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REVIEW

Aging of hematopoietic stem cells: DNA damage and mutations?

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Aging in the hematopoietic system and the stem cell niche contributes to aging-associated phenotypes of hematopoietic stem cells (HSCs), including leukemia and aging-associated immune remodeling. Among others, the DNA damage theory of aging of HSCs is well established, based on the detection of a significantly larger amount of γH2AX foci and a higher tail moment in the comet assay, both initially thought to be associated with DNA damage in aged HSCs compared with young cells, and bone marrow failure in animals devoid of DNA repair factors. Novel data on the increase in and nature of DNA mutations in the hematopoietic system with age, the quality of the DNA damage response in aged HSCs, and the nature of γH2AX foci question a direct link between DNA damage and the DNA damage response and aging of HSCs, and rather favor changes in epigenetics, splicing-factors or three-dimensional architecture of the cell as major cell intrinsic factors of HSCs aging. Aging of HSCs is also driven by a strong contribution of aging of the niche. This review discusses the DNA damage theory of HSC aging in the light of these novel mechanisms of aging of HSCs.

Hematopoiesis, hematopoietic stem cells, and aging of hematopoietic stem cells

The mammalian blood system consists of many distinct types of differentiated cells with specialized functions like erythrocytes, platelets, T and B lymphocytes, myeloid cells, mast cells, natural killer cells, and dendritic cells. Many of these mature blood cells are short-lived and, thus, need to be replaced at a rate of more than one million cells per second in the adult human [1]. This continuous replenishment depends on the activity of hematopoietic progenitor cells (HPCs) and, ultimately, hematopoietic stem cells (HSCs). HSCs are defined functionally by their ability to self-renew, as well as to differentiate into more mature progenitor cells and, therefore, to provide long-term reconstitution potential of the blood in lethally irradiated recipients over serial transplantation experiments. HSCs were first described in the 1960s by Becker [2], Simonovitch [3], Till and McCulloch [4], and Wu [5] as a population of bone marrow (BM) cells capable of forming myelo-erythroid colonies in the spleens of irradiated recipient mice. Within these colonies a very small subset of cells displayed the ability to self-renew and to differentiate into all types of blood cells. HSCs reside predominantly in the BM at a frequency of 2 to 5 cells in 10^5 total BM cells. Phenotypically, HSCs can be purified to near homogeneity using combinations of cell surface marker staining or dye efflux properties with fluorescence-activated cell sorting. Common marker combinations for long-term (LT)-HSCs are Lin^- (lineage-negative), Sca-1^+, c-Kit^+, $CD34^-$, $Flk2^-$ or side population (high Hoechst efflux), Lin^-, Sca-1^+, and c-Kit^+. SLAM family markers ($CD150^+$, $CD48^-$, $CD41^-$) can be used to further distinguish myeloid biased LT-HSCs [6–8].

The number and potential of HSCs are controlled via complex regulatory mechanisms involving tight molecular and cellular control of quiescence (G0 state of the cell cycle), self-renewal (maintenance and expansion), differentiation (production of mature blood cells), apoptosis

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(clearance of damaged cells), and localization, as well as cell architecture. Under steady-state conditions, HSCs are a largely quiescent, slowly cycling cell population, where only 8% of cells enter the cell cycle per day [9]. However, in response to stress, HSCs exit quiescence, expand, and differentiate. The mostly quiescent status of HSCs is thought to be a protective mechanism against endogenous stress caused by reactive oxygen species and DNA replication [10]. Quiescence is different from terminal differentiation, senescence, and apoptosis because it is reversible and functionally unlike cell cycle arrest. When quiescence is disrupted, HSCs might display premature exhaustion, impaired self-renewal, and loss of repopulating capacity [11,12]. The balance between quiescence and proliferation is usually strictly controlled (positively as well as negatively) by the intrinsic and extrinsic (niche) mechanisms of HSCs.

