Chapter 3

Stem Cell Aging

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Additional information is available at the end of the chapter

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Abstract

Stem cells persist throughout life, replacing cells lost to homeostatic turnover, injury, and disease. However, their functions decline with age, which contributes to degeneration and dysfunction. The molecular mechanisms involved in the aging of stem cells are the same as the ones involved in the aging of somatic cells, including telomere shortening, oxidative stress, epigenetic dysregulation, miRNAs changes, alterations of DNA, RNA, proteome, and various cellular organelles. Aging impacts various pathways, such as insulin/insulin-like growth factor 1 (IGF-1), mTOR, FoxO, AMP-activated protein kinase (AMPK), sirtuin, and many others, resulting in senescent stem cells that exhibit functional and numerical impairment. Stem cells have developed special mechanisms to prevent age related damage accumulation and to sustain their stemness properties, however, these mechanisms lose their effectiveness over time. The most fatal consequence of this is found in the immune system, where both innate and adaptive immunity are affected, exhibiting a plethora of defects, including increased autoimmune disease occurrence, elevated tolerance to cancer and chronic inflammatory status. Stem cell therapies call for the best quality of stem cells grafts. Stem cell products should be devoid of cells containing a senescent phenotype, thus a comprehensive knowledge of the biology behind the senescence of stem cells should be taken into account in every cell based therapy.

Keywords: molecular mechanisms of aging, senescence, stem cell niche, epigenetic changes, telomere attrition, stem cell pool, mitochondrial changes, proteostasis, immune deterioration, shortened life span

1. Introduction – Stem cells aid regeneration and longevity

An 70 kg adult human body consists of approximately 3.72×10^{13} cells [1]. These trillions of cells are not permanent and a majority of them are constantly renewed throughout our lifetime, although some of them – such as cells in the lenses of our eyes and some of the neurons

of our central nervous system – are thought to be an exception. The frequency of renewal depends on the function of the cells and may vary from several hours to several years. A collection of the replacement rates of different cells in our body is given in **Table 1**.

The renewal of adult tissues is enabled by specialized cells that function over the lifetime of an organism, i.e., the stem cells (SCs). They persist throughout life in numerous mammalian

Cell type	Turnover time
Small intestine epithelium	2–4 days
Stomach	2–9 days
Blood neutrophils	1-5 days
White blood cells eosinophils	2-5 days
Gastrointestinal colon crypt cells	3–4 days
Cervix	6 days
Lungs alveoli	8 days
Tongue taste buds (rat)	10 days
Platelets	10 days
Bone osteoclasts	2 weeks
Intestine Paneth cells	20 days
Skin epidermis cells	10–30 days
Pancreas beta cells (rat)	20–50 days
Blood B cells	4–7 weeks
Trachea	1–2 months
Hematopoietic stem cells	2 months
Sperm (male gametes)	2 months
Bone osteoblasts	3 months
Red blood cells	4 months
Liver hepatocyte cells	0.5–1 year
Fat cells	8 years
Cardiomyocytes	0.5–10% per year
Central nervous system	life time
Skeleton	10% per year
Lens cells	life time
Oocytes (female gametes)	life time

Adapted from: http://bionumbers.hms.harvard.edu/bionumber.aspx?&id=107875

Table 1. Cell renewal rates in different tissues of the human body.

tissues, replacing cells lost to homeostatic turnover, injury, and disease. Stem cells reside in specific anatomic reservoirs, such as bone marrow, and circulate in the organism when needed. SCs represent a very small proportion in adult tissues. It is estimated that the bone marrow of a 70 kg adult human contains around 1.5–1.7 × 10^{12} cells, among them only 45–120 × 10^{6} are true hematopoietic stem cells (HSCs) that give rise to more frequent progenitors (Jazbec et al. 2017, submitted). The frequencies of stem cells in other tissues are even lower and still a matter of debate.

Adult SCs can typically self-renew and differentiate into multiple cell types within a developing and adult body. Due to their self-renewal capacity they were regarded as immortal reservoirs of youth, however, they are nonetheless susceptible to the age related damages. To prevent or reverse the accumulation of age related damage and epigenetic changes, SCs developed special mechanisms to maintain long telomeres, enhance proteostasis, avoid ROS production and defend against toxic substances. In spite of that, their functions decline with age in a number of tissues, including blood, forebrain, skeletal muscle, skin and all the other tissues as reviewed by Schultz et al. [2].

Declines in stem cell functions not only contribute to degeneration and dysfunction of aging tissues, but also negatively affect the life span of the organism [3, 4]. Some strong evidence for SCs as regulators of longevity comes from animal studies. For instance, if in *C. elegans* germline stem cells (GSCs) are eliminated, this almost doubles its lifespan [5] and such a phenomenon is highly conserved [6]. Similarly, if the fruit flies are modified with overexpression of a PGC-1α homolog or a heat-shock response transcription factor and moderate repression of insulin/IGF or JNK signaling, this directly extends their life span [7], implying that improved stem cell function leads to better tissue function, and that stem cell aging underlies the aging of tissues and organs.

In humans, there is considerable evidence supporting the fact that young stem cells perform better than old ones. Proof of this concept is best documented is the recent multicenter study on the success of hematopoietic stem cell transplantation, which is currently the most popular and efficient cell therapy for malignant diseases. In more than 6000 cases of allogeneic bone marrow transplantation between 2007 and 2011, it was clearly shown that patient survival was significantly better after grafts from young donors (aged 18–32 years) were used. For every 10-year increment in donor age, there was a 5.5% increase in the hazard ratio for overall mortality [8]. This is probably one of the most important findings in this field, suggesting that for regenerative purposes, and other stem cell therapies, grafted stem cells should be young and devoid of senescent defects.

2. The biology of stem cell aging

Adult stem cells express several characteristic features that are specific to stem cells, as well as certain features that are found in any other somatic cell in the body. They express telomerase – an enzyme required for telomere extension that is essential for repeated self-renewal, they cycle between phases of quiescence and activation needed for the production

of progeny, their chromatin exists in a bivalent state primed for self-renewal or differentiation, they have unique metabolic requirements, they distribute their macromolecules asymmetrically during asymmetric cell divisions, and they reside in niches that regulate their behavior [9].

The molecular mechanisms that are involved in the aging of adult stem cells are the same as the ones involved in the aging of the somatic cells. Traits and mechanisms that are affected by aging are present in various populations of stem cells. The age-related decline of stem cells is mainly functional, but in some cases, a decline in stem cell numbers can also be observed. Since many of these mechanisms appear simultaneously, it is practically impossible to trace or determine a single initial damaging agent that causes the cascade of other detrimental sequences. Therefore authors agree that aging is probably the result of multifactorial derangements caused by several causative factors that act in parallel, including the formation of damaging reactive oxygen species (ROS), telomere attrition, DNA damage and mutations, epigenetic changes (alterations of histones, DNA and the consequent dysregulation of gene expression), mitochondrial DNA mutations with mitochondrial decline, changes of microRNAs, ribosomal changes and defects of RNA splicing, changes of proteostasis, changes in cellular polarity, changes in nutrient sensing and metabolism, niche deterioration, improper accumulation of various circulating factors, stem cell pool exhaustion, cellular senescence with cell cycle arrest, and altered intercellular communication (**Table 2**).

1. Formation of damaging reactive oxygen species (ROS)
2. Mitochondrial DNA mutations, decline of mitochondrial integrity and biogenesis
3. Nuclear damage and nuclear DNA mutations
4. Telomere shortening /attrition
5. Epigenetic changes/alterations of histones and DNA and consequent dysregulation of gene expression
6. Changes of microRNAs
7. Changes of RNA splicing and ribosomal machinery
8. Changes of proteostasis
9. Changes of cell polarity
10. Metabolism and nutrient sensing
11. Niche deterioration
12. Accumulation of various circulating factors
13. Stem cell pool exhaustion
14. Cellular senescence – arrest of the cell cycle
15. Altered intercellular communication

Table 2. Multifactorial causes of stem cell aging.

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2.1. Formation of damaging reactive oxygen species (ROS) and oxidative stress

The free radical theory of aging has been long accepted as the most plausible explanation for the aging process. It was first formulated in the 1950s by Harman who hypothesized that an accumulation of endogenous oxygen radicals (reactive oxygen species, or ROS) occurs, which in turn causes further mitochondrial deterioration and the global cellular damage responsible for the aging and death of all living beings [10]. This theory was then revised in 1972 when mitochondria were identified as being responsible for the initiation of most of the free radical reactions [11]. It was also postulated that life span was determined by the rate of free radical damage to the mitochondria. Mitochondrial respiration, the basis of energy production in all eukaryotes, generates ROS by leaking intermediates from the electron transport chain [12]. In all aerobic organisms, age-related oxidative stress is generated either by exposure to endogenous metabolites or exogenous sources such as radiation (UV, X-ray), and ROS accumulation is the result of an imbalance between free radical production and antioxidant defenses, such as superoxide dismutase that is responsible for scavenging superoxide anions [12, 13]. In fact, oxidative modifications have been shown to occur in DNA, protein, and lipid molecules [14].

