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Hand in hand: intrinsic and extrinsic drivers of aging and clonal hematopoiesis

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Over the past 25 years, the importance of hematopoietic stem cell (HSC) aging in overall hematopoietic and immune system health span has been appreciated. Much work has been done in model organisms to understand the intrinsic dysregulation that occurs in HSCs during aging, with the goal of identifying modifiable mechanisms that represent the proverbial "fountain of youth." Much more recently, the discovery of somatic mutations that are found to provide a selective advantage to HSCs and accumulate in the hematopoietic system during aging, termed clonal hematopoiesis (CH), inspires revisiting many of these previously defined drivers of HSC aging in the context of these somatic mutations. To truly understand these processes and develop a holistic picture of HSC aging, ongoing and future studies must include investigation of the critical changes that occur in the HSC niche or bone marrow microenvironment with aging, as increasing evidence supports that these HSC-extrinsic alterations provide necessary inflammation, signaling pathway activation or repression, and other selective pressures to favor HSC aging-associated phenotypes and CH. Here, we provide our perspectives based on the past 8 years of our own laboratory's investigations into these mechanisms and chart a path for integrative studies that, in our opinion, will provide an ideal opportunity to discover HSC and hematopoietic health span-extending interventions. This path includes examining when and how aging-associated HSC-intrinsic and HSC-extrinsic changes accumulate over time in different individuals and developing new models to track and test relevant HSC-extrinsic changes, complementary to innovative HSC lineage tracing systems that have recently been developed. **© 2020 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.**

There is a notable decline in hematopoietic and immune system function with age, leading to increased risk of infection, poor vaccination response, anemia, and increased risk of bone marrow failure and blood cancers [1]. The mature effector cells of the hematopoietic and immune systems are continually being replenished throughout life by HSCs, considered to be the "roots" of the system. Thus, to fully understand how aging alters hematopoiesis, we must strive to understand how aging alters HSC biology and perpetuates impaired function. Numerous HSC-intrinsic mechanisms have been implicated, as detailed below, but many of these mechanisms cooperate or can cause each other to occur, making it unclear which represent ideal therapeutic targets for rejuvenation. An additional key to understanding HSC aging is the fact that HSCs exist within a bone marrow microenvironment, which comprises many distinct hematopoietic and nonhematopoietic cell types as well as secreted factors, all of which accumulate aging-associated alterations as well. It is now understood that the processes of bone aging occur at a younger age than aging-associated hematopoietic and immune system decline [2], supporting the idea that changes in the bone and bone marrow may be the foundation upon which functional HSC decline occurs. We outline below some of the known bone marrow microenvironmental changes with age that affect HSC function. However, many of the cellular, transcriptional, epigenetic, and functional changes in the aging microenvironment are yet to be discovered. We argue that it is the interplay between these HSC-intrinsic and HSC-extrinsic mechanisms (Figure 1) that warrants deep investigation and innovative approaches, for example, new models to track and test key alterations in the HSC-extrinsic bone marrow

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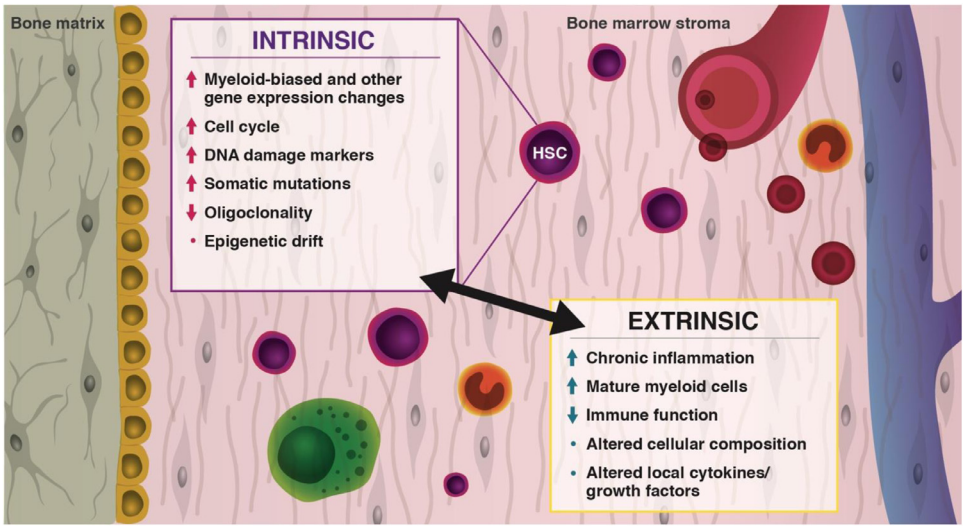

