HHS Public Access

Author manuscript

Exp Hematol. Author manuscript; available in PMC 2017 October 01.

Published in final edited form as:

Exp Hematol. 2016 October ; 44(10): 895–901. doi:10.1016/j.exphem.2016.06.253.

Aging of hematopoietic stem cells: DNA damage and mutations?

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Abstract/Introduction

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 higher 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 to young cells, and bone marrow failure in animals devoid of DNA repair factors. Novel data on the increase and the nature of DNA mutations in the hematopoietic system upon aging, 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 3D architecture of the cell as major cell intrinsic factors of HSCs aging. Aging if HSCs is also driven by a strong contribution of aging of the niche. This review discusses the DNA damage theory of HSCs aging in the light of these novel mechanisms of aging of HSCs.

Hematopoiesis, HSCs and aging of HSCs

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 need thus to be replaced at a rate of more than one million cells per second in the adult human (Ogawa 1993). 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

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first described in the 1960s by Till, McCulloch, Wu, Becker and Siminovitch as a population of BM cells capable of forming myelo-erythroid colonies in the spleens of irradiated recipient mice (Becker et al., 1963; Siminovitch et al., 1963; Till and McCulloch, 1961; Wu et al., 1968). 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 predominantly reside in the bone marrow (BM) at a frequency of two to five 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 together with fluorescence-activated cell sorting (FACS). Common marker combinations for 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 (Kiel et al. 2005; Yang et al. 2005; Goodell et al. 1996).

HSCs numbers and HSCs potential 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 (clearance of damaged cells), 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 (Cheshier et al. 1999). However, in response to stress, HSCs exit quiescence and 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 (Walter et al. 2015). 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 (Cheng et al. 2000; Wilson and Trumpp 2006). The balance between quiescence and proliferation is usually strictly controlled (positively as well as negatively) by HSCs intrinsic and extrinsic (niche) mechanisms.

In contrary to a common assumption that cell loss is tightly associated with aging, the number of phenotypic HSCs actually increases in both mouse and humans (de Haan, Nijhof, and Van Zant 1997; Rossi et al. 2005). In the aged bone marrow, there are two- to ten-fold more HSCs present when compared to young (de Haan, Nijhof, and Van Zant 1997; S. M. Chambers and Goodell 2007; Morrison et al. 1996). Aged HSCs show under stress, like for example in serial transplantation assays, a diminished regenerative potential as consequence of a lower long-term self-renewal capacity (Sudo et al. 2000; Kamminga et al. 2005; Janzen et al. 2006). Aged HSCs also present with heightened replicative stress upon cycling and decreased ribosomal biogenesis (Flach et al. 2014). Additionally, their ability to home to the bone marrow is reduced by a factor of two (Liang, Van Zant, and Szilvassy 2005). Furthermore young and aged HSCs occupy distinct niches within the bone marrow as seen in their localization relative to the endosteum (Köhler et al. 2009). Aged HSCs show impaired adhesive properties to stroma cells and in turn can be better mobilized into the blood compared to young HSCs (Köhler et al. 2009). Genome-wide 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 (Stuart M Chambers et al. 2007). Clinically, aging of the

hematopoietic system is correlated to anemia, decreased competence of the adaptive immune system and increased incidence of myeloid diseases (reviewed in Geiger, Denkinger, and Schirmbeck 2014). 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 show a markedly decreased output of cells from the lymphoid lineage, whereas the myeloid lineage output is maintained or even increased compared to young HSCs. Recent exciting developments in the field, driven primarily by large-scale high throughput sequencing approaches of human blood cells suggest a highly polyclonal hematopoiesis in young individuals that is transformed into clonal hematopoiesis upon aging, starting at the age of 70. This shift, from around 1000 active HSCs to a few active clones, might also be an additional driver for the development of hematological diseases (McKerrell and Vassiliou 2015), as mutations in genes associated with aging-associated leukemia like DNMT3A, TET2, JAK2, ASXL1, SF3B1 and SRSF2 have been found to be associated with clonality (Genovese et al. 2014; Jaiswal et al. 2014; McKerrell et al. 2015). 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 (Florian et al. 2012). Activated levels of Cdc42 activity are causative for aging of murine HSCs (Florian et al. 2012). In addition, epigenetic programs that maintain HSC function in young decline with age, demonstrated by for example changes in DNA methylation patterns upon HSC aging (Beerman et al. 2013; Stuart M Chambers et al. 2007).

