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Aging, Clonality and Rejuvenation of Hematopoietic Stem Cells

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Abstract

Aging is associated with reduced organ function and increased disease incidence. Hematopoietic stem cell (HSC) aging driven by both cell intrinsic and extrinsic factors is linked to impaired HSC self-renewal and regeneration, aging-associated immune remodeling, and increased leukemia incidence. Compromised DNA damage responses and increased production of reactive oxygen species have been previously causatively attributed to HSC aging. However, recent paradigm-shifting concepts such as global epigenetic and cytoskeletal polarity shifts, cellular senescence, as well as clonal selection of HSCs upon aging provide new insights into HSC aging mechanisms. Rejuvenating agents that can reprogram the epigenetic status of aged HSCs or senolytic drugs that selectively deplete senescent cells provide promising translational avenues for attenuating hematopoietic aging and potentially, alleviating aging-associated immune remodeling and myeloid malignancies.

Stem Cells and Aging

Aging is associated with a gradual functional decline of a variety of organs and tissues and is the highest risk factor for cancer [1, 2]. With the onset of a demographic shift towards an older population, understanding mechanisms regulating the complex process of aging is important for understanding aging-associated disease development and promoting a longer and healthier lifespan. In the past decade, it has become evident that organ aging is linked to the aging-associated decline in somatic stem cell function in various animal model systems [3-11]. Stem cells, with self-renewal and multi-lineage differentiation properties, generate distinct tissue-specific cell types during development and later support homeostatic maintenance and tissue repair. During the process of aging, cells are continuously exposed to both cell-intrinsic and -extrinsic stress factors; the effects of stress on long-lasting stem cells can cause damage accumulation and impair long-term tissue homeostasis. The

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hematopoietic system depends on the constant replenishment of differentiated blood cells by hematopoietic stem and progenitor cells (HSCs and HPCs) throughout adulthood, a process termed hematopoiesis. In this review, we focus on hematopoietic stem cell aging and discuss novel research, including age-associated HSC cytoskeletal protein **polarity shifts** (see Glossary) and global **epigenetic drifts** that add exciting new insights into HSC aging mechanisms. We further discuss how these might contribute to recently discovered common changes in aging-associated **clonal dynamics** and conclude with the likely implications and limitations of HSC rejuvenation clinical approaches.

Aging of Hematopoietic Stem Cells

Adult mammalian hematopoietic tissue homeostasis depends on the balance of HSC self-renewal with multi-lineage differentiation decisions [12, 13]. Under steady state hematopoiesis, HSCs differentiate within the bone marrow (BM) into myeloid and lymphoid progenitors, leading to a balanced and tightly controlled production of both myeloid and lymphoid lineages. By contrast, older adults present a higher prevalence of anemia and compromised adaptive immunity due to reduced T and B-lymphocyte function caused by thymus involution and to a decreased number and/or function of aged lymphoid progenitors [14, 15]. This aging-associated impairment in immunity was initially anticipated to be a consequence of reduced numbers of HSCs. Evolutionary theories on hematopoietic aging support a model in which an early peak in lymphocyte production in life is a program favored by natural selection and a decline over time is inevitable, whereas an additional decline in HSCs and lymphoid progenitor fitness further contributes to aging-associated immune remodeling [16]. Interestingly, HSC numbers are 2-3 fold times increased in C57Bl/6 aged mice (>20 months old) relative to young and are only slightly decreased in other aged mouse strains, DBA/2 and Balb/c [9, 17-20]. These mouse strain differences can be advantageously exploited through forward genetics to identify genetic regulators of HSC aging [21]. More recent publications on human HSC aging further support the view that phenotypic human HSC frequency in BM ($CD34^+CD38^-CD90^+$ cells) is more likely to be increased, not decreased, upon aging [22, 23]. While phenotypic mouse HSC numbers are elevated upon aging, their function is impaired, as demonstrated by their reduced transplantation efficiency in **competitive transplantation assays**. Using irradiated animals as recipients, aged mouse HSCs (23-28 months) exhibit reduced repopulation capacity, independent of murine genetic background [3, 24-27]. In addition, aged mouse HSCs display reduced BM homing abilities [20, 28], and adhesion to BM stroma cells, as well as increased Granulocyte Colony-Stimulating Factor (*G-CSF*)-induced mobilization [29]. Similarly, HSCs isolated from BM from older human donors (45 years and above)has been associated with reduced transplantation success in patients, indicating that human HSC regenerative capacity also declines upon aging [30].

Another aging-associated phenotype characteristic of HSCs is their myeloid-biased differentiation potential, described by the propensity of aged HSCs to contribute to a higher percentage of myeloid cells in peripheral blood, both upon transplantation and at steady state. This property is also reflected by a relative expansion of myeloid progenitor numbers in aged mice compared to young (Figure 1, Key Figure). This feature of aged HSCs is demonstrated to be a **cell-autonomous function** [3, 31]. Dorshkind *et al* propose an

