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Hematopoietic Stem Cells Through the Ages: A Lifetime of Adaptation to Organismal Demands

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Abstract

Hematopoietic stem cells (HSC), which govern the production of all blood lineages, transition through a series of functional states characterized by expansion during fetal development, functional quiescence in adulthood, and decline upon aging. We describe central features of HSC regulation during ontogeny to contextualize how adaptive responses over the life of the organism ultimately form the basis for HSC functional degradation with age. We particularly focus on the role of cell cycle regulation, inflammatory response pathways, epigenetic changes, and metabolic regulation. We then explore how knowledge of age-related changes in HSC regulation can inform strategies for rejuvenation of old HSCs.

Introduction

Hematopoiesis is a constant, vital function by which all cells of the blood and immune system are produced throughout development, adulthood, and old age. Blood production by HSCs supports oxygen transport, wound healing, and immunological responses during different stages of life, with dynamic regulations tailoring the production of particular lineages of blood cells through a complex array of intrinsic and extrinsic mechanisms. HSCs are equipped with a unique network of stress-response mechanisms that enables them to rapidly respond to organismal demand while simultaneously protecting themselves from damage. However, these pro-survival mechanisms provoke specific genetic, epigenetic, and metabolic forms of damage which accumulate over time, leading to an expanded pool of indolent and functionally degraded old HSCs.

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Declaration of Interests

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Here, we review the unique properties of HSCs that dictate their behavior, focusing on the fulfillment of distinct sets of organismal requirements during development and adult life. Through this lens, we examine how mechanisms used by HSCs during these periods engender features of aging, focusing on cell cycle regulation, inflammatory response pathways, epigenetics, and metabolism. Considering the changes affecting HSCs throughout the entire lifespan also provides the basis for strategies to forestall or reverse decline with age. As such, we conclude by reviewing current HSC rejuvenation strategies, with a focus on their mechanistic rationale for restoring youthful functionality.

Dynamic changes in HSC behavior throughout ontogeny

The hematopoietic system is a dynamic entity with HSC function evolving over the lifetime to support changing organismal demands. As the organism develops, functional changes in HSCs fulfill the distinct requirements of different stages of ontogeny. In the context of fetal and neonatal development, the prioritization of HSC expansion over blood production allows for the establishment of the hematopoietic compartment[1,2]. By contrast, during adulthood, balanced blood production and dynamic responses to stress take precedence to maintain peak efficiency during reproductive years[3] (Figure 1). In this section, we review these changes in HSC behavior with a focus on the functional characteristics that allow hematopoiesis to be tailored to different stages of ontogeny.

Fetal HSCs establish the hematopoietic compartment

In humans as well as zebrafish and murine model systems, hematopoiesis initiates during early embryonic development and occurs in successive waves that mark the transition from primitive hematopoiesis in the yolk sac to definitive hematopoiesis in the aorta-gonad-mesonephros (AGM) region and the fetal liver[4]. Primitive hematopoiesis has been extensively reviewed elsewhere[5–9], and we focus here on definitive hematopoiesis, during which HSCs emerge from the hemogenic endothelium of the AGM region and migrate to the fetal liver before homing to the nascent bone marrow (BM) cavity[10].

In the fetus, HSC behavior is driven by the fundamental requirement of establishing the hematopoietic compartment. In line with this objective, fetal HSCs demonstrate higher proliferative capacity and greater repopulation ability than adult HSCs[11–16]. This emphasis on proliferative capacity is enabled by numerous cell-intrinsic features, including active cell cycle engagement. Studies using murine models have shown that almost all HSCs from the fetal period until 3 weeks after birth are in the $G_1/S/G_2$-M phase of the cell cycle, with the G_0 fraction estimated to be less than 0.02%[17–19]. Similarly, human HSC-enriched $CD34^+/CD38^-$ cells from umbilical cord blood demonstrate increased propensity to transit through the cell cycle relative to their adult BM-derived counterparts[20]. To cope with the anabolic demands of cell division, murine fetal HSCs exhibit a greater reliance on oxidative metabolism compared to adult HSCs, with increased mitochondrial mass and oxygen consumption necessitating the buffering of reactive oxygen species by autophagic degradation[21,22]. Additionally, to mitigate the effects of elevated protein synthesis, murine fetal HSCs use bile acids from the maternal and fetal liver as chemical chaperones to suppress stress responses triggered by unfolded proteins[23]. These specific strategies allow

fetal HSCs to maintain cellular integrity while supporting the proliferative demands of the rapidly developing organism.

Importantly, cell-extrinsic factors and inflammatory signals from the developing embryo and the maternal environment directly contribute to fetal HSC emergence, specification, and function[24]. Inflammatory signaling via tumor necrosis factor alpha (TNF-α) is required for the generation of HSCs in zebrafish, with Notch and NF-κB signaling pathways becoming activated to establish HSC fate[25]. Interferon (IFN) signaling has also been implicated in HSC emergence and maturation in both zebrafish and mouse models[26,27]. Converging evidence suggests that these inflammatory signals implicated in HSC emergence are largely sterile, with primitive neutrophils, and possibly primitive macrophages, contributing to the inflammatory milieu that drives HSPC expansion[25,28–30]. However, results in zebrafish indicate that inflammatory cytokine expression mediated by the commensal gut microbiome also promotes HSPC development[31].

The expansion of murine fetal HSCs has classically been thought to occur in the vascularized fetal liver (FL) niche, with $Nestin^{+}NG2^{+}$ pericytes promoting HSC expansion[32] and hepatic stellate and endothelial cells supporting HSC maintenance[33]. However, work in mice has challenged the model that fetal HSCs expand in the FL and progressively transition to the BM during the perinatal period, instead suggesting minimal contribution of FL HSCs to the adult pool, with most adult HSCs generated via postnatal expansion in the BM[34,35]. In this context, the placenta deserves further study as an extra-embryonic niche contributing to HSC expansion, given that phenotypic HSCs are known to expand in the murine placenta from E10.5 to E11[36–38]. The high frequency of HSPCs in the human placenta similarly suggests its role as an extra-embryonic niche for HSC expansion, an idea that is further supported by the ability of stromal cell lines from human placenta to support hematopoiesis in *ex vivo* co-culture[39].

While the expansion of HSCs is critically important to the establishment of the adult HSC pool, the growing organism simultaneously has a vital need for erythroid cells to provide oxygen transport. Until recently, the mechanisms by which organisms balance these opposing requirements have been unclear. Lineage tracing experiments suggest that fetal HSCs only minimally contribute to the generation of progenitors and functional mature blood cells before birth, thereby prioritizing the growth of the HSC pool. Instead, a stem cell-independent, progenitor-based mechanism promotes the outburst of mature blood cells that support oxygen transport, stimulate rapid tissue growth, and establish the fetal immune system[1,2]. These stem cell-independent early embryonic multipotent progenitors (MPP) emerge as early as E10.5 in mice and produce multilineage progeny, with their contributions to blood production declining progressively postnatally but persisting up to 21 months of age[1]. The early embryonic MPPs also appear to be the dominant source of lifelong lymphoid output relative to HSC-derived MPPs, raising a possible connection to the decline in lymphoid production observed with age. Together, these findings start to map the numerous integrated mechanisms promoting the establishment of an HSC pool during fetal development and preparing the organism to support long-term blood production during adulthood. The extent to which these studies from mouse models are reflective of human HSC development remains to be investigated, with recent advances detailing

the transcriptional evolution of human HSCs throughout the fetal period[40] providing the foundation for further exploration.