Whereas young stem cells contain a spectrum of antioxidant mechanisms, aged stem cells display an inadequate anti-oxidant defense that is associated with functional impairment, including decreased responsiveness to physical environmental cues and decreased resistance to oxidative stress [15]. In several studies, aging stem cells from bone marrow and adipose tissue showed a significantly reduced capacity for coping with oxidative stress with increasing donor age [16, 17]. Therefore, oxidative stress is still recognized as the fundamental underlying component of the aging process, leading to dysregulation of various cellular pathways and the subsequent accumulation of toxic aggregates and cellular debris, and ultimately to the activation of cell death/survival pathways leading to apoptosis, necrosis, or autophagy, as reviewed by Haines, et al. [18].

However, recent developments have forced an intense re-evaluation of the mitochondrial free radical theory of aging after the unexpected observation that increased ROS may paradoxically prolong the lifespan of yeast and *Caenorhabditis elegans* [19–21]. In mice, genetic manipulations, which increased mitochondrial ROS and oxidative damage, did not accelerate aging as one would expect [22, 23]. Furthermore, manipulations that increased antioxidant defenses did not extend longevity [24], and lastly, genetic manipulations that impaired mitochondrial function but did not increase ROS, accelerated aging [25, 26]. There has also been other solid evidence that in response to physiological signals and stress conditions, ROS triggered proliferative and survival signals [27].

The mitochondrial theory of aging has also been challenged as it has become clear that there exists a rather complicated interplay between various other cellular compartments [28]. Dysfunctional mitochondria can contribute to aging independently of ROS, as demonstrated by studies with mice deficient in DNA polymerase γ [29, 30]. This could happen through a number of mechanisms, for example, mitochondrial deficiencies may affect apoptotic signaling by increasing the propensity of cell's death through mitochondrial membrane

permeabilization in response to stress [31], and trigger inflammatory reactions by favoring ROS-mediated and/or permeabilization- facilitated activation of inflammasomes [32]. Also, mitochondrial dysfunction may directly impact cellular signaling and interorganellar cross-talk, by affecting mitochondrion-associated membranes that constitute an interface between the outer mitochondrial membrane and the endoplasmic reticulum [33].

The mitochondrial ROS that were considered the main cause of age related defects actually contribute positively to various signaling pathways and normal cellular responses, such as adaptation to hypoxia, cellular differentiation, autophagy, inflammation, and immune responses, as reviewed recently [28, 34], meaning that ROS are also beneficial for cellular biology.

2.2. Mitochondrial DNA mutations, the decline of mitochondrial integrity and biogenesis

Mitochondrial function has a profound impact on the aging process. Mitochondrial dysfunction can accelerate aging in mammals. It was generally believed that age-related pathology was caused by defects of mitochondria related to oxidative stress, leading to the accumulation of irreparable changes of nucleic acids, proteins, and lipid molecules [14, 35]. But there are also other defects of mitochondria that develop during normal aging. Similar to the nuclear DNA, mitochondrial DNA (mtDNA) is exposed to mutations and deletions in aged cells, which are not found in nuclear DNA, and which also contribute to aging [36]. This is aggravated by the oxidative microenvironment of the mitochondria and the limited efficiency of the mtDNA repair mechanisms [37].

The mutations that can lead to mitochondrial dysfunction and death are now detectable in generated induced pluripotent stem cell (iPSC) lines, i.e., expanded clones from each individual skin or blood cell. As a result, every cell in the iPSC line contains the same mitochondrial DNA (mtDNA) mutations as the original adult cell, and can for this reason be easily sequenced. We now know that to ensure healthy mitochondrial genes, we must screen stem cells for mutations or collect them at a younger age. This may help illuminate the role of mutated mitochondria in degenerative diseases and to assess the patient-derived regenerative products destined for clinical applications [38].

Interestingly, most mtDNA mutations in adult or aged cells appear to be caused by replication errors early in life, rather than by oxidative damage. These mutations may undergo polyclonal expansion and cause respiratory chain dysfunction in different tissues [39]. Studies of accelerated aging in HIV-infected patients treated with anti-retroviral drugs, which interfere with mtDNA replication, have supported the concept of clonal expansion of mtDNA mutations that originated early in life [40].

Aging also affects the biogenesis of mitochondria. Mitochondrial biogenesis is the process by which cells increase their individual mitochondrial mass and copy their number to increase the production of ATP, as a response to greater energy needs. With aging, the reduced efficiency of mitochondrial bioenergetics may be a result of multiple converging mechanisms,

including reduced biogenesis of mitochondria. For instance, in telomerase-deficient mice, it can be a consequence of telomere attrition with subsequent p53-mediated repression of PGC-1α and PGC-1β (peroxisome proliferator-activated receptor gamma coactivator 1 – alpha and –beta, which are the master regulators of mitochondrial biogenesis) [41]. This mitochondrial decline also occurs during physiological aging in wild-type mice and can be partially reversed by telomerase activation [42]. Sirtuin 1 (SIRT1) modulates mitochondrial biogenesis through a process involving the transcriptional co-activator PGC-1α [43] and the removal of damaged mitochondria by autophagy [44]. SIRT3, which is the main mitochondrial deacetylase [45], targets many enzymes involved in energy metabolism, including components of the respiratory chain, tricarboxylic acid cycle, ketogenesis and fatty acid β-oxidation pathways [46]. SIRT3 may also directly control the rate of ROS production by deacetylating manganese superoxide dismutase, a major mitochondrial antioxidant enzyme [47]. Collectively, these results support the idea that sirtuins may act as metabolic sensors to control mitochondrial function and play a protective role against age-associated diseases [48, 49].

Interestingly, endurance training and alternate-day-fasting may improve healthspan through the capacity to avoid mitochondrial degeneration [50, 51]. It is tempting to speculate that these beneficial effects are mediated, at least in part, through the induction of autophagy, for which both endurance training and fasting constitute potent triggers [52]. However, autophagy induction is probably not the sole mechanism through which a healthy lifestyle can retard aging, since, depending on the precise diet reduction regime, additional longevity pathways can be activated [53].

The combination of increased damage and reduced turnover in mitochondria, due to lower biogenesis and reduced clearance, may contribute to the aging process [48]. Some other mechanisms can also affect the mitochondrial bioenergetics and contribute to the aging mitochondrial phenotype, among them the mutations and deletions in mtDNA, oxidation of mitochondrial proteins, destabilization of the macromolecular organization of respiratory chain, defects of the lipid membranes, and defective autophagy that targets deficient mitochondria [54].

In conclusion we could say that the importance of mitochondria in the basic biology of aging and the pathogenesis of age-associated diseases is stronger than ever, although the emphasis has moved from ROS to other causative aspects. Obviously, besides the mitochondrial dysfunction due to ROS, there exists a complex interplay of several other factors of aging, such as mDNA mutations, changes of lysosome processing, endoplasmic reticulum stress, genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, altered intercellular communication, mitochondrial biogenesis and turnover, energy sensing, apoptosis, senescence, and calcium dynamics. Mitochondria do play one of the key roles in the pathophysiology of aging and events that lead to the aged phenotype, therefore they will increasingly be targeted to prevent and treat chronic diseases and to promote healthy aging [48, 55, 56]. We expect that future studies will determine whether genetic manipulations that decrease the load of mtDNA mutations and other damaging factors, are able to extend lifespan.

2.3. Nuclear damage and nuclear DNA mutations

It is clear that in aged humans and model organisms, somatic mutations accumulate over time within all cells [57]. Other forms of DNA damage, such as chromosomal aneuploidies, copy-number variations and increased clonal mosaicism for large chromosomal anomalies have also been found to be associated with aging [58, 59]. Each time a stem cell replicates its DNA and divides, the likelihood of DNA defects and oncogenic transformations increases. Therefore the lifetime risk of cancer development in a tissue correlates with the number of divisions the stem cells of this particular tissue have undergone [60]. A variety of these DNA alterations can finally affect the essential genes that control the key transcriptional pathways. Such defect cells should be normally eliminated by apoptosis or senescence, however, if this does not happen it may jeopardize tissue and organismal homeostasis. This is especially important in stem cells because the DNA damage has a detrimental impact on their functional competence, i.e., on their role in tissue renewal [61, 62].

An accumulation in DNA damage and mutations leading to stem cell aging has been one of the earliest theories of aging [63]. DNA damage can be caused by external factors (ionizing radiation, ultraviolet radiation or environmental toxins), or by internal factors (ROS and errors in DNA replication). These factors can lead to various DNA lesions such as modifications of bases or sugar residues, the formation of DNA adducts, cross-linking of the DNA strands or the appearance of single and double-strand breaks. Among these lesions, DNA double-strand breaks (DSBs) are particularly lethal because they result in physical cleavage of the DNA backbone. DSBs can occur through replication fork collapse, during the processing of interstrand crosslinks, or following exposure to ionizing radiation [64, 65]. In spite of the fact that cells have evolved at least six different DNA repair pathways to deal with these distinct types of DNA damage [66], there is convincing evidence that with aging, stem cell DNA is also subject to damage. In HSCs, histone H2AX phosphorylation and comet tails, both of which are measures of DNA damage, increase with age [67, 68]. Phosphorylation of *H2AX*, one of several genes coding for histone H2A (one of the five main histone proteins involved in the structure of chromatin in eukaryotic cells), accumulates with age in satellite cells, i.e., stem cells of the muscles [69]. Moreover, aged HSCs display a history of replication stress and decreased expression of DNA helicases, further sensitizing them to future replication challenges [70].