Contrary to a common assumption that cell loss is tightly associated with aging, the number of phenotypic HSCs actually increases in both mice and humans [13,14]. There are two- to tenfold more HSCs present in the aged BM than in young marrow [13,15,16]. Under stress, as in serial transplantation assays, for example, aged HSCs exhibit a diminished regenerative potential as consequence of a lower long-term self-renewal capacity [17–19]. Aged HSCs also present with heightened replicative stress on cycling and decreased ribosomal biogenesis [20]. Additionally, their ability to home to the BM is reduced by a factor of 2 [21]. Furthermore, young and aged HSCs occupy distinct niches within the BM as seen in their localization relative to the endosteum [22]. Aged HSCs exhibit impaired adhesive properties to stroma cells and, in turn, can be better mobilized into the blood compared with young HSCs [22]. Genomewide expression studies comparing young and aged HSCs have identified a general downregulation of genes involved in lymphopoiesis and an upregulation of myeloid genes in aged HSCs, which is consistent with their myeloid bias [23]. Clinically, aging of the hematopoietic system is correlated to anemia, decreased competence of the adaptive immune system, and increased incidence of myeloid diseases (reviewed by Geiger et al. [24]). These aging-associated changes can be attributed at least in part to aging of HSCs. Aged HSCs are deficient in their ability to support erythropoiesis and exhibit a markedly decreased output of cells from the lymphoid lineage, whereas the myeloid lineage output is maintained or even increased compared with young HSCs. Recent exciting developments in the field, driven primarily by large-scale high-throughput sequencing approaches to human blood cells, suggest a highly polyclonal hematopoiesis in young individuals that is transformed into clonal hematopoiesis on aging, starting at the age of 70. This shift, from around 1,000 active HSCs to a few active clones, might also be an additional driver for the development of hematologic diseases [25], as mutations in genes associated with aging-associated leukemia, such as DNMT3A, TET2, JAK2, ASXL1, SF3B1, and SRSF2, have been found to be associated with clonality [26–28]. Finally, aged HSCs present with increased activity of the cell division control protein 42 (CDC42), a small Rho GTPase. The increased activity of CDC42 leads to loss of cell polarity in aged HSCs involving tubulin, CDC42, and the random nuclear distribution of acetylated H4K16 [29]. Activated levels of Cdc42 activity are causative for aging of murine HSCs [29]. In addition, epigenetic programs that maintain HSC function in young decline with age, demonstrated by changes in DNA methylation patterns on HSC aging, for example [23,30].

DNA damage in aging of HSCs

A controversially discussed cell-intrinsic factor driving HSC aging is DNA damage. HSCs are responsible for maintaining tissue homeostasis throughout a lifetime. It is therefore critical for HSCs to maintain their genomic integrity to reduce the risk of either BM failure or transformation. The paradigm of the DNA damage theory of stem cell aging states that aging-associated changes in the DNA repair system in HSCs, together with changes in cell cycle regulation caused by increased DNA damage with age [31,32], are thought to result in elevated DNA mutations, which then causally contribute to the decrease in HSCs function with age. The paradigm is in part based on the finding that mice lacking a distinct set of DNA damage repair proteins display reduced function of HSCs, including an impaired repopulating potential and an overall depletion of the HSC pool [32–41]. In contrast, in “naturally” aged mice, there is actually an expansion of the number of phenotypic stem cells as described above and not a depletion of the HSCs.

DNA damage constantly arises from DNA replication errors, spontaneous chemical reactions and assaults by external or metabolism-derived agents. It has been estimated that a single cell might undergo up to 100,000 DNA lesions per day. Endogenous sources for DNA damage include replication and recombination errors (error rate: 10^{-10}/bp), spontaneous hydrolysis, and reactive metabolites created as a by-product of cellular metabolism like reactive oxygen species (ROS) [34]. These events and metabolites can cause abasic sites, base deamination, 8-oxoguanine lesions, base oxidations, and a variety of DNA strand breaks. DNA damage can also be induced by exogenous factors, such as mutagens present in the environment, ultraviolet light from the sun, chemicals, X-rays, and gamma irradiation as well as chemotherapeutic drugs causing base modifications, interstrand crosslinks, and single- and double-strand breaks. The most severe forms of DNA damage are DNA double-strand breaks (DSBs), which arise at an estimated frequency of 10 DSBs/cell/day. These breaks can be intentional in the case of V(D)J recombination and class switch in B lymphocytes or accidental and unintentional. DSBs arise from ROS, gamma irradiation, mechanical stress, defective telomere processing, chemotherapeutic drugs, and replication fork collapse.

