Chapter 3
Biomedical Research in Aging

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Abstract Biomedical research has been instrumental in identifying key molecular and cellular changes that occur throughout the aging process, also known as the Hallmarks of Aging. Notably, these are shared between humans and several other species that have served as models for the study of aging in the laboratory. In this chapter, we discuss current knowledge regarding the significance of hallmarks such as: decay of stem cell function, acquisition of genomic instability, DNA damage, telomere attrition, deregulated nutrient sensing, chronic inflammation and cellular senescence. We further describe current methodological issues, experimental techniques and best practices for the study of each hallmark across different *in vivo* and *in vitro* systems, while also pointing at their limitations. Finally, we provide future perspectives for the improvement of experimental designs in biomedical research of aging.

Keywords Stem cells · DNA-damage · mTOR · Inflammation · Epigenetics · Senescence · SASP

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3.1 Introduction

The main goal of biomedical research is to increase elemental knowledge on the functional mechanisms that underlie both normal and pathological life processes, with a particular attention to those affecting human health. Aging is one such process and occurs widely across organisms. It involves a time-dependent decline in function that spans all organizational levels; from biomolecules to cells, tissues and organs. As is it also the case for many other biological phenomena, the fundamental factors that drive aging in humans appear to be shared to a significant extent with other lifeforms such as yeast, nematode worms, fruit flies and rodents. By studying the events affecting lifespan and time-dependent health deterioration in these models, biomedical research has led to the identification of a number of common denominators of the aging process [1]. These Hallmarks of Aging, include stem cell exhaustion, genomic instability, telomere attrition, epigenetic alterations, deregulated nutrient-sensing, chronic inflammation, and cellular senescence. Due to space constraints we are unable to discuss two other important hallmarks of aging: the loss of cellular proteostasis and mitochondrial dysfunction. These however have been extensively reviewed elsewhere [2, 3].

It is expected that improving our understanding of their role in aging will lead to the development of novel care strategies and therapeutics for preventing age-related disease in humans. For each hallmark, we present current knowledge regarding their importance in aging and focus on discussing methodological aspects such as *in vivo* and *in vitro* systems, experimental tools and techniques, as well as best practices for their application in the context aging. Finally, we will also discuss current limitations of these approaches and provide future perspectives for the field.

3.2 Stem Cells in Aging

Stem cells (SCs) are characterized by their dual ability to self-renew and differentiate into most, if not all, of a tissue´s specialized cell types [4]. Due to their ability to continually replenish a population of tissue-specific progenitors, SCs are crucial for growth during embryonic development, regeneration following injury and homeostatic tissue maintenance in adult life [5, 6]. Typically, adult SCs are thought to be essentially immortal, although they are susceptible to damage accumulation during life. In order to appropriately function, SCs also require the action of surrounding supporting cells which are collectively known as the SC niche [7].

As SCs sit atop the hierarchy of cellular differentiation, any dysfunction has significant deleterious consequences to their tissue of residence. It is therefore thought that loss of adult SCs or their functional decline over time results in the tissue and organ dysfunction that is observed in old age. This view is supported by the numerous aging phenotypes described in SCs of the intestine, brain, muscle, skin, germline and hematopoietic system, among others [8]. Although the aging

phenotypes of SCs can vary depending on the tissue context, many SCs display conserved characteristics including: support by a niche, unique metabolic requirements, telomerase expression (to prevent replicative exhaustion), cycling between quiescent and activated states, and asymmetric distribution of macromolecules during differentiation [9]. Importantly, these common SC characteristics are known to be perturbed in normal aging by the processes further described in this chapter (e.g. senescence, DNA damage, epigenetics). The role and biology of stem cells in aging has been exhaustively reviewed elsewhere [8, 10–13].

3.2.1 Identifying Stem Cells

The SC definition requires the experimental demonstration that the candidate cell is undifferentiated, able to self-renew (i.e. to divide-indefinitely) and capable to give rise to a tissue's specialized cell types [4, 14]. Experimental approaches such as flow cytometry analysis [15], *in vitro* clonogenicity and differentiation assays [16], immunohistochemistry [17], transplantation studies [18, 19] and genetic lineage tracing [20] have been employed to identify SC populations in most organ and tissue systems. The variability of the SC phenotype has led to the development of specific approaches depending on the tissue in question. Both functional and transcriptomic profiling studies have yielded a plethora of biomarkers used for the isolation and identification of various adult SC populations *in vivo* [17]. It should be noted however, that SC biomarkers should not be over-relied upon, especially in aging studies. Many SC biomarkers are commonly identified in young or developing animals and the utility of these markers in aged organisms should not be assumed to be unequivocal. For example, in the human hippocampus the expression of some neural stem cell/progenitor markers is not affected with age, however a significant decrease directly was found in the expression of the proliferation marker Ki67 and other neuronal markers [21]. This suggests that the simple presence of SC markers is uninformative with respect to their function and that conclusions should not be directly drawn from studies on younger SC compartments.

Following the identification of a potential SC population, they can be transplanted to a host animal to test their ability to self-renew and differentiate [18, 19, 22]. Coupling transplantation with genetic cell labeling allows the tracing of SC fate over time and provides the ability to ascertain a SC's potential for differentiation and self-renewal. Genetic cell ablation studies in which the putative SC is selectively killed can also be conducted to demonstrate tissue loss of homeostatic/regenerative potential [23, 24]. Following this, transplantation experiments can be performed where the candidate SC can be returned to the depleted tissue to test for functional recovery. Experiments involving Cre-Lox genetic lineage tracing [20] and/or cell ablation [25] studies must be careful to investigate whether the promoter selected to drive gene expression is specific to the SC compartment. This prevents potentially labelling/killing differentiated cells and obtaining confounding results. Specificity can be demonstrated by examining the co-localization of reporter

expression with SC and differentiation markers by immunohistochemistry soon after activation of the reporter in the putative SC compartment.