Since mutations are a common daily occurrence, our cells could not survive without DNA repair mechanisms. There are two groups of repairing mechanisms, the first acting to repair DNA single-strand breaks (mismatch repair mechanism, base excision repair mechanism, nucleotide excision mechanism), and the second acting to synchronously repair DNA double-strand breaks, i.e. homologous recombination and non-homologous end joining (NHEJ). In spite of the repair mechanisms our DNA accumulates mutations, since the genes of repair mechanisms are themselves subject to mutations [71].

Evidence that DNA damage plays a causal role in the aging process includes the observation that mice with defects in DNA damage repair display some aspects of premature aging [72], whereas enhancing DNA repair through increased expression of *SIRT6* increases lifespan [73]. In some situations, DNA damage may also reduce stem cell numbers by causing them

to undergo apoptosis, senescence or differentiation, although it is not yet confirmed whether these effects are due to an increase in stem cell longevity [2].

It is also known that deficiencies in DNA repair mechanisms cause accelerated aging in mice and underlie several human progeroid syndromes such as Werner syndrome, Bloom syndrome, xeroderma pigmentosum, trichothiodystrophy, Cockayne syndrome, or Seckel syndrome [74–76]. Moreover, transgenic mice overexpressing multidomain protein kinase BUBR1 (budding uninhibited by benzimidazole-related 1), a mitotic checkpoint component that ensures the accurate segregation of chromosomes, exhibit an increased protection against aneuploidy and cancer, and display an extended healthy lifespan [77]. These experimental data prove that artificial reinforcement of nuclear DNA repair mechanisms could delay aging [48].

In addition to genomic damage affecting nuclear or mitochondrial DNA, there is evidence that certain defects in the nuclear lamina can also change nuclear architecture and thereby cause genomic instability [78]. Nuclear lamins participate in genome maintenance by providing a scaffold for tethering chromatin and protein complexes that regulate genomic stability [79, 80]. Mutations in genes encoding protein components of this structure, or factors affecting their maturation and dynamics, cause accelerated aging syndromes such as the Hutchinson-Gilford and the Néstor-Guillermo progeria syndromes [81–83]. Alterations of the nuclear lamina and production of an aberrant prelamin A isoform called progerin have also been detected during normal human aging [84]. Since telomere dysfunction also promotes progerin production in normal human fibroblasts upon prolonged *in vitro* culture, this suggests that there exist intimate links between telomere maintenance and progerin expression during normal aging [85]. In addition to these age-associated changes in A-type lamins, lamin B1 levels decline during cell senescence, pointing to its utility as a biomarker of this process [86].

2.4. Telomere shortening

Although accumulation of DNA damage affects the genome near-to-randomly, there are some chromosomal regions that are particularly susceptible to age-related deterioration [87]. Telomeres are repetitive TTAGGG sequences and associated nucleoproteins at the ends of a chromosome that play a critical role in protecting chromosomes from degradation, undesirable recombination, and chromosome fusion [88, 89]. With each somatic cell division, telomeres shorten and this exposes cells to the aging phenotype. Due to inability of the normal DNA replication machinery to completely replicate the telomeric sequences, telomeres in somatic cells shorten with each cell division, and are thus markers for cellular aging and replicative capacity [90].

Mice with shortened or lengthened telomeres exhibit decreased or increased lifespans, respectively [91–93]. Telomere shortening is observed during normal aging both in humans and mice [94]. In humans, recent meta-analyses have indicated a strong relation between short telomeres and mortality risk, particularly at younger ages [95].

In contrast to somatic cells, embryonic and adult stem cells express telomerase, a reverse transcriptase enzyme, which catalyzes the extension of telomeric sequences, thereby avoiding telomere attrition and prolonging cellular proliferative life span. While the telomerases are normally absent from most somatic cells, they are active in the stem cells and most cancer

cells [88, 96]. Mammalian telomerase consists of a telomerase RNA component (TERC) and a telomerase reverse transcriptase (TERT) component. The latter catalyzes the synthesis of new telomeric repeats. Nevertheless, consistent decline in telomere length with age does occur in adult stem cells, suggesting that telomerase activity is insufficient to maintain the replication of these cells indefinitely [90]. So the telomeres of various stem cells, i.e., hematopoietic, neural, germinal and other, do shorten with age [97, 98].

Telomere exhaustion also explains the limited proliferative capacity of some types of *in vitro* cultured cells, the so-called replicative senescence or Hayflick limit [99, 100]. Indeed, as shown already in the 1990s, ectopic expression of telomerase confers immortality to otherwise mortal cells, without causing oncogenic transformation [101]. Similarly, telomerase deficiency in humans is associated with the premature development of diseases, such as pulmonary fibrosis, dyskeratosis congenita and aplastic anemia, which involve the loss of the regenerative capacity of different tissues [102].

Recent evidence also indicates that aging can be reverted by telomerase activation. In particular, the premature aging of telomerase-deficient mice can be reverted when telomerase is genetically reactivated in these aged mice [103]. Moreover, normal physiological aging can be delayed without increasing the incidence of cancer in adult wild-type mice by pharmacological activation or systemic viral transduction of telomerase [104].

This correlation between telomere length, telomerase activity and age is not completely clear. For example, while telomere length is negatively correlated with age in humans up to 75 years, it is positively correlated with age in the elderly, suggesting that long telomeres contribute to survival in old age [105]. Furthermore, telomere length predicted survival in elderly twins, suggesting that telomeres contribute to longevity in humans even when controlling for the influence of genetic background [106].

There is a good correlation between the expression of human TERT mRNA and the presence of telomerase activity in extracts from tissue culture cells, and normal and cancer tissues, suggesting that human TERT expression is the primary and rate-limiting determinant of telomerase activity [107]. This is important in stem cell therapies, so we have already investigated the importance of longer telomere length of the CD34+ cell grafts used for cell therapy and found that longer telomere length and higher telomerase expression agree with CD34+ cell's increased functional capacity, however the patients with longer CD34+ telomere length did not favorably respond to autologous CD34+ cell transplantation therapy [108].

2.5. Epigenetic changes and consequent dysregulation of gene expression

The regulation of the chromatin state is important for stem cell function. In Waddington's epigenetic landscape theory, stem cells stand at an undifferentiated epigenetic summit above multiple cell fates [109]. During the differentiation and aging of cells, numerous chromatin and gene expression changes appear progressively in response to cell stress, most notably in response to DNA damage signals. The changes in epigenetic modification of chromatin and histones lead to dysregulation of gene expression. The epigenetic modifications that are observed to change during aging are histone acetylation, histone methylation, and DNA methylation.

As already mentioned, of all other different types of DNA damage, the one that has the greatest lasting effect on chromatin is the double-strand breaks, which cause a dramatic redistribution of chromatin factors. This is a part of the response to damage that is not fully restored after the repair [110]. Thus, changes in chromatin caused by DNA damage might underlie the skewed lineage phenotypes exhibited by aged stem cells [111].

The epigenetic changes have now been cataloged. In mice, it has been observed that the level of histone deacetylase SIRT1 decreases with age and that decrease of SIRT1 expression correlates with premature aging in mice with increased p53 activity [112].

The expression levels of chromatin modifiers, including components of the SWI-SNF (switch/sucrose non-fermentable) and PRC (polycomb repressive complex) complexes, histone deacetylases (HDACs) including sirtuins, and DNA methyltransferases, also change with age in stem cells [113, 114]. These changes may underpin declining stem cell function. Indeed, the overexpression of enhancer of zeste homolog 2 (EZH2), a component of PRC2, improves long-term repopulating potential in HSCs [115]. Additionally, in aged HSCs, clusters of genes increase in expression levels based on chromosomal location, suggesting that epigenetic dysregulation engenders regional loss of transcriptional silencing [113]. Taken together, these findings suggest that changes in epigenetic modifications are a general trait of stem cell aging, which impacts their function.

It is interesting that with aging appear changes that reinforce self-renewal. Sun et al. conducted a comprehensive integrated genomic analysis of young (4 mo) and old (24 mo) murine HSCs by profiling the transcriptome, DNA methylome, and histone modifications. Transcriptome analysis indicated reduced transforming growth factor beta (TGF-β) signaling and perturbation of genes involved in HSC proliferation and differentiation. Aged HSCs showed increased DNA methylation at transcription factor binding sites associated with differentiation-promoting genes, combined with a reduction at genes associated with HSC maintenance. When they profiled the principal regulatory chromatin marks with the use of chromatin immunoprecipitation sequencing (ChIP-seq) they found that the H3K4me3 mark, an activating histone modification, increases with age at loci that regulate HSC self-renewal, potentially underlying the increase in HSC number observed with aging [116].

