

J. Robin Harris
Viktor I. Korolchuk *Editors*

Biochemistry and Cell Biology of Ageing: Part III Biomedical Science

Subcellular Biochemistry

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Editors

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Preface

The concept for this sub-series within the long-established Springer Nature *Subcellular Biochemistry* book series came to one of us (JRH) following reading the two popular controversial books on ageing by the late Lewis Wolpert “How We Live and Why We Die” (2009) and “You’re Looking Very Well” (2011).

The field of ageing is indeed large and expanding, so upon completing Parts I and II of Biochemistry and Cell Biology of Ageing (series Volumes 90 and 91), we realised that many exciting research areas had not been included. Thus, we embarked upon the lengthy compilation of Parts III and IV. Even so, some relevant topics remain to be included in one or more future books, and by then updates on some rapidly developing topics may be required.

This, our third book on ageing continues the pattern already established by including a range of interesting biomedical science chapters, each standing firmly on its own but clearly relating to the companion chapters and those previously published. Following the introductory chapter by Professor Vera Gorbunova, University of Rochester, 15 further chapters follow (see the **Contents** list, immediately following). It is clear that to comment here upon the content of these chapters is inappropriate: all are worthy of perusal and more detailed study as up-to-date leads into their respective research area.

We hope that this book will be of interest and value to established researchers, undergraduate and graduate students in the field of ageing and also those with a more general interest ageing. The availability of e-books and e-chapters, often via institutional subscriptions with Springer Link, now increases the access of this material to many. Part IV: Clinical Science will follow shortly and Part V: Anti-ageing Interventions has also been commissioned by Springer Nature.

Mainz, Germany
Newcastle upon Tyne

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About the Editors

J. Robin Harris is an Honorary Professor of the University of Mainz, who specialized in macromolecular electron microscopy. He has been the Series Editor of the Subcellular Biochemistry Series for many years and his broad scientific interests are reflected in the diversity of content of the Series.

Viktor I. Korolchuk is Reader in Molecular Cell Biology at Newcastle University. His scientific interests lie in the area of intracellular protein trafficking and degradation pathways. The current focus of research in his laboratory is autophagy (literally self-eating) where portions of cytoplasm are recruited into intracellular vesicles called autophagosomes and transported for degradation by lysosomal hydrolases.

Chapter 1

Introduction: Progression of the Science of Ageing



Vera Gorbunova and Andrei Seluanov

Abstract We outline the progression of ageing research from ancient history to present day geroscience. Calorie restriction, genetic mutations, and the involvement of the sirtuins are highlighted, along with pharmaceutical interventions, in particular rapamycin. At the cellular level, replicative senescence and telomere shortening are presented in the history of ageing studies. We discuss the roles of macromolecular damage in ageing including damage to nuclear, and mitochondrial DNA, epigenetic and protein damage. The importance inflammation during ageing “inflammageing” is becoming increasingly recognized. Omics-based biomarkers are now proving to be a promising approach, along with comparative studies on long-lived animals. The science is getting closer to understanding the mechanisms of ageing and developing reliable interventions to improve human health.

Keywords History of ageing · Calorie restriction · Genetic mutation · Sirtuins · Rapamycin · DNA damage · Telomere shortening · Senescent cells · Senolytic therapies · Inflammageing · Omics-based markers

Introduction

Throughout history people have been searching for ways to achieve longevity and rejuvenation. The first emperor of China ordered an official search for immortality potion. The Queen of Sheba used water of the Dead Sea to rejuvenate her skin, then Cleopatra traveled from Egypt to build in search of the fountain of youth.

This search for longevity continues to the modern times but now with the use of scientific methods. Ageing research had humble beginnings. For a long time, it was believed that ageing is not a biologically regulated process amenable to interventions. Evolutionary studies of ageing concluded that ageing is a result of declining natural selection due to increased risk of death with age (Medawar 1952). The

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second theory named antagonistic pleiotropy posits that alleles that are beneficial at young age may become detrimental at old age leading to functional decline (Williams 1957). Hence there is no selective pressure to evolve genes that promote longevity. Those who tried to study the molecular mechanisms of ageing were met with skepticism.

This situation changed when it was discovered that mouse lifespan can be extended by calorie restriction (McCay et al. 1935). Works in *C. elegans* showed that lifespan can be increased by lower temperature or food restriction (Klass 1977). In the early 80th seminal studies in worms (Johnson and Wood 1982) and mice (Eicher and Beamer 1980) identified genetic mutants that are long-lived, showing that the lifespan can be controlled by genes. In the 90th the identity of the genes responsible for lifespan extension had been revealed identifying the insulin/IGF signaling pathway as the first regulatory network of ageing (Kenyon et al. 1993). This was followed by identification of another network controlling ageing, namely sirtuins (Kaeberlein et al. 1999; Kennedy et al. 1995), that when modulated can extend lifespan in a variety of organisms from yeast to mice (Kanfi et al. 2012; Tian et al. 2019; Tissenbaum and Guarente 2001).

The success in regulating lifespan genetically instilled confidence that pharmaceutical interventions can also be developed. Multiple drug screens were performed in lower organisms identifying life extending compounds (reviewed in (Carretero et al. 2015)). Fewer compounds were found to work in mice, but some produced reliable lifespan extension. Most notable compound that consistently extended lifespan in a variety of organisms including mammals was rapamycin (Harrison et al. 2009), an inhibitor of mTOR signaling. NIH Intervention Testing Program was established to test life extending compounds in mice. In addition to rapamycin, this program has confirmed several molecules including aspirin (Strong et al. 2008), acarbose (Harrison et al. 2014), and 17-alpha-estradiol (Harrison et al. 2021) to extend lifespan in mice.

In parallel with organismal studies of ageing, our understanding of cellular ageing has been developing rapidly. For a long time, it was believed that human cells taken outside of an organism are immortal. This belief was challenged by the pioneering works (Hayflick and Moorhead 1961) showing that human fibroblasts are mortal and undergo replicative senescence after a fixed number of passages. The mechanism behind replicative senescence was first explained by Alexey Olovnikov, as an “end replication problem” (Olovnikov 1973). This was followed by the discovery of telomerase and telomere shortening (Greider and Blackburn 1985), and the demonstration that ectopic expression of telomerase can bypass replicative senescence (Bodnar et al. 1998). The discovery of telomere shortening as the mechanism of senescence raised hopes that extending telomeres will be sufficient to extend lifespan. However, the realization that telomere shortening is first and foremost a tumor suppressor mechanism, dampened enthusiasm for telomere-extending treatments. Next, senescent cells were found to promote inflammation by secreting inflammatory cytokines (Coppe et al. 2008), indicating that removal of senescent cells may be beneficial. The field of senescence was revitalized by the discovery that elimination of senescent cells in mouse models alleviates the signs of ageing and

even extends lifespan (Baker et al. 2011). This raised enthusiasm for senolytic therapies (therapies that selectively kill senescent cells) as a life extending strategy.

We are still far from understanding the underlying causes of ageing and the regulatory networks that control ageing progression. Attempts had been made to systematize the knowledge about the causes of ageing by identifying a set of hallmarks of ageing (Lopez-Otin et al. 2013). These hallmarks include damage to various cellular components, most importantly, accumulation of mutations in nuclear and mitochondrial DNA, epigenetic alterations, and damage to cellular proteins with the loss of proteostasis. Ageing is also associated with telomere shortening and induction of senescence through both telomere shortening and stress. These processes lead to stem cell exhaustion and dysregulation of intracellular communication leading to dysregulation of nutrient sensing pathways such as insulin/IGF and mTOR signaling.

Since the publication of the “hallmarks of ageing” paper, additional hallmarks have emerged. This includes increased systemic inflammation with ageing, named “inflammageing” (Franceschi et al. 2018), and the demonstration that factors controlling inflammation may have antiageing effect.

More recently, activation of junk DNA, mostly LINE and SINE transposable elements emerged as an important driver of ageing, senescence, and age-related inflammation (De Cecco et al. 2019; Simon et al. 2019). Strategies are being developed that target these genomic parasites.

While genetic mutations are difficult to reverse, the discovery of epigenetic reprogramming (Takahashi and Yamanaka 2006) opened a possibility that the epigenome can be rejuvenated. Indeed partial *in vivo* reprogramming using Yamanaka factors was able to rejuvenate mice, albeit with a risk of death and teratoma formation (Abad et al. 2013; Ocampo et al. 2016). This works were further expanded by applying partial reprogramming strategy to other tissues such as the eye (Lu et al. 2020).

The studies of ageing received a strong boost from the development of omics-based biomarkers and application of machine-learning approaches to biological data. The first such biomarker was the methylation clock that can predict biological age of the sample based on a methylation pattern of a set of CpG sites in the genome (Horvath 2013). This method allows quick assessment whether an intervention has a rejuvenating effect without waiting for the experimental cohort to die. The methylation clocks are now being combined with transcriptomic, proteomic, and metabolomic clocks to achieve a more accurate prediction of mortality.

Omics studies of ageing are rapidly developing, facilitated by progress in genome sequencing and single cell technologies. Transcriptomic Ageing Atlases are now available for the mouse (Tabula Muris 2020) and some tissues of nonhuman primates (Wang et al. 2020), at singe cell resolution, and similar studies are underway for the proteome and metabolome. The hope is that integrating these different platforms will allow identification of regulatory networks that can be modulated to delay ageing.

Genomes of many long-lived animal species have recently been sequenced (Kim et al. 2011), which substantially extended the bestiary of research models used for

ageing research. Studies in long-lived organisms hold promise for identifying novel strategies for longevity and disease resistance. A multi-species transcriptomic study identified DNA repair genes and pluripotency networks to control longevity across species (Lu et al. 2022) pointing out conserved mechanisms of longevity and novel targets for intervention.

In the past few years, ageing research has experienced a rapid growth. Many promising interventions have been shown to extend lifespan and improve health in model organisms and human studies are underway. In addition to academic science, Biotech started investing heavily into ageing research and there is an influx of novel ideas. We are optimistic that geroscience researchers are getting closer to understanding the mechanisms of ageing and developing reliable interventions to improve human health.

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Chapter 2

Chromatin Structure from Development to Ageing



Lorelei Ayala-Guerrero, Sherlyn Claudio-Galeana, Mayra Furlan-Magaril,
and Susana Castro-Obregón

Abstract Nuclear structure influences genome architecture, which contributes to determine patterns of gene expression. Global changes in chromatin dynamics are essential during development and differentiation, and are one of the hallmarks of ageing. This chapter describes the molecular dynamics of chromatin structure that occur during development and ageing. In the first part, we introduce general information about the nuclear lamina, the chromatin structure, and the 3D organization of the genome. Next, we detail the molecular hallmarks found during development and ageing, including the role of DNA and histone modifications, 3D genome dynamics, and changes in the nuclear lamina. Within the chapter we discuss the implications that genome structure has on the mechanisms that drive development and ageing, and the physiological consequences when these mechanisms fail.

Keywords Chromatin structure · 3D genome organization · Epigenetics · Development · Ageing · Differentiation · Cellular senescence

Introduction

Several theories have tried to explain why we age. Among them, the antagonistic pleiotropy theory originally proposed by Williams in 1957 provides a reasonable explanation (Williams 1957): some of the same molecular and cellular processes that contribute to morphogenesis and embryo survival, after reproductive selection

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pressure can lead to ageing phenotypes. More recently, the developmental theory of ageing integrates the observations that several aged-related pathologies are the consequence of a combination of genetic predisposition with environmental factors, and that ageing is also affected by epigenetic changes that occur during embryo development and are driven by environmental influence (de Magalhaes and Church 2005). Hence, understanding embryonic genome structure, dynamics, and developmental epigenetic regulation contributes to understand ageing.

DNA is wrapped in histone proteins in the nucleus, and these proteins are responsible for nucleosome formation to increase chromatin compaction. Depending on the post-translational modifications made to histones, the chromatin will adopt a loose or compact conformation, allowing or preventing the access of transcription factors to the DNA (Fyodorov et al. 2018).

Global changes in chromatin dynamics are essential during development, differentiation, and ageing. At the same time, transcriptional activation or repression of genes is a consequence of the changes in chromatin epigenetic modifications and nuclear organization. Among the molecular changes that are hallmarks of ageing in several species are genome instability, telomere attrition, epigenetic alterations, and cellular senescence. Interestingly, these features have in common chromatin changes. Therefore, long-term maintenance of chromatin structure is required to guarantee proper cell function. If it is compromised early or accelerated, pathologies related to ageing such as progeria occur (Ahmed et al. 2018).

In the first part of this chapter, we describe general information about the nuclear lamina, the chromatin structure, and the 3D organization of the genome. Next, we describe the role of DNA and histone modifications, 3D genome dynamics, and nuclear lamina changes first during development and then in ageing.

Nuclear Architecture

The Nuclear Envelope

The nuclear envelope (NE) is a double phospholipid bilayer composed of an outer (ONM) and an inner nuclear membrane (INM). Nuclear pore complexes (NPCs) connect this double membrane to form channels across the NE that allow macromolecular transport in and out of the nucleus. This highly regulated connection between both membranes allows the nucleus to be responsive to environmental and physiological signals through the association between the INM and the cytoskeleton outside the nucleus and between the INM with the nuclear lamina and chromatin within the nucleus (Burla et al. 2020).

The opposing forces from both sides of the nucleus provide the characteristic round or oval shape of most cell nuclei. The cytoskeleton contributes to the shape and stabilization from outside the nucleus by exerting tensile and compressive forces. Micromanipulation force measurements showed that chromatin regulates the force of the nuclear response to small deformations acting as an elastic spring,

and simultaneously, lamin A seems to offer stiffness to protect the nucleus from large deformations (Stephens et al. 2017; Wang et al. 2018b).

These data suggest that the nucleus is a semiflexible meshwork with lamins, chromatin, and the cytoskeleton regulating the nuclear shape stability and protecting the nuclear material. The stability of the nucleus is required to maintain cellular homeostasis since the perturbation of the NE leads to loss of nuclear compartmentalization, which is a hallmark of several pathologies such as cancer, laminopathies (Houthaeve et al. 2018), and pathological cellular senescence (described below).

Nuclear Lamina

Underneath the INM, the nuclear lamina (NL) is a network of type V intermediate filament proteins and nuclear membrane-associated proteins. Even though the constitution of the NL varies among different cell types, in mammals, the main lamins are lamin A/C, which are variants of the *LMNA* gene, generated through alternative splicing, and lamins B1 and B2, that are expressed from individual genes *LMNB1* and *LMNB2*, respectively. Lamin protein structure consists of three main domains, an N-terminal head, a ~45-nm alpha-helical coiled-coil central rod domain, and a globular C-terminal domain. Furthermore, the three lamins undergo different post-translation modifications such as phosphorylation, acetylation, or O-GlcNAcylation in the case of lamin A. The assembly of the lamins occurs in a hierarchically ordered polymerization pathway that involves homodimerization of lamins, followed by assembly of filaments that form a globular protofilament of 3.5 nm with immunoglobulin domains every 20 nm. These filaments form a thick yet flexible layer of 14 nm localized adjacent to the INM, underneath the NPC (Ahn et al. 2019; Turgay et al. 2017). A schematic representation is shown in Fig. 2.1.

Some of the roles of the nuclear lamina are to maintain the structure of the nuclear envelope, sustain genome architecture, influence transcription, repair, and protect the nuclei against mechanical stress. The multiple scale organization and post-translational modifications of lamins reflect the complex stabilization and the importance of their role in the 3D organization of the genome. Remarkably, mutations in lamins are associated with diseases known as laminopathies, such as Hutchinson–Gilford Progeria Syndrome (HGPS) (Houthaeve et al. 2018).

Chromosome Territories

Early studies by Carl Rabl (1885) and Theodor Boveri (1909) using optical microscopy suggested that interphase chromosomes are segregated in vertebrates and form independent units, called chromosome territories (CTs); many years later, FISH experiments (Fluorescence In Situ Hybridization) supported these observations (Bolzer et al. 2005). In recent years, Hi-C, a high-throughput chromosome

conformation capture technique used to map the 3D genome organization of the genome, confirmed that CTs are independent chromosomes with a subnuclear positioning, with limited contacts between CTs (Lieberman-Aiden et al. 2009) (a schematic representation is shown in Fig. 2.1).

Chromatin 3D Organization and Dynamics

The basic unit of the eukaryotic genomic DNA is the nucleosome, which contains 146 base pairs (bp) of DNA wrapped around a histone octamer conformed by two copies of the core histones H2A, H2B, H3, and H4. Furthermore, the H1 linker histone wraps this structure forming a chromatosome. The interaction between these units forms a 30 nm fiber, further organized into higher-order chromatin structures. The chromatin compaction level is dynamic and facilitates several processes such as transcription, replication, and DNA repair. Several factors regulate chromatin compaction, such as histone post-translation modifications. Here we refer to specific modifications using a nomenclature in which the histone, residue number, and type of modification (methylation, acetylation) are sequentially denoted. For example, the addition on one methyl group to histone H3 at lysine 4 will be referred to as H3K4me1. Chromatin compaction is regulated by additional factors, including chromatin remodelers and histone variants; based on the level of such compaction, chromatin can be roughly classified into two types: euchromatin and heterochromatin (Morrison and Thakur 2021).

Heterochromatin is a highly condensed structure mostly contained in pericentric regions and telomeres, and is localized at the nuclear periphery and surrounding the nucleolus. It is enriched in the histone marks H3K9me3 H3K27me3 and has low transcriptional activity. Furthermore, heterochromatin is classified into constitutive (CH) and facultative heterochromatin (FH). CH is highly stable heterochromatin, invariable, and primarily contained in repetitive sequences. In contrast, FH is more dynamic since it can switch to euchromatin during development and differentiation and responds to stimuli in a cell type-specific manner (Allshire and Madhani 2018). On the other hand, *euchromatin* is a less condensed structure, enriched in the histone marks H3K4me3, H3K27ac, and H3K36me. In addition, the flexible folding of euchromatin facilitates the binding of the transcriptional machinery, allowing a higher transcriptional activity (Morrison and Thakur 2021).

The dysregulation of the chromatin compaction contributes to some pathologies such as premature ageing. Also a global alteration of heterochromatin regions contributes to the establishment of cellular senescence (Narita et al. 2003; Shumaker et al. 2006; Tsurumi and Li 2012). These features are further described below in the ageing section.