The postnatal transition in HSC behavior revolves around establishing quiescence

Although fetal HSCs are primed to expand and generate the hematopoietic compartment, a significant transition occurs after birth. Postnatally, HSC behavior shifts from a constitutively proliferative state to one that prioritizes fitness during the reproductive lifespan by balancing long-term HSC survival with a demand-adapted response to stress. This abrupt transition in HSC behavior with adulthood is marked by the establishment of functional quiescence. Quiescence, a state of cell cycle and metabolic dormancy, protects HSCs from exhaustion. In mice, this marked functional change occurs between 3 and 4 weeks postnatally[18]. While HSCs from 1 to 3-week-old mice are still cycling, HSCs from 4-week-old mice and older are predominantly quiescent with more than 98% of HSCs in the G_0 fraction[17]. Adult HSCs also have overall decreased self-renewal capacity compared to fetal HSCs, as evidenced by consistently lower levels of donor chimerism after transplantation into irradiated recipients[18]. This effect is likely a direct reflection of the changes in organismal needs for the HSC compartment, namely a shift from expansion to maintenance. In humans, this transition is suggested to occur at approximately 1 year of age, based on a marked decrease in the rate of telomere attrition at that time[41]. There may also be a role for inflammatory signaling in this transition, as the evolution from a fetal to an adult transcriptional state is associated with a prenatal spike in type I interferon (IFN) signaling[42]. However, the functional importance of this burst of inflammatory signaling remains unclear, with loss of the IFN-1 receptor in murine models resulting only in altered surface marker expression on HSPCs, and not affecting overall HSPC numbers or long-term mature blood cell production in neonates or adults[43].

Although the transition to a quiescent phenotype occurs abruptly in the postnatal period, several murine studies using bulk and single-cell sequencing methods have indicated that the transcriptional underpinnings of this shift occur via progressive downregulation of fetal gene expression programs in conjunction with a gradual upregulation of adult gene expression[42,44]. While these transcriptional changes are likely connected with the differences in chromatin architecture between fetal and adult HSCs[45,46], the functional consequences of perinatal epigenetic remodeling are just beginning to be explored. Advances in technology to examine chromatin organization and reorganization will herald more insights into how non-transcriptional changes affect the transition from fetal to adult HSC behavior.

The postnatal transition is accompanied by the migration of HSCs from the fetal liver to the adult BM niche, with interactions within the marrow microenvironment providing crucial signals reinforcing HSC dormancy. Mesenchymal stromal cells (MSC), endothelial cells, nerve cells, Schwann cells and various types of hematopoietic cells found in the BM niche all play intimate roles in governing HSC behavior during homeostasis and stress responses[47]. The endosteum, which lines the inner surface of the marrow cavity, seems particularly important for maintaining a highly quiescent subset of HSCs[48,49]. Bone-lining osteoblasts have been speculated to regulate HSC quiescence, but the relative importance

of their various secreted factors, such as osteopontin, thrombopoietin and angiopoietin-1, remains unclear[50]. In the central marrow, megakaryocytes are critical for supporting HSC quiescence via the secretion of CXCL4 and TGFβ, both in terms of homeostatic maintenance and reinstatement following stress[51–53]. Similarly, sinusoidal endothelial cells, perivascular stromal cells, and non-myelinating Schwann cells are implicated in maintaining HSC quiescence through cytokines and chemokines, such as SCF and CXCL12[54–57]. These data show that the changes in the extrinsic environment following migration to the BM, along with cell-intrinsic transcriptional changes in the perinatal period, together reinforce the postnatal establishment of HSC quiescence.

Young adult HSCs balance pro-survival and stress responses for functional maintenance

In contrast to the highly proliferative state of fetal HSCs, the enforcement of quiescence in young adult HSCs is a crucial characteristic protecting the established stem cell pool and allowing for lifelong blood production. The majority of adult HSCs exist in the G_0 phase of the cell cycle[17,58], and the potential for long-term HSC engraftment resides predominantly within this fraction[59]. Genetic mouse models that perturb cell cycle dormancy and induce HSCs to proliferate lead to exhaustion of the HSC compartment[60]. However, although quiescence enforcement protects the HSC pool, it can also hinder the elimination of damaged cells. To manage these two opposite requirements, HSCs engage a delicate balance of pro-survival and stress response mechanisms to maintain a functional HSC compartment.

Preserving HSC numbers.—In line with the goal of protecting the adult HSC pool from diminution, HSCs engage pro-survival mechanisms in the face of various forms of stress. For example, murine HSCs show enhanced resistance to apoptosis following irradiation as compared to downstream progenitors[61]. This finding is underscored by decreased pro-apoptotic gene expression and increased expression of pro-survival genes within the *Bcl2* gene family[61]. Additional studies in mouse models further emphasize the importance of *Bcl2* family members in regulating HSC number and survival, with inducible *Mcl-1* deletion resulting in HSC loss[62] versus constitutive expression of *Bcl2* resulting in HSC accumulation[63,64]. These results contrast with studies showing that irradiated human cord blood-derived HSCs demonstrate a slower rate of double-stranded break repair and increased apoptosis relative to progenitors[65], highlighting different priming towards preservation versus elimination in fetal versus adult HSCs. In addition to anti-apoptotic mechanisms, murine HSCs also engage gene programs that prevent necroptosis in the face of TNF-α induced inflammatory stress, emphasizing the close regulation of cell death pathways that are implicated in adult HSC pro-survival mechanisms[66].

Protecting HSC functional integrity.—In parallel with the mechanisms preserving HSC number, other stress-response pathways are activated to protect adult HSC functionality. Induction of the cellular recycling processes of autophagy and mitophagy in response to stress are among these strategies, with loss of these mechanisms resulting in impaired self-renewal activity and diminished regenerative potential[67–69]. Autophagy serves as an essential gatekeeper of quiescence by degrading activated healthy mitochondria and terminating OXPHOS-driven pro-differentiation signals, thereby allowing HSCs to return to a quiescence-enforcing glycolytic metabolism following cell cycle activation[69]. Activation

of mitophagy via the peroxisome proliferator-activated receptor and fatty acid oxidation pathways degrades damaged mitochondria, similarly reinforcing quiescence and protecting self-renewal activity[68].

HSCs also rely on carefully tuned regulation of protein synthesis rates, with both increases or decreases in protein synthesis leading to impaired function[70]. As part of this regulatory framework, the process of protein degradation via aggrephagy plays a role in maintenance of the normal proteome and protection from accumulation of misfolded proteins specifically in HSCs[71]. Evidence also suggests that chaperone-mediated autophagy (CMA), a selective form of autophagy that regulates the proteome by targeting proteins with specific motifs for lysosomal degradation, protects HSC function in adult mice, with elimination of CMA leading to impaired self-renewal[72]. In addition, human HSCs have also been shown to activate the unfolded protein response (UPR) in response to stress-induced accumulation of misfolded proteins, which predisposes damaged cells to apoptosis[73]. However, HSCs must reach a pathologic threshold of ER stress to activate apoptotic UPR mechanisms[74]. As such, these pathways cannot fully protect from the accumulation of damage over time. We speculate that evolutionary pressure has tuned the threshold of damage needed for the clearance of adult HSCs, with an array of stress-response mechanisms mitigating low level insults to preserve HSC numbers, and only severe damage resulting in cell death, thereby optimizing the fidelity of the adult HSC pool to support effective lifelong hematopoiesis.

Regulating HSC metabolic activation.—The intrinsic wiring of adult HSCs within the hypoxic BM microenvironment serves to reinforce quiescence and protect HSCs from the cellular consequences of engaging active metabolism. Quiescent murine HSCs rely primarily on glycolysis as an energy source, limiting the mitochondrial respiration and reactive oxygen species generation associated with oxidative phosphorylation (OXPHOS)[75–77]. This quiescence-enforcing metabolic state of adult HSCs contrasts with the propensity of fetal HSCs to employ oxidative metabolism to support proliferation[21,22].

The metabolic behavior of adult murine HSCs is further regulated via the inheritance of cellular degradative machinery, with asymmetric inheritance of lysosomes in HSC daughter cells predicting future patterns of activation[78]. The asymmetric inheritance of autophagic and mitophagic machinery may similarly regulate HSC integrity[68]. Growing evidence suggests an important interplay between the metabolic wiring and epigenetic state of adult HSCs. For instance, quiescent murine HSCs have high acetyl-CoA to NAD^+ ratios, providing a substrate for histone acetyl transferases, such as CRB-binding protein, which play a role in preserving HSC self-renewal[79] . Murine HSCs also show epigenetic remodeling through DNA demethylation when undergoing metabolic activation[69]. Regulatory pathways enforcing quiescence are also starting to be identified and often connect to amino acids or other metabolic cues such as vitamin C (ascorbate)[80], vitamin A (retinoic acid)[81,82], and vitamin B3 (nicotinamide riboside)[83], which control HSC fate through epigenetic and transcriptional regulations. Ongoing work is actively mapping the cellular metabolism of quiescent and activated states[84], with vital importance for understanding the mechanisms maintaining HSC function.

