The functional characterization of microRNA-125 in hematopoiesis

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AGING OF HEMATOPOIETIC STEM CELLS IN THE MOUSE

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ABSTRACT

Hematopoietic stem cells (HSCs) are unique in their ability to self-renew and differentiate into all mature blood lineages. The equilibrium between these processes is crucial for tissue maintenance during the lifetime of the organism. However, with age the functionality of HSCs declines, resulting in development of anemias, deficiencies of immune response and increased risk of hematopoietic malignancies. Aged HSCs are characterized by preferential differentiation towards myeloid lineage, impaired self-renewal and engraftment. Recent evidence provides clues to the understanding of these processes on cellular and molecular levels. Key components contributing to stem cell aging are shifts in the transcriptome and epigenome, accompanied by dysfunction of DNA repair pathways. In this chapter we will focus on studies and conceptual models of murine HSC aging.

INTRODUCTION

Organismal aging is associated with functional decline of many tissues. In the hematopoietic system, aging is associated with a decreased immune response, an increased incidence of hematopoietic cancers, anemia, and several autoimmune disorders (1). A growing body of data supports the notion that at least some of these changes originate from dysfunctional hematopoietic stem cells (HSCs). Functional decline of HSCs is believed to be orchestrated by the interplay between cell-intrinsic and cell-extrinsic factors. Insight into the mechanism underlying HSC aging can be obtained from molecular studies in mice. This chapter provides an overview of our current understanding of the mechanisms of murine HSC aging.

CHARACTERISTICS OF MURINE HSCS

In the mouse, HSCs constitute a small proportion (< 0.01%) of all bone marrow cells. However, their extensive proliferative capacity ensures a daily production of over $10^{11}$ blood cells (2). All types of mature hematopoietic and immune cells are generated and regenerated by HSCs, in an intricate process of step-wise gradual differentiation (Figure 1). The abilities to self-renew and to differentiate into multiple lineages are the defining properties of the HSCs (also see Box 1). Morphologically, HSCs cannot be discriminated from more differentiated progenitor cells (Figure 1). However, a panel of cell surface markers, characteristically expressed on HSCs, has been identified. These include expression of Sca1, c-Kit, CD150 (one of the SLAM markers), CD201 (EPCR), and lack of the expression of lineage surface markers CD48, CD244, Flt3 and CD34. Alternatively, the ability of HSCs to efflux certain dyes (Rhodamine-123 (Rho) and Hoechst 33342) can be also used for purification (3,4). This feature of HSC results from the expression of the ABC-transporters P-glycoprotein and Abcg2 (5). Hoechst 33342 exclusion results in a characteristic appearance of primitive cells during fluorescence-activated cell sorting (FACS), known as the “side population” (SP) (6). A combination of multiple of these markers allows prospective isolation and characterization of HSCs with purity up to 50%, defined by the ability of a single cell to reconstitute hematopoiesis of a lethally irradiated animal (7-11).
Aging has been shown to increase both the relative frequency and the absolute number of HSCs in the bone marrow of C57BL/6 mice. Initial studies using unfractionated bone marrow cells had demonstrated a competitive advantage of old cells when co-transplanted with equal numbers of young cells (12), which was attributed to higher concentrations of repopulating cells in the aged population (13). More recent studies, using different combinations of phenotypic markers, confirmed a remarkable increase in the pool size of HSC upon aging (9,14-16). An example of such a phenotypic increase of primitive hematopoietic population upon aging is shown in Figure 2. The extent of such expansion was 7- to 16-fold (17). Furthermore, while the frequency of HSCs is similar among individual young mice, HSC frequencies become highly variable in old mice, indicating a possible loss of control of HSC pool size with age (9).