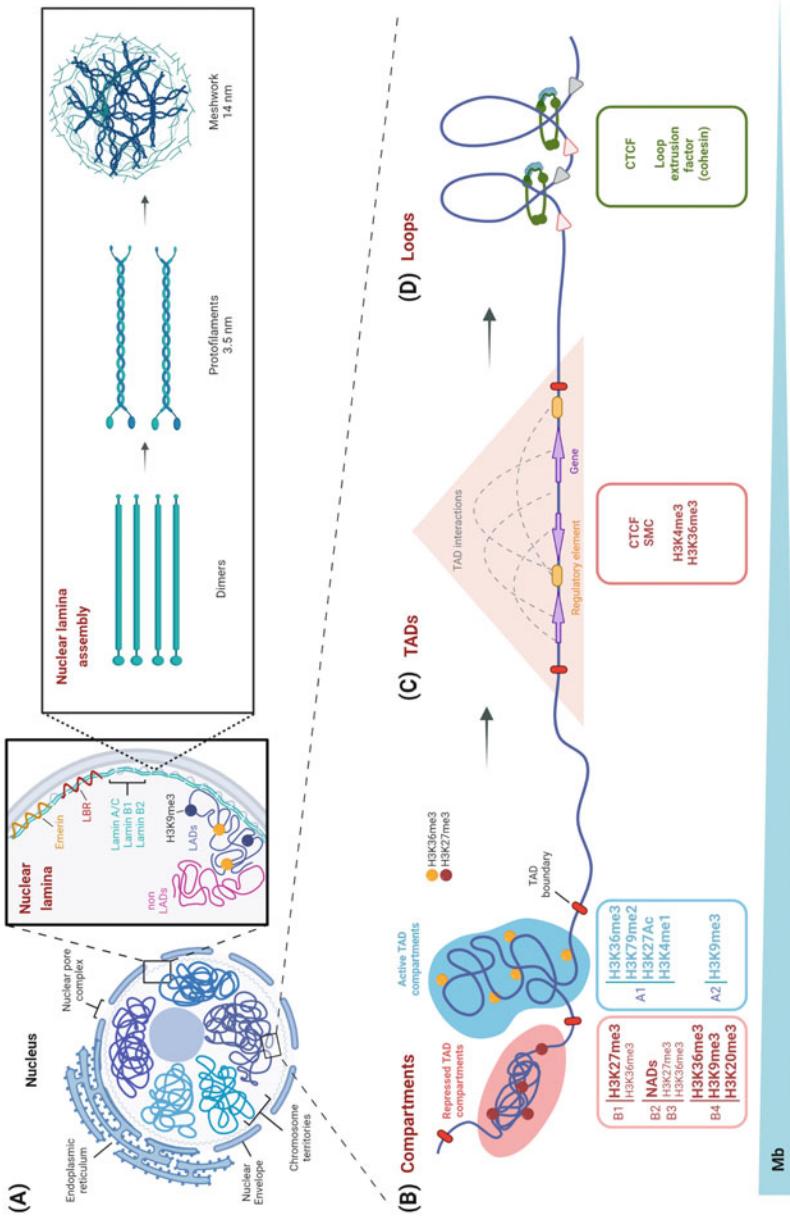


Fig. 2.1 Nuclear architecture and compartments. **(a)** The cell nucleus has a continuous nuclear envelope with the endoplasmic reticulum. The cytoskeleton of the nucleus contains a network of V-type intermediate filament proteins and nuclear lamina, which are assembled in an orderly fashion by polymerization (at the top). Chromatin has a finely regulated three-dimensional arrangement, segregated at subnuclear positions known as chromosome territories. **(b)** At the megabase scale, transcriptionally active compartments A and transcriptionally inactive compartments B are depicted together with their respective subcompartments,

Fig. 2.1 (continued) associated with different histone marks (those in bold are the most abundant, while the small ones are less abundant). (c) TADs with their respective interactions delimitated by TAD boundaries; some TAD-associated proteins are indicated in the box below. (d) At a kilobase scale, chromatin loops with proteins necessary for their formation are depicted. *LBR* lamin B receptor, *LAD*s lamina-associated domains, *NADS* nucleolus-associated domains, *TADs* topologically associated domains, *CTCF* CCCTC-binding factor, *SMC* structural maintenance of chromosomes. Created with BioRender.com

Compartments

Analysis of Hi-C data reveals that chromosomes are segregated into two types of compartments, visualized in Hi-C maps as a checkerboard-like pattern that reflects the separation of chromatin into active (euchromatin) and inactive (heterochromatin) regions inside the nucleus, since robust interactions occur between regions with the same type of chromatin status (Lieberman-Aiden et al. 2009). Principal Component Analysis (PCA) of Hi-C data confirms the segregation of the genome into two compartments. Furthermore, the identity of compartments A or B is also found detecting epigenetic features: compartment A is more accessible to digestion with the DNase I enzyme, is enriched in the H3K36me3 histone mark and in genes, and has a higher transcriptional activity. On the other hand, compartment B has a lower transcriptional activity and is enriched in highly condensed chromatin (Fortin and Hansen 2015) (a schematic representation is shown in Fig. 2.2).

Analysis of a higher-resolution contact map generated through Hi-C revealed that compartments A and B are also partitioned into smaller domains called subcompartments characterized by specific chromatin modification marks. Subcompartments A1 and A2 are gene dense, have high gene expression, and possess activation chromatin marks such as H3K36me3, H3K79me2, H3K27Ac,

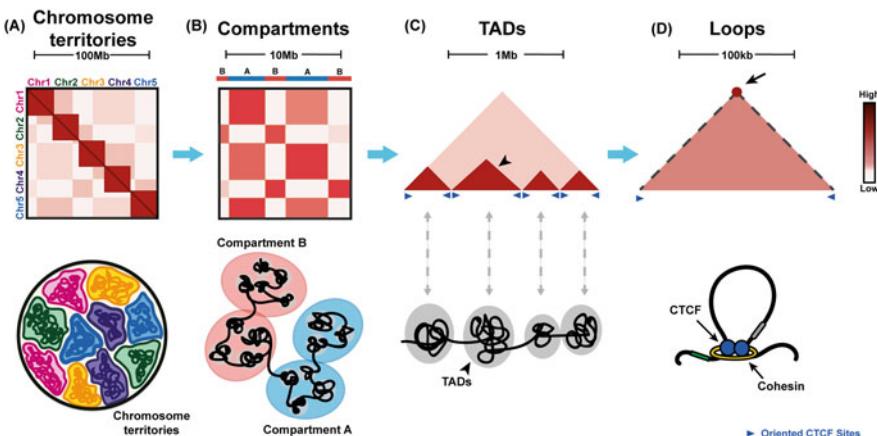


Fig. 2.2 Illustration of Hi-C interaction maps at different scales (at the top) and their interpretation (at the bottom). Genomic coordinates are indicated at map axes, and the interaction frequency between regions is color-coded represented. (a) Whole-genome Hi-C interaction maps show chromosomal territories as a high interaction frequency between regions in the same chromosome that rarely interact with other chromosomes. (b) At the megabase scale, the Hi-C interaction map shows a checkerboard-like pattern of low and high interaction frequency representing compartments A and B, in which interaction frequency is higher between two regions of the same type. (c) At the sub-megabase scale in a half Hi-C matrix, TADs are visualized as triangles of high interaction frequency (black arrowhead), separated by contact depleted regions or TAD boundaries. (d) Finally, an intense interaction frequency at the vertex of a triangle suggests the formation of a chromatin loop formed by the presence of convergent CTCF sites

and H3K4me1. However, A2 has a lower GC content and a stronger association with H3K9me3 than A1. On the other hand, subcompartments B1–B3 have other marked differences. Subcompartment B1 is enriched in H3K27me3 and has a lower correlation with the histone mark H3K36me3, and subcompartments B2 and B3 lack both H3K27me3 and H3K36me3 histone marks, but B2 is highly enriched with pericentromeric heterochromatin and is especially enriched at nuclear lamina and nucleolus-associated domains (NADs). Moreover, B3 is depleted of NADs and is enriched at the nuclear lamina. Additionally, a sixth subcompartment, B4, presents a characteristic pattern enriched with activation marks such as H3K36me3 and heterochromatin-associated marks such as H3K9me3 and H3K20me3 (Rao et al. 2014).

Topologically Associated Domains (TADs)

At the sub-megabase scale, chromatin tends to self-interact in domains called Topological Associated Domains (TADs) isolated from other TADs by regions called boundaries. In a Hi-C interaction frequency map, TADs are observed as squares of high interaction frequencies along the diagonal of the map, or triangles if half the matrix (Lieberman-Aiden et al. 2009) (Fig. 2.2c). TADs are considered the basic unit of the chromatin architectural organization since they are essential platforms to maintain and regulate dynamic cis-regulatory interactions, such as enhancers and promoters. TADs occupy 91% of the mouse genome and were thought to be highly conserved among several cell types and species, with a few exceptions (Rao et al. 2014; Dixon et al. 2012, 2015; Vietri Rudan et al. 2015; Battulin et al. 2015). However, recent single-cell Hi-C experiments reported a notable variability at this scale, challenging this model (Flyamer et al. 2017; Nagano et al. 2017; Stevens et al. 2017).

TAD boundaries have strong interaction frequencies isolating the domain that they delimit. They have high occupancy of CCCTC-binding factor (CTCF) and the structural maintenance of chromosomes (SMC) cohesin complex, among other features such as histone marks H3K4me3 and H3K36me3, repetitive elements, and highly transcribed genes (Jin et al. 2013). One of the best-accepted loop and TAD formation models is the loop extrusion model. It states that one or two cohesin complexes are loaded onto chromatin; afterward, chromatin extrudes through the cohesin ring until they stop at two convergent CTCF sites, creating a loop anchor (Beagan and Phillips-Cremins 2020; Sanborn et al. 2015) (Fig. 2.2d).

Lamina-Associated Domains (LADs)

The NL proteins interact with peripherally located chromatin regions called lamina-associated domains (LADs), and they have been identified through different

techniques such as DNA adenine methyltransferase identification, Hi-C, and FISH (Guelen et al. 2008). LADs represent a silent chromatin environment enriched in repressive histone marks such as H3K9me3 and H3K27me3 and hence genes with low expression. As well as TADs, LAD boundaries are demarcated by CTCF, active promoters, and CpG islands (Guelen et al. 2008; Pickersgill et al. 2006).

LADs are dynamic regions since they display periodic patterns of association to the NL, depending on the cell type in specific cellular processes. During terminal differentiation, the association of tissue-specific genes and lineage commitment genes to the lamina is essential to repress their expression (Peric-Hupkes et al. 2010). For example, the artificial recruitment of genes to the NL in mouse fibroblasts results in the transcriptional silencing of those genes (Reddy et al. 2008). However, similar experiments showed that not all genes recruited to the NL result in silencing (Finlan et al. 2008). These studies suggest that the peripheral position is sufficient to repress the expression of just some genes. Therefore, additional mechanisms to modulate the expression of selected genes are also necessary.

Chromatin Restructuring During Development

Early embryonic development is an extensively studied process that involves profound epigenomic changes to ensure correct spatiotemporal gene regulation. Conversely, deficiencies in epigenetic reprogramming lead to developmental defects, diseases, and in many cases, embryonic lethality.

The zygote is the earliest stage of development; this single-cell embryo arises from the fusion of two germ cells, the sperm and the oocyte. Right after fertilization both nuclei provided by each germ cell are visible and are called “pronuclei.” They fuse to give rise to the nuclear zygote creating the new individual genome, which remains silenced for a period that varies in different species. In mice, the zygotic genome activation (i.e., transcription from embryonic genome) occurs at the two-cell stage and in humans between the 4-cell and the 8-cell stage (before that only maternal mRNA is being translated). Development continues to form a *morula* and then a *blastocyst*, an early developmental stage before implantation, where cells commit to develop either extra-embryonic structures (derived from the trophoectoderm) or the cells that will give rise to the whole embryo (derived from the inner cell mass). Cells from the inner cell mass can be isolated and reproduced *in vitro* maintaining their pluripotent capacity to differentiate into all the cell types in the organism and are the so-called embryonic stem cells (ESCs). Establishing the zygotic and early development involves epigenetic changes such as DNA global demethylation, chromatin remodeling, genome reorganization, and transcriptional changes (Fig. 2.3a).

DNA Methylation

DNA methylation is one of the most frequent epigenetic marks in the mammalian genome. It commonly occurs in CpG dinucleotides found mainly in promoter regions of different genes. Depending on its abundance and the site where DNA methylation occurs (inter- or intragenic regions), it can interfere with the correct binding of transcriptional factors and different histone modifications that favor or reduce transcriptional repression (Field et al. 2018). DNA methylation is catalyzed by DNA methyltransferases (DNMTs), resulting in 5-methylcytosine (5mC), and this methylation can be removed by 10–11 translocation cytosine dioxygenases (TETs) that catalyze the oxidation of 5mC in intermediates such as 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), with 5hmC being the most stable and abundant in the mammalian genome (Meng et al. 2015; Unnikrishnan et al. 2019). DNA methylation regulates gene repression, genomic imprinting, transposon silencing, and the X chromosome inactivation, essential processes for development and genomic stability (Bird 2002; Zeng and Chen 2019).

DNA methylation is known for being relatively stable in somatic cells. However, two major global demethylation and remethylation waves occur during germ cell development and early embryogenesis. First, the 5mC reprogramming in the germline ensures sex-specific methylation patterns; eventually, oocytes arrested in metaphase of the second meiotic division (MII, where they remain until fertilization) show moderate levels of 5mC, while sperm shows high levels of 5mC since approximately 90% of CpGs are methylated. Moreover, parental genomes preserve this tendency in the first stages of the zygote pronuclei (Kobayashi et al. 2012; Smallwood et al. 2011).

On the other hand, the methylation reprogramming in the 1-cell early mouse embryo is dynamic and involves the loss of parental methylation marks with few exceptions in imprinting control regions and some retrotransposon prior to implantation. Interestingly, parental genomes have different demethylation mechanisms since the maternal genome goes through passive demethylation that occurs through subsequent rounds of replication. In contrast, the paternal genome undergoes active demethylation by enzymatic modification of 5mC. Ultimately, DNA methylation reestablishes upon implantation (Cardoso and Leonhardt 1999; Gu et al. 2011; Inoue et al. 2011) (Fig. 2.3).

The change from pluripotency to lineage-specific differentiation is associated with wide *de novo* DNA methylation. Mouse *mesenchymal stem cells* (mESCs) display low DNA methylation levels due to the *de novo* methylation and the increase of hydroxylation. This demethylation ensures the plasticity and the pluripotent state of ESCs. However, lineage specification is demarcated by losing the ESC plasticity and acquiring a cell-type-specific identity. There is a global gain of methylation in differentiated cells to silence pluripotency-associated genes. Simultaneously, there is a loss of methylation in lineage-specific genes to allow cellular identity, reported in many differentiation models (Kaaij et al. 2013; Meissner et al. 2008; Stadler et al.

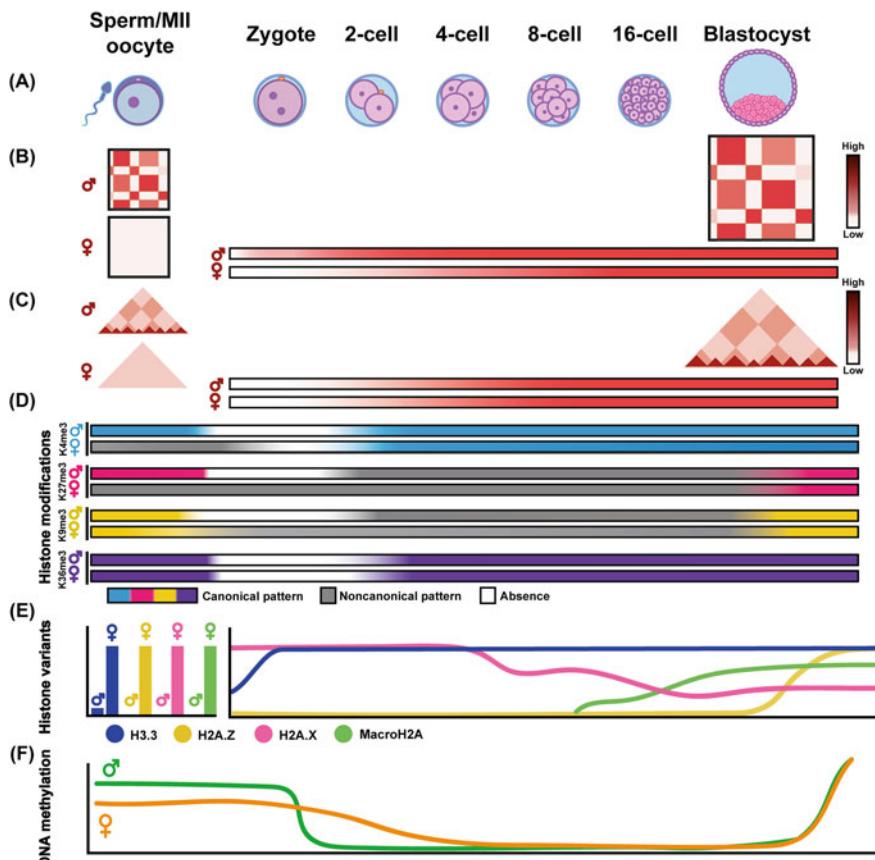


Fig. 2.3 Epigenetic dynamics and genome folding changes during early mouse development. (a) Schematic representation of early mouse development, from gametes to blastocyst, is shown. (b) A Hi-C interaction map represents changes in 3D genome organizations such as compartments and (c) as TADs with color-represented interaction frequency. The bars show the gaining of each pattern during early development in both maternal and paternal genomes. While sperm show a typical pattern of TADs and compartments, oocytes are depleted of both conformations. TADs and compartments appear weak during the first stages and gradually appear as more robust patterns at later stages of embryo development. (d) The gaining of the canonical histone H3 modifications is color-coded represented, with gray regions representing the non-canonical pattern and white areas showing the absence of the histone modification. While sperms show a canonical distribution erased during the first stages in the paternal genome, oocytes and the early stages of the zygote maternal genome show a non-canonical broad domains distribution of H3K4me3 and H3K27me3. After the 2-cell stage, both maternal and paternal genomes reestablish the canonical pattern of H3K4me3. On the other hand, the non-canonical pattern of H3K27me3 is present in both parental genomes and remains until embryo's implantation. During the first stages, H3K9me3 is mainly found at LTRs, silencing their expression in a non-canonical pattern, and they are reestablished in gene promoters in both parental genomes after implantation. H3K36me3 is lost during the first stages of embryo development and deposited on active genes during the 2–4 cell stage. (e) The dynamics of histone variants are color-coded, represented by bars in the gametes and by lines after the zygote stage. While protamines in mouse sperm replace most histones, H3.3, H2A.Z, H2A.X, and MacroH2A are stably expressed in MII oocytes. H3.3 is gained and reestablished during early embryo development, while H2A.X is reduced in the 4–8 cell stage and maintains a low expression onwards, and

2011; Zhao et al. 2014). Interestingly, inhibition of DNMTs in stem cells decreases the differentiation capacity of cells but does not interfere with their self-renewal potential. These results suggest that methylation is crucial for lineage commitment (Chen et al. 2003; Schmidt et al. 2012; Tsumura et al. 2006) (Fig. 2.3f).