Controlling blood regeneration.—Although young adult HSCs maintain a quiescent state during steady-state hematopoiesis, they are responsible for orchestrating a dynamic response to stress, including regeneration following blood loss[85], treatment with myeloablative agents[53], and infectious challenges[86]. In this context, Type 1 IFN signaling directly activates dormant HSCs, leading to cell cycle entry and allowing for a robust proliferative response to stressors[86,87]. However, this proliferative response is transient, with the HSC pool rapidly returning to protective quiescence[88]. The inflammatory cytokine TNF-α also contributes to HSC pro-survival responses, while simultaneously inducing myeloid progenitor apoptosis[66]. The effect of TNF-α in protecting against HSC exhaustion in the face of inflammation may be mediated by activation of the transcription factor PU.1, which represses cell cycle and protein synthesis programs. Similarly, activation of PU.1 after stimulation with the pro-inflammatory cytokine interleukin (IL)-1 limits HSC expansion and mediates a return to quiescence[89,90]. Altogether, the ability of young adult HSCs to preserve their number and functional integrity while responding dynamically to inflammatory stress allows the hematopoietic system to maintain homeostasis during the reproductive lifespan.

The future of HSC ontogeny research

The tremendous progress in our understanding of dynamic changes in HSC function, supported by technological advances such as single-cell sequencing and *in vivo* lineage-tracing methods, have laid the foundation for further exploration into the regulators of HSC behavior in different developmental stages. In the fetal context, additional investigations to uncover the interconnected layers of genetic, epigenetic, and metabolic regulation that cue the transition toward HSC quiescence will be important. In the adult context, gaining a better understanding of the cell intrinsic programs controlling stress-response pathways and reinforcing quiescence, or allowing HSCs to return to quiescence upon activation, will help developing a working knowledge of functional quiescence and adult HSC maintenance. Additionally, developing a more detailed understanding of the three-dimensional spatial organization of the BM niche, which represents one of the most notable barriers in the field, will improve our assessment of the cell-extrinsic programs regulating adult HSC behavior. Importantly, such studies will provide the essential basis to explore how cellular programs become dysfunctional in aging and disease.

HSC aging: normal mechanisms of maintenance giving rise to dysfunction

Although young adult HSCs employ finely tuned mechanisms to balance pro-survival and stress responses, these maintenance strategies provoke vulnerabilities that manifest with continued age, leading to decline in function. In this section, we examine HSC biology through the lens of antagonistic pleiotropy, whereby the same mechanisms that are important for reproductive fitness form the basis for functional decline during aging[91]. We focus on the insults that HSCs face because of their unique job requirements, and how this contributes to the declining functionality of old HSCs (Figures 2 and 3). As in previous stages of life, we highlight the central role of cell cycle regulation, niche cell reliance, inflammatory responses, cellular memory, and distinct metabolic regulation in driving HSC aging features including expansion of the HSC pool, decreased self-renewal, myeloid-biased

differentiation due to loss of lymphoid cell production, and genomic instability leading to clonal hematopoiesis (CH) and oncogenic transformation.

Cell cycle regulation and DNA damage repair

The dynamic regulation of cell cycling that predominantly maintains a state of quiescence is crucial for protecting adult HSCs from age-dependent exhaustion due to over-proliferation, as well as from the genetic and metabolic insults associated with activation. However, residence in G_0 also restricts murine HSCs from engaging homologous recombination and repairing DNA with high fidelity, instead limiting them to fallible end-joining repair mechanisms[61]. HSC aging is also associated with downregulation of mini-chromosome maintenance genes and a loss of dormant origins, which cause old murine HSCs to experience delayed cell cycling and severe replication stress upon cycle entry, leading to further mutational accumulation and an inability to mount appropriate regenerative responses[92]. The repression of cell cycling and cell cycle gene expression is shared in human HSCs, and has been connected to reduced mitogenic signaling[93]. Nevertheless, intact DNA damage repair machinery[94] and reduced apoptotic priming[95] allow old HSCs to survive in the face of physiological levels of genotoxic stressors, which allows for the persistence of the compartment while also preventing clearance of mutated stem cells. Thus, although repression of cell cycling and specific mechanisms of DNA damage repair are necessary to ensure lifelong HSC survival, they inevitably lead to genomic instability during aging. This is particularly relevant to understanding the mechanistic basis for human genomic studies showing that somatic mutations associated with CH and leukemic transformation are increasingly prevalent with advanced age[96]. Indeed, some recurrent CH mutations are directly attributable to end-joining based repair mechanisms[97]. Still, CH-associated mutations are nearly ubiquitous at low variant allele frequencies (VAF) in healthy humans over 50 years old and do not uniformly expand to higher VAFs, implying that environmental factors are also crucial in this respect[98,99]. Given emerging evidence from mouse models that genetic lesions cooperate with external signals, in particular heightened inflammation, to drive CH[100–103], we speculate that both are needed to drive clonal expansion.

Niche cell alterations and inflammaging

Cellular and molecular changes to BM niche cells are already apparent at mid-life, well before the emergence of the most striking features of HSC aging, such as numerical expansion and defective transplantation[104,105]. Although the order of events is not yet fully detangled, the specific relationships between niche alterations and hematopoietic aging features are beginning to be dissected. Changes in the cellular composition and functionality of the BM vasculature and lymphatic system with age play a direct role in driving defective hematopoietic regeneration of old murine HSCs[104,106,107]. Similarly, the nervous and mesenchymal components of aged murine BM contribute to myeloid-biased hematopoiesis and HSC dysfunction via localized production of pro-inflammatory cytokines[104,108,109]. Recent work has added mechanistic understanding to the crosstalk between mesenchymal and hematopoietic cells in aging. For example, an osteolectin-expressing subset of MSCs that is responsible for supporting osteogenesis and lymphopoiesis declines with age[110], whereas the remaining osteoprogenitor cells secrete IL-1 that reprograms central

marrow MSCs, causes defective hematopoietic regeneration, and impairs HSC function[104]. Furthermore, reduced insulin-like growth factor (IGF)-1 production by murine BM MSCs during middle age drives an age-related, myeloid-biased HSC phenotype[105]. These interweaved dependencies illustrate the complex influence of BM niche cell ecology to HSC aging, and future work will uncover additional cellular and molecular mediators of this crosstalk. For one, BM-resident T cells directly regulate HSCs[111] and themselves decline in function with age[112]. We therefore speculate that alterations to BM T cells are an important unstudied driver of hematopoietic aging.

Chronic inflammation is a well-appreciated driver of organismal aging[113] and is implicated in impairing HSC self-renewal activity[66,87,89,114]. Recent work shows that temporally discrete inflammatory events accelerate murine HSC aging and lead to irreversible attrition of self-renewal capacity[115]. Age-related inflammation in the BM microenvironment was first reported over a decade ago[116]. Since then, different niche cell types, including macrophages[117], MSCs[108] and osteoprogenitors[104], as well as adrenergic innervation[118] and dysbiosis of the gut microbiome[119], have all been implicated as important sources of chronic inflammation promoting age-related HSC dysfunction. To return to the lens of antagonistic pleiotropy, whereas the ability of HSCs to respond to inflammatory signals enables rapid responses to infection and injury, it also renders HSCs susceptible to maladaptive, low-grade inflammation within the aged marrow cavity. Heterogeneity within the HSC compartment regarding the potential of an individual HSC to engage in differentiation versus self-renewal leads to variable responses to inflammation in different HSC subsets[120,121]. This adaptive behavior allows the HSC compartment *en masse* to mount a response to inflammatory stress while also retaining self-renewal capacity long-term. However, it likely leads to the selective attrition of those HSCs that are primed to differentiate[122], resulting in a compartment that is less conducive to activation with age. It therefore seems likely that HSC responses to acute inflammatory episodes over the lifetime of the organism, as well as to chronic inflammation in the BM niche that progressively worsens with age, will favor the retention of a biased subset of HSCs.