In satellite cells of muscles, H3K4me3 levels modestly decrease with age, whereas levels of the repressive modification H3K27me3 significantly increase with age. It has also been shown that the expression levels of histones themselves decrease with age [117]. The levels of H4K16Ac, another activating modification, decrease with age in HSCs; inhibition of cell division control protein 42 homolog (CDC42) restores H4K16Ac levels to that of young HSCs and reverses phenotypes of HSC aging in transplantation assays [118].

It is not known whether the epigenetic changes in stem cell products affect their clinical efficiency. In our recent study we intended to gain insight into the methylation status of CD34+ enriched cell products intended for autologous CD34+ cell transplantation in patients with cardiomyopathy. We found that the global DNA methylation and hydroxymethylation status as well as the target methylation profile of 94 stem cell transcription factor genes in CD34+ enriched cell products did not differ significantly as compared to initial leukapheresis products. The epigenetic landscape of different cell products can tell us little about the functional capacity and regenerative properties of CD34+ cells (Rozman et al. [108]).

2.6. Changes of microRNA

Impairments in stem cell function that occur during aging are globally mirrored in the epigenome and transcriptome of HSCs, including the microRNAs. MicroRNAs (miRNAs) are small noncoding evolutionarily conserved RNAs that regulate gene expression primarily at the posttranscriptional level. They act by binding to specific sequences in the 3′ untranslated region of their target genes and causing the transcripts to be degraded by the RNA-induced silencing complex (RISC). The human genome encodes over 1000 miRNAs that appear to target about 60% of other genes. MiRNAs are important posttranscriptional regulators of gene expression and play important and diverse roles in almost all biological and metabolic processes, including early development, cell proliferation, cell cycle regulation, apoptosis, fat metabolism, signal transduction, aging and diseases, as reviewed recently [119].

In stem cells, miRNAs influence properties such as potency, differentiation, self-renewal, and senescence. Different kinds of stem cells possess distinct miRNA expression profiles. Among other things, miRNAs regulate a number of cell functions such as defense mechanisms against ROS, DNA repair, and apoptosis. These properties, and the assumption that miRNAs act as some kind of general switch, make them highly relevant in research on aging [120], especially since specific miRNA expression profiles could be used to terminally differentiate cells from stem cells in order to treat various diseases, including myocardial infarction, neurodegenerative diseases, blood diseases, and muscle diseases [121].

miRNAs regulate the state of stem cells by directly targeting three prime untranslated region (3′-UTR) of pluripotency factors in the section of messenger RNA. For instance, miR-145 miRNA represses the pluripotency of human embryonic stem cells (ESCs) through targeting octamer-binding transcription factor 4 (*Oct4*; also known as *Pou5f1*), sex determining region Y-box 2 (*Sox2*), and kruppel-like factor 4 (*Klf4*) [122]. In addition, miRNAs target the coding regions of transcription factors to modulate stem cell differentiation. miR-296, miR-470, and miR-134 regulate mouse ESC differentiation by targeting the coding regions of *Nanog*, *Oct4*, and *Sox2* [123]. Other classified miRNAs also regulate pluripotency, self-renewal, reprogramming, and differentiation of stem cells [124–128].

miRNAs act as key regulators of hematopoiesis during the proliferation and differentiation of HSCs in mammals. Ectopic expression of AAAGUGC seed-containing miRNAs enhance the primary hematopoietic progenitors [129]. miR-181, miR-223, and miR-142 are preferentially expressed in hematopoietic tissues, with miR-181 significantly promoting B-lymphocyte differentiation [130]. miR-125a is conservatively expressed in long-term HSCs and can increase the number of HSCs by targeting the apoptosis factor Bax1 [131]. Furthermore, overexpression of miR- 125b leads to lethal myeloid leukemia in mice [132]. See the recent review of Li et al. [119].

Besides regulating the ESCs, miRNAs exert several other actions that indirectly impact stem cells and regeneration. For instance, let-7 family and miR-15a/16-1 cluster function as regulators of the cell cycle and tumor suppressors. While miR-29a and miR-29b regulate progression through the cell cycle [133], miR-9 and miR-124a play a critical role in specification of the neural progenitors from ESCs [134, 135].

On the other hand, miRNAs also modulate development of other tissues, such as cardiovascular differentiation of cardiomyocyte progenitor cells and stem cells, including the differentiation of cardiomyocytes, vascular smooth muscle cells, and endothelial cells. They are involved in the regulation of cardiovascular differentiation of human-derived cardiomyocyte progenitor cells, the cardiovascular differentiation of ESCs and iPSCs, in cardiac differentiation of ESCs after myocardial infarction, vascular endothelial growth factor (VEGF) signaling and angiogenesis, which has great therapeutic value for the future regenerative medicine, as reviewed recently by Li et al. [119]. Some other observations comment on the important role miRNAs play in brain development, as well as in later stages of mammalian neuronal maturation and synapse development. Conversely, dysregulation of miRNAs expression has been implicated in developmental defects, cancers and nervous system diseases, as recently reviewed by Murashov [121].

Lee et al. have measured the expression levels of 521 small regulatory miRNAs in young and old animals of six mouse strains and found that expression levels of three miRNAs (miR-203-3p, miR-664-3p, and miR-708-5p) were associated with lifespan. Pathway analysis of binding sites for these three miRNAs revealed enrichment of key target genes involved in aging and longevity pathways including mechanistic target of rapamycin (mTOR), forkhead box protein O (FOXO) and mitogen-activated protein kinase (MAPK), most of which also demonstrated associations with longevity [136].

In conclusion, one could infer that miRNAs have critical roles in stem cell reprogramming, pluripotency maintenance and differentiation, as well as some other important cellular functions. In the future, miRNAs may greatly contribute to stem cell clinical therapy and have potential applications in regenerative medicine.

2.7. Changes in RNA splicing and ribosomal machinery

RNA splicing is the editing of the nascent precursor messenger RNA (pre-mRNA) transcript into a mature messenger RNA (mRNA). After splicing, introns are removed and exones are joined together. Splicing usually takes place immediately after transcription, and is carried out in a series of reactions catalyzed by the spliceosome, a complex of small nuclear ribonucleoproteins (snRNPs). This results in an mRNA molecule, which can be translated into protein. Splicing enables one gene to generate multiple proteins allowing organisms to generate complexity from a relatively limited number of genes.

In healthy aging, splicing homeostasis takes place, while deregulation of the splicing machinery is linked to several age-related chronic illnesses. Certain studies point out that defective splicing machinery and de-regulation of RNA splicing acts as a driver of the aging process itself. Studies on the roundworm *C. elegans* show that with age they lose muscle mass, their cutickle thickens, they wrinkle, and they experience declines in fertility and immune functions. The pre-mRNA splicing homeostasis is a biomarker and predictor of life expectancy in this worm. Recently, Heintz and her colleagues found that splicing could also play a major role in the aging process of humans. Using transcriptomics and in-depth splicing analysis in young and old animals they found defects in global pre-mRNA splicing

with age that are reduced by caloric restriction via one particular component of the splicing apparatus, called splicing factor 1 (SFA-1)—a factor also present in humans. They also showed that SFA-1 is specifically required for lifespan extension by caloric restriction and by modulation of the target of rapamycin complex 1 (TORC1) pathway components 5′ AMP-activated protein kinase (AMPK), RAGA-1, and ribosomal protein S6 kinase (RSKS-1/S6 kinase), and demonstrated that overexpression of splicing factor 1 (SFA-1) extends lifespan. Together, these data demonstrate a role for RNA splicing homeostasis in caloric restriction longevity and suggest that modulation of specific spliceosome components may prolong healthy aging [137].

The ribosomal machinery that is responsible for protein synthesis (translation), i.e., linking amino acids in the order specified by mRNA molecules, consists of two major components: the small ribosomal subunit, which reads the RNA, and the large subunit, which joins amino acids to form a polypeptide chain. Ribosomes contain ribosomal RNA (rRNA) molecules and a variety of highly conserved ribosomal proteins, and similar to other cellular compartments, these are particular targets of aging. After a comprehensive integrated genomic analysis of young and aged cells, consisting of the profiling of transcriptome, DNA methylome, and histone modifications of young and old murine HSCs, Sun et al. found an increased transcription of ribosomal protein and RNA genes, and hypomethylation of rRNA genes [116]. Indeed, inhibition of ribosomal proteins or their regulators has been shown to extend life span in yeast and worms [138, 139]. Although the research has not been focused on the plicing in stem cells we can expect that the splicing homeostasis in stem cells is similarly affected by aging.

2.8. Proteostasis

The proteostasis or homeostasis of the proteome is a complex system that takes care of the proper folding, functioning, and degradation of cellular proteins. Mechanisms, by which proteostasis is ensured, include regulated protein translation, chaperone assisted protein folding, and protein degradation pathways. Adjusting each of these mechanisms to the requirements of proteins, which need to be correctly folded, is essential for maintaining all cellular functions.