Histone Variants

During development, histone variants substitute the canonical histones (H3, H4, H2A, H2B). In particular, variants such as H2A.Z, MacroH2A, and H3.3 have a dynamic expression and perform vital roles during embryonic development, ESC differentiation, and lineage commitment. A summary of the developmental expression and function of some histone variants are presented in Table 2.1 and Fig. 2.3e.

H2A.Z H2A.Z is a variant of the core histone H2A; it shares 60% identity and is highly conserved during evolution. Its expression is first detected in the inner cell mass during the early blastocyst stage before implantation, particularly in pericentric domains (Rangasamy 2003). It is essential to embryo development since H2A.Z loss in mice and *Drosophila* is lethal (Faast et al. 2001; van Daal and Elgin 1992). It is enriched at promoters and enhancers of the Polycomb complex target genes in mESC (Hu et al. 2013). Interestingly, these genes are marked as bivalent by H3K4me3 and H3K27me3; this occupation is necessary for efficient self-renewal and differentiation (Hu et al. 2013; Mikkelsen et al. 2007). Furthermore, H2A.Z occupies a different subset of genes in lineage-committed cells, which could mean that histone variants are another regulatory layer of bivalent promoters in ESCs and that H2A.Z redistribution is necessary for cell fate transition (Creyghton et al. 2008).

In mESCs, the incorporation of H2A.Z enhances HP1a to form a more condensed structure in pericentric heterochromatin (Fan et al. 2004; Rangasamy et al. 2004). Contrastingly, it has also been reported that the binding of H2A.Z promotes chromatin accessibility by destabilizing the nucleosome structure and facilitates nucleosome removal (Hu et al. 2013).

In *Drosophila*, H2A.Z is expressed throughout the first 12 h of embryogenesis and decreases in subsequent stages. Ultimately, it remains with a constant expression through embryogenesis. It is preferentially located at the heterochromatic chromocenter of polytene chromosomes (Leach et al. 2000). In *Drosophila* embryos, 65% of genes activated during zygote gene activation are enriched in H2A.Z, which

Fig. 2.3 (continued) H2A.Z is only expressed later at the blastocyst stage. On the other hand, macroH2A starts expressing during the 8–16 cell stages. (f) DNA methylation dynamic is shown in both the paternal and maternal genomes. After fertilization, parental genomes show global demethylation during the zygote stage, with the maternal genome showing a mostly passive loss, and the methylation is later reestablished after the blastocyst stage

Table 2.1 Dynamic of histone variants gene expression during early mouse development

Histone variant	Change	Function	References
H2A.Z	First detected in the ICM during early blastocyst stage in regulatory regions and heterochromatin. Its expression increases in further stages	Formation of embryonic-specific pericentromeric heterochromatin. Essential for embryo development	Rangasamy (2003), Hu et al. (2013), Fan et al. (2004)
H2A.X	Reduction of expression from blastocyst onward	Important for embryo development and the maintaining of proliferative state of ES cells	Nashun et al. (2010), Celeste et al. (2002), Adiga et al. (2007)
MacroH2A	Activation of expression between the 8-cell and the 16-cell stage, highly enriched afterwards in developmental genes	Regulation of gene repression through binding of chromatin remodeling factors Hallmark of X inactivation	Costanzi et al. (2000), Pasque et al. (2012), Barrero et al. (2013), Gaspar-Maia et al. (2013)
H3.3	Incorporation of maternal H3.3 in the paternal pronucleus post-fertilization and stable expression onwards	Dual function, associated with transcriptional activation and repression in the early mouse embryo and mESC	Torres-Padilla et al. (2006), Martire et al. (2019), Santenard et al. (2010)

precedes zygote gene activation and RNAPII loading onto chromatin, suggesting that H2A.Z is necessary to initiate zygote gene activation (Ibarra-Morales et al. 2021).

H2A.X The classically known function of H2A.X is its role to recruit repair factors after its phosphorylation in response to DNA double strand breaks. Nevertheless, it has additional physiological functions. In mice, H2A.X is abundant during the first cell divisions, and its expression reduces and remains lower from blastocyst onward (Nashun et al. 2010). However, it is not essential for embryonic development since mouse embryos deficient in H2A.X are viable. However, they carry developmental defects such as having a reduced size, lymphocyte deficiency and male mutants are not fertile (Celeste et al. 2002).

H2A.X has not been linked to cell fate transitions but contributes to maintaining the highly proliferative state of ES cells through its function in the DNA damage response pathway (Adiga et al. 2007; Bassing et al. 2003).

MacroH2A MacroH2A is also a histone variant of the canonical H2A. It is undetectable during the first developmental stages and appears between the 8-cell and the 16-cell stage. Afterward, it is highly enriched in the trophectoderm and differentiated somatic cells in the mouse (Costanzi et al. 2000; Pasque et al. 2012). However, it is not particularly essential for development since mice lacking MacroH2A1 and MacroH2A2 isoforms show normal early development, although they have reduced prenatal and postnatal growth. MacroH2A1.2 is the predominant

isoform with low levels in mESCs, but it increases during differentiation. mESCs from mice lacking MacroH2A1 and MacroH2A2 isoforms have no effect on their self-renewal capacity (Barrero et al. 2013).

MacroH2A1.2 is found on developmental genes of terminally differentiated genes occupied by H3K27me3 marks. It is associated with the repression of genes through the interference of the binding of transcription factors and nucleosome remodeling (Barrero et al. 2013; Gaspar-Maia et al. 2013). Correspondingly, the loss of macroH2A altered the activation of differentiation genes in ESC (Creppe et al. 2012).

It is also a hallmark of X inactivation since it is enriched on the Xi. Its deposition depends on the inactive-X-specific transcript (*Xist*) localization between the 8-cell and the 16-cell stages (Costanzi et al. 2000). Unexpectedly, the knockdown of MacroH2A in mice does not affect the X inactivation (Pehrson et al. 2014; Changolkar et al. 2007).

H3.3 H3.3 is one of the most conserved histone variants in eukaryotes. In mice, sperm-derived H3.3 is extruded from the zygote after fertilization, and the maternal H3.3 is incorporated into the paternal pronucleus in the first 2 h post-fertilization (Torres-Padilla et al. 2006).

H3.3 was first associated with active chromatin since 75% of H3.3 occupies regions of active genes in mESC. Moreover, it is a mark of transcriptional activation, enriched in transcribed genes, promoters, and regulatory elements in *Drosophila* and mammals (Trovato et al. 2020). In this sense, it acts as a nucleosomal cofactor for p300 when H3.3 is phosphorylated at serine 31. Moreover, the loss of H3.3 in mESCs shows a reduction of H3K27ac at enhancers (Martire et al. 2019). Besides its association with transcriptional activity, H3.3 has also been associated with heterochromatin formation. In this regard, H3.3 phosphorylated in lysine 27 is essential for heterochromatin propagation during the zygotic S phase in the mouse embryo (Santenard et al. 2010). In both cases, such contrasting activity could be related to different histone marks and the association of H3.3 to cofactors.

On the other hand, H3.3 contributes to the recruitment of repressive complexes such as PRC2 at bivalent promoters of developmental genes and establishes H3K27me3 (Banaszynski et al. 2013). Interestingly, H3.3 is also enriched in the body of active genes but not repressed genes, which correlates with high gene expression in mESCs (Goldberg et al. 2010). Moreover, during mESC differentiation, there is a relocation of H3.3 since it is particularly delocalized from telomeres (Wong et al. 2009).

H3.3 has also been linked to regulating the balance between self-renewal and differentiation of mESCs (Banaszynski et al. 2013). Its depletion in mice embryos causes dysfunction of heterochromatin at telomeres, centromeres, and pericentromeric regions (Jang et al. 2015; Wong et al. 2009). Moreover, loss of H3.3A results in postnatal death of 50% at day 6.5 of gestation, and the surviving animals show several defects such as reduced growth rates (Couldrey et al. 1999).

In *Drosophila* zygotes H3.3 deposition onto the chromatin is mediated by HIRA in the paternal genome, provided by the maternal genome (Bonnefoy et al. 2007;

Loppin et al. 2005). Also, the replacement of H3.2 with H3.2K27 showed that H3K27me3 is required for gene repression during early development. Interestingly, *Drosophila* embryos can develop to adulthood in the absence of H3.3. However, *Drosophila* mutants of H3.3A and H3.3B have reduced viability and both males and females are unfertile (Hödl and Basler 2009; Sakai et al. 2009).

CENP-A CENP-A (centromere protein A) is another variant of histone H3 basally located in the centromere. It is involved in the nucleosomal packaging of centromeric DNA and mitosis. The dynamics of CENP-A during early development and differentiation remain to be analyzed. However, CENP-A appears to be essential for mouse embryonic development since targeted deletion of the gene encoding mouse CENP-A causes mitotic defects and embryonic lethality at embryonic day 6.5 (Howman et al. 2000).

Histone Modifications

Histone modifications are essential epigenetic regulators for embryonic development in mammals that influence the interaction of transcriptional regulators with chromatin. Interestingly, in *Drosophila*, methylation is not a central epigenetic regulator since the level of 5mC is particularly low. Only 0.034% of the total cytosine of the adult fly genome is methylated. The lack of methylation in *Drosophila* suggests that histone modifications and variants are primary sources of epigenetic regulation (Rasmussen et al. 2016; Takayama et al. 2014). A summary of histone modifications along mouse early development is presented in Table 2.2 and Fig. 2.3d.

H3K4me3 H3K4me3 is a hallmark of active promoters since it recruits chromatin remodelers and transcriptional machinery. Gametes show different patterns of H3K4me3; in sperms, most histones are replaced by protamines. However, the residual histones are still subject to modifications. In particular, H3K4me3 is enriched in CpG-rich promoters (Gold et al. 2018; Hammoud et al. 2009). On the other hand, metaphase MII oocytes show a non-canonical pattern of H3K4me3 (ncH3K4me3), where it appears as broad domains at promoters and distal non-transcribed sites, particularly in partially methylated DNA domains, which are deposited by the methyltransferase MLL2 (KMT2B) (Dahl et al. 2016; Hanna et al. 2018; Liu et al. 2016; Zhang et al. 2016).

After fertilization, paternal H3K4me3 is erased, and maternal H3K4me3 is inherited by zygotes briefly. Afterward, both parental H3K4me3 are erased and reprogrammed to the canonical pattern in the two-cell stage. In the following stages, H3K4me3 appears dynamically along with the development and correlates with positive gene expression (Dahl et al. 2016; Lepikhov et al. 2008; Lepikhov and Walter 2004; Liu et al. 2016; Zhang et al. 2016).

Implantation of the mouse embryo involves a genome-wide wave of de novo DNA methylation, except for regions primed for transcription, occupied by H3K4me3; this may indicate that the H3K4me3 pattern form when DNA is

Table 2.2 Histone modifications dynamics during early mouse development

Histone modifications	Change	Function	References
H3K4me3	Dynamic apparition along the mouse development, the occupation correlates with positive gene expression. Shows a non-canonical pattern in the early embryo and recovers the canonical pattern after the 8-cell stage	Activation of gene expression Barrier against repression in post-implantation embryo	Dahl et al. (2016), Hanna et al. (2018), Liu et al. (2016), Zhang et al. (2016), Greenfield et al. (2018)
H3k27me3	Appearance of non-canonical broad domains in early embryo and a more localized occupation is gained after blastocysts stage, in a canonical pattern	Gene repression and modulation of chromatin compaction	Zhang et al. (2016), Chen and Zhang (2020), Liu et al. (2016)
H3K9me3	Enriched in LTRs in early embryo and reestablished in promoters in post-implantation embryo	Remodeling of heterochromatin, repression of repetitive sequences, and regulation of lineage specification	Allshire and Madhani (2018), Becker et al. (2016), Burton et al. (2020), Peaston et al. (2004), Wang et al. (2018a)
H3K36me3	Loss of occupation in parental genomes after fertilization and reappearance upon ZGA	Modulation of transcription, splicing, DNA replication and repair. Important for the establishment of maternal imprints	Wagner and Carpenter (2012)

unmethylated and might provide a template for the genome-wide methylation pattern, protecting genes from repression in later developmental stages (Greenfield et al. 2018). Contrarily, loss of H3K4me3 correlates with DNA hypermethylation at specific CpG of developmental promoters during mESC differentiation into neurons, and genes that gain H3K4me3 show loss of DNA methylation (Meissner et al. 2008).

H3K27me3 H3K27me3 is a repressive mark found at promoters of developmental genes, deposited by the Polycomb Repressive Complex (PRC), and modulates chromatin compaction. Interestingly, H3K27me3 also has a non-canonical pattern in the mature oocyte. It is also found in partially methylated DNA domains, but it resides in different subregions than H3K4me3 (Liu et al. 2016; Zhang et al. 2016). In sperms, H3K27me3 is enriched at developmental gene promoters (Brykcynska et al. 2010; Erkek et al. 2013; Hammoud et al. 2009).

After fertilization, the paternal H3K27me3 is lost and subsequently regained in megabase broad gene desert regions, but with a low signal; this pattern remains until the blastocyst stage (Zhang et al. 2016). Maternal H3K27me3 is lost at promoters of

developmental genes, but the distal H3K27me3 in partially methylated DNA domains remains until the blastocyst stage (Liu et al. 2016; Zhang et al. 2016).

H3K27me3 starts appearing at canonical polycomb target promoters in the blastocyst, and a more robust occupation is gained in post-implantation embryo, particularly in epiblasts cells. Distal ncH3K27me3 also disappears at this stage. The maternal inherited H3K27me3 domains function as an imprinting mark, similar to DNA methylation-mediated imprinting (Chen and Zhang 2020; Inoue and Zhang 2011).

H3K27me3 and H3K4me3 Bivalency Both repressive and active histone marks demarcate bivalent promoters. In mESCs, bivalent promoters with H3K27me3 and H3K4me3 were observed on the same allele (Meissner et al. 2008). Interestingly, most bivalent high CpG promoters become monovalent in more committed cells. For example, in neural progenitor cells, 46% of bivalent high CpG promoters resolve to only H3K4me3, and concordantly genes show increased expression. On the other hand, 14% resolve to H3K27me3, and 32% lost both marks. In both cases, genes show low expression. The 8% remaining keep the bivalent pattern and remain repressed (Mikkelsen et al. 2007). These results might indicate that bivalency is necessary for a lineage-specific expression pattern and to poise developmental genes.

H3K9me3 H3K9me3 is a repressive mark mostly found in constitutive heterochromatin. In the mouse embryo, H3K9me3 regulates the development by remodeling heterochromatin, repressing repetitive sequences, and regulating lineage specification (Allshire and Madhani 2018; Becker et al. 2016).

Upon fertilization, the paternal pronucleus shows no detectable H3K9me3, but it starts accumulating in later stages (Burton et al. 2020; Wang et al. 2018a). Interestingly, global demethylation makes a significant fraction of repeat-rich sequences such as LTR retrotransposons accessible for transcription, vital for zygote gene activation. In consequence LTRs are silenced by epigenetic modifications, including repressive histone marks such as H3K9me3 and H3K27me3 in later developmental stages. Concordantly, during early embryo development, the establishment of H3K9me3 in LTRs correlates with their repression, while it is mostly absent in gene promoters of both parental genomes (Burton et al. 2020; Wang et al. 2018a). Further, in post-implantation embryos, H3K9me3 is reestablished in promoters, regulating lineage-specific gene expression (Nicetto et al. 2019; Burton et al. 2020; Wang et al. 2018a).

Interestingly, in ESCs, H3K9me3 is present in constitutive heterochromatin and transposons and lineage-specific genes, impeding precocious activation of lineage-incompatible genes. Subsequently, this mark is removed in specific genes to allow cell-specific gene expression (Becker et al. 2016). These results indicate that H3K9me3-mediated heterochromatin is a barrier to cell fate and must be reprogrammed after fertilization.

H3K36me3 H3K36me3 is a histone mark involved in DNA replication, transcription, splicing, and DNA repair. It is often found in the body of active genes and is

deposited by the histone methyltransferase SETD2 (Wagner and Carpenter 2012). After fertilization, both parental genomes lose H3K36me3, which reappears in later stages during zygote gene activation (Xu et al. 2019).

In mouse oocytes it is essential to guide DNA methylation through the recruitment of the DNA methyltransferase DNMT3A/B and, thus, establish maternal imprints (Bartolomei and Ferguson-Smith 2011; Chotalia et al. 2009; Xu et al. 2019). Also, it inversely regulates the deposition of H3K4me3 and H3K27me3 since SETD2 depleted oocytes exhibit both marks invasion in former H3K36me3 regions; also, these cells exhibit aberrant DNA methylome, the loss of maternal imprinting, and embryonic lethality (Hanna et al. 2018; Xu et al. 2019).

Nuclear Remodeling in Development

The 3D chromatin organization is dynamic during eukaryotic development and differentiation. During development, epigenetic reprogramming correlates with a reorganization of the high-order chromatin structure, and various studies have employed chromosome conformation capture techniques such as Hi-C to study genome dynamics during these developmental processes.

Studies in germ cells have found that despite sperm cells having a highly compacted genome, consequence of the exchange of histone for protamines and the loss of nucleosomes in large regions of the genome during spermiogenesis, they conserve 3D genome features such as TADs and an organization in A and B compartments. However, the tight packaging of the sperm genome gives rise to the enrichment of long-range contacts compared with fibroblast (Battulin et al. 2015; Jung et al. 2017; Ke et al. 2017). On the other hand, the oocyte organization is dynamic during gametogenesis. TADs, compartments, and loops weaken throughout gametogenesis and disappear in the metaphase II oocyte, where it remains until fertilization (Du et al. 2020; Ke et al. 2017).

After fertilization, the genome undergoes a dramatic reorganization. Early-stage mouse and *Drosophila melanogaster* embryo have relaxed TADs and compartment organization, followed by a gradual establishment of higher-order chromatin in later stages of the embryo, especially after zygotic genome activation (Du et al. 2020; Ke et al. 2017). Eventually, the number of TADs found at the 8-cell stage mouse embryo is similar to TADs found in mESC (Ke et al. 2017).