Figure 1. Crosstalk between HSC-intrinsic and HSC-extrinsic mechanisms associated with hematopoietic aging and immune function.

microenvironment, if we are to uncover fundamentally critical nodes in this network that can be targeted to extend healthy hematopoietic and immune system function into old age.

Traditionally defined HSC-intrinsic mechanisms associated with aging and CH

Phenotypic HSC expansion, heterogeneity, and myeloid lineage bias

One of the earliest reported phenotypes of HSC aging in mice and humans, consistently reproduced by many groups since, is the expansion of long-term (LT)-HSCs based on cell surface marker phenotyping [3,4]. Counterintuitively, despite this increase in proportion and absolute number of phenotypic LT-HSCs in the bone marrow, aging is associated with a decline in HSC function as determined by serial transplantation assays [5], and old HSCs exhibit reduced ability to home to the bone marrow [3,6]. Transcriptome analyses using bulk and/or single-cell RNA sequencing (scRNA-seq) to compare cell surface marker-defined young and old HSCs have identified signatures indicating increased HSC self-renewal and altered cell cycle [7–12]. Increased cell division and replicative stress in old HSCs [13], in turn, are thought to change their epigenetic state and lead to a decline in HSC fitness. Largely considered to be an HSC-intrinsic mechanism, dysregulation of numerous pathways responsive to extracellular signals has also been observed in old HSCs, including transforming growth factor (TGF)-β, Notch, NF-κB, Wnt, and mTOR, which are predicted to have significant effects on HSC function as they have all been found to have important roles in regulating quiescence, self-renewal, and differentiation [3,14].

Integral to interpretation of the aforementioned literature and the studies described below is the fact that the HSC population is intrinsically heterogeneous, consisting of a limited number of distinct subsets of HSCs, and the composition of the HSC compartment has been reported to be altered with aging [15]. HSCs exhibit defined heterogeneity with respect to their self-renewal capacity, differentiation potential, maturation rate, lineage potential, and life span [15]. This heterogeneity has been further demonstrated by recent advances in lineage tracing, coupled with single-cell transcriptomics and functional analyses [16,17]. Considered a hallmark of HSC aging, myeloid-biased differentiation was first functionally identified by Sudo et al. [6]. Subsequently, work from Muller-Sieburg et al. [18] and single-cell transplantation studies by Dykstra et al. [19] revealed that the myeloid-biased differentiation program already exists in a subset of HSCs in young mice. Later, several groups provided evidence that most of the "aged" behavior of HSCs could be explained by an accumulation of myeloid-biased HSCs in the aged HSC compartment [20–22]. More recently, identification of platelet-biased HSCs and their accumulation with aging have been reported as well [7,11]. Another critical caveat to consider in the literature is the reliance on cell surface marker definitions of HSCs to isolate this rare cell population from the total bone marrow to perform functional and molecular assays. Data from three independent laboratories (Gerald de Haan, Connie Eaves, and Hiromitsu Nakauchi) have functionally demonstrated through single-cell transplantation of aged HSCs that various cell surface marker combinations considered to highly enrich for HSCs (ESLAM, LSK/CD48/CD34/EPCR/CD150, and CD34KSL) result in functional HSC frequencies of between 10% and

40% [5,23,24]. Thus, many of the above-described HSC aging-associated phenotypes may in fact be a result of alterations in "contaminating" non-HSC populations interrogated in these assays. Furthermore, while cell surface marker phenotype-function studies have been robustly performed on prospectively isolated HSCs and progenitor cell populations from young mice, this has been done to a lesser extent in old mice, and it stands as an assumption that an "apples-to-apples" comparison is being made. The recent discovery of "latent HSCs" in old mice contained within phenotypically defined myeloid-restricted progenitors, with demonstrated long-term self-renewal capacity and multilineage potential in secondary transplantation [24], calls into question what else might be lost from our current phenotypic definition of HSCs in aged individuals. In these respects, studying HSC heterogeneity in the context of aging may be more straightforward using emerging single-cell tools [25] rather than relying on phenotypically defined HSCs.

