore, HoxB5-mCherry reporter mice were created by in-frame knock-in of mCherry into the *Hoxb5* gene locus 5′ of the endogenous stop codon.[28] α-catulin–GFP⁺ reporter mice were created by knock-in of GFP into the first exon of the α-catulin gene, a gene with high expression in HSCs. Both heterozygous and homozygous mice were viable with no obvious hematological phenotype.[29] Finally, FYVE, RhoGEF And PH Domain Containing 5 (Fdg5) fused to the mCherry reporter driven by the endogenous promoter labeled HSCs in mice as demonstrated by HSC surface marker expression and transplantation.[30] This allele was then further modified to allow temporal induction via the Cre recombinase (Cre) fused to a mutant estrogen ligand-binding domain (ERT2) that requires the presence of tamoxifen for activity. All these mouse models demonstrated that only a subpopulation of HSPCs were true long-term, functional HSCs. Overall, the use of reporter mice allows for easy isolation and identification of HSP populations and thereby enables their study even in the bone marrow niche and in vivo. However, since all reporters are fused to genes identified to mark only a subset of HSCs, conclusions about HSC heterogeneity become difficult.

The concept of myeloid-biased HSPC output upon aging, which was demonstrated by both flow cytometric approaches discussed above and lentiviral barcoding studies in mice,[31] has more recently been questioned by a study using genetic barcoding in young and old macaques, thereby being able to trace the clonal output of HSPCs. This study demonstrated a delayed output from multipotent clones with increased age.[32] Moreover, and in contrast to mouse studies, persistent output from both myeloid and B-cell-biased clones was noted, providing a model resembling clonal hemopoiesis in humans. However, genetic barcoding requires ex vivo manipulation of HSPCs and transplantation, which might induce biases. A novel approach using a multicolor, noninvasive in vivo labeling approach in mice was used to assess the clonal complexity over the murine lifespan. In this study, clonal complexity declined with age, leading to a reduced repopulating activity and lymphoid potential.[33] Similar in vivo labeling studies in macaques are needed to establish if this myeloid-biased output applies to species closer to humans.

HUMAN LT-HSC ISOLATION STRATEGIES UPON AGING

Human HSCs with long-term repopulation ability were originally shown to be contained in a subpopulation of CD90$^+$CD34$^+$Lin$^-$ cells.[34] More impure HSPC populations are also often defined as CD34$^+$CD38$^-$ cells but are known to be highly heterogeneous, only containing a small fraction of true LT-HSCs and several progenitor cell populations.[35] The addition of several markers helped to resolve a more purified HSC population in humans with Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$ markers now commonly used.[36,37] Since only a subset of CD90$^+$ cells shows serial transplantation capacities in humans, Notta et al[38] tracked the expression of adhesion molecules, revealing CD49f as a new marker able to distinguish long-term multi-lineage engraftment. The addition of CD49f$^+$ to Lin$^-$CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$ thereby further enriches LT-HSCs.[38]

In humans, increases in phenotypic HSCs and HSPCs have also been observed with aging. This HSC expansion was shown to be apparent at several sites in human bone marrow (BM), suggesting an overall phenotypic HSC/HSPC increase in humans.[39,40] In addition, this study reported a decrease in human common multipotent lymphoid progenitor (MLP) using the Lin$^-$CD34$^+$SSC^{low}CD38$^-$CD90$^-$/low CD45RA$^+$ markers with no change in the common myeloid progenitor/megakaryocyte–erythroid progenitor (CMP/MEP) as defined by Lin$^-$CD34$^+$SSC^{low}CD38$^+$CD90$^-$CD45RA$^-$ or the MPPs, characterized by Lin$^-$CD34$^+$SSC^{low}CD38$^-$CD90$^-$CD45RA$^-$ upon aging.[39] This study supports reports in mice of a decrease in MLPs upon aging. A different study reported the proportion of multipotent CD34$^+$CD38$^-$ cells to increase in the bone marrow of individuals above 70 years.[41] In addition, myeloid-biased outputs of old human LT-HSCs were identified using in vitro colony forming assays and by in vivo transplantation into mice when compared with the colony outputs of young human LT-HSCs.[41] Those findings were confirmed in a study of individuals 65 years old and older and compared with young bone marrow donors with more aged HSCs in a nonquiescent state.[42]

A different study traced human HSCs through the lifespan using human cord blood and young and aged bone marrow.[40] This group also observed a gradual increase in the frequency of HSCs, linked to functional impairment of those cells such as a decrease in lymphoid output. Moreover, CLPs were also reduced in numbers with MEPs increasing along the human lifespan, thereby mimicking findings in mice.[40]

EXAMINATION OF CELL-INTRINSIC CHANGES UPON AGING

Intrinsic age-related changes in HSPCs can manifest themselves as DNA damage, the accumulation of senescent cells, decreased levels of autophagy, and metabolic alterations. To characterize DNA damage in single HSPCs, assessment of the phosphorylation status of Serine 139 of the histone variant H2AX (termed γH2A) is often employed using microscopy by counting γH2A foci. Newer approaches now allow for γH2A detection via flow cytometry, thereby allowing for easier assessment of heterogeneity. γH2A is an early marker of the formation of DNA double-strand breaks.[43] Several studies of DNA damage-related changes in murine HSCs examined not only γH2A levels but also markers of single-strand DNA breaks such as replication protein A and ataxia telangiectasia and Rad3-related protein (ATR) interacting protein, which were all upregulated when comparing young and old HSCs.[44,45] Alternative methods to detect DNA damage are poly-ADP-ribose and TdT-mediated dUTP nick end labeling staining, which quantifies DNA fragmentation or by using alkaline comet assays to measure the number of DNA breaks.[44] In humans, the accumulation of γH2A was noted to increase with advancing age in CD34$^+$ cells.[46]

Senescence markers, such as cell cycle inhibitors cyclin dependent kinase inhibitor 2A (commonly known as p16^{INK4A}) and cyclin dependent kinase inhibitor 1A (commonly known as p21), and senescence-associated beta galactosidase (SA-ß gal) expression (via C12FDG or 9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One-7-yl) β-D-Galactopyranoside [DDAO] detection) are commonly used to detect senescent cell populations. These markers are now regularly detected by flow cytometry, either as a single marker or in combination,[47,48] thereby allowing for the characterization of senescence heterogeneity in hematopoietic cells. The onset of senescence in the hematopoietic compartment has been reported by several groups in the context of normal aging and when overexpressing oncogenes,[49,50] for example, Kirschner and colleagues[50] reported a p53-driven senescence signature in a subset of aged HSCs in mice, pointing to differences in the engagement of the senescence program. When HSPCs overexpressing the oncogene *BRAFV600E*, which has extensively been characterized as an oncogene driving senescence in solid cells,[51] were transplanted into mice, a senescence program driven by the senescence-associated secretory phenotype (SASP) and the accumulation of DNA damage became apparent.[49] Tumor necrosis factor alpha (TNFα) was identified as a key component of the SASP, leading to myeloid-restricted hematopoiesis in this context.[49] In a mouse model where p16^{INK4A} positive, senescent HSPCs accumulated with age or upon irradiation, a marked rejuvenation effect could be detected when ablating p16^{INK4A}-positive HSPCs by administration of the senolytic drug ABT263.[52] Irradiation-induced senescence of HSCs in the bone marrow led to increases in SA-ß gal and expression of p21. Upon ablation of irradiation-induced senescent HSCs, both long-term engraftment as measured by primary and secondary transplantations, and clonogenicity improved, hence demonstrating a functional improvement of hemopoiesis.[52]

In humans, the most widely studied blood cells to display senescence are T cells with widespread consequences for cancer therapies.[53] Senescent T cells display a flattened phenotype, stain positive for SA-ß gal, and display increased DNA damage alongside the activation of p53/p21 and Rb/p16 pathways.[53] Senescent T cells were first identified in the blood of elderly people, linking T-cell senescence with aging.[53] Whether senescence of other blood cell populations plays a role in human hematopoiesis remains to be elucidated.