Despite many advances that have been made in the identification of the phenotype of HSC, “stemness” of a (murine) HSC can only be demonstrated retrospectively by its ability for robust multilineage repopulation of a myeloid-related host. An HSC is often defined by the ability of a cell to support at least 1% lymphomyeloid chimerism in a recipient animal for 4 months post-transplantation (18,19). Primitive cells contributing to peripheral blood cells less than 1% are usually unable to sustain robust blood production in the long-term, or upon secondary transplantation. Therefore they are not considered bona fide

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**Figure 1.** Simplified representation of hematopoietic hierarchy and effects of aging (adapted from Verovskaya and de Haan (90)). HSCs residing in the bone marrow produce all the mature hematopoietic and immune cells that are found in the blood. The majority of HSCs resides in a quiescent G0 state (top left) and becomes activated into cycling and differentiation. During the gradual process of multilineage differentiation, HSCs lose the ability to self-renew (as indicated by round arrows), and become more committed to a certain lineage. In aged mice, HSCs have been found to more actively cycle. Moreover, hematopoiesis becomes skewed towards production of myeloid cells while lymphoid production becomes deficient. Regeneration of red blood cells and platelets is also decreased with lifespan. Processes activated with age are indicated with red arrows, while decreased activity is depicted with blue arrows. LT-HSCs indicate long-term repopulating HSC, ST-HSCs – short-term repopulating HSCs. Several described intermediate progenitor states are shown on the figure: MPP – multipotent progenitor cells, LMPP – lymphoid-primed multipotent progenitors, CMP – common myeloid progenitors, CLP – common lymphoid progenitors, MEP – megakaryocyte-erythroid progenitors, GMP – granulocyte-macrophage progenitors.

**BOX 1**

Hematopoietic stem cell – adult multipotent stem cell that is able to self-renew and differentiate into all mature types of immune and hematopoietic cells. Functionally defined HSC – a cell that can support long-term robust multilineage repopulation of a host, whose own hematopoietic system was ablated (e.g. by irradiation). This definition is retrospective. Criteria for defining stemness differ from lab to lab and are based on either total donor cell presence (chimerism) in blood of transplant recipient, or chimerism in specific mature blood populations. The time-scale for defining long-term repopulation varies, but usually constitutes at least 4 months of activity after transplantation. A very common functional HSC definition is its ability to support production at least 1% of myeloid and lymphoid cells for four months after transplantation. Phenotypically defined HSC – hematopoietic cell expressing a panel of cell surface markers characteristic for HSCs. Phenotypic definitions of HSCs become more and more stringent over the years, coinciding with discoveries of novel markers. Marker combinations allowing to achieve isolation of highly enriched HSC populations include among others CD150^+^CD244^−^CD48^-^ (SLAM) (7), CD45mid Rho^-^SP (8), LSKCD34 CD150^-^ (37), lineage-Sca1^-^c-Kit^-^ (LSK) CD48^-^CD34^-^EPCR^-^CD150^-^ (9), CD45^-^EPCR^-^CD48^-^CD150^-^ (ESLAM) (78), SP LSK (34). CD45 is expressed on hematopoietic cells and allows to distinguish them from non-hematopoietic populations.

HSC clone – all progeny of a single HSC

Clone size – proportion of differentiated cells in blood generated by an HSC clone
stem cells. In contrast to studies where whole bone marrow cells were used, transplantsations of purified aged HSCs demonstrated a two-to-seven fold decrease in functional stem cell frequency in vivo (9,14,15,20). Moreover, compared to young, aged HSCs have been shown to exhaust more rapidly upon serial transplantation (21). Finally, the ability of HSCs to generate cells of all different lineages changes as a function of age and switches towards production of myeloid cells concurrent with decreased lymphopoiesis (14,16,22) (indicated in Figure 1).

NOT ALL STEM CELLS ARE EQUAL

Historically, it has been assumed that the HSC pool is homogeneous and all HSCs are uniform in their ability to produce cells of all lineages (as shown in Figure 1). However, this concept was challenged by clonal studies demonstrating profound heterogeneity among HSCs regarding three of their key properties: 1) repopulation potential; 2) cycling activity and 3) developmental program.