Parental genomes remain separated until the 8-cell stage due to the formation of two bipolar spindles around pronuclei that keep them apart in the mouse zygote. As a result, both parental genomes undergo allele-specific reprogramming (Reichmann et al. 2018). Low-input and single-cell Hi-C studies in the two-cell stage of mouse embryo have reported that both paternal and maternal TADs and compartments are markedly weak, compared with those in later stage embryos, and interestingly, the paternal compartmentalization is stronger than the maternal genome, which could reflect the nuclear architecture in oocytes and sperms (Collombet et al. 2020; Ke et al. 2017).

Conversely, another study applied single-nucleus Hi-C (snHi-C) experiments in the G1 phase of the mouse zygote. It showed that both parental genomes contain TADs and loops at similar strength when averaging contact maps over previously annotated TADs and loops in CH12-LX cells (Flyamer et al. 2017; Rao et al. 2014). Additionally, A/B compartmentalization was detected in the paternal but not the maternal genome, agreeing with previous work (Flyamer et al. 2017).

Interestingly, TADs present after the 8-cell stage of the mouse embryo showed different derivations since some show bias for the maternal or paternal genome, and most of these domains disappear by the 4-cell stage. In contrast, other domains are progressively gained symmetrically on the two parental genomes at different stages of development (Collombet et al. 2020).

After the 4-cell stage, a rearrangement of A and B compartments has been reported in mice embryos, and the expression of genes changes concordantly. The expression of genes changing from compartment A to B tends to decrease, and genes changing from B to A compartments tend to increase. This change correlates with the unmethylated CpGs status since unmethylated promoters are enriched in A compartments, which have higher accessibility (Ke et al. 2017).

Although the chromatin conformation establishes around zygote gene activation, the formation of domains is not dependent on transcription. A study showed that the inhibition of zygote gene activation with α -Amanitin in 2-cell embryos does not block the establishment of high-order chromatin structures (Ke et al. 2017). Similar results were reported in *Drosophila* since TAD formation is independent of transcription but depends on Zelda for TAD boundary formation. Concordantly, the removal of Zelda leads to the loss of insulation of TAD boundaries (Hug et al. 2017). Notably, blocking DNA replication in 2-cell mice embryos does inhibit the establishment of high-order chromatin structures (Ke et al. 2017).

On the other hand, lineage specification is highly regulated and requires a coordinated spatiotemporal expression of lineage-specific genes. For example, during mESC differentiation into neurons, there is an increase in compartment size with a decrease in interaction strength between A-type domains and an increase in contacts in B-type domains. This compartment rearrangement could be explained by the segregation of heterochromatin observed during cell differentiation (Bonev et al. 2017). On the other hand, less prominent TAD domains changes were observed since most of the boundaries are conserved through differentiation, with a slight increase in insulation. Interestingly, interactions between enhancers and promoters showed to be highly dynamic, and this was established accordingly with gene expression (Bonev et al. 2017).

Similar results were reported during human ESCs differentiation since 36% of the genome displays compartment switching. Interestingly, these changes in compartments correlated modestly with gene expression since most of the genes remain unaffected. Also, minor TAD boundaries were affected, although intra-TAD interactions were dynamic (Dixon et al. 2015) (see Fig. 2.3).

Lamins Regulate Nuclear Structure and Chromatin Organization During Development

The nuclear periphery fulfills a vital role in regulating gene expression during development and differentiation. Changes in the composition of the NL, the association with chromatin, and the epigenetic landscape are part of the regulation of gene expression through these processes.

DNA adenine methyltransferase identification experiments in the mouse embryo showed that LADs are established de novo after fertilization, previous to TAD consolidation (Borsos et al. 2019). However, their establishment in the mouse embryo is dynamic since 42% of LADs detach from the nuclear periphery through early development, but 70% of these LADs regain the association with the NL in blastocysts. Surprisingly, 39% of LADs show colocalization with compartments A instead of compartments B in the 2-cell stage, yet are associated with transcriptional repression and adopt a more conventional pattern from the 8-cell stage onwards (Borsos et al. 2019).

The paternal genome has broad domains, while the maternal genome has smaller and more fragmented LADs with fewer lamina contacts. Also, it was reported that the establishment of LADs does not correlate with DNA replication or H3K9me2/me3. Interestingly, the overexpression of KDM5B, an H3K4me3 demethylase, causes the loss of LADs on the paternal genome at the 1-cell stage, with no effect on maternal LADs (Borsos et al. 2019).

These results indicate that during early development, the organization of LADs is established de novo and matures through development in later stages, with the parental LAD architecture converging after the 8-cell stage of the mouse embryo. Nevertheless, their emergence is prior to TAD formation, which could help prime repressive regions.

On the other hand, cell differentiation is characterized by the spatial segregation of euchromatin and heterochromatin, where heterochromatin tends to accumulate to the nuclear periphery and around nucleoli (Mattout et al. 2015). ESC differentiation into neuronal precursors and differentiated astrocytes entail a dynamic association of LADs with the NL, whereas up to 35% of the genome switches between A and B compartments. Furthermore, genes that detach from the NL do not activate immediately but are more likely to become active in subsequent differentiation steps (Peric-Hupkes et al. 2010).

Although the precise mechanism by which the association of chromatin to the NL regulates the 3D organization of the genome is not yet known, it has been shown that the lamin A/C and lamin B receptor (LBR) are required to tether heterochromatin to the nuclear periphery. For example, the loss of these proteins in the NL leads to global relocalization of the heterochromatin to the nuclear center during the terminal differentiation of rod photoreceptors in nocturnal mammals. The same pattern is observed during neutrophil extracellular traps (NETosis), a form of cell death used as an immune response in terminally differentiated neutrophils (Chen et al. 2016; Solovei et al. 2013).

Programmed Cellular Senescence

Cellular senescence was originally described when studying ageing, cellular damage, or stress. However, a similar phenotype of cellular senescence also occurs during embryo development, and hence has been coined programmed cell senescence. Among the hallmarks of cell senescence are the expression of inhibitors of cell cycle progression such as p21^{Waf1} and p16^{INK4A}, an increase in the activity of a lysosomal protein that has β -galactosidase activity and is coined senescence-associated β -galactosidase (SA- β -gal), and the secretion of molecules such as growth factors, metalloproteinases and cytokines, collectively known as senescence-associated secretory phenotype (SASP). The establishment of programmed senescent cells depends on p21^{Waf1} since *Cdkn1a* (gene coding for p21^{Waf1})-deficient mice have a reduced number of senescent cells and an altered patterning of the neural tube. The presumed function of SASP molecules during development is to contribute to morphogenesis, as programmed cellular senescence is observed in organizing centers during tissue remodeling (Muñoz-Espín et al. 2013; Storer et al. 2013). Programmed cellular senescence is conserved in evolution, as it has been found in mouse, humans, chicken, quail, Xenopus, axolotl, zebrafish, and naked mole-rat embryos (Davaapil et al. 2016; Gibaja et al. 2019; Muñoz-Espín et al. 2013; Nacher et al. 2006; Storer et al. 2013; Villiard et al. 2017; Zhao et al. 2018).

During mouse embryonic development, some tissues with programmed cell senescence identified by SA- β -gal activity are mesonephros, the endolymphatic sac of the inner ear, the apical ectodermal ridge, the tip of the tail, and the closing neuronal tube (Muñoz-Espín et al. 2013; Storer et al. 2013); programmed cellular senescence also occurs in the spinal cord and notochord (Domínguez-Bautista et al. 2021). Programmed senescent cells appear dynamically through mouse embryo development and after 2–3 days are removed by infiltrated macrophages. It is possible that senescent cells complement apoptosis sculpture function during development (Wanner et al. 2020). Nevertheless, not all programmed senescent cells are eliminated by macrophages. Interestingly, some senescent cells lose their hallmarks of SA β -Gal activity and p21^{Waf1} expression and re-enter the cell cycle *in vivo* during embryogenesis (Li et al. 2018).

Programmed senescent cells have increased heterochromatin markers H3K9me3 and HP1 γ , while no DNA damage has been detected, nor activation of p53 or expression of p19^{ARF}, which are typical markers of age-associated cellular senescence (Muñoz-Espín et al. 2013; Storer et al. 2013; Domínguez-Bautista et al. 2021). This might explain why programmed cellular senescence in embryos has a physiological function, as opposed to the detrimental role persistent senescent cells have in ageing and age-related diseases (detailed below).

Chromatin Remodeling in Ageing

Ageing is a biological process characterized by a progressive loss of physiological functions that leads to an accumulation of damage at the tissue, cellular, and molecular levels over time, increasing the likelihood of death. Among the hallmarks of ageing, genome instability, telomere attrition, epigenetic alterations, and cellular senescence (Lopez-Otin et al. 2013) have in common chromatin changes. So, it is important to understand what molecular modifications occur in ageing that lead to chromatin remodeling and what are their functional impacts on the cell.

Persistent Cellular Senescence

As discussed in the previous section, transient cellular senescence has a physiological role during embryonic development. It also contributes to wound healing and tissue regeneration. In the adult, DNA damage induces cellular senescence and since senescent cells no longer divide, they prevent the onset of tumors in a cell-autonomous way. Nevertheless, in ageing senescent cells instead of being transient, they accumulate most likely because the immune system stops removing them. Persistent senescent cells increase with age in many tissues and secrete senescence-associated secretory phenotype (SASP) molecules that become harmful in the long term. Among the molecules secreted are chemokines, cytokines, growth factors, angiogenic factors, matrix metalloproteinases, etc. giving rise to several pathological processes associated with ageing (Sacco et al. 2021). Age-associated cellular senescence shares similarities with programmed cell senescence but has some distinctive features. They have in common a phenotype characterized by a lack of response to mitotic and apoptotic stimuli, mediated by the expression of cell cycle inhibitors p21^{WAF1} and p16^{INK4A} and members of the BCL2 family of anti-apoptotic proteins, as well as an increase in the activity of the enzyme SA- β -Gal. Among the hallmarks specific for cellular senescence during ageing are an accumulation of lipofuscin, sustained DNA damage response, and alterations in nuclear morphology due to degradation of lamin B and its receptor, which lead to characteristic changes in chromatin structure. In the long term, there is a change in the composition of the SASP molecules and they promote tumor development and chronic inflammation (Paramos-de-Carvalho et al. 2021). The persistent accumulation of senescent cells drives age-related phenotypes (reviewed in (Sacco et al. 2021)).

Cellular senescence can be caused by different stressors such as telomere dysfunction, oxidative stress, radiation, proteasome inhibition, metabolic dysfunctions, autophagy failure, and DNA damage (Schumacher et al. 2021), and it is classified depending on the nature of the stimulus that induced the senescent phenotype. For example, replicative senescence is induced by telomere shortening that causes damage to its loop-shaped structure, which triggers the activation of the DNA damage response pathway (Zhu et al. 2019). When the senescent phenotype is

acquired before cell duplication exhaustion, as induced by chronic exposure to different stressors such as oxidative stress, UV radiation, DNA damage, calcium deregulation, and misfolded proteins, it is called stress-induced premature senescence (SIPS). These stressors increase protein expression of pathways related with inflammation or cell cycle regulation such as p38MAPK, p53, p21^{WAF1}, and p16^{INK4A}, among others. Activation of oncogenes such as *K-ras*, *B-raf*, *PTEN*, and *NF1* induces also the senescent phenotype coined oncogene-induced senescence (OIS) (Tan et al. 2014).

Changes in Chromatin Structure and Histone Variants in Cellular Senescence

In most healthy cells, heterochromatin is found in the nuclear periphery bound to lamin B1 and its receptor, and euchromatin is located in the center of the nuclei. During cellular senescence the reduction of lamin B1 and its receptor causes a detachment of heterochromatin and a loss of its spatial location, leading to a global modification of chromatin structure in which there is an inversion in the location of heterochromatin and euchromatin (Lukasova et al. 2017).

A global loss and local gains of heterochromatin are also related to senescence; for example, decreased trimethylation of H3K9 due to lack of proteins such as HP1 α or SUV39H1 induces SIPS in human embryonic stem cells (Zhang et al. 2015). On the other hand, comparing chromatin conformation in normal human replicating fibroblasts with senescent fibroblasts, the latter lose heterochromatin in promoters and enhancers of active genes and gain heterochromatin in gene-poor regions; constitutive heterochromatin in centromeric and pericentromeric regions become more open, while the peripheral heterochromatic compartment is reduced and the centromeric structure is enlarged (De Cecco et al. 2013).

During ageing and senescence, heterochromatin marks are distributed from areas of constitutive heterochromatin (such as telomeres) to areas of facultative heterochromatin (Zhang and Adams 2007). Particularly in cellular senescence, there is an accumulation of heterochromatin domains known as senescence-associated heterochromatin foci (SAHFs) enriched by marks such as H3K9me3, H4K20me2, H3K27me3, and HP1 proteins that together contribute to cell cycle arrest, as they recruit retinoblastoma protein (RB) and prevent genes regulated by the transcription factor E2F from being expressed (Narita et al. 2003; Chandra et al. 2015). Also, in WI38 cells, the formation of SAHF is mediated by the overexpression of HMGA2, a protein responsible for chromatin structure, and by the reduction of the WNT pathway, which inhibits glycogen synthase kinase three beta (GSK3 β) (Shi et al. 2017). In addition, increment of nuclear pore components such as nucleoporin TRP contributes to chromatin reorganization leading to SAHF formation, as observed in an OIS model in IMR90 cells (Boumendil et al. 2019). Finally, macro histone H2A, a variant associated with gene silencing, is also enriched in SAHF and is associated

with the inactivation of genes with proliferative function and the activation of genes that contribute to SASP (Hao et al. 2022).

Chromatin remodeling during ageing contributes to the establishment of cellular senescence. For example, the reduction of marks such as H3K9me occurs in promoters of *IL6* and *IL8* genes, which are two essential components of the SASP (Hao et al. 2022). Moreover, accumulation of histone variant H2A.J in response to DNA damage in senescent human fibroblasts has been associated with the upregulation of proinflammatory immune response and SASP-associated genes (Contrepois et al. 2017). Additionally, a study comparing the gene expression and DNA methylation profile of cells with replicative senescence and OIS showed that genes related to cell cycle progression were minimally expressed and those related with immune response were highly expressed; only replicative senescence active gene expression was consistent with hypomethylation in the promoters of genes, immune genes related to SASP (Sakaki et al. 2017).

DNA Methylation

The first studies on DNA methylation indicated a global loss of methylation with ageing (Bollati et al. 2009). However, subsequent studies comparing the methylation status in the intestine of aged mice showed that ageing leads to a more complex epigenetic dysregulation, with gains and losses of methylation found in different regions of the mouse genome, the most relevant being a hypermethylation in promoters of tumor suppressor genes such as *Cdkn2a*, *Cdkn1c*, *Rassf1*, among others (Maegawa et al. 2010). Several studies have identified differential DNA methylation patterns associated with ageing in humans and other animals. While age-related hypermethylated DNA regions are preferentially observed in bivalent promoters, CpG islands, and Polycomb target genes, other regions are preferentially hypomethylated. Additionally, age-associated methylation changes are tissue-dependent since no changes have been found in the methylation levels of adipose tissue (Thompson et al. 2010) and in old rat livers (Guarasci et al. 2018). Even though DNA methylation patterns vary among tissues, there are specific CpG sites that consistently undergo DNA methylation changes with ageing and in age-related diseases. Combining DNA methylation data with chronological age information, several research groups have used supervised machine learning methods to “learn” which CpG sites predict chronological age, and developed so-called “epigenetic clocks” (Unnikrishnan et al. 2019; He et al. 2021). It has been interesting to observe that in very long-lived people or supercentenarians, the methylation of their genome is lower and slower (Horvath et al. 2015b), while in pathologies that develop characteristics of ageing, such as Down syndrome or progeria, methylation is higher and accelerated (Horvath et al. 2015a; Bejaoui et al. 2022). Accordingly, experimental interventions such as caloric restriction (Maegawa et al. 2017) or rapamycin treatment (Cole et al. 2017) decrease the apparent age of mice and monkeys, increase their lifespan, and have delayed DNA methylation.

One of the explanations for epigenetic deregulations in ageing is the decrease in the expression of DNMTs proteins (Uysal and Ozturk 2020), which would explain hypomethylation and a decrease in the enzymatic activity of TET proteins (Borkowska et al. 2020), leaving hypermethylated zones. Consequently, the entire gene expression pattern of the cell is altered, contributing to cellular dysfunction in ageing.

Histone Variants and Modifications

Nucleosome composition rearranges with age, as there is a global loss of constitutive histones such as H2A, H3, and H4 in aged human fibroblasts (O’Sullivan et al. 2010). Also, in ageing, there are changes in the expression dynamics of different histone variants (Yi and Kim 2020); for example, in post-mortem aged human brains, as well as in old mice brains, there is an accumulation of the H3.3 variant and a decrease in the H3.2 variant (Maze et al. 2015). Furthermore, in the hippocampus of old healthy mice, the H2A.Z variant increases and promotes the expression of early expression genes necessary for forming contextual memories such as *Arc* (Stefanelli et al. 2018). In turn, in different tissues of old mice and primates, macro histone H2A (mH2A) and heterochromatin one beta-protein (HP1 β) are increased, which are associated with facultative heterochromatin. Both increase the expression of genes coding for SASP molecules, characteristic of senescent cells that accumulate in ageing (Kreiling et al. 2011). These findings suggest that the decrease in nucleosomal histones and the increase in specific histone variants associated with heterochromatin formation contribute to the structural changes in chromatin described in ageing.

In addition to the decrease or increase in specific histone variants during ageing, there is also a global loss of heterochromatin accompanied by a decrease in histone-associated proteins such as HP1 (Lee et al. 2020). Specifically, in mouse cells, the HP1 protein recognizes the di- or trimethylation of lysine 9 of histone 3 (H3K9me2/3), which is a characteristic mark of heterochromatin that contributes to transcriptional suppression and is also involved in the regulation of heterochromatin by regulating the stabilization of protein methyltransferases and demethylases that participate in the methylation dynamics of H3K9. Interestingly, aberrant levels of H3K9me2/3 have been found in both cancer and ageing, possibly associated with decreased HP1 and, consequently, changes in chromatin organization and decreased transcriptional suppression (Maeda and Tachibana 2022). A summary of histone variants along ageing is presented in Table 2.3.