Autophagy plays a critical role in keeping aged HSCs quiescent, thereby guaranteeing long-term regeneration potential. Ho et al[54] reported heterogeneity in autophagy levels in murine HSCs with age, with two-thirds of all HSCs exhibiting low levels of autophagy, increased mitochondrial levels, and an activated metabolic state resulting in age-related deficiencies in HSCs. Autophagy can be readily assessed by levels of microtubule-associated protein 1A/1B-light chain 3 (LC3). A cytosolic form of LC3 (LC3-I) is conjugated with phosphatidylethanolamine to form lipidated LC3 (LC3-II), which can be used as a

surrogate marker of autophagy. Following fusion of autophagosomes and lysosomes, the cargo within autophagosomes is degraded by lysosomal hydrolases.[55] A number of methods have been described that allow assessment of autophagy flux using flow cytometry and thereby assist in dissecting heterogeneity in autophagy levels in rare cell populations such as aged HSCs.[56]

Mitochondrial activity and mitochondrial membrane potential (MMP) have been reported to be another factor leading to HSC heterogeneity with age,[57] where old murine HSCs with high MMP resemble more closely HSCs from young mice. Although membrane potential can be readily assessed using flow cytometric dyes such as the cationic cyanine dye DilC1(5) or tetramethylrhodamine methyl ester, it is recommended that these dyes are used together with inhibitors of efflux pumps for accurate measurements in different HSPC populations.[57]

A relatively new method for single-cell flow-based detection of metabolic changes is called Single Cell ENergetIc metabolism by Profiling Translation inHibition (SCENITH).[58] This method facilitates the interrogation of metabolic changes in multiple blood cell types simultaneously ex vivo and will be useful to address age-related changes in energy consumption of blood cells without the need for cell culture. In essence, SCENITH uses a puro-fluorescent antibody to measure protein synthesis as a surrogate for ATP consumption since ATP and protein synthesis levels are tightly coupled.[58] Puromycin incorporation is measured by direct immunofluorescence and used as readout by flow cytometry. By incubating samples with inhibitors of known metabolic pathways, a reduction in protein synthesis is indicative of an energy dependency of this cell population on the inhibited pathways. SCENITH, therefore, measures metabolic dependencies indirectly and allows for single-cell resolution.

Examination of aging-related changes in the bone marrow microenvironment

Extrinsic age-related changes in the bone marrow niche in mice have also been reported widely with a shift in the number of subsets of mesenchymal stem cells (MSCs) and epithelial cell (EC) subtypes.[59] For example, Nestin$^-$GFP^{bright} platelet-derived growth factor receptor beta (PDGFRβ)$^+$ Neuron-glial antigen 2$^+$ MSCs alongside a cluster of differentiation 31 (CD31)^{hi} Endomucin (Emcn)^{high} type-H ECs can be detected by flow cytometry and have shown to be reduced in endosteal regions upon aging of the bone marrow, with no changes to numbers of Leptin receptor (LepR)$^+$Nestin$^-$GFP^{low} MSCs and CD31^{lo}Emcn^{lo} type-L ECs in the central bone marrow.[59–61] However, the expression of the cell surface receptor LepR increases in human MSCs with increased age.[62] C-X-C motif chemokine ligand 12-abundant reticular (CAR) cells, which are a form of primitive MSCs, are essential for HSC maintenance and form a niche for both HSCs and immune cells, which are produced in the bone marrow.[63] Ablation of CAR cells in mice led to reduced HSC numbers and cell size, and enhanced quiescence alongside an increase in the expression of myeloid differentiation genes.[64] Upon aging, CAR cells are thought to upregulate chemokine (C-C motif) ligand 2, mediating the emigration of inflammatory monocytes from the bone marrow into circulation, at least in the context of bacterial lipopolysaccharide (LPS) stimulation.[63]

Other features of the aging bone marrow niche include the accumulation of senescent cells and increased inflammation, termed inflammaging.[7,59–61] Inflammaging is driven by senescent endothelial and stromal cells that accumulate in the bone marrow during aging and secrete increased levels of proinflammatory chemokines and cytokines.[7] This process is thought to promote HSC aging and majorly contribute to myeloid skewing. For example, transforming growth factor beta (TGFß) and interleukin (IL)-6 signaling increases in aged murine bone marrow stromal cells and has been shown to be elevated in plasma and serum samples of aged human individuals.[65–67] In mice, the inhibition of either TGFß or IL-6 led to the reversal of age-related decreases in lymphoid output and in erythroid progenitor activity, respectively.[67]

TNFα is an important extrinsic and intrinsic regulator of HSC function. In aging mice, accumulation of plasma cells led to an increase in the release of proinflammatory cytokines (such as TNFα) with old plasma cells displaying a Toll-like receptor-responsive gene signature, resulting in increased production of inflammatory cytokines.[68] Moreover, plasma cells could directly regulate inflammation-related gene expression in bone marrow stromal cells.[68] A study examining inflammation in both mice and humans demonstrated an increase in circulating monocytes with age due to elevated TNF levels in the circulation. Lymphocyte antigen 6 complex locus G (Ly6C)$^+$ monocytes from old mice and CD14$^+$CD16$^+$ intermediate/inflammatory monocytes from older adults were also shown to increase production of inflammatory cytokines such as IL-6 and TNF in steady state and during stimulation with bacterial products.[69] Cell intrinsically, TNFα promotes HSC survival and myeloid differentiation in mice through nuclear factor of kappa B signaling, poising HSCs toward myeloid cell production, a mechanism that is active in aging HSCs.[70]

IL-1 signaling has been identified as a key proinflammatory cytokine in the bone marrow niche in mice where IL-1 exposure led to accelerate proliferation and myeloid differentiation of HSCs through PU.1-mediated gene expression.[71] Moreover, IL1ß signaling stemming from the aging, damaged endosteum drives the proinflammatory phenotype of the central bone marrow, resulting in HSC dysfunction in old mice.[72] Interestingly, the effects of IL-1-driven proinflammatory aging effects in the niche were reversible upon targeting of IL-1 signaling.[71,72]

A recent study demonstrated impaired mitochondrial function in aged HSPCs when stressed with LPSs.[73] HSPC malfunction during stress was supported by the accumulation of senescent, p16^{INK4A}-positive MSCs that naturally occur with aging in the bone marrow. In turn, mitochondrial impairment in HSPCs could be improved by senolytic depletion of bone marrow-resident senescent mesenchymal stromal cells, highlighting the close interplay between aging HSCs and their niche.[73] As mentioned above, the detection of senescence is well established via various SA-ß gal substrates or p16^{INK4A} levels by flow cytometry and can readily be used to detect and isolate senescent MSCs.[74]

Another recent study characterizing alterations in the bone marrow niche highlighted a role of the middle-aged bone marrow in driving HSC aging due to decreasing levels of insulin-like growth factor 1 (IGF1).[75] This increase in IGF1 stimulated a myeloid-biased output, as detected by flow cytometry, which could be rescued in an in vitro culture setting by IGF1 stimulation. Therefore, interplay between HSCs and the altered aging bone marrow niche leads to significant functional HSC decline.