Replicative history, epigenetic drift, and trained immunity

HSC aging is associated with the accumulation of epigenetic changes. Studies in both mice and humans indicate that these age-related epigenetic changes include shifts in histone marks, such as global increases in the active mark H3K4me3, global decreases in the repressive mark H3K27me3[123,124], and loss of H4K16ac polarity[125]. Although global DNA methylation in HSCs is mostly stable during aging, site-specific alterations in DNA methylation are observed in old HSCs and correlate with methylome changes induced by proliferation[126]. Consistently, HSCs seem to carry memory of their activation history, with progressive cell divisions causing loss of self-renewal[127].

Perhaps unsurprisingly, clonal tracking experiments have revealed that epigenetic heterogeneity plays a role in the disparate response of genetically normal murine HSCs to various stressors[128]. The functional consequences of epigenetic changes encompass an effect on HSC self-renewal capacity[123] and may also account for the predominance of old HSC subsets that are responsible for altered responses to inflammatory challenges[129].

Additionally, studies have provided evidence for trained immunity occurring at the level of HSCs and their immediate downstream progeny[130–132], where epigenetic changes following inflammatory challenges reinforce and expedite the transcriptional response to subsequent insults. These findings are again consistent with the theme of antagonistic pleiotropy, in that epigenetic memories of previous inflammatory challenges enable organismal fitness during adulthood by providing improved myelopoietic responses to pathogens, but also likely engender aging features by encoding chromatin states that bias toward the myeloid lineage.

HSC metabolism, proteostasis, and autophagy in aging

Mirroring the context-dependent regulation of cell cycling, response to inflammatory signals, and epigenetics, HSCs depend on discrete metabolic requirements that undergo marked alterations upon activation and during aging[22]. In particular, HSCs necessarily switch from glycolysis to OXPHOS to achieve the anabolic demands of activation and differentiation[133]. Notably, genetic models that interfere with transcriptional enforcement of glycolysis and activate OXPHOS lead to loss of HSC quiescence and self-renewal, paralleling functional features of aging[134,135]. Furthermore, there is a close coupling between HSC cell cycle activity and metabolic requirements[22]. This interlinked relationship is relevant to aging in part because asymmetric mitochondrial segregation imbues daughter cells with an additional form of memory associated with declining regenerative potential[136]. Given the intimate connection between metabolism and epigenetics, an important interplay likely exists between these two HSC regulatory mechanisms. Thus far, the metabolic-epigenetic axis, as it pertains to HSC aging, has been examined mainly through investigations of sirtuin (SIRT) genes, which are NAD+-dependent deacetylases regulating both histone architecture and cell signaling pathways with well-known connections to aging biology[137]. SIRT1 is involved in the activation of FOXO3A, which enforces HSC quiescence and stress resilience throughout life[67], and SIRT1 deletion promotes an accelerated HSC aging phenotype[138]. SIRT2 is required to repress the NLRP3 inflammasome downstream of mitochondrial stress[139]. SIRT3 and SIRT7 both suppress mitochondrial metabolism, with SIRT7 specifically tuning protein translation and inducing a mitochondrial UPR that maintains HSC metabolism during aging[140,141]. Proteostasis more generally is also important for HSC aging, with evidence suggesting that middle-aged murine HSCs depend on the stress response gene HSF1 to maintain low translation rate and engraftment capability[142], and that mechanisms of proteostasis are altered in old HSCs[71].

Autophagy has long been associated with organismal aging, with interventions that increase lifespan, such as calorie restriction or rapamycin treatment, directly linked to autophagy induction[137]. Work over the past decade has demonstrated the specific relevance of autophagy to HSC aging, with disruption of macroautophagy[69] as well as CMA resulting in premature aging phenotypes[72]. Still, a subset of old HSCs retains the ability to engage autophagy via a FOXO3A dependent pro-survival circuit[67]. Mouse studies using autophagy reporter systems have confirmed the functional importance of this process, showing that those HSCs with robust autophagy engagement are the most fit[69] . In contrast, old HSCs that fail to engage autophagy display metabolic deregulation and impaired self-renewal activity[69,71]. Future work will indicate what triggers specific subsets of old HSCs to

engage autophagy. Old HSCs also show reduced CMA activity, with genetic mouse models demonstrating impaired glycolysis and loss of proteostasis in CMA-deficient HSCs[72]. Collectively, this work illustrates that the mechanisms that regulate HSC metabolism undergo marked alterations during aging. We speculate that metabolically protective processes such as autophagy and CMA engagement become increasingly important upon aging to compensate for increased cellular stressors.

The future of HSC aging research

Studying HSC aging is critical because an accurate understanding of the underlying mechanisms will pave the way for root-cause therapies for age-related disease. The implications of such studies extend beyond the blood system, as hematopoietic aging contributes to solid tissue senescence[143]. Accordingly, understanding and targeting the defects arising in HSCs will likely have important consequences in other aging tissues. Although much has been uncovered regarding individual drivers of HSC aging, these processes are often studied in isolation, and future work will begin to examine their relationships to each other. HSC aging is a consequence of many cellular alterations, and such integrated approaches are needed to develop a comprehensive understanding of the process as a whole. Application of advanced technologies will also provide fresh insight into important aspects of HSC aging. For instance, clonal barcoding technologies have revealed unique features of fetal and adult HSC biology[1,144], and it will be exciting to see what they will uncover in the aging context. Spatial transcriptomics applied to the aging BM niche is similarly promising in its potential to resolve the relationship between specific niche cell alterations and transcriptional features of aged HSCs. Additionally, CRISPR-enabled genome engineering broadens the feasibility of genetic studies on HSC aging in model organisms, providing the opportunity for more mechanistic insight. Lastly, much of the groundwork for understanding the connection between aging and the development of CH and hematological malignancies has been established in mouse models, which will guide translation into human hematopoietic systems with the goal of improving outcomes for individuals suffering from age-related blood diseases.

Intervention strategies for rejuvenating aged HSCs

Given that age-related changes in HSCs are thought to be key contributing factors to infectious susceptibility, reduced vaccination efficacy, leukemic emergence, and even solid tissue senescence, a focus has been placed on mitigating or reversing these changes for systemic functional benefit[145]. In general, studies have predominantly been performed in mouse models and have focused on restoring the engraftment potential and reverting the lineage-biased differentiation of old HSCs. Attempts at HSC rejuvenation have employed a diverse array of environmental, metabolic, and epigenetic strategies targeting mechanisms associated with HSC functional decline. These rejuvenation attempts can be broadly categorized into two types of approaches: 1) preventing HSC functional decline by diminishing the effect of aging, for example by reducing inflammation, preventing epigenetic alteration, or forcing metabolic reprogramming; and 2) restoring overall HSC functionality, for example by removing damaged HSCs. Here, we summarize the state of these efforts and their potential for translational applications aimed at improving immune

responses in older individuals and mitigating the development of age-associated blood disorders (Table 1).

Unique challenges to HSC rejuvenation

Experiments suggesting that the rate of aging can be modulated and delayed in model organisms via dietary, pharmacological, and genetic means have resulted in growing scientific and public interest in interventions aimed at functional rejuvenation of aged tissues[146]. Some approaches have been described as “universal” rejuvenation strategies, due to broad successes in rejuvenation across multiple tissues. For example, heterochronic parabiosis rejuvenates muscle function, bone healing, and neurogenesis in aged mice[147–149]. Additionally, life-long caloric restriction improves neural stem cell activity and reduces cardiovascular disease in aged mice and primates[150]. Exercise has also been described as an anti-aging strategy, with effects spanning multiple tissues and leading to reduced risk of cardiovascular disease as well as improved muscle function, cognition, and neurogenesis[151]. However, these bloodborne systemic rejuvenation approaches have not been successful in functionally rejuvenating old HSCs[152,153] or improving other aspects of the aging blood system, such as diminished thymic function[154]. For instance, the lodging and long-term residence of old HSCs in the young BM cavity, which results from cross-over observed in heterochronic parabiosis pairs, does not restore old HSC regenerative potential[152]. Additionally, transplantation of old murine HSCs into young recipients does not revert age-associated DNA methylation changes[153], and young plasma injection does not dampen chronic inflammation in the aged BM microenvironment[152]. This lack of success likely reflects unique aspects of the biology of HSCs and their niche compared to other tissues and stem cell populations and demonstrates the interdependency of cell-intrinsic deregulations and cell-extrinsic environmental changes in damaging old HSC function. Given the limited effectiveness of broad, systemic rejuvenation approaches, strategies that take advantage of our growing knowledge of age-related changes in HSC properties and in the BM niche, such as the metabolic-epigenetic axis, will be important to continue developing promising interventions. Meanwhile, the field will continue to grapple with the cost and time required to age relevant mouse models, which limits mechanistic insight into the genetics of the aging process, and the challenge of translating findings in model organisms to humans, which age on a different timescale.