In previous paragraphs it has been already explained that in aged subjects, stem cells display a thoroughly altered proteome. Many studies have demonstrated that proteostasis is altered with aging and that accumulation of misfolded or damaged proteins is an important determinant of the aging process [140]. Indeed, many different proteins involved in cytoskeletal organization, anti-oxidant defense, and other functions are age-dependent and associated with functional impairment of the cell functions, including decreased responsiveness to physical environmental cues and decreased resistance to oxidative stress [15]. Chronic expression of unfolded, misfolded or aggregated proteins contributes to the development of some age-related pathologies, such as Alzheimer's disease, Parkinson's disease and cataracts [141]. Since the passage of altered proteins to progenitor cells during asymmetric division could compromise development and cause aging, proteostasis maintenance in stem cells has an important role in organismal aging [142].

During the evolution the cells developed a variety of mechanisms that maintain and promote proteostasis and slow down the aging. This is performed by an array of quality control mechanisms that preserve the stability and functionality of the proteome. Various mechanisms for the correction of folded proteins have developed, such as the heat-shock family of proteins, as well as the corrective mechanisms for the degradation of misfolded proteins in proteasome or the lysosome [140, 143]. Moreover, there are regulators of age-related proteotoxicity, such as modifier of protein aggregation (MOAG-4), that act through an alternative pathway distinct from molecular chaperones and proteases [144]. The stress-induced synthesis of cytosolic and organelle-specific chaperones is significantly impaired in aging [145]. All these systems function in a coordinated fashion to restore the structure of misfolded polypeptides or to remove and degrade them completely, thus preventing the accumulation of damaged components and assuring the continuous renewal of intracellular proteins.

As previously mentioned, there are several approaches for maintaining or enhancing proteostasis aimed at activating protein folding and stability mediated by chaperones. A number of animal models support a causative impact of chaperone decline on longevity. In particular, transgenic worms and flies overexpressing chaperones are long-lived [146, 147]. Also, mutant mice deficient in a co-chaperone of the heat-shock family exhibit accelerated aging phenotypes, whereas long-lived mouse strains show a marked up-regulation of some heat-shock proteins [148].

Moreover, activation of the master regulator of the heat-shock response, the transcription factor heat shock factor 1 (HSF-1), increases longevity and thermotolerance in nematodes [149], while amyloid-binding components can maintain proteostasis during aging and extend lifespan [150]. Pharmacological induction of the heat-shock protein Hsp72 preserves muscle function and delays progression of dystrophic pathology in mouse models of muscular dystrophy [151].

Small molecules may be also employed as pharmacological chaperones to assure the refolding of damaged proteins and to improve age-related phenotypes in model organisms [152].

For the degradation of unneeded and misfolded proteins there are special protein complexes, named proteasomes, which degrade them with proteolysis, a chemical reaction that breaks peptide bonds. The degradation process yields peptides of about seven to eight amino acids long, which can then be further degraded into shorter amino acid sequences and used in synthesis of new proteins.

Stem cells can also maintain high levels of autophagy and proteasome activity to clear damaged proteins. For example, autophagy is greater in HSCs and skin stem cells than in surrounding differentiated cells [153]. Although proteasome activity has yet to be characterized in adult stem cells, it has been shown that human ESCs exhibit high proteasome activity [142]. Fly oocytes, which require similar long-term proteome-protection mechanisms as stem cells, maintain high activity of large multi-protein complex 26S proteasome with age, despite the decline of its activity in the somatic cells [154].

The activities of the two principal proteolytic systems implicated in protein quality control, namely, the autophagy-lysosomal system and the ubiquitin-proteasome system, decline with

aging [155, 156], supporting the idea that collapsing proteostasis constitutes a common feature of old age. In relation to the proteasome, activation of epidermal growth factor (EGF) signaling extends longevity in nematodes by increasing the expression of various components of the ubiquitin-proteasome system activators accelerates the clearance of toxic proteins in human cultured cells [157]. Moreover, increased expression of the 26S proteasome subunit RPN-6 by the FOXO transcription factor DAF-16 confers proteotoxic stress resistance and extends lifespan in *C. elegans* [158].

Regarding autophagy, transgenic mice with an extra copy of the chaperone-mediated autophagy receptor lysosome-associated membrane protein 2a (LAMP2a) do not experience aging-associated decline in autophagic activity and preserve improved hepatic function with aging [159]. This is a promising example of genetic manipulations that improve proteostasis and delay aging in mammals [159]. Functional decline in the cellular proteolytic machinery leads to the formation of an autofluorescent protein called lipofuscin, which can be used as a biomarker of aging [160]. Based on the given data it is obvious that SCs are a subject of age related changes of proteostasis and further studies will probably focus on proteostasis maintanance in SCs.

2.9. Changes of cell polarity

In order to prevent the accumulation of damaged components, stem cells developed diverse mechanisms such as the asymmetric segregation of damaged proteins and enhanced proteostasis. After a symmetric division, stem cells produce two daughter cells with the same fate, whereas after asymmetric division they produce one daughter stem cell and one differentiating daughter cell. During the asymmetric division, damaged components such as damaged DNA, replicating circular DNA, carbonylated proteins and damaged organelles are distributed into the differentiating cell, whereas the daughter stem cell remains youthful [161, 162]. In a similar way, stem cells have been shown to asymmetrically segregate damaged proteins and mitochondria into the progeny, which retains the stemness of the mother cell [163, 164]. A similar evolutionary principle enables that the parental strand of DNA is always sequestered in the daughter stem cell, whereas the strand synthesized during S phase, which might contain errors from replication, is directed to the differentiating daughter cell [165]. In this way the non-random strand segregation serves to avoid mutations and to control the inheritance of epigenetic state [166]. It was shown that the distribution of epigenetic modifications on mitotic chromosomes differs, which means that the bias is generated non-randomly during chromatid segregation. In *Drosophila* male GSCs, the histone modifications present in the stem cells are distinct from those in the differentiating daughter cells, which helps to retain pre-existing histones in the mother stem cell while imparting newly synthesized histones to the daughter cell. This retention of pre-existing histones in the stem cells is a prerequisite for maintaining their ability to self-renew. Different epigenetic modifications potentially lead to variations in the otherwise equivalent chromatids that segregate during asymmetric cell divisions [167].

There is accumulating evidence that other organelles are also non-randomly distributed between daughter cells. Numerous organelles have been widely studied for their asymmetric segregation in non-mammals and mammals, such as mitochondria, centrioles of the centrosome, and midbody, as well as different protein complexes [168].

The asymmetric division of stem cells first requires that a cell be polarized and several studies demonstrate that aged germinal stem cells (GSCs) and HSCs are less able to perform such polarized divisions, suggesting that loss of polarity contributes to stem cell aging [169]. Other data on HSCs suggest that changes in age-related Wnt signaling are a cause of this loss of polarity [170]. This process also appears to occur in satellite cells [171]. There is certain disagreement as to whether polarized division occurs in other stem cell populations, such as intestinal, hair follicle, neural or germline stem cells, as reviewed by Yennek and Tajbakhsh in 2013 [172].

2.10. Changes in metabolism and nutrient sensing

Metabolic status plays an important role in stem cell aging [2]. Similar to other cells, stem cells generate energy via glycolysis or oxidative phosphorylation. Quiescent stem cells generally rely upon glycolysis, perhaps because this reduces the abundance of ROS [142]. Many adult stem cells also reside in hypoxic niches, perhaps as a part of a mechanism to limit ROS production [173].

For the provision of necessary energy, proliferating stem cells rely on the oxidative phosphorylation, which predisposes them to oxidative damage and cellular dysfunction. Therefore the molecules that scavenge ROS or enable the overexpression of the transcription factor NRF2, which regulates the response to oxidative stress, reduce the aged phenotype of old cell.

2.10.1. Caloric restriction

The most robust longevity-extending intervention across species is caloric restriction (CR). For example, CR increases the abundance of satellite cells in muscles [174] and improves the function of many stem cell populations, including HSCs in mice [175] and GSCs in flies [176].

CR also promotes ISC self-renewal in mice by induction of the enzyme BST1 in Paneth cells, which form the niche. BST1 then converts NAD+ to the paracrine signal cyclic ADP ribose (cADPR), which is sensed by the ISCs [177]. Pathways and factors implicated in mediating the response of stem cells to CR that extend lifespan, include insulin and IGF-1 signaling (IIS) pathway, target of rapamycin (TOR) signaling, AMPK, sirtuins and FOXO transcription factors [178].

2.10.2. Glucose metabolism

Recent studies also show that HSCs and satellite cells increase glucose and glutamine metabolism during activation [179] — an alteration that mimics the Warburg effect in cancer cells. Similarly, in skeletal muscle, aging is associated with pseudohypoxia and Warburg-like metabolism, which compromise cellular function [180] and promote oncogenic transformation [181].