SINGLE-CELL CULTURE APPROACHES TO STUDY HETEROGENEITY OF THE AGING HEMATOPOIETIC COMPARTMENT

Single-cell culture approaches are a powerful tool to assess heterogeneity in growth kinetics, to ascertain mutational profiles and hierarchies, and to assess, at least to a certain extent, lineage commitment of single HSPCs upon aging. Single-cell growth kinetics can be recorded manually by plating single HSPCs into 96-well plates in liquid cultures. Quantification of growth kinetics can be achieved by counting manually at regular intervals or by combining automatic image acquisition with tissue culture incubation. In all cases, image analysis can be automated through artificial intelligence methods such as Ilastik.[76–78] This system allows the determination of the growth potential of individual HSPCs in liquid cultures and assesses heterogeneity in proliferation capacity over a period of 7–10 days.[50,79] Using these methods, several groups reported aging-related changes in the growth potential of murine HSCs

in the context of *Janus Kinase* 2 (JAK2) mutations. One group showed accelerated growth in young HSPCs carrying the *Jak2V617F* mutation compared with wild-type (WT) cells with aged *Jak2V617F*-mutated HSPCs no longer leading to increased proliferation.[50] Another paper used single-cell culturing of murine *Jak2V617F*-mutated HSCs and quantitative analysis of resulting clones alongside mathematical modeling to determine fate choices in vitro.[79] This study reported a reduced self-renewal capacity of *Jak2V617F*-mutated individual HSCs with progenitor expansion being intact.[79] Moreover, a skew toward proliferation and differentiation of HSCs in the context of the *Jak2V617F* mutation was noted, demonstrating that the acquisition of this mutation alone is insufficient to drive clonal expansion. In both studies, single murine HSCs were cultured in serum-free media containing stem cell factor (SCF) and IL-11. This combination of growth factors has been shown to retain long-term repopulating activity of murine HSPCs, which could be serially transplanted.[80]

To assess stem and progenitor cell potential in vitro over a larger time span, the long-term culture initiating cell or the cobblestone assay can be conducted,[81] giving a measure of stem and progenitor cell potential in vitro. These assays rely on stromal cells to support HSPC growth and were used to show reduced proliferation potential of aged murine HSCs in vitro.[16]

In humans, several studies deployed whole-genome sequencing of single HSPC-derived colonies to address aging-related changes in clonal output.[82,83] Using these methods, clonal dynamics of single HSPCs and mutation acquisition was determined. In humans, a much reduced clonal diversity was noted with increasing age in healthy individuals.[83] Of note, different cytokine cocktails were used to expand single HSPCs in vitro in those studies, thereby making a direct comparison of results difficult, especially when addressing clonal lineage outputs. Lee-Six et al[82] expanded cells in human Fms-like tyrosine kinase 3 (Flt3) ligand, human SCF, human IL-3, and human IL-6 with the addition of growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage CSF, erythropoietin (EPO), and thrombopoietin (TPO) after 10 days in culture. This cocktail supports the proliferation of human HSPCs and leads to differentiation toward megakaryocyte-erythrocyte progenitors, granulocyte-macrophage progenitors, and CMPs.[82] Mitchell et al[83] used an expansion protocol that promotes the proliferation and differentiation of granulocytes, monocytes, erythroid cells, and natural killer cells. Supplements used were SCF, FLT3, TPO, EPO, IL-6, IL-3, IL-11, GM-CSF, IL-2, IL-7, and lipids.

CHIP is defined as the expansion of HSCs in healthy aged individuals that results from genetic alterations. Although mostly inconsequential, the constant rate of the acquisition of mutations in HSCs (17 mutations/year)[83] leads to an increasing probability, with respect to age, of a variant occurring in a gene that dysregulates the tightly maintained mechanism of hematopoiesis. Whole-genome sequencing of human single HSPC-derived colonies in the context of CHIP demonstrated that clones without known driver mutations showed a similar growth rate compared with clones harboring known driver mutations.[84] In a different study, to determine the speed of growth (fitness) of clones with CHIP mutations in vivo over a 12 year period in humans, targeted error-corrected sequencing was deployed. The group identified gene-specific fitness effects in clonal outgrowth, with mutations in splicing genes exerting the highest fitness effects and biggest expansion over time.[3,85] For those same splicing mutations, reconstructed growth trajectories derived from in vitro data correlated with expansions of clones in vivo in humans for the same mutation, highlighting the power of identifying clonal dynamics with age using in vitro approaches.[84]

However, culturing of murine and human HSPCs longer term is currently challenging and often results in in vitro differentiation upon prolonged culture.[86] In recent years, several groups reported improvements in the long-term culturing of HSPCs, which are crucial for the culture of single cells.[87,88] Until recently, liquid cultures of murine or human HSPCs used a variety of cytokines in combination with fetal bovine or human serum, which typically allows for HSPC cultures to be maintained for 1 to 2 weeks in vitro. However, HSPC expansion does not typically take place under those conditions.[86]

In mice, Wilkinson et al[89] reported a cell culture system, which supports the long-term ex vivo expansion of functional HSCs. In this system, high levels of TPO, low levels of SCF, and fibronectin with the addition of polyvinyl alcohol resulted in ex vivo expansions of functionally defined HSCs over 30 days, forming a first step to facilitate murine studies of HSPC aging in vitro.

Other groups reported several cell-engineering approaches to mimic the bone marrow niche, thus resulting in improved culturing of human HSPCs.[87] Resident Nestin$^+$MSCs are prominent cell types, supporting HSC homing to the bone marrow niche. However, in 2D systems, MSCs often undergo senescence following serial expansion.[90] To circumvent this problem, a recent bone marrow-like niche model was based on the formation of multicellular spheroids in low-density type I collagen, allowing Nestin$^+$MSCs to stay quiescent in culture for up to 14 days.[87] A more complex 3D model for co-culture of human HSPCs was based on organoid generation from induced pluripotent stem cells, providing mesenchymal and myeloid cells alongside sinusoidal-like structures.[91] The authors go on to demonstrate engraftment of healthy and malignant blood cells into organoids ex vivo. A recent study achieved long-term ex vivo expansion of human cord blood HSCs in a cytokine-free environment.[92] Chemical agonists directed towards phosphoinositide 3-kinase activator and TPO-receptor were combined with the pyrimidoindole derivative UM171 to expand functional human HSCs. Taken together, exciting advances in new tissue culture methods of human and mouse HSPCs will hopefully allow for more long-term follow up of single HSPCs in an aging context in vitro by recreating culture conditions more akin to the in vivo environment. In the future, combining more complex single-cell growth assays with genotyping of human cells carrying clonal hematopoiesis mutations will be useful to delineate mutation-specific growth behaviors.[2]

SINGLE-CELL OMIC APPROACHES TO ELUCIDATE THE HETEROGENEITY OF THE AGING HEMATOPOIETIC COMPARTMENT

The ability to interrogate individual cells within the heterogeneous hematopoietic compartment has allowed researchers to study in detail the changes that occur over time at the genomic, epigenomic, transcriptomic, and proteomic levels (Figure 3). Changes in the HSC compartment during aging are associated with a predisposition to various disease states, including cancer.[93] We are now entering an era where various types of single-cell omics data can be derived from a single cell in parallel enabling a multiomics view of HSPC aging. Newer platforms from companies such as 10× Genomics and Nanostring now allow expression data to be correlated with histological data to provide spatial context.

Transcriptomic

Although earlier studies have shown that aging of the HSC compartment is characterized by lineage bias and decreased cell function,[94] the elucidation of the precise molecular mechanisms and underlying heterogeneity has only been possible by using single-cell RNA-sequencing (scRNA-Seq) approaches. ScRNA-Seq is now routinely used to identify and classify cellular populations of the HSC compartment based on their transcriptional signatures.