Changes in modifications made to different histone variants have also been reported in ageing and age-related diseases (Saul and Kosinsky 2021). For example, the H4K16ac acetylation mark regulates chromatin compaction both in ageing and in Alzheimer’s disease (AD); in post-mortem human brains, it was found that this mark is redistributed in normal ageing, having gains and losses specifically in the lateral temporal lobe, a structure important for memory. However, unlike normal ageing,

Table 2.3 Histone variants change during ageing

Variant	Model organism	Tissue/cell type	Change	Function	References
H2A.Z	Human and mouse	Mouse brain and pancreatic ductal adenocarcinoma cell lines (PDAC)	Increases in hippocampus of old mice and in PDAC cell lines	In mouse brain suppress memory function by reducing learning-gene expression patterns. In PDAC cell lines, H2A.Z isoform depletion promotes senescence	Stefanelli et al. (2018), Avila-Lopez et al. (2021)
H2A.X	Mouse	Mouse embryonic fibroblast (MEF), mouse liver, lung, spleen, intestine, and brain	Age-dependent increase of H2A.X foci in various mice tissues. Increase in 24-mo mice after being irradiated (10 gray)	H2A.X as an indicator of DNA damage and cellular senescence in different aged mouse tissues. Increase in 24-mo mouse brain irradiated and it has a relation with upregulation of 53BP1 and caspase 3 activation	Wang et al. (2009), Gionchiglia et al. (2021)
H2A.J	Human and mouse	Human skin samples, and senescent human fibroblasts, mouse epidermis, brain, liver, kidney, and hair follicle	Age-dependent accumulation of H2A.J in human epidermis, and in senescent human fibroblast with DNA damage. Also accumulates with age in a mouse organ-specific fashion	Promotes inflammatory gene expression related to SASP and affects proliferative capacity during human skin ageing	Contreipois et al. (2017), Rube et al. (2021)
MacroH2A	Human, mouse, and primates	Human fibroblasts and U2OS cell line, aged mice liver and lung, and muscle of aged baboons	Increasing during ageing and replicative senescence. Also is enriched at telomeres and sub-telomeric regions of U2OS human cell line	Increase heterochromatinization and upregulate gene expression related to SASP. In U2OS cell line promotes telomere stability	Kreiling et al. (2011), Kim et al. (2019)
H3.3	Human and mouse	Human brain, mouse brain, liver, kidney, and heart	Accumulates in neurons and glia of aged mice and humans. Also, in mouse somatic tissues	Regulates cell type-specific gene expression. Decreases global levels of H3 methyl modifications	Maze et al. (2015), Tvardovskiy et al. (2017)

this mark increases in the brains of patients with AD, having more gains than losses in the same region increasing gene expression of different genes (Nativio et al. 2018). Also, in the brains of mice models of AD, there is a loss of acetylation of different histones such as H2BK5ac, H3K14ac, H4K5ac, and H4K12ac, which consequently decreases the expression of essential genes for learning and memory such as *Arc*, *Bdnf*, *GluR1*, *GluR2*, *Syp*, among others (Graff et al. 2012). In brains of older people compared to people with AD, a reduction of H3K18ac and H3K23ac marks has also been found (Zhang et al. 2012) (Zhang et al. 2012). For a comprehensive review of the different histone modifications and variants in ageing, see (Yi and Kim 2020). A summary of histone modifications during ageing is presented in Table 2.4.

Finally, other important factors that change during ageing are histone modifying enzymes such as HDACs and HATs. These proteins move to different chromatin sites with age, modifying the canonical transcription of genes associated with DNA damage repair. For example, in young cells, remodeling proteins such as SIRT1 can rapidly translocate to the site of DNA damage and then return to their original loci; however, with the passage of time and the increase in DNA-damaging events, this relocation does not occur, leading to loss of cell identity, cell dysfunction, and ageing (Kane and Sinclair 2019).

Nuclear and 3D Genome Remodeling in Ageing

The previously mentioned epigenetic modifications in histones and changes in DNA methylation determine changes in chromatin architecture and, therefore, nuclear remodeling. Both in ageing and cancer, there are nuclear remodeling and changes in the three-dimensional conformation of the genome. One of the first studies on the organization of the genome in a model of ageing was done in fibroblasts from patients with the Hutchinson–Gilford Progeria Syndrome. This disease is caused by the accumulation of progerin, a mutated form of lamina A, that modifies the distribution of the repressive mark H3K27me3 in heterochromatin. As a consequence, the lamina-heterochromatin interaction is altered. This is accompanied by a global loss of the compartmentalization of active and inactive chromosome domains (McCord et al. 2013). Years later, after a comprehensive analysis of chromatin accessibility using the assay for transposase-accessible chromatin (ATAC-seq/-seq) in skin fibroblasts from patients with Hutchinson–Gilford Progeria Syndrome, greater accessibility of LADs was found in chromatin, related to a change in the expression of Hutchinson–Gilford Progeria Syndrome specific genes such as *EDIL3* and *IGFBP7* (Kohler et al. 2020).

The histone 3 variant, CENP-A (centromere protein A), participates in the correct position of the centromeres and the kinetochore assembly. In both aged and genotoxic stress-induced senescent murine cells CENP-A levels are reduced, leading to centromeric aberrations and genome instability (Hedouin et al. 2017). In tumor and aged cells, the increased number of cell divisions compromises the

Table 2.4 Histone modifications change during ageing

Modification	Model organism	Tissue/cell type	Change	Function	References
H3K9me3	Human	HGPS skin fibroblast	Decrease in HPGS fibroblast	Abnormal nuclear morphology in HPGS fibroblast and correlates with impaired DDR	Zhang et al. (2016)
H3K9me2	Mouse and rat	Brain	Accumulates in aged mouse hippocampus, and in a rat CA1 region	Promote age-related memory decline in rats	Morse et al. (2015), Butler et al. (2019)
H3K79me2	Mouse	Brain	Increase in cortex and hippocampus of 12-mo SAMP8 mice	Probably contributes to alteration of the gene expression and/or protein abnormalities reported in the brain of SAMP8 mice	Wang et al. (2010)
H3K4me2	Rhesus macaque	Brain	Increase in pre-frontal cortex of rhesus macaque	Positively associated with transcription suggesting an age-related shift to open chromatin. Changes related to stress responses such as DDR	Han et al. (2012)
H3K4me3	Rat	Brain	Increase in CA and DG regions of 19–22 months rats	Promote age-related memory decline	Morse et al. (2015)
H4K16ac	Human	Brain	Enrichment in normal ageing and losses in AD lateral temporal lobe	Positively correlated with cell death-gene expression, promote overactive transcription in ageing	Nativio et al. (2018)
H4K12ac	Mice	Brain	Loss of acetylation in an AD mouse model	Decreases memory-gene expression such as <i>Bdnf</i> , <i>GluR1</i> , <i>Glur2</i> , and <i>Syp</i>	Graff et al. (2012)
H3K18ac	Human	Brain	Reduction of acetylation in the AD temporal lobe compared to controls	Probably contributes to epigenetic modifications and late-onset cognitive loss in AD	Zhang et al. (2012)
H3K27ac	Human and mouse	Brain	Hyperacetylation in human brain prefrontal cortex and in a mouse brain	Upregulated genes have inflammation-related functions and contributes with brain inflammaging	Cheng et al. (2018)

compartmental organization of the genome. For example, late-passage human fibroblasts undergo a remodeling of compartments A and B; colorectal cancer cell lines have a loss in the segregation of compartments A and B and gain in interactions with intermediate compartments, which is characterized by the epigenetic mark H3K27me3 (Johnstone et al. 2020).

Changes in the macrodomains of the genome such as the switch between compartments A and B and changes in TADs have been studied in the context of senescence associated with ageing (Chandra et al. 2015; Rocha et al. 2022). For example, in OIS local lamina-heterochromatin is lost and there is a gain in repression marks such as H3K9me3. These cells also have SAHFs (Chandra et al. 2015). Likewise, studies carried out with Hi-C in replicative senescence found a loss of long-range interactions (>2 Mb) and an increase in short-range interactions (<2 Mb); in turn, these cells undergo a change between compartments B (inactive) and A (active) allowing a greater expression of genes related to cell cycle arrest, such as *CDKN2A* (Criscione et al. 2016).

All the changes mentioned above lead to nuclear remodeling that responds to changes in the three-dimensional organization of the genome. Changes in the NE are highly dynamic due to membrane rupture, reorganization, sealing, or cell growth. It has recently been proposed that this NE remodeling is coordinated with endoplasmic reticulum lipid synthesis, and some enzymes responsible for lipid synthesis regulate NE biogenesis and remodeling (Barger et al. 2022). This becomes highly relevant in ageing since the NE must be remodeled with greater demand to contend with the chromatin changes described above. NE remodeling could cause deregulation of lipid synthesis both locally and from the endoplasmic reticulum, overproducing nuclear membrane and causing NE remodeling defects such as nuclear invaginations, a phenotype observed in aged nuclei and with senescence associated with ageing (Bahmanyar and Schlieker 2020; Pathak et al. 2021).

The Role of Lamins in Regulating Chromatin Organization During Ageing

In addition to having a structural role, the nuclear lamina is essential for the organization and regulation of chromatin. In ageing, there is a reduction in lamina B1 and its receptor (Lukasova et al. 2017), while in both ageing and laminopathies, changes in the nuclear lamina cause nuclear deformations or invaginations. Notably, in Tig3ET cells with OIS, a decrease in lamin B1 and lamin A/C, and other NE proteins alters nuclear morphology, leaving a nucleus more susceptible to apoptosis or necrosis (Lenain et al. 2015).

Another vital function of lamins is that they affect the correct nuclear localization of proteins. For example, the emerin protein is concentrated in the inner nuclear membrane due to its interaction with A-type lamins; also, the histone-binding protein HP1 interacts with lamin A/C for its correct localization (Ranade et al.

2019). Furthermore, lamin A/C interacts with p53 protein, which promotes the degradation of repressor proteins such as PRC1, increasing the expression of genes repressed by this complex, such as the *CDKN2A* gene that codes for the p16^{INK4} protein (Yoon et al. 2019). In turn, lamin B1 also regulates the correct nuclear localization of specific proteins such as HP1, and the decrease in this lamina is related to the loss of the H4K20me2 repression mark and, consequently, an increase in expression of proinflammatory genes that are part of the SASP (Lukasova et al. 2018). Finally, nuclear lamins contribute to the correct location of the telomeres. In particular, lamin A/C interacts with TRF2 protein, altering its location and promoting the telomeres concentration in the nuclear periphery rather than in the center, which is a peculiarity feature of aged or senescent cell phenotype (Wood et al. 2014). Furthermore, in cellular models of OIS, telomeres are also distributed more towards the periphery, where they interact with the nuclear lamina (Lenain et al. 2017).

All these changes in the nuclear lamina lead to the destabilization of nuclear integrity; in cellular senescence, small fragments of damaged DNA can be expelled from the nucleus into the cytoplasm; these are known as cytoplasmic chromatin fragments (CCFs) (Lukasova et al. 2018). These CCFs indicate the destabilization of nuclear integrity associated with the previously described changes in nuclear lamina proteins. Furthermore, mutations in *LMNA* gene cause the production of a truncated version of Lamin A named progerin, and cause Hutchinson–Gilford progeria syndrome (Ahmed et al. 2018; Cenni et al. 2020), and increased expression of progerin is associated with both ageing and age-associated diseases (Cenni et al. 2020; Scaffidi and Misteli 2006) (Fig. 2.4).

Alterations in the nuclear lamina induce changes in the pattern of DNA expression during ageing, and this could increase the damage events to genetic material and causes instability of the genome. Particularly in ageing organisms, the ability to repair such damage decreases, and therefore there is an accumulation of DNA damage with ageing (Oberdoerffer and Sinclair 2007).

Finally, a common denominator between pathologies such as cancer, progeroid diseases, neurodegenerative diseases, and physiological ageing is that there is a significant decrease in the ability to repair damaged DNA causing an accumulation of DNA damage. This prolonged damage compromises the genetic information and, consequently, the transmission of information from one generation to another is altered. At the molecular level, DNA damage can affect the long-term nuclear structure (forming of buds and micronuclei) which has been associated with mutagenesis, chromosomal rearrangements, errors in DNA replication, point mutations, translocations, telomere dysfunctions, and change in chromatin structure, that collectively contribute to genome instability (Ou and Schumacher 2018). All together, these alterations at the molecular level can modify cell fate, inducing the senescent phenotype or increasing cell death; they cause inflammation, cancer, neurodegenerative diseases, and accelerate ageing. Therefore, it is vital that the cell frequently monitors the integrity of the DNA and, if it finds any alteration, activates the DNA damage response, which recruits different damage repair pathways (Jackson and Bartek 2009).

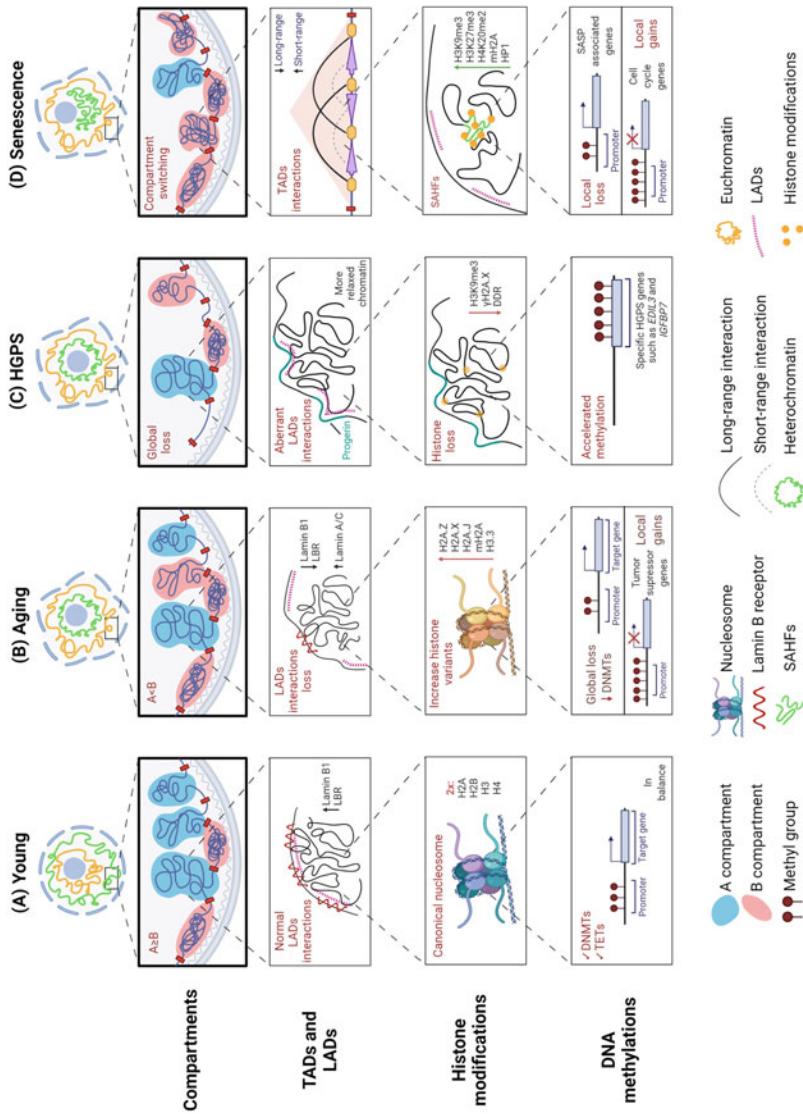


Fig. 2.4 Dynamics of epigenetic and genome organization changes during ageing and age-associated diseases. The most significant changes at each level of organization are schematically presented. (a) Young cells with a higher proportion of A compartments, without alterations in the nuclear laminae, a canonical pattern of histones, and a correct balance in the genome methylation. Heterochromatin is mainly located perinuclear and euchromatin is enriched in the center of the nucleus. (b) Aged cells have alterations in nuclear morphology, loss of lamin B1 and its receptor (LBR), an inversion in the location of heterochromatin and

Fig. 2.4 (continued) euchromatin, increase in B compartments, loss of interactions with LADs, increase in histone variants in the nucleosomes, and in general there is a global loss and local gain of methylation in different zones of the genome. **(c)** Cells with Hutchinson–Gilford Progeria Syndrome (HGPS) have alterations in nuclear morphology associated with global loss of compartments, aberrant LADs interactions by progerin, decreased some histone marks related to DNA damage such as γH2AX; they also present accelerated methylation in their genome. **(d)** Senescent cells (showing the general changes found in cells with replicative and oncogene-associated senescence) have altered nuclear morphology, changes in the ratio of compartments (with B being the most abundant) and favored short-range over long-range interaction. This chromatin remodeling culminates in senescence-associated heterochromatin foci (SAHFs) enriched in repressive histone marks. They have local methylation losses in genome regions related to proinflammatory pathways (SASP molecules) and local methylation gains in genes related to cell cycle progression. *LBR* lamin B receptor, *LADs* lamina-associated domains, *TADs* topologically associated domains, *DNMT3* DNA Methyltransferases, *TET3* Ten-eleven translocation proteins, *SASP* senescence-associated secretory phenotype. Created with BioRender.com

Concluding Remarks

Development and ageing are orchestrated by similar molecular and cellular processes, involving chromatin structure and genome 3D organization in such a way that studying development can shed light into ageing, and vice versa. Chromatin conformation dynamics and the plethora of molecular mechanisms behind genome structure modulation have a direct impact on determining gene expression programs during development and also during healthy ageing.

Cellular senescence is an example of the antagonistic pleiotropy theory of ageing, occurring from embryonic development through adulthood and in the elderly, and having both a beneficial and a detrimental effect in organisms. A key aspect of detrimental cellular senescence is the secretion of SASP molecules with tumorigenic and inflammatory functions. Since the regulation of the expression of the genes coding for such molecules is mainly mediated by epigenetic changes, understanding the mechanisms that lead to altered chromatin structure and 3D genome structure could provide tools to either prevent or revert age-related gene expression. Should it be possible to modulate the molecules secreted by senescent cells, the persistent presence of senescent cells could be switched from detrimental to beneficial. This possibility would be particularly useful for treating senescent neurons, which cannot be replaced.