3.2.2 Technical Considerations for Studying Stem Cell Aging

Aging can impact SC populations in several ways, including a reduction or increase in SC numbers, reduced proliferative capacity and skewed, or absent, differentiation capacity [8, 12, 13]. These consequences are not mutually exclusive and vary depending on the tissue. SC function is impacted upon by their present intrinsic properties as well as the extrinsic effects of their niche and circulating factors [14]. As these intrinsic and extrinsic factors are affected with age, they are important variables to consider and disassociate when investigating SC aging, especially in the context of cultured SCs in which many extrinsic factors will be unlike those *in vivo*. Comparisons of the impact of aging on adult SCs from different tissues has revealed varying characteristics which are both cell-intrinsic and extrinsic [8].

In vitro analysis for speculative SCs is most commonly performed through clonogenicity and differentiation assays. Clonogenicity assays assess the ability of a population of cells to form colonies during *in vitro* culture [16]. However, this technique has several limitations when used in isolation. For example, non-SC populations in the tissue of interest may have sufficient proliferative potential to form colonies or the culture media may not have the necessary composition to promote SC growth. The latter point is also pertinent for *in vitro* differentiation experiments in which SCs are induced to differentiate into a more specialized cell type along a defined lineage. Media composition not mimicking the extrinsic SC niche cues may prevent effective differentiation, or conversely, a cultured cell may be induced to differentiate in an artificial manner not resembling an *in vivo* scenario [26]. In regard to aging studies, providing the same extrinsic factors to young and old SCs may be informative in understanding the intrinsic or extrinsic nature of a given aging phenotype. However, the failure to adequately reproduce the systemic and niche derived factors can result in misleading interpretations. For example, Neural SCs (NSCs) decrease in number during aging, resulting in reduced neurogenesis [27, 28]. Nevertheless, functional *in vitro* analysis did not show significant differences between young and aged NSCs, suggesting that a cell-extrinsic mechanism is involved in their regulation [29].

This notion is supported by experiments conducted through heterochronic parabiosis, an experimental surgical procedure in which two age-mismatched animals have their circulatory systems surgically attached. Using this approach, it was shown that proliferative decay of aged NSCs can be ameliorated by a younger animal´s systemic factors [30–32]. Additionally, heterochronic parabiosis experiments provided evidence that systemic factors can also regulate tissue aging in muscle and liver [33, 34]. A potential improvement to this technique would be to

couple it with genetic cell labeling to evaluate blood chimerism or the contribution of SC populations from the experimental animals to tissue regeneration.

Transplantation studies have been a powerful tool in SC biology. They allow the *in vivo* assessment of self-renewal and differentiation of immunophenotypically-defined cell populations through transplantation into their target tissue [18, 19, 22]. This technique has significant advantages over *in vitro* assays due the normal physiological support that an *in vivo* system offers. Aging research has taken advantage of this system by performing transplantation experiments between animals differing in chronological age (known as heterochronic transplantation) [18, 35]. This approach has revealed the importance of intrinsic factors in regulating the function of hematopoietic stem cells during aging [18, 22, 36–41], whereas cell-extrinsic mechanisms predominantly seem to affect the function of satellite SCs in aging skeletal muscle [34, 35, 42, 43].

3.3 Genomic Instability, DNA-damage and Telomere Attrition

The occurrence of somatic mutations is common throughout a cell´s lifetime. They can be the result of endogenous events such as DNA replication errors or oxidative stress, or from extrinsic physical, chemical or biological insults [44]. If left unchecked, these errors can lead to gene-specific misregulation, or trigger the activation of cell cycle arrest or cell death pathways. The impact of these events at a cellular level are thought to contribute to the aging phenotype by limiting cellular fitness, depleting stem cells pools and thereby limiting regenerative capacity. This ultimately leads to organ function impairment, predisposing to numerous age-associated pathologies [45]. While the types of lesions arising from these genotoxic insults are highly diverse, complex mechanisms exist to safely repair most of them [44]. Although the accumulation of DNA damage is a hallmark of aging [1], it remains unclear whether it is the result of an increased incidence of genotoxic insults, impaired DNA repair capacity or more likely, a combination of both [46].

DNA damage can be assessed using the comet tail assay [47] or by immunostaining against proteins that accumulate upon DNA damage such as 53BP1 or γH2AX [48]. While global changes in the expression of these proteins can be detected in whole-cell lysates by Western Blot (WB), the visualization and quantification of discrete foci can be achieved by immunofluorescence and high-resolution microscopy [49, 50]. In the case of telomere length, a number of approaches are available including Southern Blotting (SB) of telomere restricted fragments, fluorescent *in situ* hybridization (FISH), qPCR and co-staining of DNA-damage markers with telomere specific proteins (e.g. TRF1 or TRF2) [51].

3.3.1 Human Disorders of Premature Aging and DNA-damage Repair

Research exploring many of the premature aging, and DNA-damage repair syndromes in humans has furthered the understanding of the consequences of accumulating DNA damage with age. While the direct contribution of the specific pathognomonic defects to physiological aging remains unclear, collectively they serve as both evidence of a causative role of DNA damage in aging, as well as providing targets for the generation of *in vivo* models to study the aging process (Table 3.1).

3.3.2 Animal Models for the Study of Premature Aging Driven by DNA-damage

In terms of modelling human aging *in vivo*, mice are the most commonly used species as they share a similar aging phenotype. They display however, disproportionately long telomeres in comparison to humans, which may limit their relevance in the context of telomere attrition-driven DNA damage. Arguably, simply allowing mice to grow old naturally derives the most physiologically relevant model, but this is time consuming, costly and susceptible to heterogeneity. There are therefore a host of murine models in which this process is accelerated (Table 3.2). Some of these are closely based on the genetics of human premature aging syndromes, such as Hutchinson-Gilford Progeria Syndrome (HGPS) and Werner's Syndrome (WS), which recapitulate many aspects of the human disease and display several specific features of aging. Others are more global models, such as Telomerase Reverse Transcriptase (TERT) deficient mice [59, 60], that generate critically short telomeres after serial generations, or *Bub1b*$^{H/H}$ mice [60], that have impaired mitotic checkpoint function and develop a rapid, global aging phenotype. The multifactorial nature of aging is exemplified by the crossing of telomerase-deficient and WS mice, where the combination of telomere attrition and impaired DNA repair results in a more rapid progeria phenotype than either model alone [62], suggesting that interplay between multiple pathways is responsible for DNA damage-driven aging.