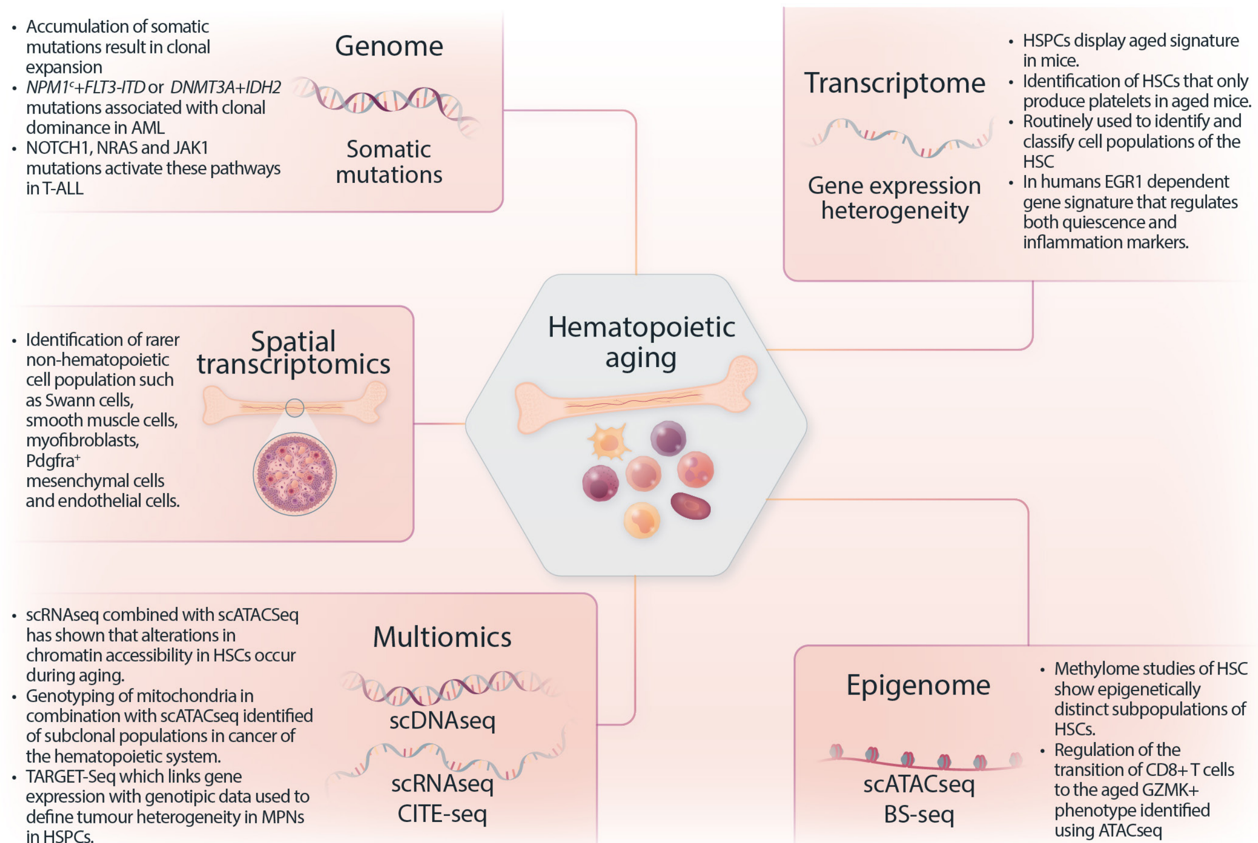

Figure 3. Single-cell omics approaches to detect changes in the aging hematopoietic compartment. A multitude of cell types in the hematopoietic compartment can be assessed for changes caused by aging by using omics-based approaches. The transcriptome can be assessed by single-cell RNA-sequencing to elucidate heterogeneity in gene expression. Somatic mutations can be measured on the single-cell level to characterize clonal structure. Multiomics enable simultaneous interrogation of the transcriptome and the cell surface proteome, changes in DNA methylation or other DNA alterations, such as single nucleotide polymorphisms. CITE-Seq = cellular indexing of transcriptomes and epitopes by sequencing; FLT3 = Fms-like tyrosine kinase 3; GZMK = granzyme K; HSC = hematopoietic stem cell; IDH2 = isocitrate dehydrogenase 2; ITD = internal tandem duplication; JAK1 = Janus Kinase 1; MPNs = myeloproliferative neoplasms; NOTCH1 = Neurogenic locus notch homolog protein 1; NPM1 = Nucleophosmin 1; NRAS = NRAS Proto-Oncogene; scATAC-Seq = single cell-level is the assay for transposase accessible chromatin using sequencing; scRNA-Seq = single-cell RNA-sequencing; HSPC = hematopoietic stem and progenitor cell.

First, using scRNA-Seq in mice, it has been shown that during HSC aging, a distinct subpopulation of old HSCs displaying *Tumor Protein (TP) 53*-mediated functional decline emerges.[50] The authors revealed heterogeneity in the proliferation capacity of HSPCs in aged mice, leading to subclones displaying an aging signature on the transcriptomic level with the majority of aged HSPCs being transcriptionally similar to HSPCs from young mice.[50] Second, a new class of HSCs that exclusively produce platelets was identified by scRNA-seq in aged mice, suggesting that platelet bias contributes to age-associated decrease in lymphopoiesis.[95] Finally, a meta-analysis of aged murine HSC single-cell transcriptomic data provided an aging signature highly enriched for membrane-associated transcripts.[96]

Using a transgenic approach to introduce pathogenic germline variants of telomerase complex genes together with scRNA-seq in mice demonstrated that telomere attrition results in the upregulation of the innate immune signaling response, and the subsequent metabolic activation and differentiation toward the megakaryocyte lineage that limits HSC renewal capabilities.[97] Performing scRNA-seq on Lin$^-$CD34$^+$ HSPCs that harbored heterozygous pathogenic germline telomerase reverse transcriptase mutations recapitulated these findings in humans.[97]

In humans, a comprehensive meta-analysis of several scRNA-seq data sets identified an EGR1-dependent gene signature in aged Lin$^-$CD34$^+$CD38$^-$ HSCs, regulating both quiescence and inflammation markers.[98]

Genomic

The accumulation of somatic mutations during aging has been shown to result in a fitness advantage for certain HSCs that results in clonal expansion, along with a reduction in clonal hematopoietic diversity and functional decline.[3,93] Single-cell DNA sequencing (scDNA-Seq) encompasses a number of techniques that have been used to detect various genomic alterations such as single nucleotide polymorphisms, insertions, and deletions.[99] Many scDNA-Seq techniques are error prone due to technical biases arising from uneven sequence coverage, allelic dropout, and the introduction of errors during amplification steps in sequence library construction.[100] Despite the error-prone nature of scDNA-Seq data, genotyping studies targeting the somatic mutations of single cells in human clonal hematopoiesis and acute myeloid leukemia have revealed the dominance of a small number of clones that frequently contain co-occurring mutations in epigenetic regulators, thereby promoting clonal expansion. For example, the co-occurence of mutations in *Nucleophosmin 1* and an internal tandem duplication mutation in the *Flt3* gene or the combination of mutations in DNA methyltransferase 3 alpha (DNMT3A) and isocitrate dehydrogenase (NADP^{+}) 2 were found to be associated with clonal dominance.[101] This study was also able to show that clonal complexity evolves and is tightly linked with disease progression.

Another scDNA-Seq technique using a targeted panel of 110 human genes revealed T-cell acute lymphoblastic leukemia as a heterogeneous disease at diagnosis, which undergoes clonal evolution during treatment with chemotherapeutic agents such that minor clones detected at diagnosis then become dominant clones during the latter stages of disease.[102] Half of all T-cell acute lymphoblastic leukemia patients in the study were found to have greater than 2 mutations in *Neurogenic locus notch homolog protein 1* in both major and minor subclones, and similar findings were obtained for *NRAS Proto-Oncogene* and *Janus Kinase 1*, suggesting the importance of activating these pathways during disease progression. How the mutational profile of T cells evolves over the human lifespan remains to be elucidated.

Epigenomic

Numerous epigenetic changes have been identified during aging and the development of senescence including DNA methylation, histone modification, and chromatin remodeling.[103] Single-cell bisulfite sequencing (scBS-Seq) has been applied to study the methylome of cells within the HSC compartment in both mouse and human and has revealed that epigenetically distinct subpopulations, which were identified first by assessing differentially methylated regions and then overlaid with single transcriptome data, of HSCs do co-exist.[104] In mice, this approach resulted in the identification of stem cell populations expressing plasma membrane proteins and CD82, a known marker for LT-HSC maintenance. In humans, CD49f$^+$ cells and MMPs could be similarly identified.[104]

An increasingly used technique to assay chromatin accessibility (ChA) at the single-cell level is the assay for transposase-accessible chromatin using sequencing (scATAC-Seq).[105] This approach enabled the finding that aged murine HSCs undergo symmetric divisions that result in daughter stem cells with reduced regenerative capacity and lymphoid potential.[106] scATAC-Seq was also used for the identification of a number of hallmarks of immune aging in mice and human cells,[107] most notably a T-BOX-regulated program that drives the transition of CD8$^+$ T cells toward the granzyme K (GZMK)$^+$ aging phenotype.