interesting evolutionary hypothesis to explain the aging-associated loss of lymphoid progenitor cells whereby the energy-inefficient process of **antigen receptor rearrangement** is minimized in order to conserve energy [14, 32]. They suggest this process would reduce the risk of transformations caused by DNA cleavage and ligation during ***V[D]J* rearrangement,** when lymphoid progenitors are highly susceptible to chromosomal translocations and transformation, particularly in an aged microenvironment [14]. Consistent with this theory is the fact that pediatric hematopoietic malignancies largely represent lymphoid leukemias, while older individuals, in addition to presenting a high frequency of chronic lymphocytic leukemia (CLL), they also exhibit a higher incidence of myeloid malignancies such as myelodysplatic syndrome (MDS) and acute myelogenous leukemia (AML). Aged human HSCs also exhibit reduced lymphoid potential, although an associated myeloid lineage increase is still a matter of debate [22, 23]. A myeloid-biased HSC lineage fate might be directly linked to increased myeloid leukemia, given that the transcriptional profile and epigenetic changes in aged mouse HSCs show a significant overlap with those of leukemic stem cells [33]. Consistently, young (1-2 month) mouse BM cells transduced with the ***BCR-ABL* oncogene,** an oncogene linked to chronic myelogenous leukemia (CML), develop a lymphoid disease (defined by both CD19 expression and blast morphology in spleen cells) upon transplantation, whereas older BM cells can manifest as either CML or myeloid leukemia, further supporting the idea that an aging-associated HSC myeloid bias exists [34]. More recently, aging of the mouse HSC microenvironment, or niche, has been found to contribute to HSC aging and myeloid skewing, in part via increased levels of the chemokine **RANTES,** secreted from the aged niche [35]. In these studies, *ex vivo* treatment of HSCs with RANTES resulted in fewer T-cell progeny, and *Rantes* knockout mice rescued the aging-associated myeloid-biased lineage differentiation. Moreover, computational modeling approaches, in combination with transplantation experiments, have established a critical link between niche aging and an indirect induction of HSC aging [16, 36, 37]. Combined, these studies imply that both HSC cell intrinsic and extrinsic aging-associated changes contribute to the altered HSC lineage potential and function with age.

Mechanisms Contributing to HSC Aging and Disease

Environmental (such as aging microenvironment, niche interactions, radiation) and cell intrinsic stress (such as metabolic and replicative stress), along with altered stress responses in aged HSCs, result in DNA damage and an increased frequency of genomic mutations [38]. In several tissues, aging-associated genomic mutations have been associated with cancer development, including leukemia [1].

An Altered DNA Damage Response

Aging-associated cancer incidence has long been associated with a compromised DNA damage response (DDR). Quiescent HSCs can utilize non-homologous end joining, an error prone DNA repair pathway that compromises genomic integrity and leads to chromosomal translocations and fusion genes [39]. The association between DNA repair pathways, aging, and genomic instability stems from studies of diseases such as Fanconi anemia (FA), where cells are defective for DNA repair pathways. FA patients and *FANCD1* deficient mice present BM failure and increased cancer incidence [40]. Further evidence for DNA damage

pathways impacting HSC function has been derived from studies in mice deficient for other DNA repair pathways. These mouse HSCs have been shown to exhibit reduced reconstitution ability, self-renewal, and to ultimately undergo stem cell exhaustion [41]. However, one of the striking characteristics of 'DNA repair pathway'-deficient mice is a decreased number of HSCs from increased apoptosis and decreased proliferation. This contrasts the observation that HSC numbers increase upon aging, suggesting that the molecular mechanisms driving HSC aging are likely not solely driven by defective DNA damage pathways. Our laboratory and those of others have shown that aged HSCs present elevated DNA damage markers, such as a 2-3 fold higher number of **γH2AX foci** and 'elongated tails' in comet assays, which are used as surrogate markers for double-stranded breaks and DNA damage [41-44]. One caveat to these studies is that they were performed in germ-free laboratory mice lacking additional environmental stress factors. Secondly, these conclusions were largely based on *γH2AX* foci formation as a DNA damage 'marker' when *γH2AX* foci are also associated with stalled replication forks and ribosomal biogenesis [44], bringing into question the idea that aged HSCs have more DNA damage than young HSCs. Finally, Moehrle *et al* recently demonstrated *in vivo* that aged HSCs repair double-strand breaks as efficiently as young HSCs without accumulating additional DNA mutations [43], data which further question the impact of aging on hematopoietic genetic instability. Consequently, additional studies are warranted to investigate to what extent DDR and impaired genomic integrity impact HSC aging.

Reactive oxygen species (ROS)

Maintaining appropriate levels of ROS (oxidative stressors) is implicated in cellular aging (see Box 1). Indeed, maintaining a delicate balance between ROS generators and quenchers has been shown to be critical for HSC maintenance and DNA integrity, as well as for preventing ROS-mediated damage to cells [45]. HSCs are quiescent and have an inherently low metabolism rate and generate low levels of ROS. However upon aging, ROS levels accumulate and can result in ROS-induced oxidative free radical damage [46]. Emphasizing the significance of ROS levels in HSC activity, Jang *et al* isolated ROS^{low} and ROS^{high} mouse HSCs and demonstrated that ROS^{low} HSC retained their long-term self-renewal activity while ROS^{high} HSC failed to serially transplant [47]. Treatment of HSCs with the ROS inhibitor N-acetyl cysteine (NAC) or with a p38 MAPKinase inhibitor (an intrinsic cellular stress signal in response to ROS) rescued ROS^{high} HSC colony formation, demonstrating a role of ROS and p38 MAPKinase in ROS-mediated HSC maintainance [47]. Additional evidence for ROS regulation of HSC activity stems from a series of studies on the **Forkhead O (FOXO)** subfamily of transcription factors. Mice with a hematopoietic-specific deletion of *Foxo1, Foxo3a,* and *Foxo4* present fewer HSCs and progenitors and increased ROS levels than control mice [48, 49]. In these murine studies, ROS^{high} HSCs did not display elevated levels of DNA damage, suggesting a likely role for ROS in cell signaling rather than DNA damage in its contribution to HSC exhaustion upon aging.