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 due to increased DNA damage with age (Pietras, Warr, and Passegué 2011; Rossi et al. 2007), 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 (Geiger, de Haan, and Florian 2013; Ito et al. 2004; Navarro et al. 2006; Nijnik et al. 2007; Parmar et al. 2010; Prasher et al. 2005; Reese, Liu, and Gerson 2003; Rossi et al. 2007; Ruzankina et al. 2007; Zhang et al. 2010). In contrast though, 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, Ito et al. 2004). 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 like for example mutagens present in the environment, ultraviolet light (UV) from the sun, chemicals, X-rays and gamma irradiation as well as chemotherapeutical drugs causing base modifications, interstrand crosslinks, single-and double strand breaks. The most severe forms of DNA damage are DNA double strand breaks (DNA DSBs) which arise at an estimated frequency of 10 DSBs/cell/day. These breaks can be intentional in case of V(D)J –recombination and class switch in B-lymphocytes or they are 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 (d'Adda di Fagagna 2008; Blanpain et al. 2011). The first step after a DNA DSB occurs involves proteins sensing the damage and activating the DDR. Following 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 (Bartek and Lukas 2007; Inomata et al. 2009; Wang et al. 2012). In case of DNA DSBs two distinct repair pathways are usually activated, homologous recombination (HR) and non-homologous end joining (NHEJ). Due to 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 (Khanna and Jackson 2001). 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 (Mohrin et al. 2010). In contrast to this observation, Insinga et al. showed an up-regulation 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 (Insinga et al. 2013). More committed progenitor cells however, underwent p53 dependent apoptosis as a consequence of irradiation demonstrating the difference in DNA damage response between stem cells and their progeny. Additionally, Beerman et al. (Beerman et al. 2014)

demonstrated that quiescent HSCs acquire DNA damage upon 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 for example demonstrate that DNA DSBs are very potent inducers of cellular senescence, implying that persistent DDR signaling confers senescent growth arrest (Leonardo et al. 1994; Nakamura et al. 2008). In addition, differentiation in response to DNA damage occurs in various tissues including HSCs, melanocytic stem cells and embryonic stem cells (Inomata et al. 2009; Li et al. 2012; Wang et al. 2012). In HSCs DNA damage induced by telomere attrition or DNA DSBs leads to an up-regulation of the transcription factor Batf resulting in induction of lymphoid differentiation of HSCs (Wang et al. 2012). 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 (Lee et al. 1998). 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 show increased IR sensitivity and decreased T-cell numbers. HSCs from these mice show increased ROS levels and present with a decrease in number and function upon aging leading to progressive BM failure (Barlow et al. 1996; Ito et al. 2004; Ito et al. 2006). Knockout of any component of the MRN complex as well as deletion of BRCA2 result in embryonic lethality. A hypomorphic $Rad50^{k22m}$ mutation in mice leads to early death accompanied by B-cell lymphoma and BM failure. This is mostly due to p53-dependent apoptosis and loss of HSC function (Bender et al. 2002). Inactivation of genes involved in NHEJ demonstrated their essential function in lymphocyte development since components of NHEJ are critical for V(D)J recombination. Mice with a LigIVy288c hypomorphic mutation are immunodeficient and display severe HSC defects like impaired repopulating potential and decreased self-renewal (Nijnik et al. 2007). Mice devoid of Ku70 or Ku80 end-binding proteins show self-renewal defects, impaired differentiation and proliferation potential as well as increased apoptosis within the HSC compartment (Rossi et al. 2007; Kenyon and Gerson 2007). In a mouse model where phosphorylation sites of DNA-PKcs were mutated (3A mutation, three 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 due to severe DNA damage. Since 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 wild-type mice presented with decreased HSC frequency, repopulating capacity and proliferation (Dumble et al. 2007). 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 physiological 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 heterozygousity assays) revealed a minor increase in these parameters upon aging in steady state hematopoiesis (Rossi et al. 2007; Rübe et al. 2011; Beerman et al. 2014; Moehrle et al. 2015). Elevated levels of γH2AX foci in aged HSCs though are also associated with replication stress as well as ribosomal biogenesis stress and might therefore not be unequivocally associated with DNA damage (Flach et al. 2014b).

In general though, aged murine hematopoietic cells (either BM or PB, and independent of the type of mutation assay) show a 2–3 fold increase in mutation frequency in hematopoiesis compared to young (Dempsey, Pfeiffer, and Morley 1993; Vijg et al. 2005; Moehrle et al. 2015), which is also in the range of changes in mutation frequency recently reported for human hematopoietic cells, determined via deep-sequencing approaches (Cancer Genome Atlas Research Network 2013; Welch et al. 2012; Genovese et al. 2014; Jaiswal et al. 2014; McKerrell et al. 2015; Xie et al. 2014; Papaemmanuil et al. 2013). Interestingly, while the overall increase in mutation frequencies in blood cells was found to be again in the range of 2–3 fold, a set of genes including DNMT3A, TET2, JAK2, ASXL1, SF3B1 or SRSF2 were frequently mutated, both in aging associated leukemia but also in aging-associated changes in clonality. These genes function in primarily in epigenetic regulation and splicing, but are usually not associated with DNA repair or the DDR, implying that these pathways are not selected upon aging of HSCs.