Cheung et al[108] have developed epigenetic landscape profiling using cytometry by time-of-flight technique, which enables single-cell profiling of a wide array of chromatin markers in human immune cells and found consistent increases in cell-to-cell variability of these markers as a signature of immune cell aging.

Multiomics

Multiomics studies are now possible where combinations of genetic, epigenetic, and/or proteomic data can be derived from a single cell at the same time. One of the most commonly used methods combines scRNA-Seq with ATAC-Seq. Using this method, a recent study reported alterations in ChA in murine HSCs, which occurred with aging.[109] These differentially open accessible regions target transcription factors related to external stress, suggesting the ability to epigenetically inscribe HSCs to prime their response to external stimuli.[109]

Several recent studies investigated features of age-related clonal hematopoiesis by using a variety of single-cell multiomics approaches. Cellular indexing of transcriptomes and epitopes by sequencing (CITE-Seq) allows the integration of single-cell expression data with cell surface markers.[110] Taking a multiomics approach utilizing both CITE-Seq and scATAC-seq, it has been shown that aging of the immune system in the mouse results in a subpopulation of highly clonal age-associated GZMK-expressing CD8$^+$ T cells, which express markers of exhaustion and tissue homing.[107]

Another single-cell multiomics technique called TARGET-Seq enables the linking of gene expression with genotypic data. This technique enabled the definition of transcriptional and genetic tumor heterogeneity in human patients with myeloproliferative neoplasms (MPN), comparing mutated HSPCs with normal, age-matched controls in the context of *JAK2V617F*[111] and in *TP53*-mutation driven clonal evolution in MPN.[112] Interestingly, WT HSPCs from MPN patients differ substantially in their transcriptomic profiles when compared with age-matched HSPCs from normal donors, indicating that environmental factors, such as the diseased bone marrow, might influence transcriptomic changes.

An alternative approach uses a combination of genotyping of targeted loci with single-cell ChA (GoT-ChA) and was able to show both cell-intrinsic and cell state-specific shifts within mutant human hematopoietic HSPCs in the context of *JAK2V617F*-mutated MPN.[113] Contributing further to MPN biology by using scRNA-Seq and genotyping of *JAK2V617F* mutation status showed increases in the expression of interferon-response genes.[99,114]

In human cells, genotyping of the mitochondrial genome at the single-cell level has been recently combined with scATAC-Seq to study cell population dynamics and clonal properties in cancer of the hematopoietic system, showing that it is possible to infer subclonal population structures using natural genetic mitochondrial DNA barcodes.[115]

The simultaneous single-cell profiling of gene expression, cell surface protein markers, and somatic mutation status is now possible and has been used to investigate the impact of splicing aberrations in human age-related clonal hematopoiesis by combing Genotyping of Transcriptomes (GoT), utilizing long-read single-cell transcriptome profiling in addition to proteo-genomics using CITE-Seq.[116] Assessment of bone marrow progenitor cells derived from patients with defective RNA splicing, due to mutations of the core RNA splicing factor 3b subunit 1 (SF3B1), was shown to have a fitness advantage over WT SF3B1 cells, resulting in the expansion of a mutant population of erythroid progenitor cells, which displayed an upregulation of genes involved in the cell cycle and mRNA translation.[116] Moreover, another study using a multiomics approach to interrogate human clonal hematopoiesis combined scBS-Seq with scRNA-Seq and targeted *DNMT3A* genotyping. This study showed that mutations within DNMT3A resulted in a myeloid over lymphoid bias and that these *DNMT3A* mutations revealed patterns of selective hypomethylation of key hematopoietic transcription factors that results in the disruption of early progenitor states.[117] Finally, advances in scRNA-Seq methodologies that incorporate a mitochondrial transcriptome enrichment step have enabled the detection of mitochondrial variants with high confidence and have been used to study age-related clonal hematopoiesis dynamics, identifying skewed immune cell expansions in primary human clonal hematopoiesis.[118]

Cellular metabolism has long been known to affect aging with caloric restriction protecting against age-related disease.[119] The use of multiomic approaches that include single-cell metabolomics will be required to elucidate the complex role that metabolism plays during HSCs aging.[120,121] Application of newer single-cell proteomic techniques, such as SpaceM, that combine single-cell metabolomics with spatial information is one such technique[122] that can be applied to human aging studies in the future. The requirement of a multiomic view to further our understanding of how aging affects humans has given rise to various initiatives to gather multimodal single-cell data from various human tissues, such as the Human Cell Atlas (www.humancellatlas.org).

Spatial

As the aging bone marrow niche provides a heterogeneous microenvironment for a variety of cells and growth factors, single-cell approaches that provide spatial information in combination with other omics data are becoming increasingly important as they enable the identification of different cell types and how those cell types interact within the bone

marrow and with HSCs.[7] A caveat in the use of scRNA-seq to investigate the bone marrow microenvironment is that the frequency of resident cell populations is known to vary by orders of magnitude.[123] A number of strategies have been used to overcome this difference in cell number that broadly fall into either positive or negative selection strategies. Using a dual strategy of depletion/enrichment of high/low abundance cell types[124] were able to identify 32 clusters of cells that included rarer nonhematopoietic cell populations such as Schwann cells, smooth muscle cells, myofibroblasts, PDGFRA^{+} mesenchymal cells, and endothelial cells. Another profiling study using a depletion strategy selecting for Lin^{-} cells was able to identify 17 clusters of cells that included endothelial cells, MSCs, osteolinage cells, chondrocytes, fibroblasts, and pericytes.[125] Lineage-specific Cre-labeling has been used to positively select for cell populations representing major niche subsets including endothelial, perivascular, and osteo-lineage subpopulations.[126] Technologies, such as 10× Genomics' Visium Spatial Gene Expression system, allow the mapping of whole transcriptome data with the morphological context in tissue sections. It has been used to generate a comprehensive tissue atlas of the developing human immune system incorporating scRNA-Seq, antigen-receptor sequencing, and spatial transcriptomics across a number of tissue types to show heterogeneity across organs and gestation of both myeloid and lymphoid cells.[127] NanoString's GeoMx Digital Spatial Profiler, which can link both gene or protein expression spatially in tissue sections, has been used to investigate the BM immune microenvironment using diagnostic bone marrow trephine samples that had been dual-stained for CD3+/CD45+ to identify immune infiltrate cells. The study showed that aplastic anemia samples cluster separately from both normal and myelodysplastic samples.[128] Laser-capture microdissection sequencing has also been combined with scRNA-Seq to transcriptionally survey the endosteal, sinusoidal, and arteriolar bone marrow niches at the molecular and cellular levels to not only determine the localization of various cell types within the bone marrow but to show the spatial sources of key growth factors and cytokines.[124] These techniques now pave the way toward exploiting well-documented changes in the bone marrow microenvironment with increasing age at increasingly single-cell resolution.

DISCUSSION

Overall, single-cell approaches, such as flow cytometry, transplantation, and colony assays, have been used for decades in hematology. However, omics-related single-cell approaches are a relatively new addition to the tools available to the field of hematology with some methods now widely used to better understand heterogeneity caused by the aging process. With senescence targeting via senolytics and rejuvenation approaches,[129–131] now widely investigated in a variety of model systems, understanding the molecular pathways and exact cell types affected by those interventions becomes increasingly important for translation into humans. Changes caused by aging are well documented in the hematopoietic compartment and the bone marrow microenvironment, however, have thus far mostly been studied in isolation. The advancement of better in vitro cell culture models combined with the development of single-cell and spatial approaches now paves the way toward a better understanding of direct changes in cell type interactions in the HSC compartment with advanced age.

AUTHOR CONTRIBUTIONS

KK conceived and supervised the review. KK, FY, and CN wrote the manuscript. GVH edited the manuscript and gave feedback.

DISCLOSURES

The authors have no conflicts of interest to disclose.

SOURCES OF FUNDING

KK is funded by a Blood Cancer U.K. grant (23001), an MRC grant (MR/W000148/1), a Tenovus Scotland grant (S19-07), and CRUK Scotland Centre funding (CTRQQR-2021\100006). FY and KK received funding from NHS GG&C Endowments.