Glucose is the main nutrient in the cell, whereas insulin informs cells about the presence of glucose. The intracellular signaling pathway that governs insulin is the same as that elicited by IGF-1, which is, together with the growth hormone (GH), produced by the anterior pituitary, and is the secondary mediator of the somatotrophic axis in mammals. For this reason, IGF-1 and insulin signaling are known as the "insulin and IGF-1 signaling" (IIS) pathway. GH and IGF-1 levels decline during normal aging, as well as in mouse models of premature aging [182]. Remarkably, the IIS pathway is the most conserved aging-controlling pathway

in evolution and among its multiple targets are the FOXO family of transcription factors and the mTOR complexes, which are also involved in aging and conserved through evolution. Similarly, genetic polymorphisms or mutations that reduce the functions of GH, IGF-1 receptor, insulin receptor or downstream intracellular effectors such as protein kinase B (PKB), also known as AKT, mTOR and FOXO, influence longevity both in humans and in model organisms, further illustrating the major impact these pathways have on longevity [53].

Multiple genetic manipulations of the IIS pathway, which attenuate signaling intensity at different levels, consistently extend the lifespan of worms, flies and mice. Genetic analyses indicate that this pathway mediates part of the beneficial effects of CR on longevity [183].

Mice with an increased dosage of the tumor suppressor protein phosphatase and tensin homolog (PTEN) exhibit a general down-modulation of the IIS pathway and an increased energy expenditure that is associated with improved mitochondrial oxidative metabolism, as well as with an enhanced activity of the brown adipose tissue [184]. In line with other mouse models with decreased IIS activity, PTEN-overexpressing mice, as well as hypomorphic phosphatidylinositol-3-kinase (PI3K) mice show an increased longevity [185].

Organisms with a constitutively decreased IIS pathway can live longer because they have lower rates of cell growth and metabolism, and a lower rates of cellular damage. Similarly, the aged organisms decrease their IIS pathway in an attempt to extend their lifespan. However, defensive responses against aging eventually exhaust and later on they even aggravate aging [186].

2.10.3. Other nutrient-sensing systems: mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK) and sirtuins

Besides the IIS pathway, three additional related and interconnected nutrient-sensing systems that participate in glucose –sensing: mammalian TOR (mTOR), for the sensing of high amino acid concentrations; AMPK that senses low energy states by detecting high AMP levels; and sirtuins, which sense the low energy states by detecting high NAD+ levels [187].

The mTOR kinase is part of two multiprotein complexes, mTORC1 and mTORC2, that regulate essentially all aspects of anabolic metabolism. Genetic down-regulation of mTORC1 activity in yeast, worms and flies extends longevity and attenuates further longevity benefits from CR, suggesting that mTOR inhibition phenocopies CR [188]. In mice, treatment with rapamycin also extends longevity in what is considered the most robust chemical intervention to increase lifespan in mammals [189].

Genetically-modified mice with low levels of mTORC1 activity, but normal levels of mTORC2 activity, have an increased lifespan [190], and mice deficient in ribosomal protein S6 kinase beta-1 (S6 K1), which is a main mTORC1 substrate, are also long-lived [191]. This means that the down-regulation of mTORC1/S6 K1 acts as the critical mediator of longevity in relation to mTOR.

It seems that the intense trophic and anabolic activity, signaled through the IIS or the mTORC1 pathways, is a major accelerator of aging. Although inhibition of TOR activity clearly has beneficial effects during aging, it also has some undesirable side-effects, such as impaired wound healing, insulin resistance, cataract formation and testicular degeneration in mice [192]. In order

to determine the extent to which beneficial and damaging effects of TOR inhibition can be separated from each other, it will be crucial to understand the mechanisms involved.

There are two another nutrient sensors, AMPK and sirtuins, which act in the completely opposite direction of the IIS and mTOR. Instead of signaling nutrient abundance and anabolism, they signal nutrient scarcity and catabolism. Accordingly, their up-regulation promotes a healthy aging. AMPK activation has multiple effects on metabolism and, remarkably, shuts off mTORC1 [193]. There is evidence indicating that AMPK activation may mediate lifespan-extension following metformin administration to worms and mice [194, 195].

The role of sirtuins in lifespan regulation has been discussed above (see section 2.2 on DNA mutations). In addition, SIRT1 can deacetylate and activate the PPARγ co-activator 1α (PGC-1α) [43]. PGC-1α orchestrates a complex metabolic response that includes mitochondriogenesis, enhanced anti-oxidant defenses, and improved fatty acid oxidation [196]. Moreover, SIRT1 and AMPK can engage in a positive feedback loop, thus connecting both sensors of low-energy states into a unified response [197].

Collectively, currently available evidence strongly supports the idea that anabolic signaling accelerates aging, and decreased nutrient signaling extends longevity [183]. Consistent with the relevance of deregulated nutrient-sensing as a hallmark of aging, CR increases lifespan or healthspan in all investigated eukaryote species, which are unicellular and multicellular organisms of several distinct phyla, including non-human primates [198]. What is more, a pharmacological manipulation that mimics a state of limited nutrient availability, such as rapamycin, can extend longevity in mice (Harrison et al. [189]). All of these reflects in stem cells, however, the exact mechanisms in the metabolism of stem cells awaits further clarification.

2.11. Niche deterioration

In the context of a tissue, adult stem cells reside in a special microenvironment referred to as the "niche". The niche allows interaction between the stem cells and different extrinsic signals. In some instances, these signals are mediated via direct cell to cell communication or cell to matrix interaction. Another category of signals comprises of diffusible signaling ligands which regulate various transcription programs in the stem cells. These interactions are crucial, as they are able to regulate whether stem cells are quiescent, self-renew, or commit to differentiation [199].

Similarly to the stem cells themselves, the BM niche changes substantially with age. The niche consists of mesenchymal stem cells (MSCs), stromal cells, osteoblasts, adipocytes, and other cells, as well as extracellular matrix. The proliferative capacity of human MSCs has been shown to decline with age [200]. Certain other authors noticed a prominent increase in adipocytes in the aged BM, which is associated with lower HSC potential [201].

Mechanisms of niche aging are probably the same as in other cells. Khatri et al. recently showed that accumulation of excessive ROS in BM stromal cells suppress BM cellularity by affecting microenvironment in aged mice. Treatment of these mice with a polyphenolic anti-oxidant curcumin has quenched ROS, rescued stromal cells from oxidative stress-dependent cellular injury, and improved hematopoietic reconstitution in old (18 months) mice. This

implicates the role of ROS in perturbation of stromal cells function upon aging, which in turn affects BM's reconstitution ability in aged mice. Rejuvenation therapy using curcumin, prior to transplantation of HSCs and progenitor cells could be an efficient strategy for successful marrow reconstitution in older mice [202].

The question remains as to whether aged BM niche cells induce age-related changes in HSCs. Evidence suggests that aging in the microenvironment influences HSC engraftment, as aged HSCs demonstrate a lower engraftment after transplantation [203]. Hematopoietic cells engrafted in subcutaneous implantation of BM stroma from both aged and young mice exhibit lower spleen colony-forming units (CFU-S) capacity [204]. Furthermore, young HSCs transplanted to aged niches exhibit impairment in homing and decreased potential for differentiation, failing to efficiently repopulate an old niche [205].

Another characteristic of aged HSCs is an altered differentiation potential tending toward higher myeloid/platelet output and lower lymphoid output. Skewing toward myeloid differentiation is attributed to the niche microenvironment, since the transplantation of young HSCs to aged recipients resulted in a tendency toward higher myeloid output [206]. Transplantation experiments on old recipients show that granulocyte-macrophage progenitor (GMP) expansion is comparable regardless of donor age. Also, the differentiation of B-cells depends on the BM microenvironment [207] and it was shown that aged HSCs occupy different niches to young HSCs [208].

One of the mechanisms of aging in the hematopoietic system are the changes in adhesion between HSCs and niche cells. Expression of various adhesion molecules in HSCs alters with age so the aged HSCs express low levels of integrin α4, integrin α5 and VCAM-1, and high levels of P-selectin and integrin α6 compared to young HSCs [209]. In *Drosophila*, the age-dependent E-cadherin decline in the stem cell-niche junction that regulates the adhesion of GSCs to the niche was shown to contribute to the aging of stem cells [210]. Another authors similarly showed that the aged HSCs exhibit less adhesion to the stromal cells compared to the young ones [211]. Another group has shown that an overexpression of CDC42, a small Rho GTPase that is involved in adhesion signaling, causes premature aging phenotypes in these cells [212].

Age-related changes in niche cells may also be attributed to changes in their metabolic state. MSCs obtained from old human BM have an elevated level of ROS along with p21 and p53 expression, indicating cellular senescence [17]. As already mentioned, high oxygen tension causes senescence in cultured human BM MSCs, whereas the continuous hypoxia make the human MSCs to exhibit higher self-renewal divisions without cellular senescence [213]. Compared to MSCs cultured in low oxygen, MSCs cultured in higher oxygen levels utilize oxidative phosphorylation, suggesting that the generation of ROS might influence MSC senescence.