Genomic instability, DNA damage and telomere attrition are core features of aging. While the study of human syndromes of DNA damage and their related animal models has provided useful insights into the phenotypic consequences of these

Table 3.1 Human premature aging disorders associated with DNA damage

Name	Predominant clinical features	Genetic lesion	Similarities / Discrepancies with physiological aging (PA)	Reference
Laminopathies				
Progeria (Hutchinson-Gilford Progeria Syndrome (HGPS)	Growth impairment, cardiovascular disease, skeletal dysplasia, lipodystrophy, alopecia, skin and nail defects, joint contractures, premature death (2^{nd}–3^{rd} decade).	Sporadic autosomal dominant point mutations in the LMNA gene, resulting in activation of a cryptic splice site and an in-frame 50AA deletion.	HGPS is a prototypic premature aging syndrome.	[52, 53]
RecQ disorders				
Werner's Syndrome (WS)	Short stature (absent adolescent growth spurt), skin atrophy, bird-like faces, lipodystrophy, hair greying, cataracts, Achilles tendon ulceration, type 2 diabetes, cardiovascular disease, osteoporosis, hypogonadism, malignancy, premature death (5^{th} to 6^{th} decade).	Loss of function (LOF) mutation in WRN gene	Generally considered a premature aging syndrome. Minor disparities include: 1) Cataracts seen in WS are typically posterior sub-capsular cf. nuclear with PA. 2) The malignancies seen with WS are not those typical of PA. 3) The increased CVD seen is WS is not associated with hypertension as it is in PA. 4) Osteoporosis is more common in distal limbs in WS rather than the vertebral column as in PA.	[54]
Telomeropathies				
Dyskeratosis Congenita	Core features (≥80%): leukoplakia, nail dystrophy, hyperpigmentaion, bone marrow failure, premature death.	Numerous mutations: Mutations in DKC1 gene, a telomerase component gene (TERC, TERT, NOP10, NHP2, or TCAB1) or a shelterin component (TINF2).	DKC encompasses a spectrum of disorders with the common feature of impaired telomere maintenance. As a result, this group is clinically heterogeneous, and while many features of DKC mimic PA, the predominant feature, and most frequent cause of death is bone marrow failure.	[55, 56]

(continued)

Table 3.1 (continued)

Name	Predominant clinical features	Genetic lesion	Similarities / Discrepancies with physiological aging (PA)	Reference
Other DNA damage syndromes				
Cockayne syndrome (CS)	Cachectic dwarfism, severe neurological manifestations (microcephaly, cognitive deficit), cataracts, sensorineural deafness, pigmentary retinopathy, photosensitivity, joint contractures, accelerated hypertension, aortic root dilatation and cardiomyopathy, premature death (2nd decade)	Two main groups: CSA due to LOF mutations in ERCC8; CSB due to LOF mutations on ERCC6.	Hearing loss and deafness in CS are akin to PA, as is hypertension with its associated end organ damage. Although mitochondrial pathology is a feature of PA, it appears significantly accelerated in CS. No associated increase in malignancy in CS in contrast to other nucleotide excision repair defects and PA.	[57]
Fanconi anemia (FA)	Myelodysplastic syndrome, bone marrow failure, acute myeloid leukemia, osteoporosis, sarcopenia, immune deficiency, endocrine dysfunction, increased susceptibility to malignancy.	19 implicated genes, principally DNA repair genes.	FA is a collective term for a clinically heterogeneous group. The predominant feature of FA is BMF, MDS and AML. Typically, MDS and AML are PA associated diseases with median onset of 50 and 70 years respectively in PA, but these occur prematurely (10 and 30 years) in FA. FA therefore recapitulates bone marrow aging in compartmental fashion rather than global aging.	[58]

Related syndromes/disorders that are not completely considered to reflect premature aging or DNA damage have not been included. HGPS, Hutchinson-Gilford Progeria Syndrome; PA, physiological aging; WS, Werner's Syndrome; CVD, cardiovascular disease; DKC, Dyskeratosis Congenita; CS, Cockayne syndrome; LOF, Loss of function; FA, Fanconi anemia; BMF, bone marrow failure; MDS, myelodysplasia; AML, acute myeloid leukemia