It was shown that individual HSCs display a marked diverse and unpredictable self-renewal capacity and repopulating activity upon transplantation into irradiated host mice (8,18). For instance, upon single-cell transplantation, purified cells can generate highly variable numbers of circulating white blood cells ranging from 1% to >80% chimerism (8,18).

Further, the HSCs are highly heterogeneous in regards to their cycling rate (23,24). While HSCs have a high proliferative capacity, their turnover is slow, what protects HSCs from accumulating mutations and prevents their exhaustion (25-27). Analysis of cell cycle in the primitive compartment has shown that 70% of phenotypically defined HSCs are found in quiescent G0 stage, opposed to under 10% of progenitor population (23). Multiple other studies supported this notion (23,28,29). Several reports suggest presence of at least two populations within the HSC pool – “homeostatic” HSCs that have a turnover time of approximately one month and “dormant” HSCs that are entering the cell cycle once in around 170 days (25, 24).

Moreover, a highly variable ability of HSCs to produce myeloid and lymphoid progeny is well documented. This was first reported in retroviral marking studies where different vector integration profiles were observed in myeloid and lymphoid tissues (30). Later, experiments in animals transplanted with limit-

Figure 2. Number of phenotypically defined HSCs increases with age. Here we show the gating strategy for isolation of LSK CD48+ CD150+ EPCR+ CD34+ cells (of young ~4 months old and 1 of ~25-months old animal) that have robust repopulating potential in vivo (9). Note that percentages of primitive cells are higher for an old mouse at all gating stages.
ing dilutions of bone marrow cells (31), and in single-cell transplant recipients (8) demonstrated the presence of at least three distinct developmental types of behavior of HSCs. These studies established that individual cells can preferentially support myeloid cell production (so-called α, or myeloid-biased HSCs), lymphoid cell development (γ or lymphoid—biased HSCs) or be equally prone to generate both lineages (β or balanced HSCs). High expression of CD150 has been demonstrated to correlate with a myeloid potential of HSCs (32). Lymphoid-biased HSCs have been shown to have a relatively short lifespan and to exhaust readily upon secondary transplantation (33). On the contrary, myeloid-biased HSCs had the longest lifespan and the highest self-renewal capacity of all HSC types (33).

Distinct gene expression profiles appear characteristic to HSCs with distinct developmental programs. CD150 (SLAM) and SP profiling in phenotypically isolated HSCs allowed their sub-fractionation into lymphoid-biased and myeloid-biased subsets (34) (Box 1). Serial transplantation experiments revealed that a lower SP<sub>LSK</sub> CD150<sup>high</sup> population preferentially contributed to LT-HSC, MPP and CMPs (Figure 1), while upper SP<sub>LSK</sub> CD150<sup>low</sup> cells produced significantly more CLPs. Micro-array analysis carried out on those two populations revealed that pathways involved in cell cycling and metabolism were highly activated in lymphoid-biased HSCs (34).

During aging, the proportion of myeloid-biased HSCs in the bone marrow increases at the expense of lymphoid biased and balanced HSCs (34-37). Such an expansion of myeloid-primed HSCs is likely to result from a combination of higher self-renewal potential (35), different response to cytokines, and lower metabolic activity (34) of this population. Moreover, while the pool of young HSCs is largely quiescent, a substantially higher fraction of aged HSCs is cycling (14,16,20,38) (Figure 1).

**CHANGES IN HOMING AND MIGRATION OF STEM CELLS UPON AGING**

The ability to engraft bone marrow and to migrate throughout the body are essential properties of HSCs that allow them to exit the fetal liver and repopulate bone marrow in the initial stages of development. Studies in 1960s demonstrated that a small number of repopulating cells is also present in the blood of mice in steady state conditions (39). These circulating cells are capable of reentering available HSC niches, as demonstrated by experiments with pairs of parabiotic mice that share their circulation (40) and by transplantation of bone marrow cells into non-irradiated recipients (41). Homing of cells to myeloablated or unmanipulated niches is a prerequisite in experimental or clinical bone marrow transplantations, where intravenous cell injection is sufficient for grafting and regeneration of hematopoietic system (42).