Mitochondria maintain mitochondrial DNA (mtDNA), which is regulated separately from nuclear DNA. ROS generated from mitochondrial respiration (metabolic ROS) are in close proximity to mtDNA and can elicit, under certain circumstances, oxidative mtDNA damage. Interestingly, little is reported on mitochondrial DNA repair systems and whether or not

aging has an impact. mtDNA mutations drive premature aging-like phenotypes in mouse HSCs, but not HSC physiological aging phenotypes [50], demonstrating that mtDNA damage does not causally contribute to HSC aging. As documented for HSCs, mtDNA mutation frequencies are increased in several other human tissues (such as eye, skeletal muscle, heart and neurons) and are most likely due to oxidative damage [51-53]. Recently, more mtDNA mutations altering mitochondrial function have been detected in fibroblasts, blood and fibroblast-derived iPSCs of aged individuals (60-72 years) compared to younger controls (25-50 years) [54].

Compromised DDR and increased ROS levels resulting in DNA or mtDNA mutations have been historically considered as primary HSC aging pathways. However, recent revolutionizing concepts in the field, including changes in aging-associated global epigenetics, protein polarity shifts, HSC senescence, and clonal selection add essential insights into HSC aging mechanisms.

Global Epigenetic Shifts and Changes in Polarity

Comparative transcriptional profiles of young and aged HSCs indicate that in aged HSCs myeloid differentiation linked genes, such as *Runx1, Hoxb6,* and *Osmr* are upregulated while lymphopoiesis genes are downregulated [24]. These global gene expression changes are in accordance with age-induced myeloid skewing at the level of gene transcription and they also raise the interesting possibility that aging is a consequence of altered epigenetic transcriptional regulation. Indeed, several laboratories, including ours, have report changes in the global epigenetic profile of aged mouse HSCs [33, 55-57].

DNA methylation is an epigenetic marker largely associated with gene repression. By gene ontology (GO) analysis, Sun *et al* found in aged HSCs that regulatory gene regions associated with HSC differentiation were hypermethylated, while genes associated with regulating HSC maintenance were hypomethylated [55], consistent with impaired differentiation potential and increased numbers of aged HSC. Other studies have demonstrated aging-associated hypermethylation of HSC genes regulated by the polycomb repressive complex 2 (*PRC2*) as well as genes regulated by repressive H3K27Me3 histone modifications [42, 56]. For example, genome-wide promoter occupancy of activation-associated H3K4Me3 and repressive H3K27Me3 histone marks has been described for aged HSCs, correlating with the transcriptional activity of affected loci [55]. These aging-associated epigenetic changes include changes in both repressive and active marks without a directional genome-wide shift towards activation or repression upon aging, leading researchers to coin the term “epigenetic drift” or “shift”. Further evidence for an aging-associated HSC epigenetic shift stems indirectly from comparative gene expression analyses, demonstrating deregulation of several components of chromatin organization and epigenetic maintenance genes in aged mouse HSCs, including genes of the PRC2 complex [58]. Finally, the Tom Misteli's laboratory has recently revealed that regions of high H3K4methylation in translocation prone regions of human HSCs facilitate chromosomal breakage and increased translocation frequency [59]. This suggests that epigenetic drift might also predispose aged HSCs to acquire distinct genomic translocations, a hypothesis that awaits experimental verification.

In another study, Florian *et al* demonstrated a significant decrease in aged mouse HSC acetylated histone 4 on lysine 16 (H4K16Ac) cellular levels, which is associated with active gene expression. Surprisingly, H4K16Ac also exhibits altered nuclear spatial distribution (polar in young and apolar in aged HSCs) that is controlled by aging-associated elevated activity of the small RhoGTPase, Cdc42.[57]. A switch from canonical to non-canonical Wnt-signaling (Wnt5a) has also been shown to result in elevated activity of Cdc42 in aged HSCs and interestingly, *ex vivo* Wnt5a treatment of young HSCs can induce an aged HSC phenotype [60]. Inhibition of elevated Cdc42 activity with the pharmacological inhibitor CASIN was found to increase H4K16Ac levels and restore its polar spatial distribution in aged mouse HSCs, correlating with their rejuvenated function [57]. Collectively, these studies imply that changes in the appearance and nuclear spatial distribution of epigenetic marks can contribute to epigenetic drift with aging.