Table 3.2 *In vivo* models of DNA-damage driven premature aging

Disease /Target	Genotype /Lesion	Phenotype	Reference
Disease Specific Models			
Laminopathy models			
HGPS	*Lmna*^{HG} knock-in mice. Accumulation of farnesylated, uncleaved progerin.	Heterozygote (*Lmna*$^{HG/+}$) shows: phenotype onset by 6–8 weeks, premature death (50% by 27 weeks), lipodystrophy, kyphosis, osteoporosis, hair loss, low birth rate. Homozygote (*Lmna*$^{HG/HG}$) shows: low birth rate, severe skeletal abnormalities, death by 3–4 weeks.	[63, 64]
HGPS	Lmna c.1827C>T;p. Gly609Gly. Point mutation resulting in splice variant akin to HGPS. Progerin accumulation.	Heterozygote (*Lmna*$^{G609G/+}$) shows: phenotype onset by 8 months, death by 7–9 months. Homozygote (*Lmna*$^{G609G/G609G}$) shows: phenotype onset from 3 weeks, death by 3–4 months. General features: impaired growth, lordokyphosis, osteopenia, lipodystrophy, cardiovascular dysfunction, vascular calcification, endocrine dysfunction, accumulation of senescent cells, accumulation of cellular DNA damage.	[65, 66]
DNA repair syndromes			
WS	*Wrn*$^{\Delta hel/\Delta hel}$. Homozygous helicase domain deletion. (Note that *Wrn*$^{null/null}$ do not exhibit a phenotype).	Mild premature aging phenotype, higher DNA mutation rate, higher reactive oxygen species levels, hallmarks of a metabolic syndrome (visceral obesity, hypertriglyceridemia, type 2 diabetes, increased cardiovascular risk), increased rate of malignancy, limited lifespan (10-15% shorter than controls).	[67–70]
WS	*Wrn*$^{\Delta hel/\Delta hel}$ / *Terc*$^{-/-}$. Homozygote helicase deletion crossed with telomerase deficient mice, manifesting in critical short telomeres.	Initial phenotype features from 4 months in *G4-6Terc*$^{-/-}$ / *Wrn*$^{\Delta hel/\Delta hel-}$ and include: hair greying, alopecia, osteoporosis, type 2 diabetes, cataracts, impaired wound healing, impaired glucose tolerance, increased rate of malignancy, limited lifespan (median survival 24 weeks compared to 96 weeks in control).	[62]
CS	*Csb*$^{m/m}$. Homozygous truncation of the CBS (ERCC6) gene resulting in CBS deficiency.	Deficient TC-NER photosensitivity, growth impairment, mild neurological dysfunction, deafness, age-dependent blindness, increased susceptibility to skin cancer, normal lifespan.	[71, 72]

(continued)

Table 3.2 (continued)

Disease /Target	Genotype /Lesion	Phenotype	Reference
Non-disease specific models			
Telomerase deficient mice	*mTR*$^{-/-}$. Homozygote deletion of the telomerase RNA component. Telomere attrition manifesting with critically short telomeres by generation 4-6.	Sequential telomere shortening with generations. No phenotype in early generations. Later they show: grey hair, alopecia, skin ulceration and dermal fibrosis, weight loss, increased susceptibility to cancer, impaired wound healing, impaired stem cell repopulation capacity, myeloid skewing, limited lifespan (18 months vs 24 in control).	[59, 60, 73–76]
Bub1b (DNA replication/cell cycle checkpoint)	*Bub1b*$^{H/H}$. Hypomorphic Bub1b. Note that *Bub1b*$^{-/-}$ is embryonically lethal, while *Bub1b*$^{-/H}$ die perinatally.	Impaired checkpoint and DNA repair, accumulation of DNA damage. Normal at birth, onset at 3–4 months, progressive aneuploidy, cachectic dwarfism, lordokyphosis, lipodystrophy, cataracts (nuclear), impaired wound healing, infertility, reduced lifespan (median 6 months).	[61]
Mitochondrial DNA damage	PolgA^{mut} /PolgA^{mut} knock-in.	Results in accumulation of mitochondrial DNA damages and DNA deletion. Normal at birth, onset 25 weeks, kyphosis, alopecia, weight loss, osteoporosis, anemia, cardiomyopathy, impaired fertility, increased mitochondrial mass and impaired mitochondrial function, reduced lifespan (median 48 weeks).	[77]

HGPS, Hutchinson-Gilford Progeria Syndrome; CVD, cardiovascular disease; DNA, deoxyribonucleic acid; WS, Werner's Syndrome; CS, Cockayne syndrome; TC-NER, transcription-coupled nucleotide excision repair; NER, nucleotide excision repair; AT, Ataxia-telangiectasia; RNA, Ribonucleic acid

aberrations, the process of aging is complex and multifactorial, meaning that there is not (nor is there ever likely to be) a single robust model.

3.4 Epigenetics of Aging

Epigenetic mechanisms can control gene expression in a heritable manner without altering the underlying DNA sequence. Histone modifications, chromatin reorganization, DNA methylation and other epigenetic mechanisms are capable of

Table 3.3 Epigenetic alterations in aging, with comments on experimental data sources and model relevance

Epigenetic alteration	Description / Evidence of relevance	Model system note	Reference
Histone Loss	General loss of histones in aging cells across multiple species. Overexpression of histone in yeast extends lifespan.	Observational data from human and murine samples. Functional data from yeast.	[79, 80]
Histone modifications	**Methylation** Yeast and worms show global decrease in repressive (H3K27me3) and increase in activating methylation marks (H3K4me3), with resulting changes in gene expression in aging.	The changes observed in yeast and worms are often not recapitulated in flies and mammals, raising questions about their validity in those systems. The relationship between histone methylation and aging in flies and mammals is less clear.	[82, 102]
	Acetylation Increased acetylation is observed with aging. Overexpression of deacetylases extends lifespan.	The global changes in acetylation seen are conserved across species. Mouse models of overexpression of sirtuin show varying degrees of resistance to aging, and depletion of SIRT6 results in a progeria phenotype.	[83, 85, 87, 88, 103–107]
Chromatin re-organization	Global heterochromatin loss, with focal reorganization and SAHF formation in senescent cells.	Human aging and progeria models show global heterochromatin loss with focal reorganization. Senescent cells undergo chromatin reorganization and SAHF formations, directly contributing to the phenotype. Modulating HP1 in *Drosophila* alters lifespan.	[92, 94]
DNA Methylation	Global hypomethylation associated with aging, but with regions of focal hypermethylation. No evidence that manipulating DNA methylation alters aging or lifespan.	Lower complexity organisms (yeast, worms and flies) have no, or limited, DNA methylation. Mammalian systems are therefore better placed to study DNA methylation. All findings are observational.	[95, 96]

DNA, deoxyribonucleic acid; SAHF, senescence-associated heterochromatin foci; HP1, heterochromatin protein 1

dynamically altering gene expression in virtually every cell type and tissue. A host of epigenetic changes occur with aging (Table 3.3) [78], and these have attracted particular attention because of their reversible nature. This makes them potential therapeutic targets for limiting the effects of aging and extend both health- and lifespans.