Several studies demonstrated that upon aging the engraftment and homing ability of HSCs decreases ~2-fold. Liang et al. demonstrated diminished short-term (24 hour) homing of old HSCs by limiting-dilution competitive transplantation of young and aged HSCs (22). Recently, our lab confirmed this observation by direct measurement of fluorescent cells in bone marrow after co-transplantation of HSCs isolated from old and young differentially colored transgenic donors (9).

On the contrary, levels of both mobilized progenitors and transplantable HSCs were ~ 5 fold higher in peripheral blood and spleen of granulocyte-colony stimulating factor (G-CSF) treated 25-month old mice compared to young 3-month old animals (43). Xing et al. proposed that lower adhesion of aged HSCs to stromal cells and increased activity of small Rho GTPases that are involved in regulation thereof play a role in this change (43).

Interestingly, data generated in our lab demonstrates that on a clonal level young and aged HSCs are similar in their migratory behavior (79). When purified HSCs were labeled with DNA barcodes and transplanted into irradiated hosts, barcoded clones of both young and old HSCs were unequally distributed across the skeleton. However, administration of G-CSF led to redistribution of HSC around the skeleton. At the same time, clonal analysis demonstrated that the pool of old HSCs was composed of multiple small clones, whereas the young HSC pool contained fewer, but larger clones (80).

The observed functional decline in aged HSCs is reflected in their transcriptome and epigenome, which will be discussed in the next section. A final issue that we will discuss is how HSCs reside in an hypoxic environment, and how oxidative metabolism, production of reactive oxygen species (ROS) and DNA repair pathways are linked to diminished self-renewal and proliferative abilities of old HSCs.
GENE EXPRESSION CHANGES

The increased number of HSCs in aged animals, accompanied by defects in their repopulating ability, as discussed above, suggests that changes in the transcriptional and epigenetic landscape occur with time. However, data describing the transcriptome of old HSCs are limited (15,44,45,81) and highly variable. Our understanding of the epigenome is even more restricted, with only 2 studies recently been published (46,81). In this section, we will discuss molecular mechanisms of HSC aging identified thus far.

To date, three groups have independently used microarray analysis to compare gene expression profile in young and aged HSCs. Categories of genes that were up-regulated with age in HSCs included those involved in NO-mediated signal transduction, stress response (protein folding), and the inflammatory response. Age-dependent repressed genes were enriched for those involved in the preservation of genomic integrity, e.g. chromatin remodeling and DNA repair (15,47). Surprisingly, while results of all studies indicate that hundreds of genes are differentially expressed, only 5 of those genes were overlapping among these reports. Of note, all of these studies used pooled samples from multiple mice to initiate the analysis. While differences in the panel of markers used for selection of HSCs (Box 1) could contribute to such discrepancies, it is also likely that the pronounced heterogeneity of individual aged mice that we discussed above, can explain these differences. In the future, analysis of individual mice will be instrumental for answering this question. Nevertheless, below we will further discuss molecular players that were indicated in those and other studies.

Genes that have been found to be involved in HSC aging thus far, include receptors, chromatin modifiers, transcription factors, genes involved in the DNA damage response and cell proliferation. Two of the highly up-regulated genes involve the surface adhesion molecules P-selectin and Inter Cellular Adhesion Molecule 1 (ICAM1) (Figure 3) (15,44). Increased levels of P-selectin and ICAM1 are implicated in a higher mobility of those cells and can interfere with HSCs engraftment in bone marrow, which is strictly dependent on adhesion. Of these genes, P-selectin has been shown to serve as a marker for physiological stress, including inflammation, and it is therefore interesting that it has now been shown to be activated upon aging. It mediates the leukocyte–vascular endothelium interaction involved in leukocyte extravasation, and therefore appears important for HSCs migration (48). At the same time, a decreased expression level of chromatin modifiers (SWI/SNF and PRC2 core members) implicates reorganization of the chromatin structure (44,46,49). Epigenetic perturbations may propagate skewing towards the myeloid lineage and be involved in anemia in aged individuals, because of hypermethylation of promoter regions of genes regulating lymphopoiesis and erythropoiesis (46).