Increased markers of DNA damage and somatic mutations

DNA damage and acquisition of somatic mutations are well-studied intrinsic hallmarks of aging that span tissues, organs, and species [26]. Increased markers of DNA double-strand breaks (DSBs) have been observed in old HSCs in both mice and humans using γH2A.X staining and the comet assay [27,28]. While this was originally considered to mean that there are more DSBs and DNA damage in old HSCs, functional studies testing radiation-induced DNA damage have revealed that the DNA damage response (DDR) is similarly robust in young and old HSCs. Thus, these cells are protected from persistent or accumulating DNA damage [29], despite transcriptional studies indicating that expression of DDR genes and pathways is reduced in old HSCs [30]. While γH2A.X staining is still commonly employed as a marker of old HSCs, the functional relevance of this marker is questionable [31,32]. Despite robust DDR in young and old HSCs, it has been clearly demonstrated using next-generation sequencing of primary or cultured human cells from donors of different ages that hematopoietic cells acquire mutations in a roughly linear fashion with time. These mutational rates are similar in human HSCs and multipotent progenitor cells (MPPs), indicating that proliferation and self-renewal are unlikely to underlie somatic mutation acquisition [33,34]. The rate of mutation in hematopoietic stem and progenitor cells (HSPCs) has been estimated at 14.2 to 17 mutations genomewide per year [33,34], or one protein-coding mutation per 10 years of life [35]. Mutagenesis in these cells appears be driven by intrinsic, stably acting processes over time, including the deamination of methylated cytosines resulting in C→T base substitutions [33,34,36,37]. Genomic context matters: while many mutations are neutral [35], some can give a cell a selective advantage, that is, clonal hematopoiesis (CH). This advantage can potentially arise from increased HSC self-renewal and proliferation, leading to dominance of mutated over nonmutated cells in an overall expanding pool of HSCs, or decreased cell death, leading to dominance in a steady or shrinking pool of HSCs. Distinct mutations associated with clonal advantage may operate through one or both of these general principles (reviewed in [38–41]).

Chromatin state and epigenetic drift

Two key chromatin-based alterations are observed in old HSCs: (1) a shift in epigenetic and chromatin organization, and (2) somatic mutations occurring in epigenetic machinery that are predicted to result in further epigenetic changes. Examining DNA methylation data sets generated from young and old mouse HSCs by different groups, few loci commonly gain or lose DNA methylation with age across these studies [12,42], supporting an "epigenetic drift" model where epigenetic patterns become more heterogeneous within the HSC population with aging. Epigenetic drift has also been described in human HSCs, with significant reductions in H3K4me1, H3K27ac, and H3K4me3 [43]. In human CH, two of the most frequently mutated genes are involved in regulating DNA methylation: the DNA methyltransferase 3α (*DNMT3A*) and tet methylcytosine dioxygenase 2 (*TET2*). *DNMT3A* catalyzes the addition of a methyl group to cytosine bases (5mC), and *TET2*, conversely, is involved in a multistep DNA demethylation process through the intermediate 5-hydroxymethylcytosine (5hmC). In mouse models, null alleles in either of these genes cause a block in differentiation and resultant expansion of HSPCs caused by increased self-renewal capacity [44–48]. However, the mechanisms connecting genotype to phenotype remain largely unexplained. In *TET2*-mutant CH, a loss of total levels of 5hmC and gains of 5mC were observed [49]. In *Dnmt3a*-null mice, HSCs did not differ in the total amount of 5mC but had focal gains and losses of 5mC compared with controls. These local differences in DNA methylation did not correlate with changes in gene expression [45], commonly observed in other studies [50]. As DNMT3A and TET2 are recruited to various genetic loci and can interact with other epigenetic proteins and complexes, their loss or altered function may lead to gradual shifts in chromatin state, changing the transcriptional output in a stochastic, or potentially progressive manner. This may explain why studies to date have found no convincing overlap in dysregulated gene signatures or DNA methylation patterns explaining their clonal dominance and argues that

different experimental approaches are needed to understand the importance of epigenetic drift as a driver of HSC aging and CH.