3.4.1 Histone Alterations

Age-associated loss of histone proteins has been described in a number of organisms, including humans [79, 80]. While the mechanisms controlling this global histone loss remain unclear, it has been possible to show that increasing the histone supply extends cell lifespan in yeast, suggesting that the histone dosage is critical [79].

Global changes in histone modifications can be assessed with modification-specific antibodies visualized by immunostaining or WB. Their functional effects however, are best characterized by identifying genes, promoters or other DNA regions with which they interact. This is best achieved with chromatin immunoprecipitation (ChIP) and qPCR (ChIP-PCR) or Next Generation Sequencing (NGS) (ChIP-seq).

The modification of histones by the addition or removal of a methyl or acetyl group directly regulates gene expression. Histone methylation can have either activating or repressive effects on gene expression. In the nematode worm *C. elegans*, researchers found that aging involved a global gain of activating histone methylation marks (H3K4me3) and loss of repressive marks (H3K27me3), and that restoring their normal levels could extend lifespan [81, 82]. However, the global gain of activating marks and loss of repressive ones in aging does not appear to be preserved in other species such as flies or mammals [78].

In contrast to histone methylation, the relationship between histone acetylation and aging is better established. Evidence suggests that acetylation increases with age and inducing hypoacetylation extends life- and healthspan. For example, spermidine is a naturally occurring polyamine that induces histone H3 deacetylation through inhibition of histone acetyltransferases. The levels of spermidine decline with age, and administration of supplemental spermidine extends lifespan in yeast, flies, cultured human immune cells and mice [83]. In addition, the role of sirtuins, which are NAD-dependent protein deacetylases, has also been extensively studies in yeast, worms and mammals. The overexpression of Sir2 was first shown to extend lifespan in yeast [84], then worms [85] and flies [86]. Mammalian models also seem to show a similar trend, as mice overexpressing different sirtuins display phenotypes related to delayed aging as improved health, resistance to DNA- and metabolic damage, diminished age-associated changes in the HSC compartment and even lifespan extension [87–89].

3.4.2 Chromatin Reorganization

The physical structure of chromatin has significant impact on gene transcription [90]. Heterochromatin is tightly packed DNA and while there are exceptions, these regions are typically not transcribed. Besides histone-modifying enzymes, a number of other factors are capable of controlling heterochromatin and nucleosome organization. Notably, some of these are specifically altered in aging, including

heterochromatin protein 1a (HP1a), polycomb group proteins and the NuRD complex [91]. The 'loss of heterochromatin' model of aging proposes that heterochromatin domains established during embryogenesis are gradually lost with age, leading to transcription of age-associated genes. The role of impaired heterochromatin maintenance aging is supported by data from some of the progeria syndromes and functional studies in flies [92, 93]. However, it is important to note that this model of global loss of heterochromatin is an oversimplification. As an example, one of the most striking features of senescent cells is their chromatin reorganization into senescence-associated heterochromatin foci (SAHF), which can be directly observed with DNA-specific fluorescent dyes such as 4´6-diamino-2-phenylindole. SAHF occur in a coordinated fashion, and directly contribute to the establishment of the senescence phenotype, by regulating specific target genes [94]. The relevance of senescent cells in aging is discussed in Sect. 3.7.

3.4.3 DNA Methylation

In addition to histone modifications, DNA can be directly modified to regulate expression. Methylation of DNA occurs at cytosine-guanine rich regions (known as CpG islands) and is associated with repressed gene expression. While the techniques to study DNA methylation (e.g. bisulfite conversion and sequencing) are relatively straightforward [95], many of the model organisms used to study aging (e.g. yeasts, worms and flies) have little or no DNA methylation [78], significantly hindering functional studies.

The most compelling evidence of an association with aging and DNA methylation comes directly from human studies. With increasing age, mammalian cells undergo global DNA hypomethylation with focal areas of hypermethylation [95], particularly at tumor suppressor genes and polycomb targets [97]. Many of these features are largely recapitulated in mouse and human progeria syndromes [98, 99], and it has been shown that DNA methylation profiles can accurately predict chronological age, age-related pathologies and mortality [100, 101]. This data is however observational, and no experimental data currently exists supporting a direct causative association between DNA methylation and aging.

3.5 Deregulated Nutrient Sensing

Nutrient sensing is a well-orchestrated, evolutionary conserved process that is essential for the survival of all living beings [108]. Whether it is a simple prokaryote or a complex eukaryote, all organisms have the ability to not only sense changes in environmental cues, but also to efficiently utilize nutrients present in the environment for the generation of cellular energy and the building blocks of cells [109]. A host of nutrient sensing pathways ensure a fine balance between anabolism when

nutrients are abundant, and catabolism, during times of nutrient scarcity. Therefore, it is unsurprising that deregulated nutrient sensing is implicated in a range of pathologies (e.g. obesity, diabetes and cancer) and that is also considered one of the Hallmarks of Aging [1]. Nutrient sensing mechanisms vary greatly, and in animals they can range from insulin and insulin-like growth factor signaling (IIS) to the mechanistic target of rapamycin (mTOR) pathway and the somatotropic axis [108, 109]. The IIS and mTOR pathways will be the primary focus of this section, as are tightly linked and are amongst the most evolutionary conserved pathways that are involved in regulating the aging process.