Further, aged HSCs are likely to display a more euchromatic genome state, since genes encoding chromatin compaction tend to be down-regulated in old HSCs (besides the above-mentioned SWI/SNF remodelers, expression of related chromatin remodeling genes such as Smarca-4 and Smarca-1, as well as histone deacetylases Hdac-1, -5, and -6 and a DNA methyltransferase Dnmt3b is decreased) (44).

Moreover, recent RNA-Seq-based report on epigenomic state of old HSCs documents an increased occurrence of the transcription activating mark-H3K4me3 across self-renewal genes, decreased methylation of HSC maintenance genes combined with hypermethylation of gene promoters associated with differentiation, reflecting phenotypical HSC ageing (81). Interestingly ribosomal biogenesis seems to be a particular target of aging with hypomethylation of rRNA genes.

Figure 3. Molecular mechanisms of murine HSC aging. The figure summarizes molecules/pathways reported to be changed in aged HSCs. Increased expression is shown with red arrows, and decreased expression with blue. Changes in cellular localization of Cdc42 are depicted: while in young cells it is polarly distributed, in old HSC polarity is lost (77).
The expression of P-selectin, one of the few proteins to be consistently up-regulated in aged HSCs, is regulated by NF-κB (the p65 isoform). In HSCs from aged mice it has been observed that p65 accumulates in the nucleus, indicating transcriptional activation. Increased abundance of this protein in the nucleus of aged HSC may induce up-regulation of Toll-like receptor 4 (TLR-4), as well as higher levels of proinflammatory molecules in the plasma of aged individuals (50,51). Such higher levels of proinflammatory cytokines (TNFα, IL-1β), together with increased expression of inflammation-related genes, support the concept of inflammation playing a significant role in the aging process (44).

Two other genes that have often been associated with the aging process are p16<sup>Ink4a</sup> and p19<sup>Arf</sup>, which are tumor suppressors that induce loss of HSCs without inducing apoptosis (52). Furthermore, p16<sup>Ink4a</sup> has been proposed as a biomarker of aging as its expression increases with age in mammalian tissues, including murine bone marrow (53). The presence of p16<sup>Ink4a</sup> restricts the number of HSCs, and affects their serial repopulation capacity, cell cycling and apoptosis (54). However, a causal role for p16<sup>Ink4a</sup> in the aging process is still under discussion since an effective repression of this gene by H3K27me3 mark in young and old HSCs was recently reported (81). Moreover, in the study from Attema et al., no increased levels of p16<sup>Ink4a</sup> transcripts in individual aged HSCs were found (55). The expression of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> is mediated by the activity of Polycomb Repressive Complex 2 (PRC2). A recent study showed up-regulation of PRC2 core component in aged HSCs, suggesting in fact increased repression of this locus upon aging (49,56). This is in line with findings that have shown hypermethylation of loci targeted by PRC2 during aging (46).

Aside from the possible, if not likely, contribution of epigenetic mechanisms underlying stem cell aging, there are also many studies that indicate a causal role of genetic and genomic alterations in the aging process. A decline in the expression of genes involved in DNA repair, resulting in an increased rate of non-repaired DNA damage, is thought to be a driving factor in aging. The role of these pathways is described in the next section.

**THE ROLE OF OXIDATIVE METABOLISM AND DNA REPAIR IN HSC AGING**

HSCs reside in the bone marrow and are supposed to replenish blood cell production for the entire lifetime of the organism. Both cell-extrinsic localization and cell-intrinsic DNA damage repair mechanisms allow HSCs to maintain their functionality. HSCs appear not be lodging randomly in the bone marrow, but in fact preferentially reside in a low oxygen environment which: 1) reduces molecular damage that results from oxidation by ROS, 2) determines their energy status, 3) induces pathways that are active under hypoxic conditions and 4) decreases the rate by which DNA mutations accumulate. Apart from preventing damage by avoiding exposure to oxygen, HSCs implement efficient mechanisms of DNA repair to remove deleterious mutations and prevent these to propagate to their progeny. To accomplish this ability, HSCs can employ two independent pathways, depending on the type of the DNA damage and their cell cycle status. We will discuss these parameters in more detail in this section.