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Chapter 3

The Nuclear Envelope in Ageing and Progeria



Adrián Fragoso-Luna and Peter Askjaer

Abstract Development from embryo to adult, organismal homeostasis and ageing are consecutive processes that rely on several functions of the nuclear envelope (NE). The NE compartmentalises the eukaryotic cells and provides physical stability to the genetic material in the nucleus. It provides spatiotemporal regulation of gene expression by controlling nuclear import and hence access of transcription factors to target genes as well as organisation of the genome into open and closed compartments. In addition, positioning of chromatin relative to the NE is important for DNA replication and repair and thereby also for genome stability. We discuss here the relevance of the NE in two classes of age-related human diseases. Firstly, we focus on the progeria syndromes Hutchinson–Gilford (HGPS) and Nestor–Guillermo (NGPS), which are caused by mutations in the *LMNA* and *BANF1* genes, respectively. Both genes encode ubiquitously expressed components of the nuclear lamina that underlines the nuclear membranes. HGPS and NGPS patients manifest symptoms of accelerated ageing and cells from affected individuals show similar defects as cells from healthy old donors, including signs of increased DNA damage and epigenetic alternations. Secondly, we describe how several age-related neurodegenerative diseases, such as amyotrophic lateral sclerosis and Huntington’s disease, are related with defects in nucleocytoplasmic transport. A common feature of this class of diseases is the accumulation of nuclear pore proteins and other transport factors in inclusions. Importantly, genetic manipulations of the nucleocytoplasmic transport machinery can alleviate disease-related phenotypes in cell and animal models, paving the way for potential therapeutic interventions.

Keywords HGPS · Laminopathy · Neurodegeneration · NGPS · NPC · Nuclear lamina · Nuclear pore complex · Nucleocytoplasmic transport · Progeria

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Introduction

Eukaryotic cells protect their genome inside a membrane-bound organelle, the nucleus. The nucleus is the main feature used to differentiate eukaryotes from prokaryotes, whose genetic material is not separated from the cytoplasm. The nuclear envelope (NE) is a complex structure composed of a double bilayer membrane (Ungrich and Kutay 2017). The outer membrane faces the cytoplasm and is continuous with the endoplasmic reticulum (ER), and the inner membrane is in contact with the nucleoplasm. At numerous points along the NE, both membranes are fused, and associated with nuclear pore complexes (NPCs), which control the traffic of macromolecules (“cargo”) between the nucleus and the cytoplasm (Kutay et al. 2021; Schuller et al. 2021). NPCs are supramolecular structures of more than 110 MDa, and composed of multiple copies of more than 30 different nuclear pore proteins or nucleoporins (nups; Fig. 3.1a). NPCs allow the free diffusion of molecules smaller than 40 KDa, whereas larger molecules depend on active transport. Structurally, the NPC is composed of multimeric subcomplexes that form rings in the plane of the NE. These includes a transmembrane subcomplex, an inner ring, a central channel and cyto- and nucleoplasmic rings. The NPC also contains filamentous cytoplasmic units and a nucleoplasmic basket structure. Many nucleoporins contain hydrophobic intrinsically disordered domains rich in phenylalanine-glycine (FG) dipeptides that establish a permeability barrier in the centre of the NPC (Schmidt and Gorlich 2016). In order for cargo to cross the NPC, they interact with nuclear transport receptors of the importin beta family (aka karyopherins). The transport receptors come in two flavours, importins and exportins, based on their functions as receptors for nuclear import or nuclear export cargoes. The interactions between nuclear transport receptors and their cargo are regulated by the small GTPase Ran. In the nucleoplasm, Ran is bound to GTP because of the nuclear activity of its guanine exchange factor RCC1, which stimulates disassembly of nuclear import complexes and assembly of nuclear export complexes (Fig. 3.1b). In contrast, the activity of the Ran GTPase activating protein RanGAP at the cytoplasmic filaments of the NPC stimulates the conversion of RanGTP to RanGDP, which favours disassembly of export complexes and assembly of import complexes (Schmidt and Gorlich 2016).

Beneath the inner nuclear membrane lays the nuclear lamina, a meshwork made of lamin proteins, which constitute its principal component, and lamin-associated inner nuclear membrane proteins (Fig. 3.2a). Lamins are type V intermediate filaments classified in A- and B-type lamins. The genomes of animal species encode from one to many lamin proteins. In broad terms, the more complex is the organism, the more genes coding for lamins harbour its genome. The nematode *Caenorhabditis elegans* contains a single gene, *lmn-1*, coding for a B-type lamin (Liu et al. 2000). *Drosophila melanogaster* cells express two genes, Dm, coding for a B-type and LamC, an A-type lamin (Rzepecki and Gruenbaum 2018). Mammalian A-type lamins are encoded by the *LMNA* gene, which through alternative splicing yields lamin A and C. Besides, it produces the minor variants A Δ 10 and C2. B-type lamins

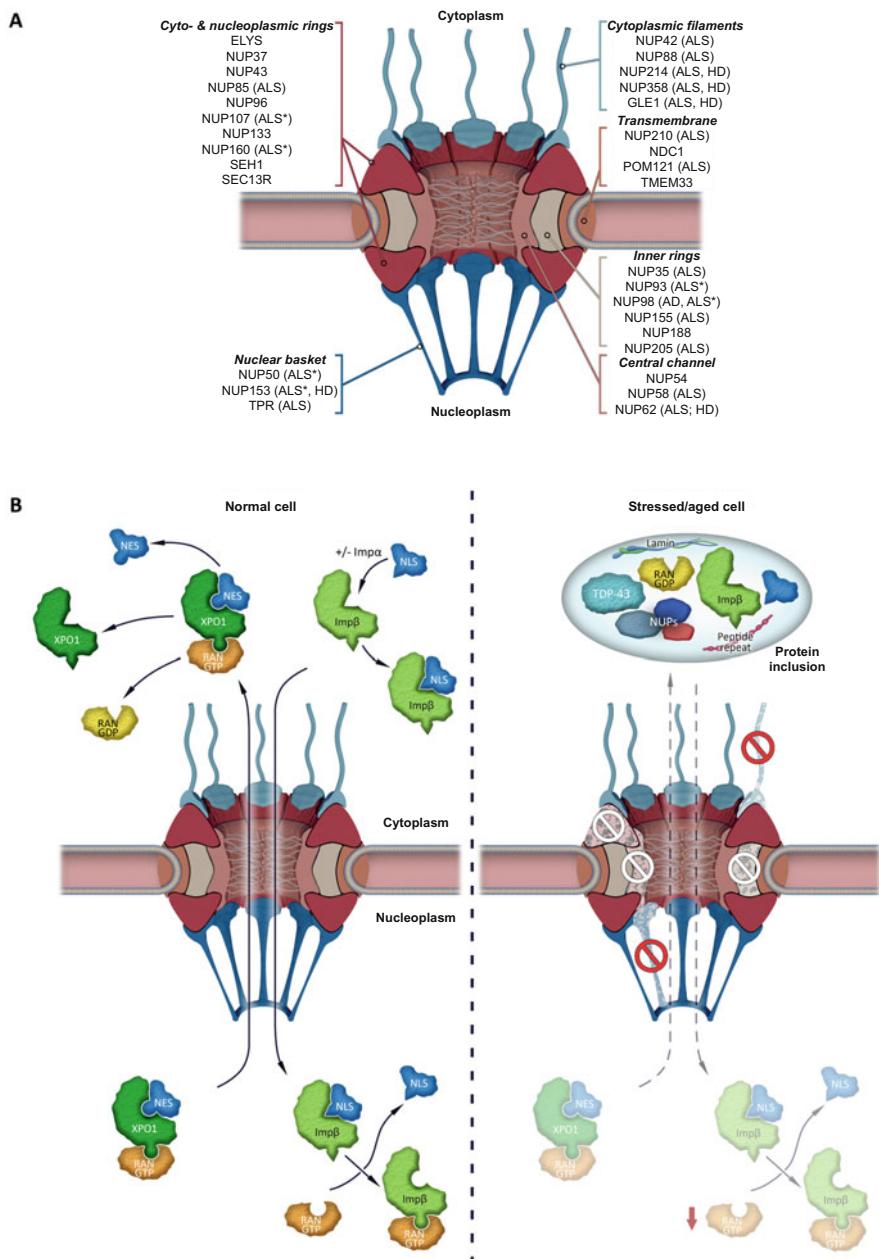


Fig. 3.1 Nuclear pore complexes and nucleocytoplasmonic transport. **(a)** NPCs are composed of multiple copies of ~35 nucleoporins (nups) that form stable subcomplexes with distinct localisation and function. Nucleoporins associated with age-related neurodegenerative diseases are indicated (AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; HD, Huntington's disease; asterisks denote nucleoporins that suppress and/or enhance phenotypes in disease models). **(b;** left) The NPC forms a selective barrier for transport between the nucleus and the cytoplasm. Translocation of

are encoded by *LMNB1* and *LMNB2*, the latter rendering lamin B2 and B3 (Ho and Lammerding 2012). The initial protein product from the *LMNA* gene, prelamin A, is subjected to extensive post-translational modifications before yielding the mature lamin A protein (Fig. 3.3a). The first modification consists in the farnesylation of the cysteine residue within the CAAX motif at the carboxyl terminus, which anchors prelamin A to the inner nuclear membrane. Then, a second modification is carried out by the zinc-metalloprotease ZMPSTE24 that cleavages the peptide bond after the farnesylated cysteine. The cysteine is next carboxymethylated by isoprenylcysteine carboxylmethyltransferase (ICMT) and finally ZMPSTE24 releases the last 15 residues, including the farnesyl moiety. This produces the mature version of lamin A, which assembles as a polymer in the nuclear lamina but is also present in the nucleoplasm.

As aforementioned, the nuclear lamina also consists of lamin-associated membrane proteins, which are part of a diverse catalogue of NE transmembrane (NET) proteins (Worman and Schirmer 2015). The most studied class features the presence of the LEM (LAP2, Emerin, MAN1) domain. LEM domain-containing proteins connect the NE to chromatin (Barton et al. 2015). A mention apart deserves yeasts, since they do not have a nuclear lamina but express LEM proteins (Steglich et al. 2012). Lamin B receptor (LBR) also associates with chromatin through interaction with heterochromatin protein 1 (HP1), whereas barrier-to-autointegration factor (BAF) offers a bridge between LEM proteins, lamin and chromatin (Sears and Roux 2020).

The NE is physically connected to the cytoskeleton by means of the linker of nucleoskeleton and cytoskeleton (LINC) complex, which provides communication between the nuclear interior and the cytoplasm and is required for nuclear positioning within the cell. The LINC complex is divided into two subcomplexes: KASH domain proteins at the outer nuclear membrane and SUN domain proteins at the inner nuclear membrane, which interact with the cytoskeleton and the nuclear lamina, respectively (Khilan et al. 2021).

Nuclear shape is tightly regulated, and certain cell types feature distinctive nuclear shapes; e.g. granulocytes (Deolal and Mishra 2021; Manley et al. 2018). Nonetheless, the NE is a highly dynamic structure, as its size and shape are modified along the cell cycle, with major changes taking place during cell division. When cells enter mitosis, its nucleus divides following one of two main processes, named open

Fig. 3.1 (continued) proteins carrying nuclear export signals (NES) or nuclear localisation signals (NLS) through the NPC is facilitated by interaction with export (XPO1) and import (Imp β) receptors, respectively. These interactions are regulated by the GTPase Ran and the distribution of RanGTP in the nucleus vs. RanGDP in the cytoplasm confer directionality of the transport processes. Imp β can bind NLS cargoes either directly or via the Imp α adapter. (b; right) In cells experiencing stress, for instance due to certain neurodegenerative diseases, several defects in the nucleocytoplasmic transport machinery are observed. These include reduced levels of nuclear RanGTP, detachment of nucleoporins from NPCs and formation of cytoplasmic inclusions that contain RNA-binding proteins (e.g. TDP-43), nucleocytoplasmic transport receptors, lamins, nucleoporins and other aggregation-prone polypeptides (e.g. poly-(glycine-arginine) dipeptide repeats)

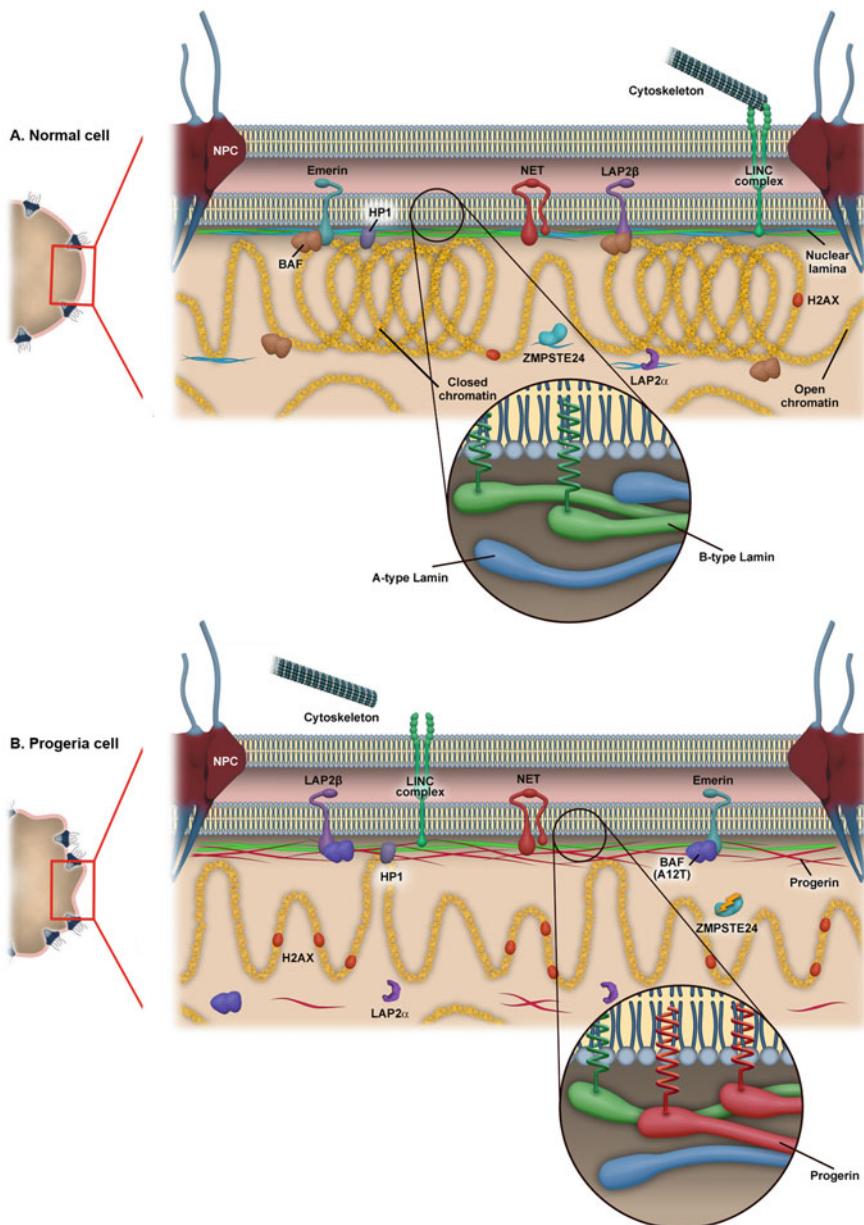


Fig. 3.2 The nuclear envelope and its alterations in progeria. **(a)** The NE is comprised by a double bilayer membrane, NPCs, integral NE transmembrane (NET) proteins and nuclear lamins (lamin A in blue; lamin B including farnesylation anchor in green). NETs containing a LEM (LAP2, Emerin and MAN1) domain interact with BAF which serves as bridging factor between the NE and chromatin. Most chromatin at the NE is transcriptionally inactive (closed heterochromatin). **(b)** The NE in cultured cells from progeria patients is irregular (left) and accumulates less heterochromatin. A higher frequency of H2AX foci is often observed, indicative of increased levels of DNA damage. In Hutchinson–Gilford progeria syndrome, cells express an aberrant lamin A isoform

or closed mitosis, or in an intermediate mode. If cells divide according to an open mode, the NE disassembles when mitosis begins, and reassembles after successful chromosome segregation between daughter cells. NE breakdown includes dismantling of the nuclear lamina and NPCs through phosphorylation of lamins, nucleoporins and other NE proteins by cell cycle-dependent kinases, thus coupling NE disassembly to the cell cycle. On the contrary, in closed mitosis, chromosome segregation during anaphase proceeds while nuclear membranes are preserved. In this case, components that trigger chromosome movements must be imported through NPCs (e.g. tubulin to polymerise microtubules) (Dey and Baum 2021).

The nucleus is the compartment where the genome is replicated and transcribed, while the cytoplasm hosts translation of mRNAs into proteins and many post-translational modifications. The NE constitutes a selective barrier that separates nuclear content and processes from those in the cytoplasm. However, the NE serves many other interesting functions that are essential for cell survival, integrity and development. The NE affects these processes through its ability to regulate the genome at multiple levels. The nuclear lamina determines gene expression, as spatial localisation of genes inside the nucleus often affects their expression (van Steensel and Furlong 2019). Some genomic regions are tethered closely to the nuclear lamina. In general, heterochromatin, containing silenced genes and repetitive sequences, is enriched at the nuclear periphery in contact with the nuclear lamina. On the contrary, actively expressed genes within euchromatin are positioned more internally. Proper 3D position and dynamics of the genome are tightly controlled during cell differentiation (Lochs et al. 2019; Cabianca et al. 2019). Chromosomal ends or telomeres are also tethered to the nuclear periphery (Gonzalez-Suarez et al. 2009). During the first prophase of meiosis, chromatin condenses into characteristic karyosomes: chromosomal ends attach to the nuclear membrane through the LINC complex, leading to bouquet formation. This enables chromosomal movements and facilitates that chromatids from homolog chromosomes pair longitudinally to form the synaptonemal complex (Zetka et al. 2020). The NE also plays important roles to preserve genome integrity. Tethering of telomeres to the NE protects them against attrition, a characteristic hallmark of ageing (Lopez-Otin et al. 2013). Moreover, the NE participates at different levels to repair DNA damage. Upon double strand breaks (DSB), the nuclear lamina is necessary for efficient activating of the DNA damage response (DDR) and the two main downstream repairing mechanisms, homology recombination and non-homologous end joining. DDR components, such as the DNA repair protein 53BP1, are imported through NPCs and the nuclear lamina stabilises and localises them to DNA repair foci. Finally, the nuclear lamina participates during the orchestration of genome replication, which initiates simultaneously at many origins in the nuclear interior and later at the nuclear periphery (Pentzold et al. 2021).