HSC Clonal Contribution to Hematopoietic Aging

Hematopoiesis is regarded as a polyclonal event with the perception that most HSCs are equipotent when contributing to hematopoiesis through life, and that multiple clones may be activated to support blood production. HSCs are clearly heterogeneous with respect to function and activity in **limiting dilution transplantation experiments** [3, 31]. However, paradigm-shifting research on HSC clonality and the heterogeneous contribution of HSC clones to hematopoiesis has recently challenged this view. Higher levels of clonality, where only a few clones actively contribute to the production of peripheral blood cells, has been observed with hematopoietic aging [61-63]. Historically, an aging-related progression towards hematopoietic clonality is demonstrated by skewing towards individual X-chromosome inactivation associated with myeloid bias in aged female individuals [64, 65]. Two recent studies track mouse HSC clonality *in situ* by either utilizing sleeping beauty transposase-mediated mobilization of genomic transposons, or Tie2-Cre mediated YFP labeling of HSCs, monitoring both steady state and stress-induced hematopoiesis. Sun *et al,* using quasi-random insertional labeling of mouse HSCs in a transplantation model (during regeneration) demonstrated that multiple clones could contribute to hematopoiesis during development, although later in adulthood, long-lived progenitors (rather than HSCs) were mostly contributing to hematopoiesis [62]. A surprising finding was that granulopoiesis could be polyclonal while lymphopoiesis seemed to be mono or oligoclonal in young animals, even during steady state [62]. Busch *et al,* similarly concluded that short-term HSCs could contribute to steady state hematopoiesis during adulthood by performing limiting dilution clonal tracking experiments *in vivo;* these short-term HSCs were thought to increase the direct contribution of HSCs to hematopoiesis with aging [63]. They further imply, based on sophisticated mathematical models, that the aging-associated myeloid bias might be a consequence of reduced multipotent progenitor (MPP) flux to common lymphoid progenitors (CLP) rather than a change in the HSC pool composition [63]. Verovskaya *et al,* using cellular barcoding and high-throughput sequencing to monitor clonal contribution to regenerative hematopoiesis, concluded that young mice present fewer active HSC clones producing high numbers of progeny, while in aged mice more active HSC clones are observed, but which produce fewer progeny [61]. These findings appear contradictory, but one explanation might be that clonality in steady-state hematopoiesis might differ from

clonality in a regenerative setting such as BM transplantation. To reconcile these findings, another potential explanation could be that upon aging, the HSC pool increases, along with the potential for diversity and clonality. However, this HSC pool might not contribute to hematopoiesis *in vivo* and their progeny might thus not be observed in peripheral blood. Clonality further needs to be experimentally addressed both directly at the level of HSCs, and with respect to the active contribution of HSCs to the peripheral blood pool.

Recently, several groups have identified somatic mutations in blood cells of healthy older adults using large datasets from high-throughput targeted sequencing methodologies [66-68]. Such somatic DNA mutations are rare, but they are also detected in younger individuals. They increase in frequency upon aging, reaching approximately 10-20% of the clonal contribution in individuals aged 65-85 years [67, 68]. Interestingly, clonal hematopoiesis in these older adults has been largely associated with mutations in only three genes, implying a causal relationship for these genes in aging-associated clonality: *DNMT3a*, a DNA methyltransferase enzyme; *TET2*, a DNA demethylase; and *ASXL1*, polycomb group (*PcG*) protein, a transcriptional repressor. Mutations in these genes have also been associated with MDS and AML [67, 68]. *DNMT3a*, *Tet2*, and *ASXL1* encode for proteins that epigenetically regulate transcription, raising an interesting novel hypothesis of "**epigenetic clonality**" with aging, where clones with certain epigenetic profiles are preferentially maintained by the aging microenvironment. McKerrell *et al* detected *DNMT3a* and *JAK2* mutations, as well as mutations in splicesosome genes such as *SF3B1 and SRSF2* in healthy adults [66]. Of note, *DNMT3a* and *JAK2*-associated HSC clonal expansion was found to be linear with age, while mutations in the splicesosome genes were linked to an exponential clonal expansion occurring after 70 years of age, implying that the aged environment could preferentially select for splicesosome-defective clones [66, 69]. Thus, HSC aging, aging-associated genomic instability, epigenetic shifts and clonality, as well as disease development are complex interwoven processes which appear to be causally linked to HSC aging and aging-associated changes in hematopoiesis.

In conclusion, compromised DNA damage and increased ROS levels are believed to contribute to HSC aging and aging-associated diseases, but direct evidence for such associations is absent. Instead, recent research supports the idea that these factors are more than likely, not directly involved in aging and aging-associated diseases affecting HSCs. Novel findings rather suggest a prominent role of changes in the epigenetic landscape and polarity of HSCs with aging. Clonality shifts are also a likely novel hallmark of aging in the hematopoietic system and in aging-associated diseases such as certain forms of leukemia. Moroever, the hypothesis that epigenetic and polarity shifts drive HSC aging is further supported by the mechanisms that promote HSC rejuvenation, as discussed below.

HSC Rejuvenation

The concept of stem cell rejuvenation is tightly linked to the finding that differentiated cells can be reprogrammed into induced pluripotent stem cells (iPSCs). This suggests that the differentiated -- and perhaps aged -- state, might also be reversible by changing the epigenetic landscape. Wahlestedt *et al* demonstrated that iPSCs generated from aged murine HSCs that were re-differentiated back into HSCs were functionally highly similar to young