REFERENCES

1. Olson OC, Kang YA, Passegué E. Normal hematopoiesis is a balancing act of self-renewal and regeneration. *Cold Spring Harb Perspect Med*. 2020;10:a035519.
2. Terradas-Terradas M, Robertson NA, Chandra T, et al. Clonality in haematopoietic stem cell ageing. *Mech Ageing Dev*. 2020;189:111279.
3. Robertson NA, Latorre-Crespo E, Terradas-Terradas M, et al. Longitudinal dynamics of clonal hematopoiesis identifies gene-specific fitness effects. *Nat Med*. 2022;28:1439–1446.
4. de Magalhães JP. How ageing processes influence cancer. *Nat Rev Cancer*. 2013;13:357–365.
5. McKerrell T, Vassiliou GS. Aging as a driver of leukemogenesis. *Sci Transl Med*. 2015;7:306fs38–306fs38.
6. de Haan G, Lazare SS. Aging of hematopoietic stem cells. *Blood*. 2018;131:479–487.
7. Matteini F, Mulaw MA, Florian MC. Aging of the hematopoietic stem cell niche: new tools to answer an old question. *Front Immunol*. 2021;12:738204 .
8. Ya-Hsuan H, Simón M-F. Microenvironmental contributions to hematopoietic stem cell aging. *Haematologica*. 2020;105:38–46.
9. Ikuta K, Weissman IL. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc Natl Acad Sci USA*. 1992;89:1502–1506.
10. van de Rijn M, Heimfeld S, Spangrude GJ, et al. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. *Proc Natl Acad Sci USA*. 1989;86:4634–4638.
11. Morrison SJ, Weissman IL. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity*. 1994;1:661–673.
12. Yilmaz OH, Kiel MJ, Morrison SJ. SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. *Blood*. 2006;107:924–930.
13. Purton LE. Adult murine hematopoietic stem cells and progenitors: an update on their identities, functions, and assays. *Exp Hematol*. 2022;116:1–14.
14. Challen GA, Boles NC, Chambers SM, et al. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. *Cell Stem Cell*. 2010;6:265–278.
15. Christensen JL, Weissman IL. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proc Natl Acad Sci USA*. 2001;98:14541–14546.
16. Dykstra B, Olthof S, Schreuder J, et al. Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *J Exp Med*. 2011;208:2691–2703.
17. Beerman I, Bhattacharya D, Zandi S, et al. Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proc Natl Acad Sci USA*. 2010;107:5465–5470.
18. Cho RH, Sieburg HB, Muller-Sieburg CE. A new mechanism for the aging of hematopoietic stem cells: aging changes the clonal composition of the stem cell compartment but not individual stem cells. *Blood*. 2008;111:5553–5561.
19. Benz C, Copley MR, Kent DG, et al. Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. *Cell Stem Cell*. 2012;10:273–283.
20. Dykstra B, Kent D, Bowie M, et al. Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell*. 2007;1:218–229.
21. Young K, Borikar S, Bell R, et al. Progressive alterations in multipotent hematopoietic progenitors underlie lymphoid cell loss in aging. *J Exp Med*. 2016;213:2259–2267.
22. Gekas C, Graf T. CD41 expression marks myeloid-biased adult hematopoietic stem cells and increases with age. *Blood*. 2013;121:4463–4472.