Age-related changes in the stem cell niches can influence HSC mobilization from the BM, which is extremely important in the clinical settings. Several authors, including ourselves, have noted that the collection of stem cells from aged patients results in low yields of mobilized HSCs intended for therapy [214, 215]. It is interesting that in various animal models an opposite effect was demonstrated since the granulocyte colony-stimulating factor (G-CSF)-induced mobilization resulted in increased numbers of HSCs in aged mice [211]. The authors deduce that differences in mobilization potential according to age are influenced mainly by

the niche in which the HSCs reside and that the clonality of HSCs may largely be influenced by specific niche cells at different anatomical sites [216].

Various studies utilizing heterochronic transplantation and parabiosis experiments showed that aging can be also be caused by extrinsic mechanisms, i.e., it is caused by factors external to the cell itself. This was shown in satellite cells [217], NSCs [218, 219], and GSCs [220]. In flies, the cells that form the niche of the GSCs themselves decline in abundance with age, possibly because of decreased self-renewal [221, 222].

Aged niche cells can also fail to send proper signals to stem cells, namely through morphogen and growth factor signaling, thereby affecting cell fate decisions. For example, increased fibroblast growth factor 2 (FGF2) in the aged satellite cell niche of mouse muscle impairs self-renewal [223]. Markers of inflammation also increase in the aging niche, for example in hair follicle stem cells, and impair stem cell function [224].

Taken together, stem cells require support cells that constitute the niche to maintain proper function. Thus, aging of the stem cell niche can also critically modulate stem cell function.

2.12. Influence of various circulating factors

The concentrations of various circulating factors exerts important influences on stem cell aging. Many of these factors have been identified by rejuvenating effects of blood or plasma derived from either young or calorically restricted animals. Among such factors are insulin and IGF-1, which have been already discussed (see paragraph 2.10.). Reduced signaling from these molecules is believed to mediate much of the longevity-extending effects of CR in mice. An opposite example is the TGF- β molecule, the levels of which increase during aging in mouse and human sera, which impairs the function of satellite muscle cells and NSCs [225]. By contrast, growth differentiation factor 11 (GDF11) has been suggested to improve the function of satellite cells and NSCs, and its levels appear to decrease during aging [218]. The validity of the effects of GDF11 on satellite cells, however, has been questioned by other studies, although it is worth noting that the dose of GDF11 and the skeletal muscle injury models used in the various studies differed [226]. Whether GDF11 actually declines with age has also been questioned, based in part on the argument that GDF11 detection methods cross-react with myostatin (ibid.), although a recent study using additional methods and controls also reports that GDF11 declines with age in mice [227]. Finally, the latest reports infer that high levels of GDF11 cause reductions in body and heart weight in both young and old animals, suggestive of a cachexia effect with the conclusion that elevating blood levels of GDF11 in the aged might cause more harm than good [228].

An important debate regarding the decline in stem-cell function is the relative role of cell-intrinsic pathways compared to cell-extrinsic ones [229]. Recent work has provided strong support for the latter. In particular, CR increases intestinal and muscle stem functions through cell-extrinsic mechanisms [174]. Similarly, when muscle-derived stem cells from young mice are transplanted to progeroid mice, this extends their lifespan and improves degenerative changes even in tissues where donor cells are not detected, suggesting that their therapeutic benefit may derive from systemic effects caused by secreted

factors [230]. Furthermore, parabiosis experiments have demonstrated that the decline in neural and muscle stem cell function in old mice can be reversed by systemic factors from young mice [231, 232].

There is also an ancient system in each cell that relates to the homeostasis of intracellular calcium (Ca2+), which in normal cell sustains a 20,000 fold concentration gradient to the exterior of the cell, resulting in the extracellular Ca2+ acting as cellular regulator when it enters the cell via the Ca2+ channels. This gradient is sustained by specific pumping and transporting mechanisms consisting of protein molecules [233]. Anomalies of these proteins results in an increase of intracellular calcium which can cause various diseases. With age, the hampered calcium homeostasis can lead to different muscle, immune and neural related defects [234].

2.13. Stem cell exhaustion

Although stem cells are regarded as immortal, as they are not subject to replicative senescence, they are susceptible to damage accumulation over time. Besides many other changes, a decline in their relative numbers and changes in subpopulations were observed. The group of dormant and active stem cells, existing in the niches of an organism that can be considered a pool of regenerative reserve, plays an important role in prevention of disease, in regeneration and aging. For instance, a decline in CD34+ circulating progenitor cells was reported with advancing age. When 100 octogenarians were observed for 7 and 10 years it was demonstrated that the number of their circulating CD34+ cells better predicted their lifespan and cardiovascular (CV) issues related mortality then the classic cardiovascular risk factors (hypertension, smoking, hypercholesterolemia), levels of inflammatory markers, or levels of cholesterol, or some other traditional cardiovascular indexes such as FRS and CVFRs The chances of reaching an older age depended on higher numbers of CD34+ cells at baseline, thus the number of CD34+ cells could be considered as a biomarker of longevity in the elderly over 80 years [235].

On the other hand, there are reports that in certain tissues the numbers of adult stem cells even increase with age, however the number of their parent clones decreases, meaning that fewer pluripotent stem cells give rise to more frequent progeny, in order to compensate for the decrease of numbers [236]. Ruzankina and Brown suggest that mammals in fact do have a finite number of stem cell replications per life and that aging of the hematopoietic system, which is due to a finite doubling capacity of stem cells, degrades its regenerative potential as well as the potential for preventing cancer [237].

Verovskaya used cellular barcoding combined with multiplex high-throughput sequencing to demonstrate clonal behavior of young HSCs transplanted to older organisms. In their study, the majority of transplanted clones steadily contributed to hematopoiesis in the long-term, although the clonal output in granulocytes, T cells, and B cells was substantially different. The final pool of old HSCs was composed of multiple small clones, whereas the young HSC pool was dominated by fewer, but larger, clones [238].

Holstege et al. have showed that the contents of a stem cell compartment actually deplete with old age. In the nonrepetitive genome of a 115-year-old centenarian woman they found approximately 450 somatic mutations that accumulated in the last years of her life, and the distribution of these mutations suggested that the majority of her peripheral white blood cells

were offspring of only two HSC clones that were still active in her old age. The telomeres of her white blood cells were significantly shorter than the telomeres from other tissues, suggesting that the HSCs have a finite lifespan, which is the cause of hematopoietic clonal evolution at extreme ages [239].

Several recent studies have confirmed that clonal hematopoiesis is almost a "normal" part of aging, with recent reports showing 0.8%, 11% and 19.5% of normal individuals aged <60, >80 and >90 years, respectively, having demonstrable clonal hematopoiesis – so called age-related clonal hematopoiesis [240, 241]. Clonal hematopoiesis (CH) arises when a substantial proportion of mature blood cells is derived from a single dominant hematopoietic stem cell lineage. It was recently shown, in the study on 11,262 elderly Icelanders which used whole-genome sequencing, that somatic mutations in candidate driver genes are thought to be responsible for at least some cases of CH [242].

At the same time there is ample evidence that there exist many dormant HSCs, and even some other and more "primitive" types of stem cells, such as for instance the VSEL stem cells with "primitive" embryonic characteristics, which co-inhabit the BM [243]. These VSEL cells exhibit some characteristics of long-term repopulating HSCs (LT-HSCs), they may differentiate into organ-specific cells (e.g., cardiomyocytes), and probably have a role in aging since the number of these cells positively correlates with longevity in several murine models [244]. Along with others, we have found similar cells in the reproductive organs [245].

It is now becoming obvious that maintaining robust stem cell pools seems to extend not only lifespan but also healthspan [49].

2.14. Cellular senescence – A stable arrest of the cell cycle

Cellular senescence can be defined as a stable arrest of the cell cycle coupled to typical phenotypic changes [246]. This phenomenon was originally described by Hayflick in human fibroblasts serially passaged in culture [99]. The senescence that was observed by Hayflick was caused by telomere shortening [101] and some other aging-associated stimuli that trigger senescence independently of the telomeric process. It is for instance well known that the non-telomeric DNA damage and de-repression of the *INK4/ARF* locus, both of which progressively occur with chronological aging, are also capable of inducing senescence [247].

The accumulation of senescent cells with age is a simple mathematical result of the increase in the rate of generation of senescent cells and/or a decrease in their rate of clearance. In normal physiology this has detrimental consequences, but in some circumstances it also has useful effects. For instance, there is good evidence that the senescent tumor cells are subjected to strict immune surveillance and are efficiently removed by phagocytosis [248].

Among other functions, the senescent cells manifest dramatic alterations in their secretome, which is particularly enriched in pro-inflammatory cytokines and matrix metalloproteinases, which is referred to as the "senescence-associated secretory phenotype" [249, 250]. This pro-inflammatory secretome may contribute to aging (see paragraph 2.15. Intercellular Communication).