HSCs, suggesting that HSC aging might be driven, at least in part, by reversible epigenetic reprogramming [70]. Additional evidence that stem cell rejuvenation is possible has emerged from mouse caloric restriction studies. Caloric restriction extends the life span of multiple organisms, including worms, flies, and mice [71]. Prolonged fasting has been found to rejuvenate the aging-associated myeloid differentiation bias as well as the reduced long-term repopulation capacity of aged mouse HSCs. Such rejuvenation has been mechanistically attributed to reduced IGF-1 signaling and restored youthful levels of intrinsic HSC nutrient sensing [72]. Sirtuins, mitochondrial histone deacetylases, mediate caloric restriction effects in lower organisms such as *S.cerevisiae, C.elegans and D.melanogaster* [73]. In particular, Sirt3, a mammalian sirtuin that regulates the mitochondrial acetylation landscape, has been found to be reduced in aged mouse HSCs; furthermore, *Sirt3* overexpression has been reported to rescue aging-associated HSC functional defects [74]. Expression of *Sirt7* has also been shown to be decreased in aged murine HSCs, while its overexpression has been found to increase HSC reconstitution capacity and to reduce the aged HSC myeloid bias [75]. This effect was presumably mediated by the mitochondrial unfolded protein response, UPR^{mt}, a signaling pathway that regulates mitochondrial chaperon transcription and is critical for stress relief [75]. Altogether, these studies highlight an interesting role of caloric restriction and sirtuins on HSC aging and rejuvenation.

The expression of another nutrient-sensing protein, the Mammalian target of rapamycin (mTOR) has been reported as elevated in aged murine HSCs [76] and treatment with the mTOR inhibitor rapamycin has been found to reverse the aging-associated increase in HSC numbers, restoring reconstitution potential and self-renewal [76]. Transient treatment of aged mice with rapamycin was also shown to improve the vaccination response to influenza virus [76], implying that improved lymphopoiesis and adaptive immunity ensue in response to mTOR inhibition.

Another recent example of successful HSC rejuvenation comes from studies on a specific inhibitor of Cdc42 activity (CASIN) [57]. As discussed previously, CASIN treatment *ex vivo* can restore aged mouse HSC phenotypes by both regulating Cdc42 activity and epigenetic reprograming by elevating H4K16Ac levels to those of young cells, again emphasizing the strong link between epigenetic reprogramming and stem cell rejuvenation. Further supporting the role of epigenetic reprogramming in HSC rejuvenation, Satoh *et al* identified that aging-associated **immunosenescence** could be linked to a reduced expression of *Satb1*, an epigenetic regulator of lymphoid progenitors [77]. Moreover, overexpression of *Satb1* via epigenetic reprogramming could rescue aged HSC immunosenescence [77]. Thus, multiple successful approaches currently share a common underlying theme; epigenetic reprogramming appears to be a central mechanistic contributor to HSC rejuvenation (Figure 2).

Cellular senescence represents an irreversible state of growth arrest; a well-established cancer defense mechanism in solid tissues associated with aging and aging-associated diseases. The senescence-associated secretory phenotype (SASP) that senescent cells display refers to the secretion of inflammatory cytokines, matrix-metalloproteases, chemokines, and growth factors with aging-associated tissue damage and cancer promotion [78-80].

Expression of the cyclin dependent kinase (CDK) inhibitor p16 (Ink4a) is critical step for establishing and maintaining cellular senescence [81]. Consequently, p16Ink4a-positive senescent cells in aged mouse tissues, such as skeletal muscle, white adipose tissue, kidney, spleen and liver appears to shorten healthy lifespan [81]. Depletion of senescent cells within tissues was recently accomplished in a transgenic mouse model that expresses FK-506-binding-protein-caspase 8 fusion protein under control of the *Ink4a* promoter. Addition of dimerizer-activated caspase 8 was shown to kill senescent cells upon binding FK-506-BP in Ink4a positive cells. These mice presented an extended lifespan with improved kidney, skeletal muscle and heart function, as well as decreased incidence of sarcomas, and an overall increase in cancer-free survival [81]. Most interesting was the fact that depleting senescent cells using the **senolytic drug** ABT263 directly in the BM of aged mice, significantly improved the function of aged HSC in serial transplant experiments [82]. These cells exhibited reduced myeloid bias and improved long-term transplantation ability. Consequently, it would be of particular interest to test whether senolytic drugs such as ABT263 might alleviate the initiation of aging-associated leukemia (see Box 2, Figure 2, Table 1). These studies further imply a specific role for tissue-resident senescent cells in conferring aging on distinct types of cells, including HSCs.

Concluding Remarks

The availability of rejuvenating agents that target different mechanisms involved in reprogramming the aged hematopoietic system and HSCs back to a youthful status demonstrate that aging, in biological terms, is amendable, and that attenuation of HSC aging is possible (Table 1). If HSC epigenetic drift drives aging and clonality (epigenetic clonality), the use of epigenetic reprogramming agents such as CASIN and the Satb1 activator might restore a more homogenous epigenetic profile in aged HSCs and rejuvenate the HSC pool. Interestingly, epigenetic reprogramming agents might also be effective rejuvenating agents even in the scenario of DNMT3a, ASXL1 and TET2 mutant HSC clones, by directly affecting the mis-regulated epigenetic targets. This view is supported by the clinical success of epigenetic modulating agents such as azacitidine in aging-associated diseases like MDS and AML [83]. However, the efficacy of these rejuvenating agents might be limited by HSC relative age and by the extent of changes that have already taken place upon aging. Successful rejuvenation approaches target changes in HSCs that are biologically reversible. As DNA mutations are not reversible, it is not clear if a rejuvenation approach would affect HSCs with DNA mutations. Age-related accumulation of DNA mutations might pose a challenging limitation to these rejuvenation approaches and would require further study to determine if aging becomes irreversible at a certain age (see Outstanding Questions).