A variety of dietary, genetic and pharmacological approaches have been utilized to study the role of nutrient sensing in aging across a range of species. Dietary restriction is perhaps the most robust approach to improve healthspan so far, as it has been successful in doing so in all tested organisms including primates [110]. In addition, intermittent fasting and the recently demonstrated fasting-mimicking diet (FMD) have also emerged as potential avenues to improve various aspects of health span, including potentially reversing diabetes and ameliorating dementia and cancer [111–113]. Importantly, intermittent fasting also improved several markers and risk factors of aging and age-related disease in humans [114]. However, several caveats remain for interpreting the data in order to understand how these approaches can be applied efficiently in a clinical setting. Many outcomes from these fasting regimens and FMDs will depend on the duration and severity of the fasting regimen, the type of diet used to mimic fasting and the time of onset of the dietary regimen. This is crucial since severe caloric restriction could lead to malnutrition and result in deleterious effects such as immunosuppression and reduced fecundity. Another major hurdle is the practicality of adhering to strict fasting regimes for prolonged periods. Thus, there is still a need for pursuing alternative avenues. To this end, genetic and pharmacological approaches targeting key regulators of metabolic processes such as mTOR or its downstream target, ribosomal protein S6 kinase 1, may pave the way for future anti-aging therapeutics [115, 116].

Rapamycin is a naturally occurring inhibitor of mTOR and rapamycin treatment is perhaps the most robust chemical intervention able to prolong lifespan in various organisms [117]. Similar to dietary regimens, *in vivo* chemical inhibition experiments require careful consideration of dosage, length of treatment and the onset of the chemical intervention, as these will affect the robustness of the findings and their relevance for human translation. Nevertheless, rapamycin treatment was shown to extend lifespan even if given intermittently in middle-aged mice, suggesting that adult-onset treatment is sufficient [115, 118]. Important factors to consider are feeding time of the day and fed status at the moment of sacrifice. In this case, over-night fasting provides a common circumvention that ensures all animals will be at a baseline status for tissue analysis. The genetic background and housing hygiene can also have profound effects on the observed phenotype in long-term metabolic and aging experiments where the immune system can have a major influence. It should also be unsurprising that manipulating many of these metabolic pathways can result in sexually dimorphic phenotypes, thus it is imperative for any aging study to study both sexes. For example, rapamycin increased lifespan in females at lower doses than

males, possibly due to sexual dimorphisms in drug metabolism [119]. Therefore, similar chemical interventions should involve appropriate pharmacodynamic and pharmacokinetic evaluations at systemic and target tissue levels across sexes. To further highlight this, high doses of intermittent rapamycin treatment to increased male lifespan, whereas it failed to do so in females and also shifted cancer incidence towards a highly aggressive hematopoietic malignancy [118].

In addition to chemical interventions, genetic manipulation of several conserved nutrient sensing pathways has been shown to increase lifespan in different model species such as yeast, worms, flies and mice [116, 120–123]. Nevertheless, some limitations to these studies must be noted from a methodological perspective. In the case of genetically modified mice, many of the reported genetic strategies (e.g. gene deletions) are already present during embryonic development, which means that some of the observed phenotypes may be confounded by effects on both embryonic and early-life development, especially as many of these genes are essential for general metabolism and nutrient sensing throughout life. Moreover, these effects might render the experimental strategy unviable. As an example, mice with complete knockout of mTOR display embryonic lethality. Therefore, future approaches should investigate the effects of adult-onset deletion/manipulation of key genes on mammalian lifespan, as this would be the basis for future research exploring therapeutic intervention in humans. Another useful strategy for circumventing embryonic/early life effects is to generate hypomorphic alleles where gene expression is significantly diminished but not absent. As an example, mTOR hypomorphic mice are viable and show a significant extension in lifespan in both genders [124].

3.6 Age-related Chronic Inflammation

Chronic, low-level inflammation in the absence of infection (known as “inflammaging”) is tightly related to many age-related pathologies, including frailty syndrome, diabetes and cancer [1, 124, 125]. In contrast to an acute inflammatory response that is beneficial and promotes tissue repair, this persistent, smoldering inflammation associated with aging is deleterious and can cause tissue deterioration. Inflammaging is characterized by the elevated secretion of several proinflammatory factors (e.g. IL1, IL6, IL8, TNF-α, C-reactive protein and reactive oxygen species) in all tissues. A diverse range of stimuli is thought to contribute to age-related tissue and systemic inflammation such as accumulation of genomic damage, the senescence-associated secretory phenotype (SASP), a defective autophagic response as well as an exhausted immune system that can no longer efficiently clear pathogens and dysfunctional host cells [126].

There is a considerable amount of clinical data implicating chronic inflammation in aging, including that it can predict changes in body composition, metabolic balance, energetic consumption and immune response capacity, besides being a highly significant risk factor for mortality and disease in older adults [126, 127]. Nevertheless, there is a scarcity of experimental evidence supporting inflammaging

as a driver of aging and its related diseases. There are however, findings providing important causal data. A notable example lies in the NLRP3 inflammasome, a protein complex activated by a diverse range of age-dependent "danger signals", including lipotoxic free acids, extracellular ATP and reactive oxygen species. Importantly, *Nlrp3*$^{-/-}$ mice show increased healthspan with improved glucose homeostasis, bone density, muscle endurance as well as protection against immunosenescence (loss of naïve T-cells and B-cells) [128, 129]. In the future, it will be particularly interesting to study the role of inflammatory factors through heterochronic parabiosis experiments, which have previously illustrated that secreted factors present in young blood can rejuvenate cardiac, muscular and cognitive functions in old mice [32, 34, 130–132] (see Sect. 3.2, for further discussion on stem cells).

In any case, delineating the sources and causes of age-related inflammation remain major issues yet to be addressed. In this sense, cellular senescence has recently caught the field's attention as a potential culprit, or at least contributor, to age-related inflammation [126]. As further described in Sect. 3.7, senescent cells secrete a vast array of proinflammatory factors and eliminating senescent cells in aged mice through semi-genetic or pharmacological approaches leads to the reduction of proinflammatory factors Il6, Il1a and Tnf [133, 134]. Moreover, the NLPR3 inflammasome also regulates oncogene-induced senescence and its SASP, therefore some of the benefits of the global NLRP3 deletion may be attributed to preventing senescence in aging [135].