One of the first concepts suggesting a link between ROS and the aging process is the ‘free radical theory of aging’, proposed by Harman in 1950’s (57). This theory argues that the effect of ROS on cellular components (proteins, nucleic acids and lipids) may be key in determining the lifespan of the organism. Thus, if we consider that the lifespan of the organism is at least partially determined by the activity of stem cells to maintain tissue homeostasis, effective neutralization of ROS may be crucial for maintaining the stem cell pool. Indeed, several recent studies have demonstrated a role of ROS, and how managing ROS levels and, more general, metabolism affect proper HSC functioning.

The redox potential in HSCs is strictly regulated by intrinsic and extrinsic factors that will be described later in detail. The average O<sub>2</sub> concentration in BM is approximately three times lower compared to blood (58,59). This suggests that HSCs are residing in an environment with limited availability of oxygen, which propagates glycolytic metabolism (60). However the content of mitochondria in HSC is relatively high compared to myeloid cells which suggest that they are less active (56).

Although the net production from 2 glucose molecules is only 2 ATP (versus 36 – 38 ATPs during mitochondrial oxidation), this metabolic landscape
is compatible with the low energy requirements of HSC (61) and significantly reduces the rate of ROS production. Whether ROS levels in HSCs are mainly determined by the niche location or by the stem cell-intrinsic activation status remains to be further investigated.

Several studies have shown that ROS levels increase with proliferation and during early steps of lineage commitment (60,62). These experiments have combined regular FACS staining for HSCs with DCF-DA, a probe enabling the measurement of ROS activity in viable cells. Using this approach allowed the identification of two populations of ROS\textsuperscript{low} and ROS\textsuperscript{high} stem cells. The population of ROS\textsuperscript{high} stem cells showed functional decline upon serial transplantation and a typical pattern of differentiation skewing towards the myeloid lineage. This clearly mimics characteristics of aged HSCs. Moreover, these ROS\textsuperscript{high} cells express activated p38 kinase and components of the mTOR pathway, both involved in stress response. Treatment of ROS\textsuperscript{high} HSCs with inhibitors of both of these pathways was able to restore functional activity (62). The comparison of the ratio of ROS\textsuperscript{low} versus ROS\textsuperscript{high} populations in aged mice showed a strong decline of cells expressing low levels of ROS, and only a moderate increase in the ROS\textsuperscript{high} population. Therefore, a decrease in ROS\textsuperscript{low} stem cells may be responsible for the skewing of myeloid differentiation and the impaired repopulating activity (62).

The major impact of oxidation on HSCs discussed above seems to be reflected also by the presence of O\textsubscript{2} in the hematopoietic niche. Decreased oxygen availability activates Hypoxia Inducible Factor (HIF)-signaling, which regulates the redox potential in HSCs. The HIFs family of transcription factors comprises 4 proteins, among which HIF-1\(\alpha\) is the best studied. HIF-1\(\alpha\) levels are controlled by products of the Ink4a gene locus, and appears to play a protective role against senescence-induced HSCs exhaustion. Bone marrow cells deficient of HIF-1\(\alpha\) showed increased ROS levels (63). Similar phenotype with HSC exhaustion and increase in ROS content was observed during deletion of subfamily of transcription factors-Forkhead O (FoxOs 1,2,3,4) (64), which regulate the expression of genes involved in metabolism and oxidative stress (65). However the treatment of those cells with antioxidants partially rescued defects in HSCs deficient for these proteins (64). Thus, induction of cellular senescence may originate from ineffective ROS management, which on its turn may lead to DNA damage. Indeed, a recent study provides evidence directly linking cellular senescence with DNA damage (66).