The agents discussed thus far largely address HSC intrinsic rejuvenation pathways and not the critical role of the microenvironment and stem cell niche aging. Senescent cell depletion might be a promising approach to address the aging microenvironment and revert an aging-associated pre-cancerous state by not only depleting senescent cells, but also by reducing SASP and SASP-induced changes in the microenvironment (Box 2). In conclusion, rejuvenating agents might provide a promising tool for rejuvenating aged HSCs and rigorous testing of these agents either individually or in combination is warranted in preclinical

human HSC aging models. Such approaches might allow the clinical development of pharmacological drugs in order to support healthy hematopoietic aging.

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Glossary

Antigen receptor rearrangement/ VDJ rearrangement
Genes coding for variable (V), Diversity (D) and Junction (J) regions of either B-cell receptor (BCR) and antibodies (B-cells) or antigen recognizing T-cell receptor (TCR) (T cells) undergo rearrangements to generate a highly diverse repertoire of antibodies and TCRs for effective antigen recognition and immunity.

BCR-ABL
Chromosomal translocation commonly detected in chronic myelogenous leukemia (CML) generated by the fusion of the *BCR* gene from chromosome 22 and the ABL kinase domain from chromosome 9, generating the fusion protein BCR-ABL with constitutively active tyrosine kinase activity.

Cell autonomous function
Changes in function driven by cell intrinsic changes independent of cell extrinsic factors.

Clonality
HSCs have been historically deemed homogenous in their contribution to hematopoiesis during steady-state or injury. However, recent experiments identify the relative contribution of individual HSC to hematopoiesis changes with aging, resulting in an increased clonal distribution with aging.

Competitive transplantation
This is a gold standard experiment to test the function of HSCs. In it, hematopoietic stem/ progenitor cells (HSPCs) or total bone marrow (BM) cells are transplanted along with fixed numbers of competitors (wild type/normal BM) cells. Subsequently, HSPC contribution to differentiated blood cells in the BM and blood are monitored, testing for HSPC function.

Cytoskeletal protein polarity
Asymmetric versus symmetric distribution of cytoskeletal proteins in the cytoplasm of a stem cell (in this case).

Epigenetic drift
Epigenetic modifications are heritable post-translational modifications of either histones or DNA itself without alterations in the DNA sequence. Global changes in the epigenetic profile of stem cells upon aging are termed epigenetic drift.

FOXO transcription factors

FOXO, a family of Forkhead transcription factors, play a critical role in regulating longevity in model organisms and in stress response signaling.

γ-H2AX
one the sub-forms of histone 2 that constitute the nucleosome structure for DNA. During DNA double strand breaks (DNA damage), H2AX becomes phosphorylated at serine 139; this phosphorylated histone is termed γ-H2aX. Recently γ-H2aX has been shown to be associated with open chromatin structures.

Limited dilution transplantation
This is an HSC transplantation experiment where HSCs are transplanted into recipients (e.g. mice) in limited numbers (10-100 cells).

RANTES (regulated on activation, normal T cell expressed and secreted, also known as CCL5)
chemokine initially known as a chemo-attractant involved in T cell development and inflammation. Recently, it has been discovered to be secreted in bone marrow.

Senescence
Senescence is an irreversible non-replicative state of the cell.

Senolytic drugs
Drugs that selectively kill senescent cells but do not affect normal cycling or quiescent cells.

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Box 1. Reactive oxygen species (ROS)

ROS are generated, among others, by oxidative phosphorylation and thus, result from regular cellular metabolism. ROS levels are also increased by external stimuli such as inflammation, UV, radiation and chemotherapy. Prolonged exposure to ROS and ROS generated free radicals has been proposed to contribute to aging [84] via oxidization of DNA, RNA and proteins. Oxidized DNA products result in DNA damage that triggers a DNA damage repair response and in case of incomplete repair, senescence and apoptosis are initiated, which result in reduced HSC numbers and functionality. However, ROS are also critical mediators of cell signaling. Maintaining appropriate levels of ROS through a delicate balance between ROS generators and ROS quenchers is thus critical for HSC maintenance, DNA integrity and ROS mediated damage to cells [45].

Box 2. Clinician's Corner

- Rejuvenation and reprogramming of hematopoietic stem cells might improve the adaptive immune response in elderly and alleviate aging-associated myeloid malignancies.
- Combination of senolytic drugs such as ABT263, or with epigenetic modulators such as CASIN might provide rejuvenation of both cell extrinsic and intrinsic factors that contribute to HSC aging.
- Current research on rejuvenating agents that address aging-associated changes intrinsic to HSCs might have potential clinical applications for the *ex vivo* treatment of bone marrow from aged donors, in order to improve transplantation success rates.
- Current limitations in harvesting HSC rejuvenation agents for *in vivo* clinical scenarios are still not well-defined because of unanswered questions, including i) what the contribution of damaged DNA to HSC aging is and ii) what happens to DNA mutations following HSC rejuvenation.

Outstanding Questions

1. During HSC aging, cytoskeletal are protein polarity changes and epigenetic drifts just associations or are they mechanistically connected?
2. What is the role if any, of mitochondrial mutations in the pathogenesis of aging-associated leukemias?
3. How do rejuvenating agents affect the functional reversal of aged lymphoid progenitors?
4. Do rejuvenating agents target genomic instability in the aged bone marrow, and if so, how?
5. If clonal hematopoiesis drives aging, do rejuvenating agents reverse the existing clonal expansion, and if so, how?
6. Rejuvenation agents so far address stem cell intrinsic properties. However, it is important to tackle how the aged microenvironment handles/supports rejuvenated cells.
7. What age is the right age for a rejuvenation process? When is rejuvenation too late or irreversible?