Targeting niche cell alterations and inflammaging

Targeting specific cellular, molecular, and structural changes occurring in the aged BM niche has shown promise in restoring HSC function and more youthful blood production. Transplantation of young BM endothelial cells into aged recipients can improve hematopoietic regeneration, presumably by compensating for age-related decline in the number and functionality of these vascular cells[155]. BM MSCs also undergo alteration with age and perhaps could be similarly harnessed, but the translational potential is currently limited by the same barriers encountered by all cell-based therapies in clinical applications[156]. From the structural perspective, murine BM stiffens with age, and a study suggests that modulating BM stiffness *ex vivo* may be a potential rejuvenation strategy[157]. Pharmacological modulation of microenvironmental alterations has also shown some success, with a chemical agonist mimicking adaptive β3-adrenergic signaling in

the aging BM niche improving old HSC functionality and regenerative potential in transplantation assays[109]. The same compound has been effective at rebalancing lineage output in a progeroid model of aging[118], and future work will determine whether boosting β3-adrenergic signaling may also be beneficial in physiological aging. Artificial restoration of secreted factors has also shown promise, with recombinant osteopontin treatment *ex vivo* improving old HSC function[158] and *ex vivo* treatment with IGF-1 improving the myeloid bias of 12-month-old HSCs[105]. However, given that increased signaling through IGF-1 promotes organismal aging in mice and other mammalian models[146], artificial activation of this pathway may be challenging to implement in humans, unless employed in a highly targeted manner.

The low-grade, chronic inflammation of the aged BM niche provides another attractive opportunity for intervention[159]. For instance, in aged mice, normalizing overactive IL-1 signaling via the FDA-approved IL-1 antagonist, Anakinra, improves the hematopoietic response to a regenerative challenge imposed by the chemotherapeutic agent 5-fluorouracil[104]. Lifelong reduction of IL-1 signaling in mice lacking the IL-1 receptor also prevents the emergence of inflammatory MSC subsets in the central marrow and improves aged blood production and old HSC engraftment potential[104]. In addition, antibiotic suppression of the microbiota or pharmacological blockade of IL-1 signaling are able to reverse the myeloid-biased output of old HSCs[119]. Further support for modulation of the commensal microbiome as a tractable HSC rejuvenation strategy comes from a study showing that fecal transplantation from young mice improves HSC function by suppressing age-related inflammation[160]. Moreover, both IL-1[100] and microbiome-driven IL-6[161,162] signaling have been implicated in the progression of *Tet2* mutant CH in mouse models, suggesting a potential role for targeted interventions to minimize the negative health consequences associated with CH[163]. In fact, clinical trial data suggest that treatment of patients harboring *TET2*-mutant CH with the anti-IL-1β antibody canakinumab decreases the incidence of cardiovascular events[162]. Collectively, these studies suggest that targeting either specific pro-inflammatory molecules or broad cellular changes in the BM niche can maintain a more youthful blood production and forestall the progression of age-related disease. Still, such strategies do not correct the intrinsic changes occurring in old HSCs that are implicated in age-related dysfunction.

Targeting HSC epigenetic drift

Correcting the epigenetic and transcriptional alterations occurring in aged HSCs is another window for therapeutic intervention, especially given the range of small molecules available for epigenetic tuning[164]. Both genetic and pharmacological approaches have been used to alter the epigenome of aged HSCs. Inhibition of the small RhoGTPase Cdc42 can restore the spatial distribution of H4K16 acetylation in old HSCs, improving their engraftment potential and lineage bias in transplantation assays[165]. Overexpression of *Satb1*, a lymphoid differentiation transcriptional regulator, also improves the lineage output of old HSCs *in vitro*[166]. Growing evidence suggests that epigenetic reprogramming instead of epigenetic correction might be a better avenue for functional rejuvenation of old HSCs. Notably, HSCs derived from induced pluripotent stem cells (iPSC) that originated from the mature cell progeny of lineage-biased old HSCs have successfully rejuvenated lineage-balanced blood

production and engraftment potential *in vivo*[167]. This finding has coincided with broad interest in the aging field in using epigenetic reprogramming via Yamanaka transcription factor (Oct4, Sox2, Klf4, and c-Myc) overexpression to push aged cells and tissues towards an embryonic state[168]. Some success has been achieved with this reprogramming strategy in various organs[169–172], although most of these studies rely on settings of progeria or inflammatory challenge and report epigenetic "clock"-based assessments of rejuvenation in lieu of functional measurements. It therefore remains to be seen whether Yamanaka factor induction *in vivo* can delay hematopoietic aging or rejuvenate old HSC function. Future implementation of such reprogramming strategies must also include consideration of inherent associated risks, such as teratoma formation[171]. Nevertheless, there is great promise for the development of successful interventions that target epigenetic drift, particularly as our knowledge of the functional implications of these changes continues to grow.

Metabolic-targeted rejuvenation approaches

Given the important role of metabolic deregulation in HSC aging, it is not surprising that this has been a prominent target for rejuvenation approaches. Modulation of the mTOR pathway through calorie restriction or small molecule inhibition is the most well-studied anti-aging strategy and is effective in an array of model organisms[173]. In the context of HSC aging, rapamycin treatment improves engraftment in transplantation assays as well as the vaccination response of old mice to influenza virus[174]. Modulation of the mTOR pathway has also been extended to human clinical trials, with data suggesting that the elderly patients' response to seasonal influenza vaccines can be improved after treatment with an mTOR inhibitor[175]. Given the immunosuppressive effects of mTOR inhibitors, intermittent low-dose treatments will likely provide the most favorable risk-benefit ratio, if large-scale trials support their widespread implementation. Targeting of sirtuins has also garnered interest as a method of improving HSC functionality with aging. In particular, overexpressing SIRT2 improves the engraftment potential of old HSCs by dampening mitochondrial stress and NLRP3 inflammasome activation[139]. Overexpression of SIRT3 or SIRT7 also improves old HSC function in transplantation assays, by reducing oxidative stress[140] and mitochondrial protein folding stress[141], respectively. Sirtuins are NAD^+-dependent enzymes, and several studies have suggested that a systemic decline in NAD^+ levels with aging could contribute to a broad decrease in SIRT activity. To combat this, nicotinamide riboside (NR), which is endogenously converted to NAD^+, has been studied as a method to restore NAD+ levels and promote youthful metabolic function in aged HSCs. Indeed, continuously treating old mice with NR for 8 weeks shifts old HSCs toward a more youthful transcriptional state and improves their reconstitution potential[176]. However, NR treatment has not demonstrated a significant impact on other functional features of HSC aging, such as lineage-bias, and old HSCs revert to decayed metabolic activity and function upon treatment discontinuation. However, despite current limitations, rejuvenation approaches that target metabolism, such as dietary strategies, are often among the most accessible interventions, and therefore represent a critical area of investigation.

Killing dysfunctional old HSCs

Another method to improve the overall function of the aged HSC pool is to selectively ablate dysfunctional cells. Several features of old HSCs, including altered metabolism,

mutations, deregulated cell cycling, and neo-antigen presentation, can provide potential targets for removal. The metabolic heterogeneity of the aged HSC compartment, wherein a subset of autophagy-engaging HSCs retains the highest functionality upon transplantation[69], may provide one such avenue for selectively ablating dysfunctional old HSCs. Such metabolic targeting has shown promise in the context of acute myeloid leukemia (AML), where leukemic stem cells (LSCs) have different metabolic properties from healthy HSCs. For example, LSCs demonstrate greater dependence on mitochondrial respiration for their survival, leaving them vulnerable to therapies that reduce OXPHOS. Methods for selectively killing LSCs by targeting their altered metabolism include inhibiting amino acid metabolism[177], inducing cell cycle entry and exhaustion by inhibiting fatty acid oxidation[178], and BCL-2 inhibition[179]. BCL-2 and BCL-xL inhibition using the compound ABT263 attenuates age-related changes of non-malignant old murine HSCs via senescent cell clearence[180]. These targeting strategies may provide a future avenue for selective removal of dysfunctional aged HSCs with altered metabolism to improve the fitness of the remaining aged HSC pool.