Fig. 3.2 (continued) (progerin; red) that is not cleaved by the ZMPSTE24 protease and instead permanently farnesylated, whereas Nestor Guillermo progeria syndrome is caused by homozygous expression of BAF A12T mutant protein (blue). Moreover, the attachment of nuclei to the cytoskeleton is imbalanced in progeria cells

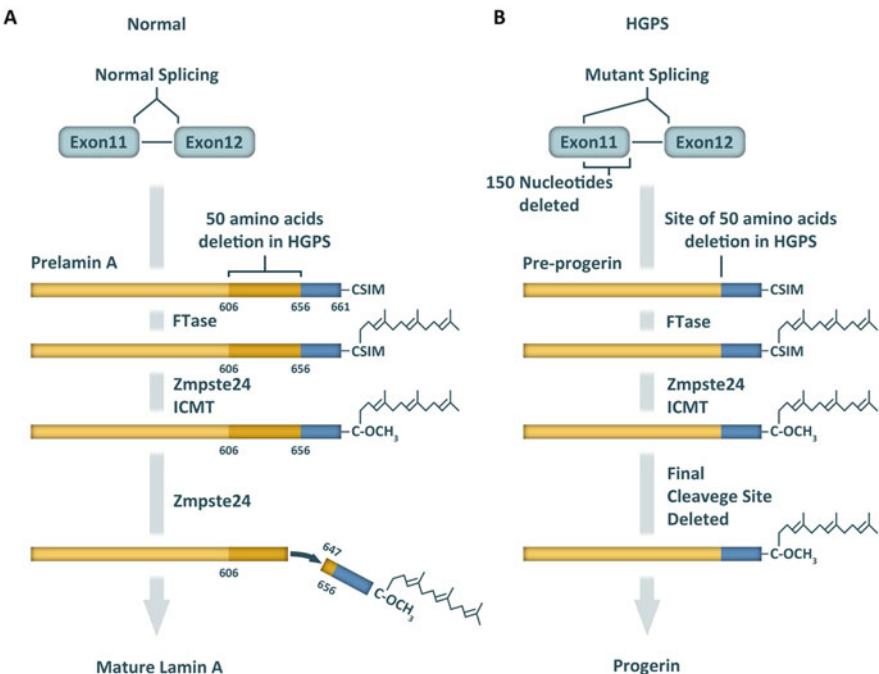


Fig. 3.3 Abnormal lamin A processing in HGPS. **(a)** Prelamin A undergoes a series of post-translational modifications including C-terminal farnesylation and internal cleavage by Zmpste24. **(b)** The most common HGPS mutation interferes with pre-mRNA splicing between LMNA exons 11 and 12 and activates a cryptic splice donor site inside exon 11. This leads to an internal truncation in prelamin A (aka progerin) including the final cleavage site for Zmpste24. In consequence, progerin remains farnesylated

The Nuclear Lamina in Ageing and Premature Ageing Syndromes

The NE is a highly dynamic structure whose composition and properties change not only along the cell cycle, but also during cell differentiation and organismal ageing. In *C. elegans*, in which somatic tissues are composed mainly of post-mitotic cells, nuclei undergo morphological alterations during the relative short life of the animals. Interestingly, most but not all genetic or environmental manipulations that extend lifespan also delay age-related morphological deteriorations (Haithcock et al. 2005; Perez-Jimenez et al. 2014). In mammalian systems, cultures of fibroblast-derived lines consist initially of cells without any apparent NE defect. After certain time of cultivation (defined by number of passages), a variable percentage of cells present deformed nuclei. This percentage increases with passages. Intriguingly, if the source of the primary fibroblast is an older donor, misshapen nuclei appear at earlier passages compared to younger donors (Scaffidi and Misteli 2006). These misshaped nuclei have irregularities in their NEs, including invaginations, blebs and

lobulations. Such alterations stem from modifications in the composition and organisation of the NE, which translate into loss of functionality of the NE. In particular, the localisation of lamin A differs between primary fibroblasts from older and young individuals. Immunostaining of fibroblasts derived from young donors reveals that lamin A is located in the nuclear periphery as well as the nucleoplasm. In contrast, fibroblasts from older persons concentrate lamin A exclusively in the nuclear lamina (Scaffidi and Misteli 2006). Furthermore, fibroblast lines from older donors present since early passages a decrease in histone H3 lysine 9 (H3K9) methylation, HP1 γ and LAP2 and a concomitant augment in H2AX foci, indicating an increased frequency of DNA damage (Scaffidi and Misteli 2006). Cells from old donors also express higher levels of the LINC protein SUN1 and suffer alteration in centrosome attachment to the NE (Chang et al. 2019). It is still unclear what triggers these changes although preventing expression of a naturally occurring variant of lamin A can reverse several of the phenotypes. The *LMNA* pre-mRNA harbours a cryptic splice donor site in exon 11, that upon activation generates a 150-nucleotide in-frame deletion in the mRNA ($\Delta 150$ -*LMNA* mRNA), and a consequent 50-residue deletion in lamin A, also known as progerin (Fig. 3.3b). Strikingly, although the levels of $\Delta 150$ -*LMNA* mRNA and progerin are similar in cells from young and old donors, inhibition of alternative splicing from the cryptic splice donor site rescues the defects in fibroblasts from old donors (Scaffidi and Misteli 2006; Cao et al. 2011). Another study focusing on muscle samples from healthy individuals aged 16–71 years also found similar levels of progerin across all ages and no evidence of increased use of the cryptic splice donor site in *LMNA* exon 11 (Luo et al. 2013). Together, this suggests that progerin expression in healthy cells is required but not sufficient to induce the observed phenotypes of cells from old donors.

Progerin, however, plays a critical role in the development of Hutchinson–Gilford progeria syndrome (HGPS). The principal feature of persons who suffer HGPS is their aged appearance. They have a short life expectancy, 14 years on average, with symptoms starting with growth retardation from their second year. HGPS patients feature hair loss, marked veins in the scalp, skin stiffness, lipodystrophy, weakened muscle function, and bone and joint abnormalities, whereas cognitive functions are normal. The major problem of HGPS regards atherosclerosis and cerebrovascular complications, which constitutes the principal cause of death (Merideth et al. 2008; Gonzalo et al. 2017). HGPS is mostly caused by the de novo mutation c.1824 C > T, which coincides with the aforementioned cryptic splice donor site in exon 11 of *LMNA*. The mutation strongly induces the use of this cryptic splice site, yielding to a pronounced accumulation of progerin at the expense of lamin A (De Sandre-Giovannoli et al. 2003; Eriksson et al. 2003) (Figs. 3.2b and 3.3b). HGPS fibroblasts accumulate significant amounts of progerin, up to 50 times more than control cells. These higher concentrations of progerin lead to more drastic alterations compared to cultured cells derived from healthy elderly donors. Severe defects appear after fewer passages in HGPS cells than in controls: HGPS nuclei suffer from more irregular NE morphology, strong decay in H3K9 methylation, HP1 and LAP2, clustering of NPCs, imbalanced connections with the cytoskeleton and an increase of H2AX foci (Goldman et al. 2004; Scaffidi and Misteli 2006; Chang et al. 2019). In

agreement with the toxic role of progerin, *LMNA* mutations that further stimulate the choice of the cryptic splice site cause more severe forms of HGPS and patients die in early childhood (Moulson et al. 2007).

Different therapies have been developed to reduce progerin production (Harhouri et al. 2018). Morpholino antisense oligonucleotides directed against *LMNA* exon 11 reduce the activation of the cryptic splice site within this exon and alleviate defects in HGPS fibroblasts and in a mouse HGPS model (Scaffidi and Misteli 2005; Osorio et al. 2011; Harhouri et al. 2016). A second subgroup of molecules aims to inhibit farnesylation of prelamin A. The farnesyl transferase inhibitor Lonafarnib extends significantly the life of HGPS patients but only modestly, perhaps because prelamin A can also be farnesylated by geranylgeranyl transferase (Gordon et al. 2018; Harhouri et al. 2018). Farnesyl transferase catalyses the prenylation of cysteine residues using farnesyl pyrophosphate. In the same manner, geranylgeranyl transferase requires geranyl pyrophosphate. Interfering with the synthetic pathway that produces both prenyl groups partially rescues HGPS-associated defects. This can be achieved with ZOPRA, that is composed by the N-bisphosphonate Zoledronate and the statin Pravastatin (Varela et al. 2008). Promising results have also been obtained with monoaminopyrimidines that inhibit both farnesyl transferase and farnesyl pyrophosphate synthase (Blondel et al. 2016). As mentioned above, prelamin A is carboxymethylated in its C terminus by ICMT. Inhibition of ICMT through expression of short hairpin RNAs in *ZMPSTE24* knockout mice and HGPS cells induces the release of prelamin A from the nuclear lamin to the nucleoplasm. Curiously, misshapen nuclei are still present, but other senescent features are delayed (Ibrahim et al. 2013). Elimination of progerin by autophagy is another therapeutic approach explored, including in clinical trials in combination with Lonafarnib (Harhouri et al. 2018; Gabriel et al. 2015; Harhouri et al. 2017). Finally, a set of drugs alleviates the defects associated with progerin expression, at least in cell culture. Inhibition of the association of N-acetyl transferase NAT10 with microtubules using Remodelin prevents progerin-derived NE alterations (Larrieu et al. 2018), whereas stress in HGPS fibroblasts can be reduced with methylene blue, N-acetyl cysteine, or inhibitors of Rho-associated protein kinase (Xiong et al. 2016; Richards et al. 2011; Kang et al. 2017). Most recently, CRISPR/Cas9-mediated inhibition of progerin expression was shown to improve health and extend lifespan of a HGPS mouse model (Beyret et al. 2019; Santiago-Fernandez et al. 2019). Efficient and safe delivery of Cas9 (or related nucleases) throughout the organism is still a major challenge for CRISPR/Cas9-based therapies, but this is a field undergoing remarkable advances.

Mutations in *LMNA* and *ZMPSTE24* can lead to other premature ageing syndromes than HGPS. Mandibuloacral dysplasia (MAD) type A is a recessive disease caused by the amino acid substitution R527H in the C-terminal immunoglobulin domain of lamin A. The mutation disrupts the interaction of lamin A with other proteins and around 10% of fibroblasts derived from patients presents nuclei with pronounced lobulation (Novelli et al. 2002). Mutation in *ZMPSTE24* generates a more severe variant of MAD (type B) (Agarwal et al. 2003). MAD individuals

display growth retardation, mandibular hypoplasia or retrognathia, osteolysis of clavicles, bulbous fingers, alopecia, lipodystrophy, and diabetes among others.

Approximately a decade ago, another progeria syndrome was described and termed Néstor-Guillermo (NGPS) after the two patients initially diagnosed (Cabanillas et al. 2011). NGPS individuals share many symptoms with HGPS patients, but they live longer, are taller, and do not suffer cardiovascular problems. Nonetheless, they develop severe osteolysis which causes chronic pain. Genetic aetiology of NGPS relies on a homozygous c.34 G > A nucleotide change in *BANF1*, that leads to an A12T amino acid substitution in BAF (Cabanillas et al. 2011). BAF forms a ternary complex with Emerin and lamins. Moreover, BAF binds to DNA, which facilitates anchoring chromatin regions to the nuclear lamina. NGPS-derived fibroblasts present nuclear irregularities and cytoplasmic accumulation of Emerin (Puente et al. 2011).

Bona fide hallmarks of ageing have to accomplish three requisites (Lopez-Otin et al. 2013). First, the phenomenon in question must appear or increase during ageing. Second, its activation in model organisms deteriorates their health. Third, its depletion ameliorates age-associated defects. Progerin fulfills the two last criteria, but it is uncertain if it should be included as a true hallmark of ageing, because its presence or increase is not clearly related to ageing. In contrast, prelamin A has been observed to accumulate during in vitro ageing of vascular smooth muscle cells, suggesting an implication of lamin processing in physiological ageing (Ragnauth et al. 2010). Moreover, progerin affects other well-established hallmarks of ageing (Gonzalo et al. 2017). Genome instability is a main feature of ageing and HGPS fibroblasts activate DNA repair factors, as indicated by the increased presence of H2AX foci (Scaffidi and Misteli 2006). Indeed, HGPS fibroblasts have defective DDR, since upon DNA damage induced by irradiation, appearance of 53BP1 foci is delayed compared to control fibroblasts. Besides, once established, 53BP1 foci persist, reinforcing that DDR is compromised in HGPS cells. Specifically, repair by homologous recombination is compromised, as HGPS nuclei contain less RAD51 foci upon irradiation compared to control cells (Liu et al. 2005). Progerin also interferes with replication, inducing replicative stress by sequestering PCNA (Hilton et al. 2017). A transcendent discovery in ageing biology was that cultured cells can only be passaged a finite number of times, named the Hayflick limit (Hayflick 1985). Only transformed or pluripotent stem cell lines can be maintained, in theory, forever. Later studies have found a strong inverse correlation between telomere length and number of viable passages. As cultured cells divide their telomeres shorten while progerin expression augments, potentially by a general increase in alternative splicing. Moreover, mutations in proteins that cap telomeres lead to progerin increase, whereas ectopic expression of telomerase, which extends telomere sequences, decreases progerin accumulation (Cao et al. 2011).

Accumulation of senescent cells are one of the nine hallmarks of ageing (Lopez-Otin et al. 2013). Senescent cells can be detected in most organs, constituting a heterogenous class of cells. Cells entering senescence activate a permanent state of no division, hyperactivation of p53, and a characteristic secretory-associated phenotype, acting as pro-inflammatory cells. Furthermore, senescent cells undergo

major morphological modifications, including enlarged cytoplasm and deformed nuclei with loss of lamin B1 being considered as a biomarker of senescence (Hernandez-Segura et al. 2018). Progerin accumulation has also been proposed as a biomarker of senescent cells (Cao et al. 2011). The downregulation of lamin B1 is due to a decrease in both transcription and translation; the latter being mediated by the annealing of miRNA23a to lamin B1 mRNA (Freund et al. 2012). Lamin B1-depleted senescent cells display large-scale chromatin alterations and compromised DNA integrity, eventually leading to release of chromatin fragments to the cytoplasm (Ivanov et al. 2013; Shah et al. 2013). Moreover, lamin B1 is required for genome stability and its depletion causes a delay in the onset of S-phase due to replication fork stalling and deregulation of genes involved in DNA repair, such as RAD51 and BRCA1 (Butin-Israeli et al. 2015).

Nuclear Pore Complex Turnover and Decline in Ageing

Several nucleoporins are extremely long-lived, which makes them particularly interesting from the perspective of ageing. While most proteins recycle within hours or a few days, pulse-chase labelling with the stable isotope ¹⁵ N revealed that for certain nucleoporins, up to two-thirds of the original proteins were present in the brains of rats for 6 months and still detectable after 1 year (Savas et al. 2012). By cell fractionation it was determined that turnover of stable nucleoporins was lower in neurons compared to glia, which correlates with the ability of glia to self-renew (Toyama et al. 2013). Interestingly, the stable nucleoporins all belong to two NPC subcomplexes, the Nup107–160 subcomplex and the Nup205 subcomplex, whereas other NPC subcomplexes turn over much faster. The members of each of the two stable subcomplexes have similar turnover kinetics among them but differ between the subcomplexes (Toyama et al. 2013). This suggests that NPCs are extremely stable structures as a unit with different exchange rates of the subcomplexes that compose the NPC. Experiments in *C. elegans*, in which all adult somatic cells are post-mitotic, similarly reported that the Nup107–160 subcomplex is expressed and incorporated into NPCs during early development and not renewed later in life (D’Angelo et al. 2009).

Proteins accumulate toxic damage over time, which is counteracted by protein degradation and synthesis. However, the slow turnover of NPCs poses a challenge particularly in post-mitotic cells, such as neurons and which is further exacerbated during ageing. Analysis of protein extracts from brains of old rats found evidence of oxidative damage of Nup93 from the Nup205 subcomplex (D’Angelo et al. 2009) and ~15–40% reduction of Nup205 subcomplex members in NPCs isolated from old rats (Toyama et al. 2013). Strikingly, when incubated with fluorescently labelled 70 kDa dextran, nuclei isolated from either old nematodes or old rats showed a dramatic increase in nuclear staining as compared to nuclei from young animals, indicating age-related deterioration of the permeability barrier function of NPCs (D’Angelo et al. 2009). Increased leakiness was also recently reported in a study that

compared muscle nuclei from old and young mice (Iyer et al. 2021). Moreover, proteomics studies comparing protein abundance between young and old rodents have detected different behaviours of individual nucleoporins and between tissues (Cutler et al. 2017; Ori et al. 2015; Rempel et al. 2020). Age-related changes in nucleocytoplasmic transport (NCT) was also inferred by comparing transcriptomes of samples from donors ranging from 0 to 89 years of age (Mertens et al. 2015). Only three deregulated genes were shared between fibroblasts, induced neurons (generated by transcription factor-based conversion of fibroblasts) and human prefrontal cortex: LAMA3, PCDH10 and RanBP17. RanBP17 is a nuclear protein import receptor of the importin beta family and reduced RanBP17 expression was found to correlate with a decrease in cells' ability to maintain nuclear and cytoplasmic reporters exclusively in their respective compartments (Mertens et al. 2015). However, the natural substrates of RanBP17 and their localisation during ageing remain unknown. Serial passages of vascular smooth muscle cells (VSMCs) in culture replicate aspects of natural ageing, such as accumulation of prelamin A and increased frequency of DNA damage. These cells also present relocalisation of Nup153 from the NE to intranuclear aggregates, disruption of the Ran gradient and a concomitant reduction of nuclear import of 53BP1 (Cobb et al. 2016). Changes in NPC stoichiometry and transport competence have also been reported in the budding yeast *Saccharomyces cerevisiae* both during chronological (in non-dividing cells) and replicative (dividing cells) ageing (Binai et al. 2014; Janssens et al. 2015; Rempel et al. 2019, 2020; Lord et al. 2015). Based on these observations, it seems clear that NPCs suffer various age-related changes that may compromise their critical role in NCT. As we discuss in the next section, this probably contributes to the development of neurodegenerative disease.

The Implication of Nucleocytoplasmic Transport in Neurodegenerative Disease

Ageing is a prominent risk factor for several neurodegenerative diseases (Hou et al. 2019). Although neurodegenerative diseases are diverse in terms of clinical and physiological symptoms, they all have signatures that overlap with multiple hallmarks of ageing (Lopez-Otin et al. 2013), in particular loss of proteostasis, mitochondrial dysfunction, genome instability and cellular senescence. For instance, signalling between the nucleus and mitochondria is essential for cellular homeostasis (Lord et al. 2015; Romero-Bueno et al. 2019; Hou et al. 2019) but NCT is frequently perturbed in multiple ways in neurodegenerative diseases (Moore et al. 2020; Liu and Hetzer 2021).