DNA damage, regardless of which type, leads to a cascade of cellular events known as the DNA damage response (DDR). The single components of these cascades can be classified into four functional groups: damage sensors, signal transducers, repair effectors, and arrest or death effectors. The outcome of DNA damage for the cell is variable and depends on potency of the damage, cell type, speed of DNA repair, p53 activation, and cell cycle stage of the cell [42,43]. The first step after a DNA DSB occurs involves proteins sensing the damage and activating the DDR. After the recognition of a DNA DSB, transducer proteins become activated, and signaling cascades are set in motion. ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) are the main transducer proteins and, once activated, promote the DDR through phosphorylation-dependent recruitment of additional factors to sites of DNA DSBs. A very important modification at DSBs is the phosphorylation of histone H2AX, referred to as γH2AX. The DNA around the phosphorylated form of the histone becomes less condensed, and a cascade of effector protein assembly is initiated. Activation of downstream targets of ATM (including Chk2 and subsequently p53), as well as ATR signaling (leading to Chk1 and CDC25A phosphorylation), can result in various different cellular outcomes like transient cell cycle arrest, apoptosis, senescence, or differentiation.

During transient cell cycle arrest, repair processes are initiated [44–46]. In case of DNA DSBs, two distinct repair pathways are usually activated, homologous recombination (HR) and non-homologous end joining (NHEJ). Because of the end-processing of DNA strands and a subsequent direct ligation of the break, NHEJ generally leads to small deletions and is thus thought to be error-prone and to result in DNA mutations [47]. In contrast, HR, which is based on a homologous DNA template (sister chromatid or homologous chromosome), is seen as an error-free process with respect to DNA mutations. HSCs mostly residing in G0 phase of the cell cycle are thought to be susceptible to undergo the error-prone NHEJ pathway to repair DNA DSBs arising from low-dose irradiation, thus making the cells vulnerable to acquiring mutations via faulty NHEJ [48]. In contrast to this observation, Insinga et al. showed an upregulation of p21 in HSCs after irradiation followed by cell cycle entry that could enable the cells to use the error-free HR pathway to repair DNA DSBs [49]. More committed progenitor cells, however, underwent p53-dependent apoptosis because of irradiation, illustrating the difference in DNA damage response between stem cells and their progeny. Additionally, Beerman et al. [50] found that quiescent HSCs acquire DNA damage on aging, but when these cells start to cycle, the damage becomes repaired.

Alternatively, if repair is impossible, cells undergo apoptosis, senescence, or in case of stem cells, also differentiation. Published data indicate that DNA DSBs are very potent inducers of cellular senescence, implying that persistent DDR signaling confers senescent growth arrest [51,52]. In addition, differentiation in response to DNA damage occurs in various tissues including HSCs, melanocytic stem cells, and embryonic stem cells [45,46,53]. In HSCs, DNA damage induced by telomere attrition or DNA DSBs leads to an upregulation of the transcription factor Batf, resulting in induction of lymphoid differentiation of HSCs [46]. DNA damage induces apoptosis by both p53-dependent and p53-independent pathways. In lymphocytes and germ cells, apoptosis even represents the primary response to DNA damage [54]. In the context of HSCs, premature differentiation, senescence, and apoptosis as possible consequences of DNA damage are double-edged swords. They can be beneficial by removing damaged cells, but might at the same time lead to depletion and exhaustion of the stem cell pool.