Studies on aged mice have revealed an overall decrease in HSC cell cycle activity, with old HSCs undergoing fewer cell divisions than young HSCs [251]. This correlates with the accumulation of DNA damage and with the overexpression of cell cycle-inhibitory proteins such as $p16^{INK4a}$ [252]. In fact, old $p16^{INK4a-/-}$ HSCs exhibit better engraftment capacity and increased cell cycle activity compared with old wild-type HSCs (ibid.). Telomere shortening is also an important cause of stem cell decline with aging in multiple tissues [253].

The accumulation of senescent cells in aged tissues has been often inferred using surrogate markers such as DNA damage. Some studies have directly used senescence-associated β-galactosidase (SABG) to identify senescence in tissues [254]. Of note, a detailed and parallel quantification of SABG and DNA damage in liver produced comparable quantitative data, yielding a total of ~8% senescent cells in young mice and ~17% in very old mice [255]. Similar results were obtained in the skin, lung and spleen, but no changes were observed in heart, skeletal muscle and kidney. Based on these data, it is clear that cellular senescence is not a generalized property of all tissues in aged organisms.

Some authors think that the amount of senescent cells increases with age and that senescence contributes to aging, but this probably undervalues the primary purpose of senescence, which is to prevent the propagation of damaged cells and to trigger their removal by the immune system. They explain that senescence is a beneficial compensatory response that contributes to clearing tissues of damaged and potentially oncogenic cells. This however requires an efficient cell replacement system that involves clearance of senescent cells and mobilization of stem cells and their progenitors to re-establish cell numbers. In aged organisms, this turnover system may become exhausted, resulting in the accumulation of senescent cells that aggravate the damage and contribute to aging [48].

Deficient proliferation of stem and progenitor cells is obviously detrimental for the long-term maintenance of the organism, but excessive proliferation of stem and progenitor cells can also be deleterious by accelerating the exhaustion of stem cell niches, which can be compensated by stem cell quiescence over the long-term. This has been demonstrated in *Drosophila* ISCs, where excessive proliferation leads to exhaustion and premature aging [256] and in p21-null mice, which present premature exhaustion of HSCs and NSCs [257].

Recent studies have shown that an increase in FGF2 signaling in the aged muscle stem cell niche results in the loss of quiescence, stem cell depletion and diminished regenerative capacity, whereas the suppression of this signaling pathway reverses these defects [223]. This opens up the possibility of designing strategies aimed at inhibiting FGF2 signaling to reduce stem cell exhaustion during aging.

As a mechanism to protect themselves from acquiring damage, many stem cells are resting for a long time in a quiescent state. During this time they are protected from replicative damage, but they are more susceptible to mutations [258]. However, although proliferating stem cells are more likely to encounter DNA damages [259], they repair that damages more accurately than do quiescent stem cells.

In addition to DNA damage, excessive mitogenic signaling is the other stress most robustly associated with senescence. A recent account listed more than 50 oncogenic or mitogenic alterations

that are able to induce senescence [260]. The number of mechanisms that implement senescence in response to this variety of oncogenic insults has also grown, but still, the originally reported $p16^{INK4a}$/Rb and $p19^{ARF}$/p53 pathways remain, in general, the most important ones [261]. The relevance of these pathways for aging becomes even more striking when considering that the levels of $p16^{INK4a}$ (and to a lesser extent also $p19^{ARFARF}$) correlate with the chronological age of essentially all tissues analyzed, both in mice and humans [262, 263]. *INK4a*/*ARF* locus was actually determined as being genetically linked to the highest number of age-associated pathologies, including several types of cardiovascular diseases, diabetes, glaucoma, and Alzheimer's disease [264]. Although the activation of p53 and *INK4a*/*ARF* is a beneficial compensatory response that prevents the propagation of damaged cells, under the stress conditions the p53 and *INK4a*/*ARF* responses can become deleterious and even accelerate aging [2].

Taken together, cellular senescence is a beneficial compensatory response to damage, but it becomes deleterious and accelerates aging when tissues exhaust their regenerative capacity. A moderate enhancement of the senescence-inducing tumor suppressor pathways may extend longevity [265], whereas at the same time, elimination of senescent cells in an experimental progeria model delays age-related pathologies [266]. Therefore, two interventions that are conceptually opposite are able to extend healthspan.

2.15. Altered intercellular communication

Beyond intrinsic cellular alterations, aging also involves changes at the level of intercellular endocrine, neuroendocrine or neuronal communication [267, 268]. As during the aging inflammatory reactions increase, immunosurveillance against pathogens and premalignant cells declines, and the composition of the peri- and extracellular environment changes, neurohormonal signaling (i.e., renin-angiotensin, adrenergic, insulin/IGF-1 signaling) is consequently deregulated, which affects various mechanical and functional properties of all tissues [48].

An important age-associated pathological finding in the intercellular communication in mammals is so called "inflammaging," i.e., an appearance of pro-inflammatory phenotype that accompanies aging. Several authors proposed that aging is accompanied by a chronic up-regulation of several pro-inflammatory responses. [35, 269, 270]. Inflammaging may result from multiple causes such as the accumulation of pro-inflammatory substances, tissue damage, the failure of the aged immune system to effectively clear pathogens and remove dysfunctional host cells, the secretion of pro-inflammatory cytokines by aged immune cells, the enhanced activation of the NF-κB transcription factor, or from a defective autophagy response. These defects and alterations result in an enhanced activation of the NLRP3 inflammasome and other pro-inflammatory pathways, finally leading to increased production of interleukin 1ß (IL-1ß), tumor necrosis factor and interferons [271]. Inflammation is also involved in the pathogenesis of obesity and type 2 diabetes, two conditions that contribute to, and correlate with aging in the human population [272]. Likewise, defective inflammatory responses play a critical role in atherosclerosis [273].

Another link between inflammation and aging derives from the finding that inflammatory and stress responses activate NF-κB in the hypothalamus and induce a signaling pathway

that results in reduced production of gonadotropin-releasing hormone (GnRH) by neurons [274]. This GnRH decline can contribute to numerous aging-related changes such as bone fragility, muscle weakness, skin atrophy, and reduced neurogenesis. These findings suggest that the hypothalamus may modulate systemic aging by integrating NF-kB-driven inflammatory responses with GnRH-mediated neuroendocrine effects.

Besides chronic inflammation, aged immune cells are prone to a multitude of deteriorating factors. Age related defects of innate immunity are observed not only in the macrophage/monocyte compartment, which is probably the main "culprit" of inflammaging, but also in other cells, i.e., NK cells, dendritic cells, and granulocytes, whereas the defects of adaptive immunity are observed in both the B-cell and the T-cell compartments. Aging of the immune system or "immunosenescence" is characterized by a time-dependent functional alteration of immunity leading to immunodeficiency [275, 276] that manifests in chronic inflammation [277], reduced resistance to infections [278], poor responses to vaccination [279], and increased incidence of autoimmunity and cancers. Similarly, the involvement of immune processes in clinical conditions, such as atherosclerosis, diabetes, and dementia, have been described [280, 281]. The impairment of the immune system exerts an influence on the increased morbidity and mortality observed in human subjects as they age [282].

There is also accumulating evidence indicating that aging-related changes in one tissue can lead to aging-specific deterioration of other tissues. Typical case are the inflammatory cytokines that can cause so called "contagious aging". In certain bystander effects senescent cells induce senescence in neighboring cells via gap junction-mediated cell-to-cell contacts and processes involving ROS [283]. The microenvironment contributes to the age-related functional defects of CD4 T cells, as assessed by using an adoptive transfer model in mice [284]. Likewise, impaired kidney function can increase the risk of heart disease in humans [285]. Conversely, lifespan-extending manipulations targeting one single tissue can delay the aging process in other tissues [286].

Defective intercellular communication underlying aging processes, including genetic, can be restored by nutritional or pharmacological interventions that may improve the cell–cell communication properties lost with aging [48]. Of special interest in this regard are the CR approaches to extend healthy lifespan [287] and the rejuvenation strategies based on the use of blood-borne systemic factors identified in parabiosis experiments [288, 289]. Moreover, the long-term administration of anti-inflammatory agents, such as aspirin, may increase longevity in mice and healthy aging in humans [290, 291]. Finally, it also appears possible to extend lifespan by manipulating the composition and functionality of the intestinal bacterial ecosystem of the human body [292]. The near future research will undoubtedly bring spectacular results in this field of human physiology that will also be translated to the clinical medicine.

3. Conclusion

Although the stem cells are often considered "a fountain of youth" they are subjected to various aging and degenerative processes. Contrary to somatic cells, they have developed a

plethora of mechanisms that prevent or delay aging and age-related pathology. Over recent decades we have witnessed an immense increase in advanced therapies. The cells used in therapeutic products must meet stringent standards of quality. The huge increase in stem cell based therapies especially demands that we use the most advanced analysis of stem cell grafts to ensure optimal performance.

The aging of stem cells is an important biological factor that contributes to the general aging of an organism. Therefore, senescence and the age related status of grafted stem cells have to be taken into account in every stem cell based therapy, as well as in tissue engineering procedure. Further research on the cellular mechanisms leading to the aging of stem cells will not only answer various burning questions related to current cell based therapies, but also pave the way to designing future counter-aging procedures.

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