Another promising method to distinguish the most dysfunctional aged HSCs might be found in the antigen presentation ability of HSCs. Both murine and human HSCs constitutively express MHC-II antigen presentation machinery[111] and, in AML, antigen-presentation correlates with stemness of LSCs. The presentation of immunogenic neoantigens by aberrant HSCs activates antigen specific $CD4^+$ T cells that drive HSC differentiation and subsequent exhaustion[111], suggesting a mechanism for selective elimination of HSCs undergoing malignant transformation. The use of PARP inhibitors for AML treatment has also been successful in inducing expression of the C-type lectin-like receptor NKG2D on LSCs, making them vulnerable to killing by NK cells[181]. These few examples highlight the therapeutic potential of killing LSCs to prevent AML development and progression and provide a roadmap on how clearance of dysfunctional old HSCs could be used for rejuvenating the aged HSC compartment. In HSC aging, enhancement of the immune response to neoantigens or induction of NKG2D ligands may allow for the selective elimination of old HSCs harboring mutations, thereby improving the functionality of the aged HSC compartment *en masse*. However, further progress in this area will rely on the identification of strategies to selectively induce expression of either neoantigens or NKG2D ligands in old HSCs, which has thus far been challenging.

The future of HSC rejuvenation

The development of translatable rejuvenation approaches for the hematopoietic system will be considerably helped by continued advances in our methods for assessing HSC function, a more rigorous definition of HSC aging, and the establishment of better criteria to claim successful rejuvenation of aged HSCs. Choosing effective intervention strategies will necessarily rely on distinguishing indispensable adaptive survival responses from targetable maladaptive features of aging. For decades, assessments of HSC function have relied primarily on transplantation, a method which is constrained by its reliance on homing and engraftment processes, despite the emergence of many new cellular and functional attributes of young and old HSC states. Other methods for assessing age, such as ‘epigenetic clocks’ for determination of epigenetic age, are limited by a lack of specificity to HSCs

and an uncertain correlation to functionality and age-related phenotypes. We anticipate that multi-dimensional assessments that include regeneration challenges, immune response to infection and vaccination, and assessments of the epigenetic and metabolic cell state will be important in validating improvements in rejuvenation approaches. With our growing understanding of HSC aging, the potential applications of HSC rejuvenation strategies to human health and disease will continue to be explored. Notably, HSCs are uniquely poised for *ex vivo* interventions, due to their ability to be isolated via pharmacological mobilization into the peripheral blood. Technological advances that allow human HSCs to be genetically edited *ex vivo* and subsequently transplanted back into the BM niche provide a mechanism for genetically driven rejuvenation strategies. Such gene therapy strategies are already being applied in other clinical areas[182], heralding new opportunities for translational approaches. Although strategies for HSC rejuvenation remain in early stages, these methods open the door to designing rational methods for future geromedical treatments.

Conclusions: toward an integrated understanding of HSC aging

The HSC compartment is governed by a complex network of cell-intrinsic and cell-extrinsic factors that enable a balance between self-renewal and differentiation to maintain long-term blood production. Here, we reviewed the features governing HSC behavior throughout ontogeny that reinforce this balance, highlighting how pro-survival mechanisms lead to maladaptation with age. As additional studies continue to dissect the genetic, epigenetic, metabolic, and inflammatory drivers of aging, new strategies to combat aging phenotypes via functional rejuvenation approaches will follow. Importantly, these future studies must account for heterogeneity in the kinetics of aging, which in mouse models may be obscured by a selection bias inherent to the prominent use of 24-month-old mice from the C57Bl6 genetic background that are fit to survive into old age. Alternative models including other strains of mice, other species, and improved human cell systems will aid in confirming the applicability of insights from established models. Longitudinal studies that encompass the full spectrum of aging - from birth, through different stages of adulthood, and into old age – have the potential to contextualize this heterogeneity, particularly given advances in sequencing technologies for in-depth profiling of genetic and epigenetic landscapes. Integration of these genetic and epigenetic profiles at different stages of life with functional features, such as repopulation ability and response to inflammation, may open the door to an era of personalized medicine strategies for HSC rejuvenation.

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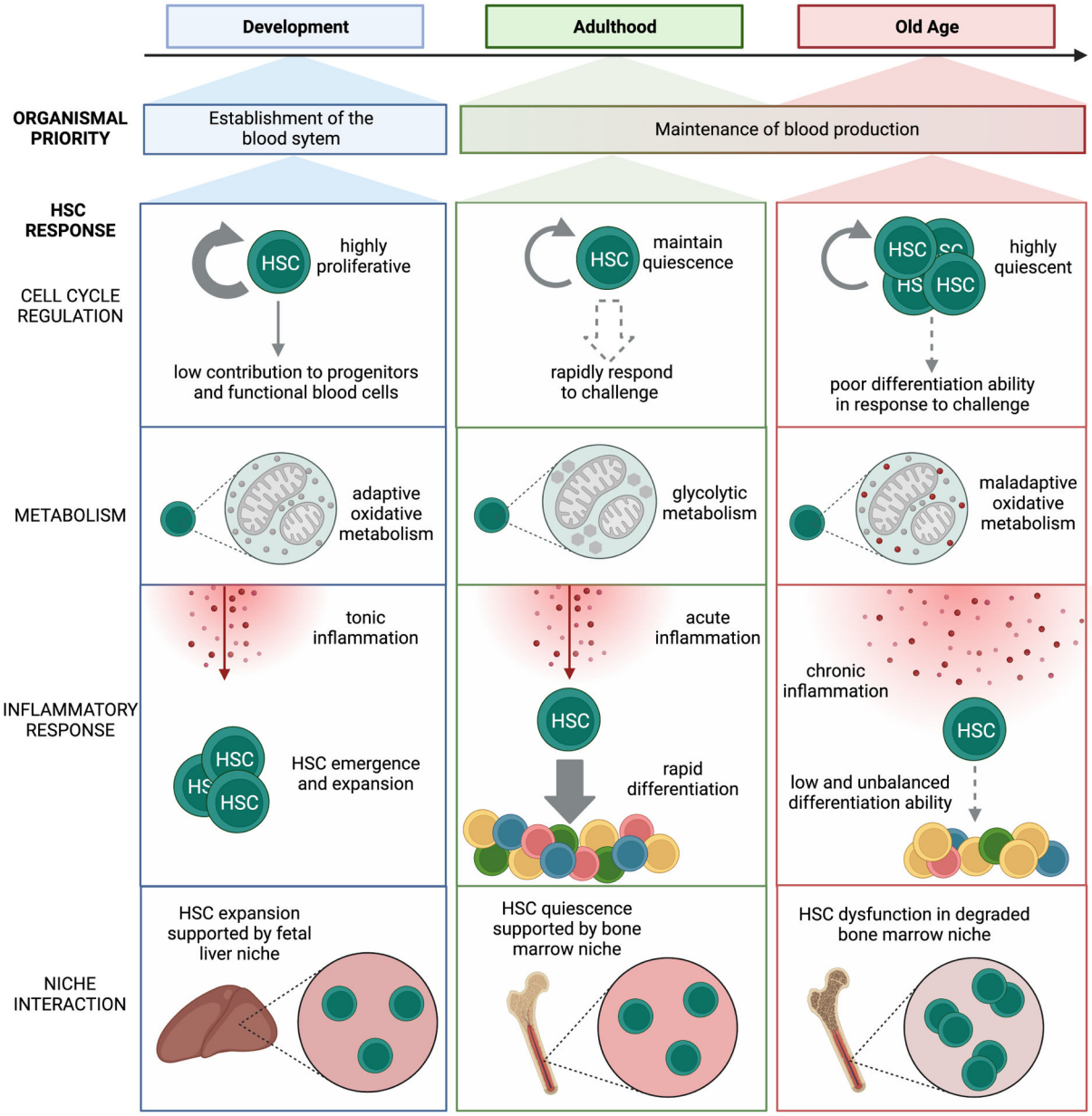

Figure 1. Dynamic responses of HSCs to organismal demands throughout the lifespan.