What kind of phenotype in the hematopoietic system is present in animals with genetic deletions in genes linked to DNA damage repair and the DDR? For example, mice deleted for the ATM gene exhibit increased IR sensitivity and decreased T-cell numbers. HSCs from these mice have increased ROS levels and present with a decrease in number and function on aging, leading to progressive BM failure [34,55,56]. Knockout of any component of the MRN complex, as well as deletion of BRCA2, results in embryonic lethality. A hypomorphic $Rad50^{k22m}$ mutation in mice leads to early death accompanied by B-cell lymphoma and BM failure. This is due mostly to p53-dependent apoptosis and loss of HSC function [57]. Inactivation of genes involved in NHEJ illustrated their essential function in lymphocyte development, as components of NHEJ are critical for V(D)J recombination. Mice with a $LigIV^{y288c}$ hypomorphic mutation are immunodeficient and display severe HSC defects, such as impaired repopulating potential and decreased self-renewal [36]. Mice devoid of Ku70 or Ku80 end-binding proteins exhibit self-renewal defects, impaired differentiation and proliferation potential, as well as increased apoptosis within the HSC compartment [32,58]. In a mouse model in which phosphorylation sites of DNA-PKcs were mutated (3A mutation, 3-alanine substitution of Thr2605, 2634 + 2643) called $DNA\text{-}PKcs^{3A/3A}$, BM failure and loss of HSCs in fetal liver could be observed. This impairment of HSC proliferation is caused by p53-dependent apoptosis resulting from severe DNA damage. Because p53 is also involved in the DDR, it is interesting to note that mice with varying p53 activities display distinct hematopoietic problems. Reduced p53 activity in heterozygous $p53^{+/-}$ mice is accompanied by an increase in HSC proliferation. A p53 hypomorphic mutation ($p53^{+/m}$) displaying higher p53 activity than in wild-type mice presented with decreased HSC frequency, repopulating capacity, and proliferation [59]. In summary though, mutations in most of the genes linked to DNA damage response so far did not result in the "aging-characteristic" initial expansion of the number of phenotypic HSCs, rendering a central role for these genes and the

pathways they represent with respect to physiologic aging of the hematopoietic system not likely.

Analyses of young and aged HSCs with respect to changes in the frequency of DNA damage and the frequency of DNA mutations (comet assay, γH2AX foci, DNA mutation frequency, loss of heterozygosity assays) revealed a minor increase in these parameters on aging in steady-state hematopoiesis [32,50,60,61]. Elevated levels of γH2AX foci in aged HSCs are also associated with replication stress, as well as ribosomal biogenesis stress, and therefore might not be unequivocally associated with DNA damage (Table 1) [20].

In general, aged murine hematopoietic cells (either BM or peripheral blood, and independent of the type of mutation assay) exhibit a two- to threefold increase in mutation frequency in hematopoiesis compared with young cells [61–63], which is also in the range of changes in mutation frequency recently reported for human hematopoietic cells, determined via deep-sequencing approaches [26–28,64–67]. Interestingly, although the overall increase in mutation frequencies in blood cells was found to be again in the range of two- to threefold, a set of genes including DNMT3A, TET2, JAK2, ASXL1, SF3B1, or SRSF2 were frequently mutated, in both aging-associated leukemia and aging-associated changes in clonality. These genes function primarily in epigenetic regulation and splicing but are usually not associated with DNA repair or the DDR, implying that these pathways are not selected on aging of HSCs.