In line with a detrimental influence of ROS on HSCs, it has been shown that endogenous DNA damage accumulates with age in normal stem cells. Multiple foci of phosphorylated \(\gamma\)-H2AX, a marker of DNA damage, were found in over 70% of old HSCs (15). However, the percentage of \(\gamma\)-H2AX positive cells decreases as the cell differentiated to more committed progenitors. The alternative explanation would be that \(\gamma\)-H2AX is a marker for impaired replication, and associated with stalled or collapsed replication forks. This hypothesis is in line with reduced expression of MDM helicase family genes that decline with age. Importantly, downregulation of members of this family in young HSCs led to their impaired engraftment-a feature characteristic of old HSCs (82). Furthermore, cellular mislocalization of PP4c (\(\gamma\)-H2AX phosphatase) in aged HSCs, leads to delayed and ineffective dephosphorylation and accumulation of \(\gamma\)-H2AX (82). This indicates that with age, DNA damage accumulates preferentially in stem cells, while this is either repaired more quickly in proliferating progenitor cells, or DNA damage-containing progenitors are eliminated. This also reiterates the notion of cell type-dependent efficiency of DNA damage repair. In the next paragraph we will further discuss how mechanisms for DNA repair are implicated in HSC aging.

To repair DNA damage, cells utilize a repair machinery that is either DNA template-dependent or template-independent. So far, it has been shown that several template-dependent mechanisms of DNA damage repair are active in HSCs. All of these are negatively affected by aging. Such repair mechanisms include non-homologous end joining (NHEJ) which occurs during G0/G1 stages of cell cycle and nucleotide-excision repair (NER) utilized to remove modified nucleotides. The effect of aging on these processes have been assessed using mutant mouse models with defects in these pathways (67). Both XPD\(^{TTD}\) mice, with deficiency of NER, and Ku80\(^{-}\)animals, where NHEJ is affected, generated numbers of old HSCs comparable to normal mice, but their functionality was severely affected upon serial transplantation (68,69).

Interestingly, a similar phenotype was observed in mTR\(^{+}\) mice, a mouse strain deficient for telomerase. Telomere shortening affected the intrinsic ability of HSCs to self-renew (68) and also limited the capacity of niche cells to support
HSC homing (70). The decline of HSCs activity in the absence of telomerase could be reverted by down-regulation of the DNA damage checkpoint – Basic leucine zipper Transcriptional Factor (BATF), which simultaneously resulted in accumulation of DNA damage in HSCs (71).

In the case of double stranded DNA breaks, template-independent DNA repair takes place. This requires the activity of the protein kinase Ataxia Telangiectasia Mutated (ATM). The analysis of ATM-/- HSCs revealed a lack of long-term repopulating capacity (72). Moreover, on the molecular level, increased ROS levels (much higher than those observed in normal aged HSCs) contributed to up regulation of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> in those cells (52,73). Malfunctioning of ATM-/- HSCs could be rescued by the long-term treatment of mice with the anti-oxidative molecule N-acetyl cysteine (NAC). This highlights the anti-aging properties of ROS quenching antioxidants. Moreover, increased cellular ROS levels activate the p38 MAPK pathway (62,72), which inhibits quiescence state in Atm-/- HSCs (72).

A schematic overview of the molecular events implicated in HSC aging is provided in Figure 3. To summarize, adult HSCs accumulate DNA damage under physiological conditions in vivo due to a plethora of intrinsic and extrinsic factors. The mechanisms that we describe above are relevant for neutralizing DNA damage in order to maintain the pool of HSCs; dysfunctioning of any of those pathways will inevitably cause HSCs exhaustion.

The functional and molecular changes that occur during aging in individual HSCs, impacts on the behavior of the entire hematopoietic stem cell pool, i.e. on how stem cells act in concert during the aging process. In the next section we will briefly discuss various currently prevailing stem cell aging models.