Trends Box

1. HSC aging is associated with global epigenetic changes and shifts in cytoskeletal protein polarity.
2. Mutations in both epigenetic modulators and splicesosome proteins have been shown to increase with age, and have been observed in healthy older individuals.
3. Specific approaches and agents aiming to reverse global epigenetic profiles of aged HSCs are demonstrating promising rejuvenating capabilities.
4. Senescent cell depletion approaches and agents have been shown to reverse or inhibit hematopoietic aging and certain aging-associated cancers in animal models.

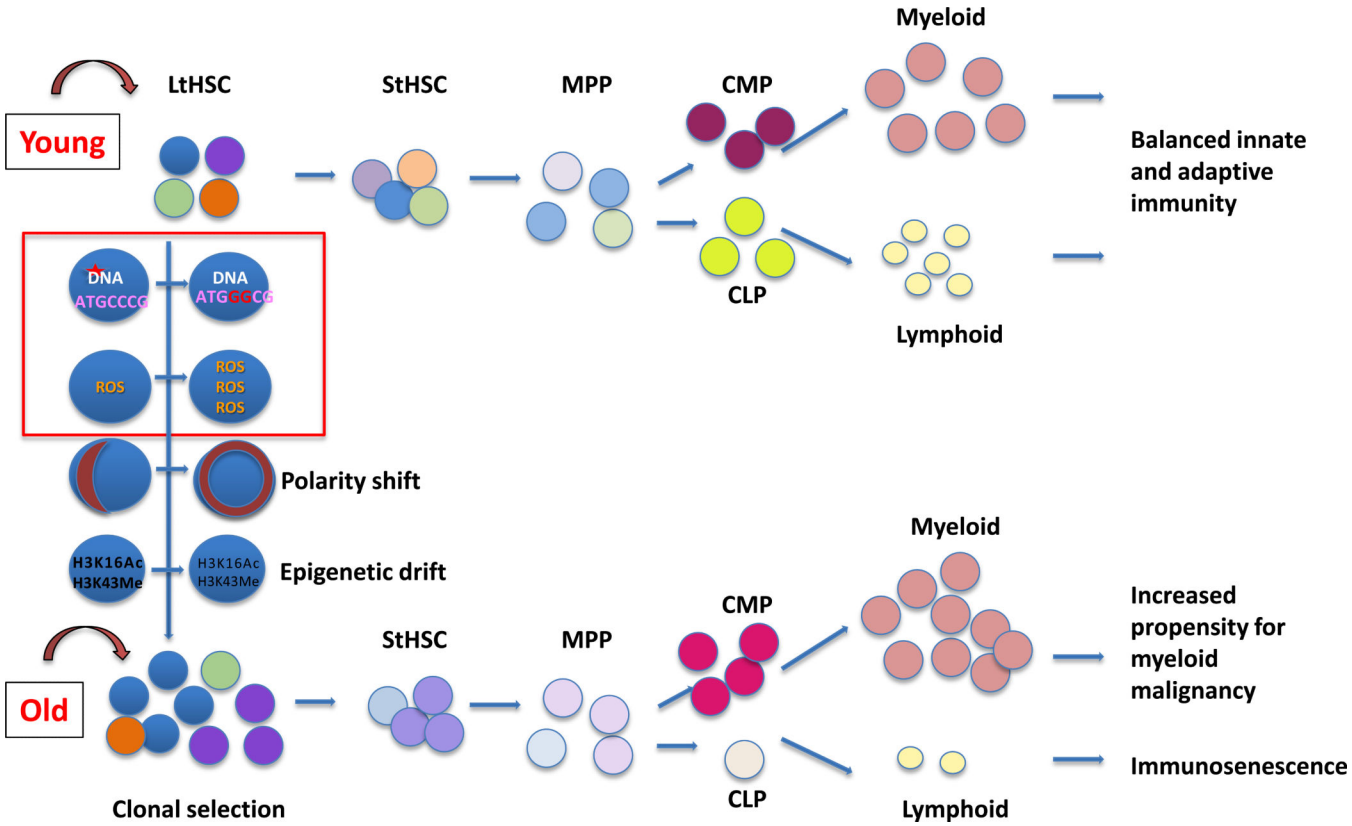

Figure 1, Key Figure. Molecular Mechanisms driving the Clinical Consequences of Aging HSCs

A compromised DNA damage response of aged HSCs resulting in translocations and fusion genes and increased accumulation of ROS, as well as ROS-associated DNA damage have been previously associated with hematopoietic aging and aging-associated disease. More recently, changes in the spatial distribution of cytoskeletal proteins and epigenetic markers termed as polarity shifts and global changes in the epigenetic landscape of aged HSCs (epigenetic drift) have been shown to drive hematopoietic aging. In addition, drifts towards clonality in hematopoiesis upon aging (clonality denoted by cells with similar color) have been described as a major contribution to the expansion of the number of phenotypic HSCs upon aging and to the aging-associated myeloid bias in HSC differentiation. Old StHSCs and MPP with altered differentiation fates (denoted by a different color from young cells) lead to lesser differentiation towards CLP than towards CMPs upon aging. [A1]Thus, aged HSCs drive, at least in part, the clinical outcomes of aging of the hematopoietic system such as aging-associated immune remodeling and increased propensity towards initiation of myeloid malignancies.