Traditionally defined HSC-extrinsic mechanisms associated with aging and CH

Spatial organization and cellular composition of the bone marrow microenvironment

Numerous studies have identified key cytokines/chemokines and cell types in the bone marrow that induce or are indispensable for proper function of HSCs. Most recently, scRNA-seq studies from three independent laboratories have begun to dissect the complex heterogeneity of the young microenvironment [51–53]. These studies have proposed new lineage relationships and subpopulations of classically defined stromal cell types, which will continue to be validated and interrogated, and this same strategy is being extended to understand how the microenvironment is altered with aging. Studies to date have identified a decrease in bone matrix, increase in vascular volume, and expansion of large adipocytes in old bone that create a physical space and environment different from that in young bone. The physical localization of HSCs and proximity to other cell types in the microenvironment are also known to be altered with aging [54]. There is emerging indication that adhesion and interaction of old HSCs with their environment are important components of HSC aging. While the true physical "niche" space in the microenvironment for LT-HSCs remains debated, it is important to consider that both physical contact with other cell types and gradients of signaling molecules produced both locally and distally may influence key HSC functions and become dysregulated with aging.

Inflamm-aging

Organismal aging is associated with chronic, low-grade inflammation, which has been termed *inflamm-aging*. Inflamm-aging is typically defined by an increase in circulating inflammatory cytokines most often measured in the blood and bone marrow. Over the past 20 years, numerous studies have identified many of these inflammatory cytokines, including RANTES, TNFα, interleukin (IL)-6, IL-1β, and C-reactive protein and have reported that old HSCs respond to these inflammatory molecules in their environment [55–58]. What causes this chronic, low-grade inflammation? Myeloid cells are considered the primary producers of inflammatory molecules in the bone marrow. As an example, aged macrophages produce more IL-1β in the microenvironment alongside having a reduced functional ability for phagocytosis [57]. This alteration ends up being a "double whammy" of IL-1β production, as old neutrophils, also producing IL-1β, are not appropriately cleared by these dysfunctional macrophages. Evidence to date supports that CH also contributes to inflamm-aging. *Tet2*-mutant macrophages have been reported to express higher levels of inflammatory cytokines at the transcript level and to secrete more cytokines ex vivo [59]. *Tet2*-deficient bone marrow engrafted into unconditioned recipients also led to increased levels of pro-inflammatory donor-derived macrophages [60]. Similarly, *Dnmt3a*-knockout mast cells secreted higher levels of IL-6, TNFα, and IL-13 after acute stimulation compared with wild-type cells [61]. As inflamm-aging is considered systemic, these alterations also have implications for other cell and tissue types, including cells within the bone marrow microenvironment and conditions associated with CH, such as atherosclerotic cardiovascular disease [59].

Inflammation and immunity as a driver of CH

Given the hypothesis that inflamm-aging is a driver of aging and the strong association of elevated inflammatory cytokines with conditions such as myelodysplastic syndrome (MDS) (reviewed in [62]), it is reasonable to speculate that inflammation may provide a positive selection pressure on HSCs carrying CH mutations or, alternatively, that mutant HSCs are not affected by inflammation whereas nonmutant cells succumb to negative pressure from inflammation. Evidence from *Tet2*-null mice indicated that in the presence of increased TNFα, *Tet2*-mutant clones continued to expand in semisolid and liquid cultures compared with control cells, and this was associated with decreased levels of pro-apoptosis genes [63]. Similarly, *Tet2*-deficient HSPCs were found to promote an IL6-dependent pro-survival pathway that could be blocked by small molecule inhibition of downstream Shp2/Stat3 [64]. Human data point to elevated IL-6 in *TET2*-mutant CH and elevated TNFα in *DNMT3A*-mutant CH [65]. Recent work in individuals with ulcerative colitis, an inflammatory bowel disease, revealed a slightly higher incidence of CH, with enrichment for *DNMT3A* mutations, and increased interferon-β (IFNβ) levels in the serum of patients with *DNMT3A*-mutant CH [66]. However, in a cohort of myeloproliferative neoplasm patients, serum cytokine levels did not differ between patients with different CH mutations, including *DNMT3A* and *TET2* [67]. More work in human patients supported by mouse models is needed to understand if, and in which direction, cause and consequence relationships exist between inflammation and specific CH mutations. It has been postulated that evasion of immune system surveillance plays a role in expansion of CH-mutant clones (reviewed in [38,68,69]). Small intestine barrier disruption or systemic bacterial stimuli were shown to be crucial for myeloproliferation in *Tet2*-knockout mice and could be blocked with antibiotics or in germ-