Age-related inflammation can be assessed by a variety of methods, but it is important to combine several approaches to obtain a broader view at tissue and organismal levels. For example, a common method to evaluate inflammation in aged mammals is to measure pro-inflammatory cytokine levels and absolute number of immune cells in the peripheral blood. However, these levels display significant variability among individuals as expected from naturally aged mice, thus a considerable sample size is needed to detect statistically significant differences. Therefore, the inflammatory response of the tissue of interest should also be assessed. This can be achieved by measuring local gene expression levels of various chemokines, cytokines and surrogate markers of immune cells by quantitative polymerase chain reaction (qPCR), WB, enzyme-linked immunosorbent assay or liquid chromatography coupled with mass spectrometry. Additionally, histological assessment of immune cell infiltration can also be conducted by immunostaining against common markers such as CD3 (T cells), B220 (B cells), F4/80 (macrophages), Ly6G (neutrophils) and MHC II [136]. This can be combined with fluorescence-activated cell sorting (FACS) to evaluate various immune cell types in more detail. Although observing cytokine and chemokine expression at the tissue level has historically proven difficult to achieve, novel variants of the RNA *in situ* hybridization technique have provided important solutions to this caveat. One example is the RNAscope® platform, which can be combined with cell-specific immunostaining in order to identify which cell type is responsible for secreting proinflammatory factors of interest in aged tissues [137].

Overall, chronic inflammation is rapidly being recognized as an important driver of aging and age-related pathology, though there is still much to learn about the

nature and origin of this inflammatory response. Novel animal models aimed at manipulating crucial inflammatory pathways will surely provide essential insight into the relationship between inflammation and health span, as well as providing evidence supporting therapeutic targeting of this deleterious inflammatory response.

3.7 Cellular Senescence in Aging

Senescence is a cellular state characterized by an irreversible cell cycle arrest and altered gene expression that has been demonstrated to act as a potent tumor-suppressive mechanism [138, 139]. This phenomenon is induced by various damaging cellular stresses including telomere shortening, a persistently activated DNA-damage response [140] and oncogene activation [141]. Senescent cells are known to induce pleiotropic effects on neighboring cells through an extensive secretome of pro-inflammatory cytokines, chemokines, extracellular matrix proteases and growth factors, which is collectively known as the SASP [142]. While canonically considered an anti-tumoral mechanism preventing the division of damaged cells, recent research has highlighted the involvement of senescent cells in numerous pathological processes, mainly aging and age-associated diseases [143]. Notably, senescent cells are observed to over accumulate in most aging tissues. This has been well characterized in the lung, liver, skin and spleen of aging mice, primates and humans [144–146].

The role of cellular senescence during the normal aging process is currently debated with two main hypotheses being considered. The first postulates that the accumulation of senescent cells and their SASP result in tissue dysfunction leading to an aging phenotype. The second view is that senescence may reduce the regenerative capacity of adult stem cells, which are necessary for tissue homeostatic balance. These hypotheses are not necessarily mutually exclusive and may act concomitantly during normal aging. Evidence supporting senescence having a causative role in aging is derived from observations that p16/INK4A-deficient mice, in which the senescence program is abrogated, have increased lifespans and reduced incidence of age-associated disease. Notably, this effect is also observed in progeroid or normal mice when senescent cells are ablated genetically or with chemical compounds that specifically target senescent cells (i.e. senolytics) [147–151]. However, further work is required to determine the mechanisms by which senescent cells accumulate during aging and their specific effects on the aging tissue microenvironment. Until recently, this area of study was confounded by the lack of available biomarkers and tools to study these processes *in vivo*.

3.7.1 Cellular Senescence: Lessons From In vitro Research

Over the past 50 years, *in vitro* research of cellular senescence has established essential techniques and principles for the study this phenomenon. As senescent cells cannot be grown in culture, an initial challenge was to discover means to induce senescence *in vitro*. A frequently employed technique is the serial passage of primary cells until they reach replicative exhaustion, which triggers telomere attrition, DNA damage and senescence [152]. Other strategies aimed at inducing either widespread or telomere-specific DNA damage, such as ionizing irradiation [140], DNA-damaging drugs [153] or depletion of the shelterin complex [154] are also robust inducers of senescence.

The detection of senescent cells relies on markers that reflect several altered cellular processes. For example, their inability to proliferate is mainly due to elevated expression of cyclin-dependent kinase inhibitors (CDKi) (e.g. p21/Cip1 and p16/INK4A). Other features include cell volume enlargement and expansion of the lysosomal compartment, which is reflected by the increased activity of Senescence-Associated β-Galactosidase (SA-β-Gal) [146]. The latter is the most widely used marker of senescence, although it is not infallible. Senescent cells are also observed to up regulate pro-survival and anti-apoptotic factors [155–159], as well as markers of DNA damage [160]. Finally, senescent cells activate the SASP as a downstream consequence of NF-κB and p38-MAPK signaling [161–163]. The complex nature of this phenotype leads then to a crucial methodological consideration: currently there is no single method able to unequivocally identify senescent cells [164].

3.7.2 Common Methods for the In vivo Identification of Senescent Cells

During the normal aging process, senescent cells are found to accumulate in tissues as evidenced by the increased detection of cells with high SA-β-Gal activity and elevated expression of p16/INK4A [146, 165–168]. To date, the best practice for the identification of senescent cells *in vivo* is combining the use of the aforementioned markers plus demonstrating absence of proliferation. However, results obtained from any single marker should not be deemed definitive, as *bona fide* senescent cells can sometimes lack even some of the most robust markers [169].

Initially, bulk cell populations or tissues can be analyzed for SASP factors, elevated SA-β-Gal activity and CDKi expression by WB or qPCR. This approach has been widely used for demonstrating the presence of senescent cell populations *in vivo* [146]. However, such strategies have significant limitations and results should be interpreted cautiously. For example, many non-senescent immune cells can display a pro-inflammatory secretome resembling the SASP. The composition of the SASP can also be highly heterogeneous depending on the senescence-inducing

stimuli or the "maturity" of the senescent cell, as is the case of some progeroid mouse strains which show elevated levels of senescence in their adipose tissues but with different SASP composition [147, 170]. Also, it is currently unknown if the length of time following the onset of cellular senescence has any qualitative of quantitative effect on the SASP. Therefore, further work is required to characterize the causes and effects of SASP heterogeneity in different cellular and temporal contexts.