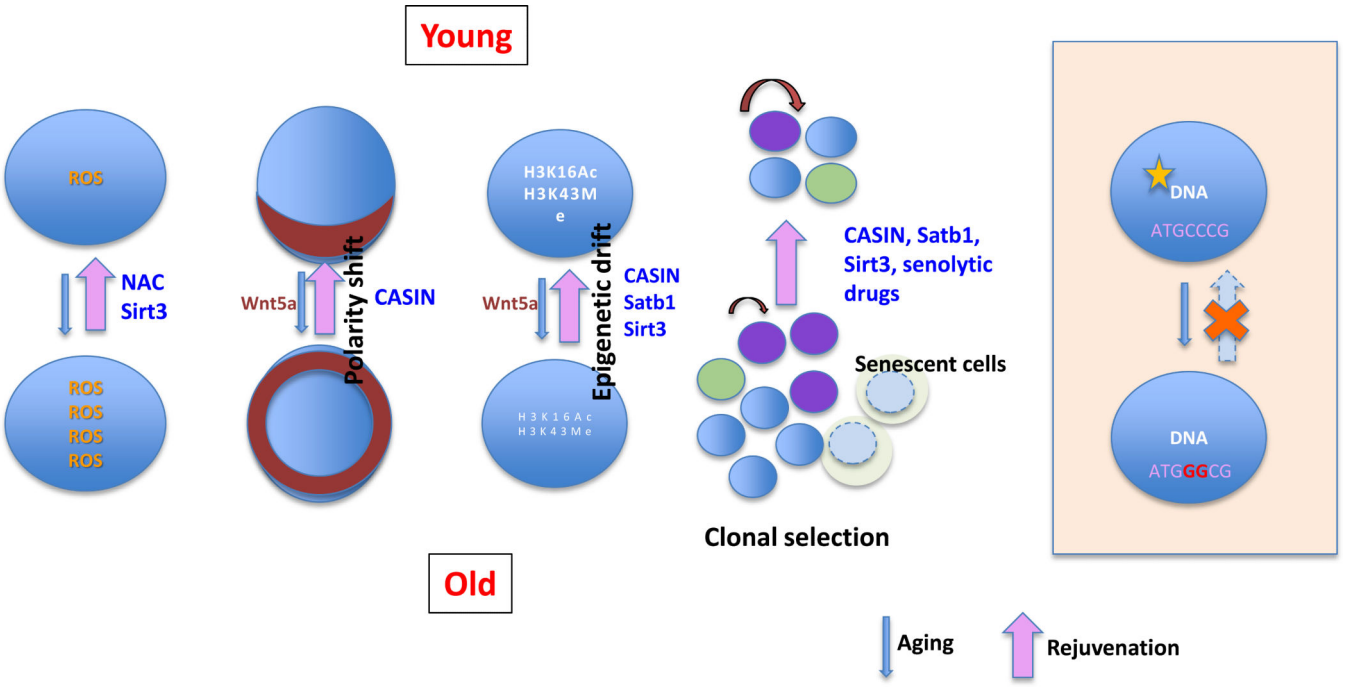

Figure 2. Rejuvenation of HSCs and Potential Pitfalls

NAC treatment and Sirt3 overexpression rejuvenates old HSCs by reducing ROS levels. Wnt5a treatment results in aging of HSCs both by altering cytoskeletal protein polarity and global epigenetic changes. The Cdc42 inhibitor CASIN can rejuvenate old HSCs by both reverting the cytoskeletal polarity shift as well as the epigenetic landscape to a young state in HSCs. Other HSC rejuvenating agents/approaches such as CASIN treatment, Satb1, Sirt3 overexpression, and senolytic drug treatment might be partially able to revert aging-associated clonality to a level seen in a young hematopoietic system. However, in general, rejuvenating agents might not be able to revert DNA mutations in HSCs associated with clonality upon aging (the rejuvenating agent potential limitation is depicted by an orange box; the blue arrow indicates aging and the pink arrow indicates rejuvenation).

Table 1

HSC Rejuvenation Pathways and Agents

Pathway Category	Rejuvenation agent/approach	Rejuvenated HSC function	Mechanism	Reference
Nutrient sensing	Caloric restriction	Myeloid bias Long term repopulation Chemoprotection	IGF-1	Cheng, C.W. *et al*, 2014
	Sirt3 overexpression	Long term repopulation competitive repopulation	ROS mitochondrial function	Brown, K. *et al*, 2013
	Rapamycin	HSC numbers Competitive repopulation Immune-suppression	mTOR	Chen, C. *et al*, 2009
Epigenetic modulators	CASIN	Myeloid bias Competitive long term repopulation	Cdc42 H4K16-acetylation	Florian, M.C. *et al*, 2012
	Satb1	Myeloid bias immune-suppression	Lymphoid progenitor gene expression	Satoh, Y. *et al*, 2013
Senescence depletion	ABT263	Myeloid bias Competitive long term repopulation	BCL2 and BCL-xL mediated apoptosis Senescent cell depletion	Chang, J. *et al*, 2016