MODELS OF HSC AGING

Originally it was considered that the age-related decline in functioning of a population of HSCs was caused by cellular aging of individual stem cells and involved a homogeneous HSC population (68,69). Such aging model was dubbed the “population shift model”. This model presumes that the HSC population is uniform and all cells gradually accumulate damage, lose their ability to produce lymphoid cells and to engraft bone marrow (74). Later, the identification of distinct developmental programs of individual HSCs and observation of changes in the representation of these HSC subsets with age, led to establishment of an alternative hypothesis. This “clonal selection model” implied that changes in the clonal make-up of the HSC pool, rather than changes in individual HSCs underlie the hematopoietic aging (36,74). This theory also assumes that individual HSCs do not change with age (74). The most recent conceptual view on aging combines both models: a changing pool of HSCs is simultaneously affected by cellular aging (9,32).

PERSPECTIVES

Several other mechanisms that may contribute to functional hematopoietic decline are currently being investigated. These include factors such as metabolism, epigenetic changes, polarity, the regulation of post-transcriptional gene expression and hematopoietic microenvironment.

Metabolism. A recent paper reports an increased activation of the mTOR pathway in HSCs and progenitors from aged mice (75). Administration of rapamycin (the inhibitor of mTOR pathway) limited the age-related expansion of the HSC pool, and also improved their functionality so that these aged HSCs became as effective as young HSCs (75). It has also been shown that autophagy is increased in old HSCs and ensures their survival (83). Additionally, loss of expression of the nutrient sensing protein SIRT7 with age, has been associated with increased HSC proliferation and skewing of hematopoiesis towards production of myeloid cells (84).

Non-coding RNAs. Furthermore non-coding RNAs seem to play a pivotal role in the aging process. In the study from Boon et al., authors studied the role of microRNAs (miRNA) in aged cardiomyocytes. MiR-34a, by suppressing the expression of its targets, leads to inhibition of telomere erosion, DNA damage response and apoptosis (76). It leaves the ground for speculation, that also during HSC aging certain miRNAs play an important role in determining their functionality and moreover specifying the cellular program to preferentially differentiate into certain lineages. No data on miRNA functioning in aged HSCs are currently available. However, a recent report documents the importance and the potential of long non-coding (Lnc-RNAs) in regulating self-renewal
and lineage-commitment in old HSCs (Luo et al., 2015). Two lnc-RNAs (referred to as LncHSC-1 and LncHSC-2) affect lineage commitment. Whereas downregulation of LncHSC-1 led to myeloid skewing at the expense of B cells, LncHSC-2 promoted T lymphopoiesis (85).

**Cell polarity.** Recent studies have demonstrated that polar localization of the small Rho-GTPase Cdc42 is characteristic for young HSC (but not more committed cells) and this polarity is lost with age due to increased activity of Cdc42.(77). The shift from canonical to noncanonical Wnt signaling has been associated with apolar Cdc42 cellular localization (86). Remarkably, administration of a chemical compound (a CASIN inhibitor), allowed restoration of both polarity and functionality of old cells, and resulted in their rejuvenation (77). Similarly reduced non-canonical Wnt5a signaling in old HSCs rejuvenates chronologically aged HSCs and restores polar localization of Cdc42 (86). The prospect that we may indeed be able to rejuvenate aged stem cells is of considerable interest for future clinical interventions.

**Microenvironment.** Finally, while this chapter focuses on cell-intrinsic mechanisms of HSC aging, recent findings indicate the potential contribution of the bone marrow microenvironment, or “niche”, to HSC aging phenotype. To support this notion, the level of inflammatory cytokine CCL5 that is shown to induce myelopoiesis at expense of lymphopoiesis, was shown to increase upon aging (87). It was also demonstrated that localization of hematopoietic progenitors in old bone changes further from endosteum and is associated with reduced cell polarity (88). A recent report also highlights the role of p19INK4d in niche remodeling upon aging (89). The authors argue that loss of p19INK4d in microenvironment with age results in expansion of myeloid-biased HSCs, possibly through changes of expressed levels of TGF-β1 by the niche cells (89). Therefore, it is likely that HSC aging is a complex interplay between changes in the HSC pool itself and the aging environment they reside in.

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