The identification of senescent cell populations through analysis of CDKi expression also has several limitations. The most widely used markers, p21/Cip1 and p16/INK4A, are also expressed by many quiescent cell populations [9, 171] or aged immune cells [172]. In the case of p16/INK4A, which is considered a more robust senescence marker, the lack of reliable antibodies has complicated its detection both *in vivo* and *in vitro*. High levels of SA-β-Gal activity are also found in maturing macrophages that can display a pro-inflammatory, SASP-like expression signature, possibly to misleading results when analyzing tissues for the presence of cellular senescence [146]. Following the identification of potential senescent tissues, the senescent cell type should be ascertained through histological approaches such as SA-β-Gal staining multiplexed with immunostaining for specific cell-type markers, as well as lack of proliferation.

3.7.3 Novel Tools and Models for the Study of Cellular Senescence

The *in vivo* identification and study of senescent cells has benefited considerably from the recent development of genetically engineered mice in which a traceable marker is expressed in cells that activate the senescence program. These "senescence-reporting mice" include p16-3MR [173], INK-ATTAC [147] and p16-LUC [165]. It should be taken into account that all of these models are based on the expression of *p16/INK4A* as a senescence biomarker, as this gene can be expressed in non-senescent cells. Furthermore, each model has intrinsic advantages and disadvantages that should be weighted according to the experimental context. p16-LUC mice contain a luciferase knocked-in downstream of the start codon of one endogenous p16/INK4A allele [165]. These mice allow for whole-body luciferase imaging, which permits the non-invasive global identification of senescent cells during aging. However, the luciferase knock-in results in disruption of one copy of p16/INK4A, which is a confounding variable that must be considered alongside appropriate controls (e.g. comparing with p16+/- animals).

The other two models, p16-3MR and INK-ATTAC, not only allow the detection of senescent cells but also permit specifically ablating them by administering certain drugs. However, the deleterious effects of continuous drug administration must be taken into account for their use in longevity studies. As an advantage, p16-3MR and INK-ATTAC do not result in the disruption of one of the endogenous p16/INK4A

alleles, while p16-3MR can also be used to non-invasively identify bulk cellular senescence in tissues due to the expression of luciferase. Another consideration is that since both p16-3MR and INK-ATTAC use different promoter elements, it may be possible that their expression differs depending on cellular context and/or senescence-inducing stimuli. The mechanism of cell ablation in both models also differs, meaning that each may be more efficient at inducing apoptosis depending on the context. As both p16-3MR and INK-ATTAC harbor fluorescent reporters, FACS isolation of senescent cell populations can be performed, which can be further investigated by qPCR and WB for SASP expression. This can permit a more refined comparison of the senescent state across varying age and tissue. Furthermore, single-cell RNA sequencing can also be performed, allowing for data to be obtained on the transcriptomic heterogeneity of individual senescent cells.

New non-genetic tools for studying cellular senescence have also been generated. For example, mesoporous silica nanoparticles capped with galacto-oligosaccharides (GOS) have been used *in vitro* to label senescent cells [174]. These nanoparticles take advantage of the increased β-Galactosidase activity of senescent cells to remove the GOS cap, specifically releasing their cargo within them. The nanoparticles can be filled with dyes such as rhodamine to allow identification of the senescent cells or with genotoxic drugs to ablate them. This tool is potentially advantageous, as it removes the need for breeding of senescence-reporters onto experimental genetic backgrounds and it possesses versatility as the nanoparticles can be filled with a variety of molecules for probing the location and function of senescent cell populations. However, further research is required to ascertain their utility *in vivo,* while the effects of off-target cargo release should also be characterized (for example in macrophages), as they could potentially impact health and lifespan in aging studies.

3.8 Conclusions

Biomedical research has been instrumental in developing our understanding of the molecular and cellular hallmarks that characterize aging, which are conserved among humans and various other species. During the last decade, both *in vivo* and *in vitro* experimental approaches have been applied in these model species, in order to characterize the role of these hallmarks, as well as their underlying mechanisms. However, many challenges remain for basic aging research from a methodological standpoint. An obvious caveat is the fact that lower life forms lack the complexity of higher mammalian systems. Therefore, some of these models lack one or more features that are considered of importance for aging in humans, such as the absence of a complete immune system. Moreover, many of these studies have been conducted *in vitro*. These systems can certainly be useful in specific contexts, such as cell-type specific models for high throughput screening for the discovery of new target genes and pathways, or the identification of novel bioactive drugs. However, in practical terms, the interpretation of findings produced in such systems should be

restricted to the processes that can be addressed by the experimental setup. Given that aging is a multifaceted, multi-system disorder, *in vivo* systems remain the most pertinent models for its study from an organismal perspective. Therefore, an important next step will be the *in vivo* demonstration of results that have been mainly produced in culture. Still, living animal models also have their limitations, one being the highly time-consuming nature of chronological aging experiments in mammalian species, our closest relatives. This difficulty has largely prevented the independent routine validation of results by different groups in diverse locations, an aspect of great importance given the inherent variability of aging studies. However, the greatest challenge will be developing a unified understanding of the role of the Hallmarks of Aging in longevity and age-related disease, as most of these discoveries have been produced in particular mutant animals or experimental models. Finally, it must be noted that translational applications are still in their infancy in this discipline. Nevertheless, the number of clinical trials as well as biotech companies involved in developing anti-aging drugs and therapeutics has grown considerably [175], remarking the importance of current and future biomedical research in aging (see Chapter 17 for a complete description of Future Research in Aging).

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