free mice, supporting a key role of the immune response [70]. Whether or not these CH-mutant cells present neoantigens that may limit their proliferation remains to be determined.

Therapy-related and environmental contributions to CH

Cancer treatment may also serve as an HSC-extrinsic, positive selection pressure for the development of CH. *DNMT3A* mutations in patients with acute myeloid leukemia (AML) had similar or higher variant allele frequency (VAF) from diagnosis to remission and relapse, indicating persistence of the mutation despite chemotherapeutic treatment [71]. In a separate study, the hotspot *DNMT3A* R882 mutation predicted the presence of minimal residual disease, and eight of nine patients with detectable *DNMT3A* mutations during remission all relapsed [72]. These data suggest that *DNMT3A*-mutant CH clones are resistant to standard induction chemotherapy and may serve as a reservoir for subsequent relapse. Mechanistically, *DNMT3A* R882-mutant cells exhibited dampened CHK1-mediated DNA repair and histone eviction, contributing to chemotherapy resistance [72]. Eskelund et al. [73] reported that 98% of mantle cell lymphoma (MCL) patients had a detectable CH mutation before therapy was initiated, indicating that chemotherapy does not induce CH mutations but provides a positive selective pressure that is associated with their expansion. CH is also common in patients with nonhematologic cancer and is associated with aging, current or former smoker status, and prior exposure to radiation therapy [74]. It is tempting to speculate that induction of inflammation in these different contexts represents a common downstream mechanism favoring selective advantage of CH-mutant HSCs and contributing to impaired function of non-mutant HSCs.

Cooperativity between HSC-intrinsic and HSC-extrinsic drivers of aging and CH

Cell signaling crosstalk between HSCs and the bone marrow microenvironment

Highlighted earlier as an HSC-intrinsic mechanism, alterations in cell signaling pathways including TGF-α, NF-κB, and mTOR have all been described in old HSCs. However, activation or repression of these pathways is also regulated in response to the presence of external ligands, and many of these factors have been reported to be altered in levels and production in the old bone marrow microenvironment [55,56,75]. Thus, HSC-intrinsic signaling and transcriptional changes with aging are acutely intermingled with HSC-extrinsic changes in cytokine and growth factor molecules in the bone marrow microenvironment with aging. In the context of CH, this cooperativity between HSC-intrinsic and HSC-extrinsic signaling is also apparent. In a model of chronic low-dose inflammation, mimicking the old bone marrow microenvironment, transcriptional profiling of *Tet2*-mutant HSPCs revealed increased non-canonical NFκB signaling, whereas wild-type HSPCs had a canonical NFκB signature [76]. These experiments point to an important interaction between the effect of HSC-intrinsic somatic mutations in altering downstream signaling potential in response to inflammation and the requisite increase in HSC-extrinsic inflammation required to observe these phenotypes and stimulate CH.