During development, HSCs primary function is to establish the adult blood system, with fetal HSCs residing in the fetal liver and engaging in oxidative phosphorylation to allow for rapid expansion. During adulthood, HSCs act to maintain organismal health and immunity via functional quiescence, a primarily glycolytic resting state that allows quick entry into the cell cycle in response to organismal demands. During aging, dysfunctional HSCs expand, survive, and maintain quiescence despite maladaptive oxidative metabolism and chronic inflammation in a degraded BM niche environment that promotes aberrant differentiation.

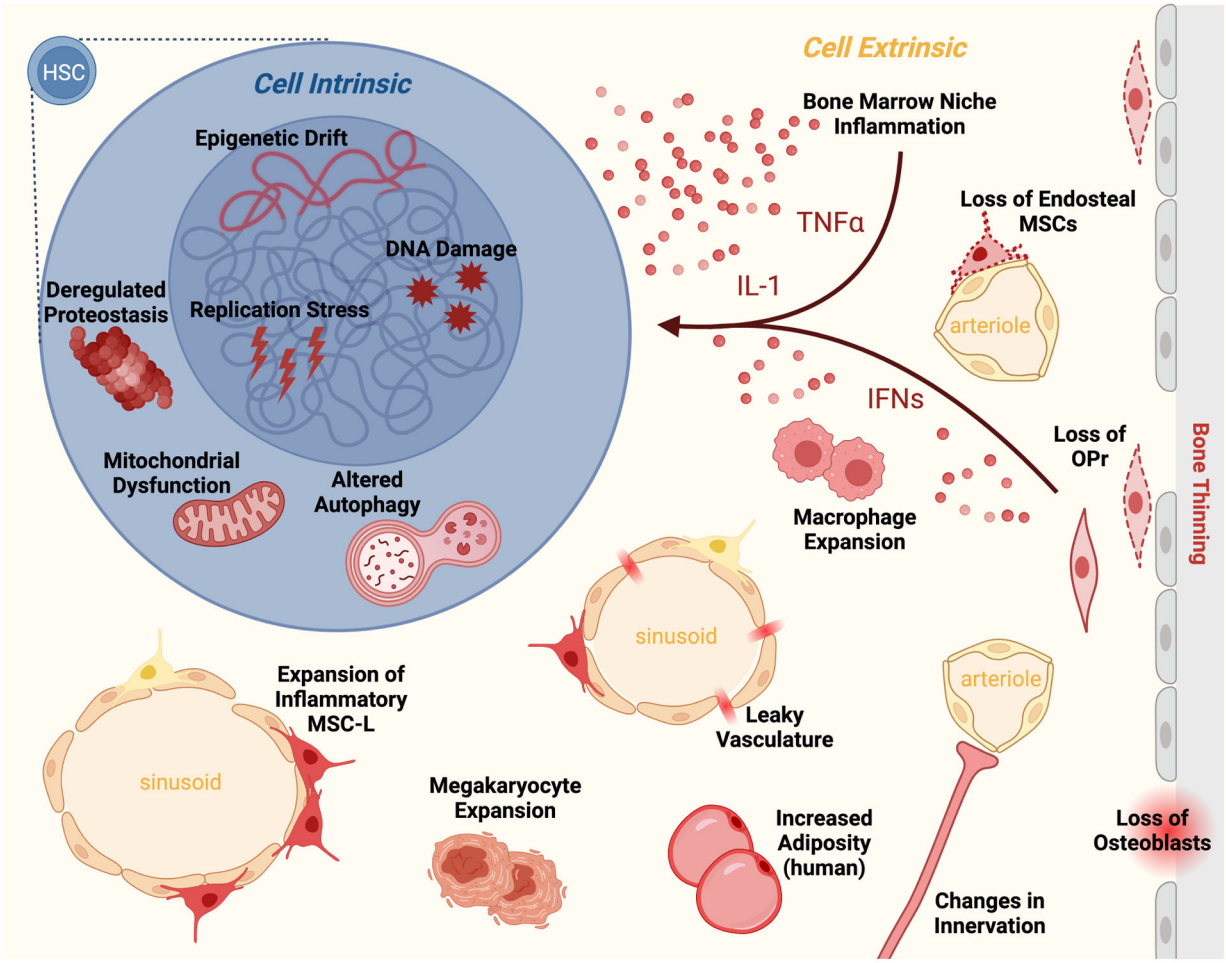

Figure 2. Cell-intrinsic and cell-extrinsic features of HSC aging.

Old HSCs display many altered cell-intrinsic features including epigenetic drift, DNA damage, replication stress, deregulated proteostasis, mitochondrial dysfunction, and altered autophagy regulation. These cell-intrinsic changes are compounded by cell-extrinsic changes in the aged BM microenvironment including loss of specific niche cells (*i.e.*, periarteriolar mesenchymal stromal cells (MSC), osteoblastic progenitors (OPr), osteoblasts), and accumulation of other cell types that contribute to an inflammatory milieu (*i.e.*, inflammatory MSCs, macrophages, megakaryocytes) rich in pro-inflammatory factors (*i.e.*, IL-1, IFNs, TNF-α, etc.). Old HSCs are also influenced by other niche changes, including altered innervation, increased adiposity, and leaky vasculature. Together, these features of HSC aging contribute to their functional decline.

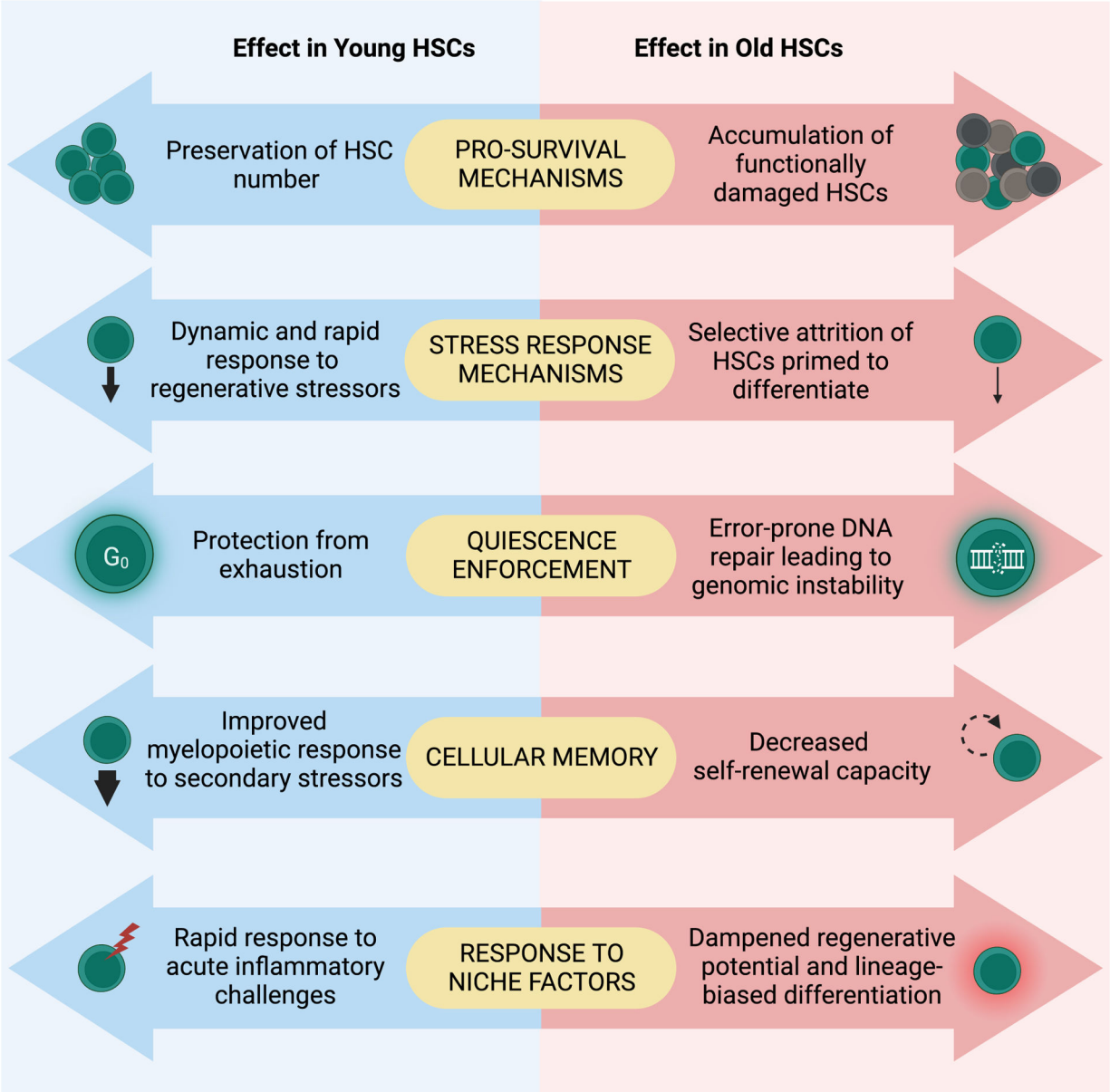

Figure 3. Antagonistic pleiotropy of HSC features during young adulthood and old age.