Although these data in aggregation confirm a mild two-to threefold aging-associated increase in the mutation frequency in hematopoiesis, the increase is linear and not exponential with respect to age, rendering a cause–consequence relationship to the exponential increase of leukemia on aging unlikely. Modeling of aging of HSCs populations based on evolutionary theories also indicates that accumulation of genetic changes within HSCs is not sufficient to alter selectivity and fitness of HSCs, and identified non-cell autonomous mechanisms, also known as changes in the niche, as the major selective driving force for aging-associated leukemia [68]. Such conclusions are also supported by the observation that while a twenty-twofold increase in the mutational load [69] initiated cancer in the presence of a mutator gene type setting [70–72], a modest two- to threefold increase in mutational load in hematopoiesis in the context of the AML-ETO oncogene did not result in leukemia initiation [73] in mice in vivo. Finally, novel data from our laboratory indicated that the quality of the DDR in HSCs does not change on aging. HSCs, both young and old, enter the cell cycle in vivo upon DNA damage, without initiating a strong G1–S cell cycle arrest as reported for fibroblasts [61]. They also do not present with a marked difference in apoptosis in vivo on induction of DSBs. Second, in response to irradiation, both young and aged HSCs responded functionally identically, as revealed in competitive transplantation–irradiation–recovery experiments, in which young and aged HSCs directly competed in the same recipient animal in vivo in response to total-body irradiation [61]. Third, there was a significant decrease in the mutation frequency in both young and aged BM 4 months after total-body irradiation, and aged animals presented with a mutation frequency similar to that of young animals [61]. One explanation for such observations might be that deeply quiescent HSCs are the ones with few mutations because of their small number of cell divisions [74], whereas HSCs that already underwent a larger number of divisions, and thus most likely have a low level of mutations, instead apoptose or differentiate in response to irradiation. Therefore, it is possible that the deeply quiescent HSCs survive irradiation and support hematopoiesis after other HSCs have been eliminated by irradiation [10], further indicating the resilience of the hematopoietic system to acquire a large number of mutations in HSCs that might directly contribute to aging of HSCs and leukemia.

Table 1. DNA damage accumulation on aging in HSCs

Increased DNA damage and DNA mutations on aging in HSCs and the hematopoietic system contribute to aging of HSCs	
Yes	No
Accumulation of γH2AX foci, indicative of persistent DNA DSBs [14]	γH2AX foci can also indicate replication and ribosome biogenesis stress in aged HSCs [20]
	In aged hair follicle stem cells, γH2AX foci are associated with persistent chromatin alterations but not DNA DSBs [78]
Comet assay experiments in aged HSCs revealed a twofold increase in DNA damage level in steady state [50,61]	DNA damage level decreased on cell cycle entry of aged HSCs [50]
Mutation frequency in human and murine BM is increased twofold upon aging [26,61,64,65]	Mutation frequency in aged BM decreases upon DNA damage [61]
Incidence of leukemia increases dramatically upon aging [77,79,80]	In aging-associated leukemia, only very few driver mutations could be identified [27,28,66,67]
Mouse models with altered DNA damage response genes exhibited BM failure and depletion of the HSC pool [32,34,36,55–59,81]	In normal aging, there is an increase in the number of HSCs [13,14]

Perspective

Because the accumulation of DNA mutations in HSCs on aging might not be linked directly to the functional decline of HSCs with age and an aging-associated exponential increase in the incidence of leukemia, what other mechanisms might contribute to these phenotypes? It could already be shown that aging of the HSC niche and environment plays an important role in selecting and expanding normal and preleukemic HSC and HPC clones on aging [75,76]. Thus, the concept of adaptive landscapes recently has been developed [77]. In this concept, the niche environment of HSCs changes on aging, influencing the functionality of HSCs. The mutations acquired over time might not influence the HSC per se. In addition to extrinsic factors, intrinsic alterations that are not mutations in DNA might ultimately contribute to HSC aging. We recently reported that HSCs change their polarity on aging, in both the cytoplasm and the nucleus. It thus might be possible that changes in the general architecture of the cell might also contribute to HSC aging. Changes in the three-dimensional arrangement of epigenetic marks and structural proteins might influence cell divisions in a way that reduces potential in daughter stem cells, for example, contributing to intrinsic HSC aging. In summary, multiple mechanisms might contribute to aging of HSCs and ultimately depend on the interplay between extrinsic and intrinsic cell factors.

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