Positive feedback loop between HSCs and mature myeloid progeny in CH

An expanded pool of myeloid-biased HSCs, mentioned previously as a hallmark of aging, has both HSC-intrinsic and HSC-extrinsic implications as a driver of CH. It remains unclear to what extent acquisition of a CH mutation is more or less likely to occur in and/or to provide a selective advantage to a particular lineage-balanced or lineage-biased HSC subset, per se. In mice, *Tet2* knockout transplants exhibited no difference in donor-derived myeloid or B-cell compartments after 12 weeks [44], while in other studies, moribund *Tet2* knockout transplants [77] and nonconditioned recipients [60] both exhibited increased myeloid skewing. Similarly, in 20-week-old conditional *Tet2* knockout mice, an expanded granulocyte–macrophage progenitor (GMP) population was observed in the bone marrow [47]. These results are similar to those for human CH, where *TET2* mutations have been more commonly found in myeloid cell types in the peripheral blood, whereas *DNMT3A* mutations were more commonly multilineage [78]. For the sake of argument, based on an assumption that CH mutations are equally likely to occur in lineage-balanced and lineage-biased HSC subsets, experimental evidence supports that, with aging, myeloid-biased HSCs become overrepresented in the population. Given the previous studies, detailed above, that have indicated that macrophages and mast cells carrying CH mutations are more pro-inflammatory and produce higher levels of inflammatory cytokines, and that inflammation can provide positive selection pressure for HSCs carrying CH mutations, this is highly likely to create a positive feedback loop. This positive feedback has the potential to be even stronger if CH mutations themselves provide a stronger selective advantage to myeloid-biased HSCs and increase production of more pro-inflammatory myeloid cell types. More experimental evidence is needed to robustly interrogate this model, which will further our understanding of the causes and consequences of myeloid bias and inflammation in CH, and to identify the most appropriate points for intervention to disrupt this putative positive feedback loop.

Interaction between environmental factors and fitness of specific variants in CH

The enrichment of certain somatic mutations in more narrowly defined patient populations points to differing clonal advantages depending on the context. For example, in CH in aplastic anemia, an autoimmune bone marrow failure syndrome, mutations in *PIGA, BCOR*, and *BCORL1* are the most common, rather than *DNMT3A* or *TET2* [79]. Prior exposure to chemotherapy or radiation in nonhematologic cancer patients is associated with prevalence of CH clones with *TP53* and *PPM1D* mutations [74], suggesting a positive selection pressure for cells carrying these mutations. Whether particular HSC-extrinsic inflammatory environments, or other extracellular stressors, select for or synergize with one or more specific HSC-intrinsic mutations remains an important and intriguing question [80]. Furthermore, such effects are likely not mutually exclusive given the positive feedback loops and cell signaling cooperative interactions noted above.

Future directions: delineating cause versus consequence

Old HSCs, at the population level, possess a catalogue of phenotypic differences compared with young HSCs, associated with reduced function, fitness, and lineage potential, but which of these are causes as opposed to consequences of aging? As many of these phenotypes have been observed by comparing young mice (3–6 months of age) with old mice (18–24 months of age), the equivalent of comparing humans 20–30 years of age with those 56–69 years of age (Figure 2), we have limited understanding of the order or sequence of events that occur to ultimately accumulate these phenotypes. This binary comparison is also accompanied by an incomplete picture of the role of biological sex, as this factor is omitted from a number of the fundamental studies described here, and it is well understood that aging and response to intervention differ between females and males [82]. Our laboratory's recent work focusing on female mice has reported that many hallmarks of HSC aging are observed as early as middle age (10–14 months of age, equivalent to 38- to 47-year-old humans) [83] and that this may be distinct in males (K. Young and J. Trowbridge, unpublished). With a concerted focus on this age point to decouple drivers of aging from mere consequences, we found that HSC-extrinsic changes in the bone marrow microenvironment, including reduced levels of insulin-like growth factor 1 (IGF1), induce and are indispensable for hallmarks of intrinsic HSC aging at middle age (K. Young and J. Trowbridge, unpublished). While we are not the first group to look at differences between hematopoiesis at middle age versus old age [30,84,85], we find alterations in the bone marrow microenvironment and HSC state at middle age to be distinct from both those at young and old age and relatively unexplored. When a putative driver of HSC aging has been identified, what comes next? If the ultimate goal is to increase health span and prevent hematopoietic and immune defects in aged individuals, we as a field need to determine which of the hallmarks of HSC aging really do cause impaired hematopoietic function. It is becoming clear from our and others' work that several hallmarks of HSC aging are not concomitant and can be separately induced or restored at both the molecular and functional levels. Pharmacological intervention strategies that have been found to rejuvenate some, but not all, hallmarks of aging in old HSCs (reviewed in [86]) have resulted in health span extension, particularly in immune system responses, lending support to the possibility of achieving this goal.