While these data in aggregation confirm a mild 2–3 fold aging-associated increase in the mutation frequency in hematopoiesis, the increase though is linear and not exponential with respect to age, rendering a cause-consequence relationship to the exponential increase of leukemia upon aging unlikely. Modeling of aging of HSCs populations based on evolutionary theories also demonstrates that accumulation of genetic changes within HSCs are not sufficient to alter selectivity and fitness of HSCs, and identified non-cell autonomous mechanisms, aka changes in the niche, as the major selective driving force for aging-associated leukemia (DeGregori 2016). Such conclusions are also supported by the observation that while a 22-fold increase in the mutational load (Geiger et al. 2006) initiated cancer in the presence of a mutator gene type setting (Kushner et al. 2008; Noronha et al. 2006; Su et al. 2005), a modest 2–3 fold increase in mutational load in hematopoiesis in the context of the AML-ETO oncogene did not result in leukemia initiation (Krejci et al. 2008) in mice in vivo. Finally, novel data from our laboratory demonstrated that the quality of the DDR in HSCs does not change upon aging. HSCs, both young and old, entering cell cycle in vivo upon DNA damage, without initiating a strong G1-S cell cycle arrest as reported for fibroblasts (Moehrle et al. 2015). They also do not present with a marked difference in apoptosis in vivo upon induction of DSBs. Secondly, 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 compete in the same recipient animal in vivo in response to total body irradiation (Moehrle et al. 2015). Thirdly, there was actually a significant decrease in the mutation frequency in both young and aged bone marrow 4 months after total body irradiation, and aged animals presented with a mutation frequency similar to young animals (Moehrle et al. 2015). One explanation for such observations might be that deeply quiescent HSCs are the ones with few mutations,

maybe due to their low number of cell divisions (Tomasetti and Vogelstein 2015), while HSCs that already underwent a higher number of divisions and thus most likely have a low level of mutations rather apoptose or differentiate in response to irradiation. Therefore it is possible that the deeply quiescent HSCs survive irradiation and they support hematopoiesis after other HSCs were eliminated by irradiation (Walter et al. 2015), further demonstrating 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.

Perspective

Since the accumulation of DNA mutations in HSCs upon aging might not be directly linked 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 HSCs niche and environment plays an important role in selecting and expanding normal and pre-leukemic HSC and HPC clones upon aging (Vas, Senger, et al. 2012; Vas, Wandhoff, et al. 2012). Thus the concept of adaptive landscapes has been recently developed (Rozhok, Salstrom, and DeGregori 2016). In this concept, the niche environment of HSCs changes upon aging, influencing the functionality of HSCs. The mutations acquired over time might not influence the HSC per se. In addition to extrinsic factors also intrinsic alterations that are not mutations in DNA might ultimately contribute to HSCs aging. We have recently reported that HSCs change their polarity upon aging, both in the cytoplasm and the nucleus. It might thus be possible that also changes in the general architecture of the cell might contribute to HSC aging. Changes in the 3D arrangement of epigenetic marks and structural proteins might influence for example cell divisions in a way that reduces potential in daughter stem cells, contributing to intrinsic HSC aging. In summary, multiple mechanisms might contribute to aging of HSCs, and ultimately depend on the interplay between cell extrinsic and cell intrinsic factors.

Acknowledgments

Work in the laboratory of H. Geiger was supported by NIH grants HL076604, DK077762, AG040118, and the DFG (SFB 1074 and 1149, KFO142), the BMBF (SyStaR), the Excellence program of the Baden-Württemberg Foundation, funds from the German Scholar organization (GSO) and the European Commission (FP7 Marie Curie Initial Training Network MARRIAGE).

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Table 1

DNA damage accumulation upon aging in HSCs

Increased DNA damage and DNA mutations upon aging in HSCs and the hematopoietic system contribute to aging of HSCs	
YES	**NO**
accumulation of γH2AX foci, indicative of persistent DNA DSBs (Rossi et al. 2005)	γH2AX foci can also indicate replication and ribosome biogenesis stress in aged HSCs (Flach et al. 2014b) in aged hair follicle stem cells, γH2AX foci are associated with persistent chromatin alterations but not DNA DSBs (Schuler and Rübe 2013)
comet assay experiments in aged HSCs showed a twofold increase in DNA damage level in steady state (Beerman et al. 2014; Moehrle et al. 2015)	DNA damage level decreased upon cell cycle entry of aged HSCs (Beerman et al. 2014)
mutation frequency in human and murine BM is increased twofold upon aging (Moehrle et al. 2015; Cancer Genome Atlas Research Network 2013; Genovese et al. 2014; Welch et al. 2012)	mutation frequency in aged BM decreases upon DNA damage (Moehrle et al. 2015)
incidence of leukemia increases dramatically upon aging (Armitage and Doll 1954; Nordling 1953; Rozhok, Salstrom, and DeGregori 2016)	in aging-associated leukemia only very few driver mutations could be identified (McKerrell et al. 2015; Papaemmanuil et al. 2013; Xie et al. 2014; Jaiswal et al. 2014)
mouse models with altered DNA damage response genes show BM failure and depletion of the HSCs pool (Barlow et al. 1996; Bender et al. 2002; Dumble et al. 2007; Ito et al. 2004; Ito et al. 2006; Kenyon and Gerson 2007; Nijnik et al. 2007; Rossi et al. 2007; Tothova et al. 2007)	in normal aging there is an increase in the number of HSCs (de Haan, Nijhof, and Van Zant 1997; Rossi et al. 2005)