23. Shin JY, Hu W, Naramura M, et al. High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. *J Exp Med*. 2014;211:217–231.
24. Gulati GS, Zukowska M, Noh JJ, et al. Neogenin-1 distinguishes between myeloid-biased and balanced Hoxb5⁺ mouse long-term hematopoietic stem cells. *Proc Natl Acad Sci USA*. 2019;116:25115–25125.
25. Yamamoto R, Wilkinson AC, Ooehara J, et al. Large-scale clonal analysis resolves aging of the mouse hematopoietic stem cell compartment. *Cell Stem Cell*. 2018;22:600–607.e4.
26. Bernitz JM, Kim HS, MacArthur B, et al. Hematopoietic stem cells count and remember self-renewal divisions. *Cell*. 2016;167:1296–1309.e10.
27. Qiu J, Papatsenko D, Niu X, et al. Divisional history and hematopoietic stem cell function during homeostasis. *Stem Cell Rep*. 2014;2:473–490.
28. Chen JY, Miyanishi M, Wang SK, et al. Hoxb5 marks long-term haematopoietic stem cells and reveals a homogenous perivascular niche. *Nature*. 2016;530:223–227.
29. Acar M, Kocherlakota KS, Murphy MM, et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. *Nature*. 2015;526:126–130.
30. Gazit R, Mandal PK, Ebina W, et al. Fgd5 identifies hematopoietic stem cells in the murine bone marrow. *J Exp Med*. 2014;211:1315–1331.
31. Verovskaya E, Broekhuis MJ, Zwart E, et al. Heterogeneity of young and aged murine hematopoietic stem cells revealed by quantitative clonal analysis using cellular barcoding. *Blood*. 2013;122:523–532.
32. Yu KR, Espinoza DA, Wu C, et al. The impact of aging on primate hematopoiesis as interrogated by clonal tracking. *Blood*. 2018;131:1195–1205.
33. Ganuza M, Hall T, Finkelstein D, et al. The global clonal complexity of the murine blood system declines throughout life and after serial transplantation. *Blood*. 2019;133:1927–1942.
34. Baum CM, Weissman IL, Tsukamoto AS, et al. Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci USA*. 1992;89:2804–2808.
35. Bhatia M, Wang JCY, Kapp U, et al. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci USA*. 1997;94:5320–5325.
36. Ortmann CA, Kent DG, Nangalia J, et al. Effect of mutation order on myeloproliferative neoplasms. *N Engl J Med*. 2015;372:1865–1866.
37. Mayani H, Dragowska W, Lansdorp PM. Characterization of functionally distinct subpopulations of CD34+ cord blood cells in serum-free long-term cultures supplemented with hematopoietic cytokines. *Blood*. 1993;82:2664–2672.
38. Notta F, Doulatov S, Laurenti E, et al. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science*. 2011;333:218–221.
39. Amoah A, Keller A, Emini R, et al. Aging of human hematopoietic stem cells is linked to changes in Cdc42 activity. *Haematologica*. 2021;107:393–402.
40. Rundberg Nilsson A, Soneji S, Adolfsson S, et al. Human and murine hematopoietic stem cell aging is associated with functional impairments and intrinsic megakaryocytic/erythroid bias. *PLoS One*. 2016;11:e0158369.
41. Kuranda K, Vargaftig J, de la Rochere P, et al. Age-related changes in human hematopoietic stem/progenitor cells. *Aging Cell*. 2011;10:542–546.
42. Pang WW, Price EA, Sahoo D, et al. Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proc Natl Acad Sci USA*. 2011;108:20012–20017.
43. Mah LJ, El-Osta A, Karagiannis TC. γH2AX: a sensitive molecular marker of DNA damage and repair. *Leukemia*. 2010;24:679–686.
44. Flach J, Bakker ST, Mohrin M, et al. Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells. *Nature*. 2014;512:198–202.
45. Rossi DJ, Bryder D, Seita J, et al. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature*. 2007;447:725–729.
46. Rübe CE, Fricke A, Widmann TA, et al. Accumulation of DNA damage in hematopoietic stem and progenitor cells during human aging. *PLoS One*. 2011;6:e17487.
47. Adewoye AB, Tampakis D, Follenzi A, et al. Multiparameter flow cytometric detection and quantification of senescent cells in vitro. *Biogerontology*. 2020;21:773–786.
48. Guerrero A, Guiho R, Herranz N, et al. Galactose-modified duocarmycin prodrugs as senolytics. *Aging Cell*. 2020;19:e13133.
49. Biavasco R, Lettera E, Giannetti K, et al. Oncogene-induced senescence in hematopoietic progenitors features myeloid restricted hematopoiesis, chronic inflammation and histiocytosis. *Nat Commun*. 2021;12:4559.
50. Kirschner K, Chandra T, Kiselev V, et al. Proliferation drives aging-related functional decline in a subpopulation of the hematopoietic stem cell compartment. *Cell Rep*. 2017;19:1503–1511.
51. Wajapeyee N, Serra RW, Zhu X, et al. Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. *Cell*. 2008;132:363–374.
52. Chang J, Wang Y, Shao L, et al. Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. *Nat Med*. 2016;22:78–83.
53. Zhang J, He T, Xue L, et al. Senescent T cells: a potential biomarker and target for cancer therapy. *EBioMedicine*. 2021;68:103409.
54. Ho TT, Warr MR, Adelman ER, et al. Autophagy maintains the metabolism and function of young and old stem cells. *Nature*. 2017;543:205–210.
55. Tanida I, Ueno T, Kominami E. LC3 and autophagy. In: Deretic V, ed. *Autophagosome and Phagosome*. Clifton, NJ: Humana Press; 2008:77–88.
56. Klionsky DJ, Abdel-Aziz AK, Abdelfatah S, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition)[1]. *Autophagy*. 2021;17:1–382.
57. Mansell E, Sigurdsson V, Deltcheva E, et al. Mitochondrial potentiation ameliorates age-related heterogeneity in hematopoietic stem cell function. *Cell Stem Cell*. 2021;28:241–256.e6.
58. Argüello RJ, Combes AJ, Char R, et al. SCENITH: a flow cytometry-based method to functionally profile energy metabolism with single-cell resolution. *Cell Metab*. 2020;32:1063–1075.e7.
59. Ho YH, del Toro R, Rivera-Torres J, et al. Remodeling of bone marrow hematopoietic stem cell niches promotes myeloid cell expansion during premature or physiological aging. *Cell Stem Cell*. 2019;25:407–418.e6.
60. Maryanovich M, Zahalka AH, Pierce H, et al. Adrenergic nerve degeneration in bone marrow drives aging of the hematopoietic stem cell niche. *Nat Med*. 2018;24:782–791.
61. Saçma M, Pospiech J, Bogeska R, et al. Haematopoietic stem cells in perisinusoidal niches are protected from ageing. *Nat Cell Biol*. 2019;21:1309–1320.
62. Laschober GT, Brunauer R, Jamnig A, et al. Leptin receptor/CD295 is upregulated on primary human mesenchymal stem cells of advancing biological age and distinctly marks the subpopulation of dying cells. *Exp Gerontol*. 2009;44:57–62.
63. Sugiyama T, Nagasawa T. Bone marrow niches for hematopoietic stem cells and immune cells. *Inflamm Allergy Drug Targets*. 2012;11:201–206.
64. Omatsu Y, Sugiyama T, Kohara H, et al. The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity*. 2010;33:387–399.
65. Daynes RA, Araneo BA, Ershler WB, et al. Altered regulation of IL-6 production with normal aging. Possible linkage to the age-associated decline in dehydroepiandrosterone and its sulfated derivative. *J Immunol*. 1993;150:5219–5230.
66. Pedersen M, Bruunsgaard H, Weis N, et al. Circulating levels of TNF-alpha and IL-6-relation to truncal fat mass and muscle mass in healthy elderly individuals and in patients with type-2 diabetes. *Mech Ageing Dev*. 2003;124:495–502.
67. Valletta S, Thomas A, Meng Y, et al. Micro-environmental sensing by bone marrow stroma identifies IL-6 and TGFβ1 as regulators of hematopoietic ageing. *Nat Commun*. 2020;11:4075.
68. Pioli PD, Casero D, Montecino-Rodriguez E, et al. Plasma cells are obligate effectors of enhanced myelopoiesis in aging bone marrow. *Immunity*. 2019;51:351–366.e6.
69. Puchta A, Naidoo A, Verschoor CP, et al. TNF drives monocyte dysfunction with age and results in impaired anti-pneumococcal immunity. *PLoS Pathog*. 2016;12:e1005368.
70. Yamashita M, Passegué E. TNF-α coordinates hematopoietic stem cell survival and myeloid regeneration. *Cell Stem Cell*. 2019;25:357–372.e7.
71. Pietras EM, Reynaud D, Kang YA, et al. Functionally distinct subsets of lineage-biased multipotent progenitors control blood production in normal and regenerative conditions. *Cell Stem Cell*. 2015;17:35–46.
72. Mitchell CA, Verovskaya EV, Calero-Nieto FJ, et al. Stromal niche inflammation mediated by IL-1 signalling is a targetable driver of haematopoietic ageing. *Nat Cell Biol*. 2023;25:30–41.
73. Hellmich C, Wojtowicz E, Moore JA, et al. p16INK4A dependent senescence in the bone marrow niche drives age-related metabolic changes of hematopoietic progenitors. *Blood Adv*. 2022;7:256–268.
74. Georgilis A, Klotz S, Hanley CJ, et al. PTBP1-mediated alternative splicing regulates the inflammatory secretome and the pro-tumorigenic effects of senescent cells. *Cancer Cell*. 2018;34:85–102.e9.
75. Young K, Eudy E, Bell R, et al. Decline in IGF1 in the bone marrow microenvironment initiates hematopoietic stem cell aging. *Cell Stem Cell*. 2021;28:1473–1482.e7.