Many features of HSCs, such as pro-survival mechanisms, stress response mechanisms, quiescence, cellular memory, and response to niche factors, are beneficial for optimal HSC function and response to organismal demands in young adulthood but become detrimental and maladaptive during aging.

Table 1.

Attempts to rejuvenate aged HSCs.

Intervention:		Methodology:	Result:	Reference:
Systemic	Rapamycin treatment	4mg/kg rapamycin injection every other day for 6 weeks in 22-month-old mice	More youthful HSC numbers and HSC cell cycling *in vivo;* improved engraftment and lymphoid output upon HSC transplantation; increased B cell progenitors and vaccine response in primary mice	Chen et al, 2009[174]
	Prolonged fasting	8 cycles of 48 hour fasting in 18-month-old mice	Decreased frequency of myeloid-biased HSCs and increased peripheral blood lymphocytes in primary mice	Cheng et al, 2014[183]
	Exposure to young bloodborne factors	Heterochronic parabiosis (1 month) or injection of young plasma (8x over 1 month) in 24-month-old mice	No effect on old HSC function (engraftment or myeloid bias upon HSC transplantation, division time *in vitro*)	Ho et al, 2021[152]
	Exercise	7-week long free access to running wheel in 18-month-old-mice	No effect on HSC function (engraftment or myeloid bias upon HSC transplantation)	Ho et al, 2021[152]
	Calorie restriction	30% calorie restriction for 9 months, analysis of 12-month-old mice	Decreased HSC number and decreased frequency of myeloid-biased HSCs in primary mice; improved engraftment and lymphoid output upon HSC transplantation	Tang et al, 2016[184]
		40% reduction in calorie intake in 3.5- to24-month-old mice	No effect on HSC function (engraftment or myeloid bias upon HSC transplantation)	Ho et al, 2021[152]
Microenvironmental	Endothelial cell transplantation	Young endothelial cells infusion for 4 consecutive days in 24-month-old-mice followed by sublethal irradiation and whole BM transplantation 1 month later	Improved peripheral blood mature cell production following irradiation; improved engraftment and lymphoid output upon whole BM transplantation	Poulos et al, 2017[155]
	Small molecule mimetic of β3-adrenergic signaling	12-week delivery with osmotic pumps in 20- to 24-month-old-mice	Improved engraftment and lymphoid output upon primary HSC transplantation and secondary BM transplantation	Maryanovich et al, 2018[109]
		8 weeks daily injection in progeroid mice	Decreased myeloid and increased lymphoid cells in peripheral blood	Ho et al, 2019[118]
	IGF1 stimulation	*In vitro* treatment of 14-month-old HSCs with 100ng/ml IGF1	Rescue of γH2AX foci, Cdc42 polarization, mitochondrial membrane potential, and morphology	Young et al, 2021[105]
	IL1R antagonism using Anakinra	3 weeks daily injection in 24-month-old mice before HSC transplantation	Increased lymphoid output in HSC transplantation; no difference in engraftment	Kovtonyuk et al, 2022[119]
		2 weeks daily injection in 24-month-old mice before immunophenotyping and HSC transplantation	Decreased HSC numbers in primary mice; no effect on HSC function (engraftment or myeloid bias upon HSC transplantation)	Mitchell et al, 2023[104]
		2 weeks daily injection starting 2 days prior to 5-FU myeloablation in 24-month-old mice	Improved regenerative response; Increased B cell and red blood cell recovery in peripheral blood; increased	Mitchell et al, 2023[104]

Intervention:		Methodology:	Result:	Reference:
			lymphoid-biased progenitors and erythroid progenitors in BM	
	Netrin-1 supplementation	10 injections over 2-week period of 4 μg of recombinant murine Netrin-1 in 18-month-old mice	Improved engraftment and lymphoid output in primary mice	Ramalingam et al, 2023[185]
	Microbiome modulation	1-year-old germ-free mice or 8-week antibiotic treatment in 2-year-old mice	Improved lymphoid output upon HSC transplantation; no difference in engraftment	Kovtonyuk et al, 2022[119]
		Oral gavage in 20- to 24-month mice with fecal slurry from 7- to 8-week-old mice	Improved B cell numbers in primary mice, improved chimerism and HSC engraftment in transplantation	Zeng et al., 2023[160]
Epigenetic	Cdc42 inhibition	*In vitro* treatment with small molecule inhibitor (CASIN) in 20- to 26-month-old mice	Rescue of Cdc42 and H4K16 acetylation polarization; improved myeloid bias upon primary HSC transplantation; improved engraftment and myeloid bias upon secondary BM transplantation	Florian et al, 2012[165]
	Satb1 overexpression	Retroviral transduction of LSK cells from 2-year-old mice *In vitro*	Improved growth of T cells, B cells and NK cells in co-culture with lymphopoiesis supporting stromal cells	Satoh et al, 2013[166]
	Yamanaka factor reprogramming	iPSC generation from HSC clones from 23-month-old mice followed by redifferentiation into HSCs ("iHSC")	T cell chimerism following transplantation of iHSC derived from aged HSC clones which previously did not exhibit T cell output	Wahlestedt et al, 2017[167]
Metabolic/ mitochondrial	Rapamycin treatment	4 mg/kg every other day for 6 weeks in 22-month-old mice	Decreased HSC numbers; improved BrdU incorporation; decreased *p16* and *Arf* expression; improved engraftment and lymphoid output upon HSC transplantation; improved vaccine response *in vivo*	Chen et al, 2009[174]
	Sirt2 overexpression	Lentiviral transduction of HSCs from 20- to 24-month-old mice *in vitro*	Decreased caspase-1 activation; improved engraftment and lymphoid output upon HSC transplantation	Luo et al, 2019[139]
	Sirt3 overexpression	Lentiviral transduction of lineage-depleted BM cells from 18- 24-month-old mice *in vitro*	Improved engraftment upon HSC transplantation	Brown et al, 2013[140]
	Sirt7 overexpression	Lentiviral transduction of murine HSCs *in vitro*	Reduced mitochondrial protein folding stress; improved engraftment and lymphoid output upon HSC transplantation	Mohrin et al, 2015[141]
	NLRP3/Caspase-1 knockdown	Lentiviral shRNA knockdown of HSCs from 20- to 24-month-old mice *in vitro*	Improved engraftment and lymphoid output upon HSC transplantation	Luo et al, 2019[139]
	Nicotinamide riboside supplementation	3mg/mL in drinking water for 8 weeks in 20- to 24-month-old mice	Decreased HSC and GMP frequency and number in primary mice; more youthful HSC transcriptome; improved metabolic parameters; improved engraftment in HSC transplantation	Sun et al, 2021[176]

Many groups have applied interventions to old HSCs, aimed at returning them to a more youthful state. These attempts span a wide range of techniques and biological mechanisms and have had mixed success. GMP, granulocyte-macrophage progenitor, iPSC: induced pluripotent stem cell, LSK: lineage^{-}/Sca-1^{+}/c-Kit^{+}, shRNA: small hairpin RNA, 5-FU: 5-fluorouracil.