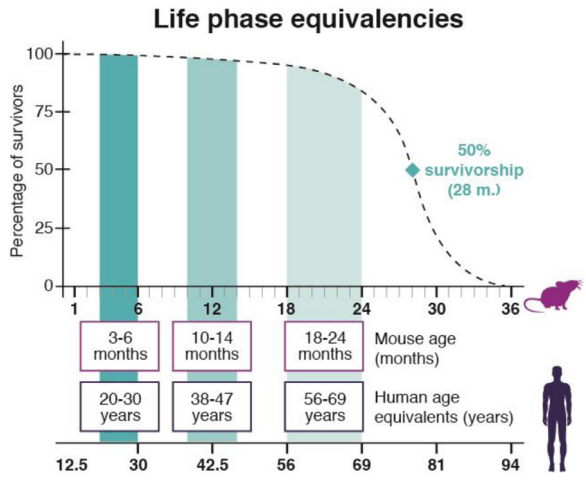

Figure 2. Age ranges for mature life history stages in C57BL/6J mice (young adult: 3–6 months, middle-aged: 10–14 months, old: 18–24 months) and relative human age equivalents. Adapted from Flurkey et al. [81] with permission.

Similar logic can apply to our understanding of CH and its associated risk for further complications, including hematologic cancer, atherosclerotic heart disease, myocardial infarction, ischemic stroke, and all-cause mortality (reviewed in [87]). Studies of human CH have indicated that from middle age to old age, there is an exponential increase in clonal expansion [49,88,89]. However, there are a multitude of potential outcomes in CH, including these three: (1) CH clones expand with lineage-balanced hematopoiesis and result in no clinical abnormalities. (2) CH clones expand the myeloid-biased HSC pool with implications for increasing risk of atherosclerotic cardiovascular disease. (3) CH clones expand and acquire additional somatic mutations that cause transformation to hematologic malignancy. One may argue that situations (2) and (3) are highly undesirable, but in fact situation (1) may be beneficial for aging hematopoiesis and immune

function if this clone is functionally superior and protected from some of the deleterious consequences of aging. How HSC-extrinsic changes in aging affect the fitness of particular mutant clones, through increased inflammation, altered niche composition, changes in the microbiome, and infection status and prior history, necessitate further mechanistic investigation. The opposite situation also requires attention: How do mutant cells signal to their environment to reinforce positive feedback loops, promoting expansion [38]?

Our perspective is that we do not yet know how much to weigh HSC-intrinsic versus HSC-extrinsic factors with respect to successful intervention to extend hematopoietic health span. Can we solve the "chicken and egg" challenge and determine which comes first? Moving forward, development of new models to track and test relevant HSC-extrinsic changes, complementary to innovative HSC lineage tracing systems that have recently been developed, will provide ideal systems to concurrently interrogate the biology of, interactions between, and effects of intervention strategies on HSC-intrinsic and HSC-extrinsic aging. For example, in the next 1–3 years, existing reporter strains for stromal lineage populations (e.g., fluorescence expression induced by Prrx1-Cre, Lepr-Cre, Nestin-Cre, Adipoq-Cre, Osx-Cre, or several others) could be used in aged versus young animals alongside HSPC reporters to determine whether altered cellular composition of the microenvironment with aging has an impact on HSPC localization. A similar strategy using these existing Cre strains to enforce expression of the diphtheria toxin receptor in stromal populations, followed by administration of diphtheria toxin to these animals, could test their necessity for HSC function in the specific context of aged mice. Importantly, for both of these strategies, several of these Cre strains will need additional engineering to be converted from constitutive to inducible alleles. Longer-term strategies to track and test relevant HSC-extrinsic changes will best be informed by comprehensive lineage tracing of the hierarchical relationships between cells of the adult and aged bone marrow microenvironment, where scRNA-seq data may be highly informative in generating a list of candidate promoters for the development of new Cre strains. In addition, development of biological sensing/recording systems for intracellular signaling in HSCs that can be coupled to HSC lineage tracing systems will allow predictions about how local levels of various cytokines in a particular HSC's environment, in the context of aging, can then affect its function. We posit that identifying the key nodes where extrinsic and intrinsic mechanisms meet may provide targets of enhanced therapeutic promise. And if we can prevent alterations early on in the process of aging, will we in fact achieve the goal of preserving healthy hematopoietic and immune function? We are just getting started and are looking forward to the next 25 years of discoveries!

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