76. Bartolović K, Balabanov S, Berner B, et al. Clonal heterogeneity in growth kinetics of CD34+CD38− human cord blood cells in vitro is correlated with gene expression pattern and telomere length. *Stem Cells*. 2005;23:946–957.
77. Berg S, Kutra D, Kroeger T, et al. ilastik: interactive machine learning for (bio)image analysis. *Nat Methods*. 2019;16:1226–1232.
78. Jeckel H, Drescher K. Advances and opportunities in image analysis of bacterial cells and communities. *FEMS Microbiol Rev*. 2021;45:fuaa062.
79. Kent DG, Li J, Tanna H, et al. Self-renewal of single mouse hematopoietic stem cells is reduced by JAK2V617F without compromising progenitor cell expansion. *PLoS Biol*. 2013;11:e1001576.
80. Broudy VC. Stem cell factor and hematopoiesis. *Blood*. 1997;90:1345–1364.
81. van Os RP, Dethmers-Ausema B, de Haan G. In vitro assays for cobblestone area-forming cells, LTC-IC, and CFU-C. In: Bunting KD, ed. *Hematopoietic Stem Cell Protocols*. Clifton, NJ: Humana Press; 2008:143–157.
82. Lee-Six H, Øbro NF, Shepherd MS, et al. Population dynamics of normal human blood inferred from somatic mutations. *Nature*. 2018;561:473–478.
83. Mitchell E, Spencer Chapman M, Williams N, et al. Clonal dynamics of haematopoiesis across the human lifespan. *Nature*. 2022;606:343–350.
84. Fabre MA, de Almeida JG, Fiorillo E, et al. The longitudinal dynamics and natural history of clonal haematopoiesis. *Nature*. 2022;606:335–342.
85. Robertson NA, Hillary RF, McCartney DL, et al. Age-related clonal haemopoiesis is associated with increased epigenetic age. *Curr Biol*. 2019;29:R786–R787.
86. Wilkinson AC, Igarashi KJ, Nakauchi H. Haematopoietic stem cell self-renewal in vivo and ex vivo. *Nat Rev Genet*. 2020a;21:541–554.
87. Lewis EEL, Wheadon H, Lewis N, et al. A quiescent, regeneration-responsive tissue engineered mesenchymal stem cell bone marrow niche model via magnetic levitation. *ACS Nano*. 2016;10:8346–8354.
88. Wilkinson AC, Ishida R, Nakauchi H, et al. . Long-term ex vivo expansion of mouse hematopoietic stem cells. *Nat Protocols*. 2020b;15:628–648.
89. Wilkinson AC, Ishida R, Kikuchi M, et al. Long-term ex vivo haematopoietic-stem-cell expansion allows nonconditioned transplantation. *Nature*. 2019;571:117–121.
90. Li Y, Wu Q, Wang Y, et al. Senescence of mesenchymal stem cells (review). *Int J Mol Med*. 2017;39:775–782.
91. Khan AO, Rodriguez-Romera A, Reyat JS, et al. Human bone marrow organoids for disease modelling, discovery and validation of therapeutic targets in hematological malignancies. *Cancer Discov*. 2022;13:364–385.
92. Sakurai M, Ishitsuka K, Ito R, et al. Chemically defined cytokine-free expansion of human haematopoietic stem cells. *Nature*. 2023;615:127–133.
93. Evans MA, Walsh K. Clonal hematopoiesis, somatic mosaicism, and age-associated disease. *Physiol Rev*. 2023;103:649–716.
94. Morrison SJ, Wandycz AM, Akashi K, et al. The aging of hematopoietic stem cells. *Nat Med*. 1996;2:1011–1016.
95. Grover A, Sanjuan-Pla A, Thongjuea S, et al. Single-cell RNA sequencing reveals molecular and functional platelet bias of aged haematopoietic stem cells. *Nat Commun*. 2016;7:11075.
96. Flohr Svendsen A, Yang D, Kim K, et al. A comprehensive transcriptome signature of murine hematopoietic stem cell aging. *Blood*. 2021;138:439–451.
97. Thongon N, Ma F, Santoni A, et al. Hematopoiesis under telomere attrition at the single-cell resolution. *Nat Commun*. 2021;12:6850.
98. Desterke C, Bennaceur-Griscelli A, Turhan AG. EGR1 dysregulation defines an inflammatory and leukemic program in cell trajectory of human-aged hematopoietic stem cells (HSC). *Stem Cell Res Ther*. 2021;12:419.
99. Ren P, Dong X, Vijg J. Age-related somatic mutation burden in human tissues. *Front Aging*. 2022;3:1018119.
100. Valecha M, Posada D. Somatic variant calling from single-cell DNA sequencing data. *Comput Struct Biotechnol*. 2022;20:2978–2985
101. Miles LA, Bowman RL, Merlinsky TR, et al. Single-cell mutation analysis of clonal evolution in myeloid malignancies. *Nature*. 2020;587:477–482.
102. Albertí-Servera L, Demeyer S, Govaerts I, et al. Single-cell DNA amplicon sequencing reveals clonal heterogeneity and evolution in T-cell acute lymphoblastic leukemia. *Blood*. 2021;137:801–811.
103. Wang K, Liu H, Hu Q, et al. Epigenetic regulation of aging: implications for interventions of aging and diseases. *Signal Transduct Target Ther*. 2022;7:374.
104. Hui T, Cao Q, Wegrzyn-Woltosz J, et al. High-resolution single-Cell DNA methylation measurements reveal epigenetically distinct hematopoietic stem cell subpopulations. *Stem Cell Rep*. 2018;11:578–592.
105. Chen X, Miragaia RJ, Natarajan KN, et al. A rapid and robust method for single cell chromatin accessibility profiling. *Nat Commun*. 2018;9:5345.
106. Florian MC, Klose M, Sacma M, et al. Aging alters the epigenetic asymmetry of HSC division. *PLoS Biol*. 2018;16:e2003389.
107. Mogilenko DA, Shpynov O, Andhey PS, et al. Comprehensive profiling of an aging immune system reveals clonal GZMK$^+$ CD8$^+$ T cells as conserved hallmark of inflammaging. *Immunity*. 2021;54:99–115.e12.
108. Cheung P, Vallania F, Dvorak M, et al. Single-cell epigenetics—chromatin modification atlas unveiled by mass cytometry. *Clin Immunol*. 2018;196:40–48.
109. Itokawa N, Oshima M, Koide S, et al. Epigenetic traits inscribed in chromatin accessibility in aged hematopoietic stem cells. *Nat Commun*. 2022;13:2691.
110. Stoeckius M, Hafemeister C, Stephenson W, et al. Simultaneous epitope and transcriptome measurement in single cells. *Nat Methods*. 2017;14:865–868.
111. Rodriguez-Meira A, Buck G, Clark SA, et al. Unravelling intratumoral heterogeneity through high-sensitivity single-cell mutational analysis and parallel RNA sequencing. *Mol Cell*. 2019;73:1292–1305.e8.
112. Rodriguez-Meira A, Norfo R, Wen WX, et al. Deciphering TP53 mutant cancer evolution with single-cell multi-omics. *bioRxiv*. 2022:03.28.485984.
113. Myers RM, Izzo F, Kottapalli S, et al. Integrated single-cell genotyping and chromatin accessibility charts JAK2V617F human hematopoietic differentiation. *bioRxiv*. 2022:05.11.491515.
114. Van Egeren D, Kamaz B, Liu S, et al. Transcriptional differences between JAK2-V617F and wild-type bone marrow cells in patients with myeloproliferative neoplasms. *Exp Hematol*. 2022;107:14–19.
115. Lareau CA, Ludwig LS, Muus C, et al. Massively parallel single-cell mitochondrial DNA genotyping and chromatin profiling. *Nat Biotechnol*. 2021;39:451–461.
116. Gaiti F, Chamely P, Hawkins AG, et al. Single-cell multi-omics defines the cell-type specific impact of splicing aberrations in human hematopoietic clonal outgrowths. *bioRxiv*. 2022:06.08.495292.
117. Nam AS, Dusaj N, Izzo F, et al. Single-cell multi-omics of human clonal hematopoiesis reveals that DNMT3A R882 mutations perturb early progenitor states through selective hypomethylation. *Nat Genet*. 2022;54:1514–1526.
118. Miller TE, Lareau CA, Verga JA, et al. Mitochondrial variant enrichment from high-throughput single-cell RNA sequencing resolves clonal populations. *Nat Biotechnol*. 2022;40:1030–1034.
119. Balasubramanian P, Howell PR, Anderson RM. Aging and caloric restriction research: a biological perspective with translational potential. *EBioMedicine*. 2017;21:37–44.
120. Ito K, Ito K. Hematopoietic stem cell fate through metabolic control. *Exp Hematol*. 2018;64:1–11.
121. Schüler SC, Gebert N, Ori A. Stem cell aging: the upcoming era of proteins and metabolites. *Mech Ageing Dev*. 2020;190:111288.
122. Rappez L, Stadler M, Triana S, et al. SpaceM reveals metabolic states of single cells. *Nat Methods*. 2021;18:799–805.
123. Gomariz A, Helbling PM, Isringhausen S, et al. Quantitative spatial analysis of haematopoiesis-regulating stromal cells in the bone marrow microenvironment by 3D microscopy. *Nat Commun*. 2018;9:2532.
124. Baccin C, Al-Sabah J, Velten L, et al. Combined single-cell and spatial transcriptomics reveal the molecular, cellular and spatial bone marrow niche organization. *Nat Cell Biol*. 2020;22:38–48.
125. Baryawno N, Przybylski D, Kowalczyk MS, et al. A cellular taxonomy of the bone marrow stroma in homeostasis and leukemia. *Cell*. 2019;177:1915–1932.e16.
126. Tikhonova AN, Dolgalev I, Hu H, et al. The bone marrow microenvironment at single-cell resolution. *Nature*. 2019;569:222–228.
127. Suo C, Dann E, Goh I, et al. Mapping the developing human immune system across organs. *Science*. 2022;376:eabo0510.
128. Koldej RM, Ritchie DS. High multiplex analysis of the immune microenvironment in bone marrow trephine samples using GeoMX™ digital spatial profiling. *Immuno-oncol Technol*. 2020;5:1–9.
129. Kirschner K, Rattanavirotkul N, Quince MF, et al. Functional heterogeneity in senescence. *Biochem Soc Trans*. 2020;48:765–773.
130. Olova N, Simpson DJ, Marioni RE, Chandra T. Partial reprogramming induces a steady decline in epigenetic age before loss of somatic identity. *Aging Cell*. 2019;18:e12877.
131. Simpson DJ, Olova NN, Chandra T. Cellular reprogramming and epigenetic rejuvenation. *Clin Epigenetics*. 2021;13:170.