RNA-Binding Proteins and Neurodegenerative Diseases

Amyotrophic lateral sclerosis (ALS) represents the best characterised connection between NPCs and neurodegenerative disease. ALS is characterized by the death of motor neurons in the brain and spinal cord, causing progressive paralysis (Taylor et al. 2016). Some ALS patients are also diagnosed with frontotemporal dementia (FTD) and the two diseases share underlying mutations and cellular phenotypes. At least 16 loci are linked unequivocally to the pathogenesis of ALS, with mutations in *SOD1*, *TARDBP*, *FUS* and *C9orf72* genes occurring with highest frequencies (Taylor et al. 2016). *TARDBP* encodes the RNA-binding protein TDP-43, which is normally nuclear or reversible enriched in cytoplasmic stress granules but trapped in cytoplasmic inclusions in cells of the central nervous system of ALS patients (Neumann et al. 2006; Winton et al. 2008). The formation of these inclusions is stimulated by ALS-linked mutations in *TARDBP* (Johnson et al. 2009), but can also be induced by mutations in other ALS/FTD genes, indicating the propensity of wild-type TDP-43 to form fibrils (Moore et al. 2020; Springhower et al. 2020). Most *TARDBP* mutations map to the intrinsically disordered region of TDP-43 and are thought to change the equilibrium between formation of physiological stress granules and toxic inclusions (Taylor et al. 2016; Moore et al. 2020). It is unclear if the pathogenic inclusions are derived from stress granules, but mutant TDP-43 can sequester multiple nucleoporins and NCT factors to stress granules (Zhang et al. 2018) (Fig. 3.1b). Furthermore, inhibition of stress granule formation rescues NCT defects and suppresses neurodegeneration in a *Drosophila* ALS model (Zhang et al. 2018). Affinity purification of soluble and aggregated TDP-43 from neuroblastoma cells revealed a strong enrichment for nucleoporins: 14 were found more frequently in the aggregates, whereas 2 interacted more strongly with soluble TDP-43 (Chou et al. 2018) (Fig. 3.1a). Nup205 was also detected in TDP-43-positive inclusions in ALS brain tissues, whereas co-aggregation of Nup98, Nup153 and Nup214 with TDP-43 in neuroblastoma cells was dependent on prion-like domains within their FG-repeat regions (Chou et al. 2018). Recently, relocalisation of Nup98 from NPC to the cytoplasm was also reported in neurons from progranulin mutant mice that represent another example of FTD with TDP-43 aggregates (Zhang et al. 2020). While it seems clear that TDP-43 can recruit nucleoporins to inclusions, alterations in NPC function may occur prior to TDP-43 mislocalisation (Coyne et al. 2021). Interestingly, TDP-43 in inclusions is not associated with mRNA, and, conversely, binding of TDP-43 to mRNA can reduce formation of inclusions (Mann et al. 2019). This suggests that the ratio between TDP-43 and mRNA is important for assembly of pathological TDP-43 inclusions and that this ratio is affected by the efficiency of NCT. Moreover, the transport receptors Importin- α (Imp α) and Imp β 1 (aka Kap β 1) which are responsible for nuclear import of TDP-43 act as chaperones to prevent aggregation of TDP-43 in vitro (Guo et al. 2018).

Other RNA-binding proteins found to accumulate in inclusions in samples from ALS and FTD patients include FET (FUS, EWS, TAF15) and hnRNP A1/A2 proteins, which also contain intrinsically disordered regions (Springhower et al.

2020). Similar to TDP-43, FET and hnRNP A1/A2 proteins are mainly nuclear although also found in cytoplasmic stress granules and their solubility is regulated by interaction with a nuclear transport receptor, in this case Transportin-1 (Kap β 2) (Guo et al. 2018). Interestingly, overexpression of Kap β 2 can rescue muscle degeneration in *Drosophila* caused by hnRNP A2 aggregation, providing in vivo evidence for the role of the NCT machinery in regulating protein homeostasis (Guo et al. 2018). Further evidence was provided in a recent study that found that expression of ALS-mutant FUS in motor neurons perturbed the distribution of Ran, led to diminished nucleoporin levels in the NE and reduced both nuclear import and export rates (Lin et al. 2021).

Peptide Repeat Toxicity and NPC Function

The most common single-gene dominant mutation responsible for ALS is an expansion of an intronic GGGGCC repeat in the *C9orf72* gene (Taylor et al. 2016). The gene harbours less than ~20 repeats in healthy persons and up to several hundred in ALS and FTD patients. Several hypotheses have been proposed to explain why the expansion induces neurodegenerations and evidence exists for at least three: (1) the expansion reduces *C9orf72* expression from the affected allele, causing haploinsufficiency, (2) formation of RNA foci consisting of stable sense and anti-sense transcripts and (3) translation of poly-dipeptides by repeat-associated non-AUG (RAN) translation (see references in (Moore et al. 2020)).

When expressed in *Drosophila*, GGGGCC dose and length-dependent toxicity as well as poly-(glycine-proline) and poly-(glycine-arginine) dipeptide repeat accumulation arising from RAN translation are observed in multiple tissues (Freibaum et al. 2015). Two genetic screens in the fly model identified many NE-associated proteins as either enhancers (E) or suppressors (S) of GGGGCC-induced phenotypes when mutated or knocked down. Hits included nucleoporins Nup107 (S in both screens), Nup50 (S/E; opposing effects in the two screens), Nup93 (E), Nup153 (E), Nup160 (S), Nup98–96 (S), nuclear transport receptors Kap β 2 (E in both screens), Crm1 (E in both screens), components of the GTPase Ran cycle (E in both screens) and several RNA-binding factors (Freibaum et al. 2015; Boeynaems et al. 2016). Moreover, expression of the expanded GGGGCC repeat interfered with nuclear protein import and RNA export both in *Drosophila* and human induced pluripotent stem cell (iPSC), thus providing evidence for a NCT defect caused by *C9orf72* mutation (Freibaum et al. 2015; Zhang et al. 2015). Further support for this was provided by a systematic screen in *S. cerevisiae* which found that overexpression of six nuclear transport receptors including KAP104 (Kap β 2) suppressed poly-(proline-arginine) dipeptide toxicity, whereas overexpression of nucleoporin NDC1 or Ran's guanine nucleotide exchange factor SRM1/RCC1 enhanced toxicity (Jovicic et al. 2015). Poly-(glycine-arginine) and poly-(proline-arginine) dipeptides are capable of sequestering multiple transport receptors and nucleoporins in insoluble aggregates, but, remarkably, the dipeptides also inhibit Kap β 1-mediated nuclear protein import

under conditions without formation of aggregates, arguing that they might also possess aggregation-independent toxic effects (Hayes et al. 2020). The disruption of NCT by poly-dipeptides also alters the distribution of the transcription factor TFEB, leading to impaired autophagy and thereby accelerated formation of aggregates and eventually neuron death (Cunningham et al. 2020).

Huntington's disease (HD) is caused by an expansion of the CAG repeat found in the *HTT* gene. This leads to the synthesis of the Huntingtin (HTT) protein with a polyglutamine (polyQ) tract with more than ~40 residues in HD patients as compared to ~10–30 residues in healthy individuals. The expansion of the polyQ tract causes HTT to form aggregates that sequester numerous other proteins including lamin B and several FG-repeat nucleoporins (Suhr et al. 2001) and perturb NCT (Woerner et al. 2016). In young HD mice, the aggregates appear initially intranuclear where they accumulate RanGAP1, Nup62, and Gle1 (Gasset-Rosa et al. 2017; Grima et al. 2017). These defects coincide with alterations in NE morphology and appearance of DNA damage foci, which become more severe during ageing. Interestingly, either overexpression of Ran and RanGAP or drug-mediated manipulation of NCT protects primary cortical neurons and *Drosophila* against the toxic effects of HTT with polyQ expansion (Grima et al. 2017).

Additional Implications of NCT in Neurodegenerative Diseases

TDP-43 inclusions are not specific to ALS and FTD: they appear frequently in HD, Alzheimer disease (AD) and Parkinson's disease (Moore et al. 2020). Similarly, the microtubule-associated protein tau is linked to several neurodegenerative diseases, including FTD and AD. Aggregation of hyperphosphorylated tau in neurofibrillary tangles is a hallmark of AD and was recently found to colocalise with Nup98 in brain samples from AD patients (Eftekharzadeh et al. 2018). In contrast to the large number of nucleoporins found in TDP-43 inclusions described above, the accumulation of phospho-tau seemed to affect Nup98 (and perhaps Nup62) more specifically, arguing that NPC composition is largely unaffected in AD. Nevertheless, physical interaction of Nup98 with Tau in the cytoplasm and at NPCs disrupts the gradient of Ran across the nuclear envelope, impairs the permeability barrier of the NPC and interferes with NCT (Eftekharzadeh et al. 2018). These defects may explain other phenotypes observed in AD and related to NCT, including mislocalisation of Impα (Lee et al. 2006).

Parkinson's disease (PD) is caused by degeneration of dopaminergic neurons. A potential involvement of NCT was proposed more than two decades ago after detecting a dramatic accumulation of the transcription factor NF-kappaB in the nuclei of mesencephalon neurons of PD patients (Hunot et al. 1997). Moreover, it was reported that inactivation of one *NUP358* allele in mice increases the sensitivity to the neurotoxin MPTP, which reproduces PD phenotypes although a direct link to impaired NCT has not been established (Cho et al. 2012).

Returning to ALS as the pathology with most evidence for a role of NCT in disease progression, a recent study found that ectopic expression of an ALS mutant form of the actin-binding protein PFN1 impairs NCT (Giampetrucci et al. 2019). Several nucleoporins including Nup358 and POM121 as well as RanGAP were delocalised from NPCs in cells expressing mutant PFN1 and the nucleocytoplasmic Ran gradient was perturbed. Although mutant PFN1 forms cytoplasmic inclusions, the detached NPC components did not accumulate in these structures (Giampetrucci et al. 2019), which differs from the TDP-43 aggregates described above (Chou et al. 2018). Restoration of actin polymerisation in PFN1 mutant cells by overexpression of a constitutively active form of mDia1 rescued the defects in nucleoporin and RanGAP localisation and NCT. Importantly, the constitutively active form of mDia1 was also able to alleviate multiple defects induced by GGGGCC repeat expansion, arguing that actin polymerisation is critically involved in ALS, regardless of the underlying mutations (Giampetrucci et al. 2019).

Concluding Remarks

Several alterations are observed in the NE during normal and premature ageing, affecting in particular the nuclear lamina and NPCs. Special attention is focused on progerin, a minor splice variant of the *LMNA* gene because mutations leading to progerin accumulation cause progeria. Nonetheless, it remains an open question if progerin constitutes a true hallmark of physiological ageing. On the other hand, loss of lamin B1 is well-established as a feature of senescent cells. NPCs are characterised by a remarkably slow turnover of several of its components, which may affect its permeability barrier function during ageing. Moreover, nucleocytoplasmic transport is impaired in multiple age-associated neurodegenerative diseases (e.g. Parkinson, Alzheimer and amyotrophic lateral sclerosis) and several nucleoporins have been identified as promising therapeutic targets. The wealth of interesting and recent discoveries described in this chapter illustrates the pace of research in a field with broad implications for human health.

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Chapter 4

Mitochondrial DNA Mutations and Ageing



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Abstract Mitochondria are subcellular organelles present in most eukaryotic cells which play a significant role in numerous aspects of cell biology. These include carbohydrate and fatty acid metabolism to generate cellular energy through oxidative phosphorylation, apoptosis, cell signalling, haem biosynthesis and reactive oxygen species production. Mitochondrial dysfunction is a feature of many human ageing tissues, and since the discovery that mitochondrial DNA mutations were a major underlying cause of changes in oxidative phosphorylation capacity, it has been proposed that they have a role in human ageing. However, there is still much debate on whether mitochondrial DNA mutations play a causal role in ageing or are simply a consequence of the ageing process. This chapter describes the structure of mammalian mitochondria, and the unique features of mitochondrial genetics, and reviews the current evidence surrounding the role of mitochondrial DNA mutations in the ageing process. It then focusses on more recent discoveries regarding the role of mitochondrial dysfunction in stem cell ageing and age-related inflammation.

Keywords Ageing · Mitochondria · DNA · Mutations · Cellular damage · Molecular damage · Free radical damage

Introduction

Ageing is the complex accumulation of random cellular and molecular damage that leads to a progressive decline in tissue and organ function, resulting in age-related disease and ultimately, death. Many evolutionary theories addressing how and why ageing occurs have been proposed and can be seen to date back to the late nineteenth century (Weismann 1891). Breakthroughs in scientific research and the revelation

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that the ageing process is modifiable by caloric restriction (Weindruch 1996), have steered biomedical research towards understanding the molecular mechanisms of ageing. Alongside this, a decline in early-midlife mortality and increase in life expectancy have occurred creating a social and economic burden on society. For many chronic illnesses such as cancer and neurodegenerative disease, ageing is the biggest risk factor and as such, establishing the existence of biomarkers, innate mechanisms and influential exogenous factors which may help or be manipulated to protect or treat age-related dysfunction and disease, is in great demand.

One of the most well-known molecular theories of ageing, the mitochondrial free radical theory of ageing (MTFRA) (Harman 1972), centres on the fact that mitochondria are the major cell intrinsic producers of reactive oxygen species (ROS) (Loschen et al. 1971; Chance et al. 1979). ROS are highly reactive molecules capable of damaging intracellular proteins, lipids and DNA and as such the MFRTA stipulated that mitochondrial respiration controlled the ageing process. However, since the original proposal of the MFRTA, the significance of mitochondrial ROS in ageing has come under scrutiny, as ROS have also been shown to have essential roles in maintenance of normal cellular homeostasis. Therefore the study of the role of mitochondria in the ageing process has broadened to include investigation of numerous essential aspects of mitochondrial physiology. Moreover, a direct causal relationship of mitochondrial alterations in the ageing process cannot be deemed from the mere presence of mitochondrial dysfunction within cells and tissues, and at present there are many contradictory theories and reports within the field of mitochondrial biology. Here we review the current evidence surrounding the role of mitochondrial dysfunction in ageing with a focus on recent developments regarding mitochondrial function in stem cell ageing and inflammation.

Mitochondrial Structure and Function

Mitochondria are semi-autonomous organelles thought to have evolved from the endosymbiosis of a free living α -proteobacterium into a eukaryotic cell (Gray 2012; Gray et al. 1999). Present in the majority of eukaryotic cells, the mitochondrion is rod-like shaped, encompassing an outer (OMM) and an inner (IMM) mitochondrial membrane with inner protruding folds termed cristae that envelope the mitochondrial matrix. The generation of high-resolution live cell microscopy has further revealed mitochondria as dynamic entities with ability to fuse and divide that enables the formation of tubular networks, enhancing mitochondrial functioning and synergy within the cell (Nunnari et al. 1997). Mitochondria are commonly referred to as the ‘powerhouse’ of the cell as they are the major site for the generation of adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS) (Mitchell 1961). Other important mitochondrial functions include iron-sulphur biogenesis and haem synthesis, one carbon metabolism, fatty acid synthesis and oxidation, regulation of calcium homeostasis, induction of apoptosis and ROS generation.

Oxidative Phosphorylation

An array of intricate metabolic pathways within a cell convert energy stored in macronutrients into ATP. Conventional carbohydrate oxidation comprises a series of metabolic pathways with shuttling of glucose into glycolysis in the cytoplasm, followed by flux through the tricarboxylic acid (TCA) cycle and OXPHOS in the mitochondria. Glycolysis synthesises 2 ATP molecules per glucose molecule, whereas in the presence of O₂ complete oxidation in the mitochondria to CO₂ and H₂O, permits the synthesis of 36 ATP molecules. The OXPHOS machinery comprises the mitochondrial electron transport chain (ETC), composed of protein-lipid enzyme complexes (I–V), present as supercomplexes denoted as ‘respirasomes’ in the IMM (Schagger and Pfeiffer 2000; Acin-Perez et al. 2008), and complex V, the ATP synthase. NADH and FADH₂, reduced in the TCA cycle, donate electrons to the ETC at complexes I and II, respectively. Transfer of electrons down the ETC yields free energy for the pumping of protons at complexes I, III and IV from the mitochondrial matrix across the IMM to the intermembrane space. Subsequently, an electrochemical gradient is established across the IMM providing the proton motive force required by complex V (ATP synthase) for its rotation and conformational change for the synthesis of ATP from ADP and inorganic phosphate (Noji et al. 1997).

Mitochondrial Genetics

Human mtDNA is a double-stranded, ~16.5 Kb circular genome located within the mitochondrial matrix (Anderson et al. 1981). MtDNA encodes 37 genes, 13 protein subunits of the mitochondrial respiratory chain, the 12S and 16S ribosomal RNA (rRNA) and 22 transfer RNAs (tRNAs) for mitochondrial translation (Nass 1966; Anderson et al. 1981). The relatively small and compact mitochondrial genome is strictly maternally inherited (Giles et al. 1980) and is thought to have evolved through the loss and transfer of genes from the α -proteobacterium genome to the nuclear DNA (Gray et al. 1999; Gray 1999). As such the remaining gene products required by mitochondria for biogenesis, maintenance and function are nuclear encoded and are imported into the mitochondria. Mitochondria are abundant within most somatic cells and as such there are approximately 1000–10,000 mtDNA copies per cell (Lightowers et al. 1997). Single or multiple mtDNA molecules are organised into compact nucleoprotein complexes, called nucleoids (Satoh and Kuroiwa 1991). The first reports of nucleoid composition suggested that the proteins located within the nucleoid core included mitochondrial transcription factor A (TFAM), mitochondrial single-strand binding protein (mtSSB), mitochondrial DNA polymerase γ (Poly), mitochondrial RNA polymerase (POLRMT) and the mitochondrial TWINKLE helicase (Bogenhagen et al. 2008), whilst proteins involved in RNA processing, translation and assembly of respiratory complexes

were said to be found in the periphery of the nucleoid (Bogenhagen et al. 2008). However, more recently, alternative models have been proposed. Using electron microscopy, cryo-ET microscopy and super-resolution microscopy it was demonstrated that the mitochondrial nucleoid consists of a single mtDNA molecule compacted by cross-strand binding of approximately 1000 molecules of TFAM per mtDNA molecule in the absence of additional proteins (Kukat et al. 2011, 2015). Furthermore, the abundance of TFAM and thus the level of DNA compaction has been shown to impact the proportion of mtDNA molecules available for transcription or replication (Farge et al. 2014).

Nucleoids are thought to be semi-protective; however, mutagenesis of mtDNA resulting in deletions, point mutations, insertions or large-scale rearrangements is seen to occur due to a range of extrinsic or intrinsic agents, and biological malfunctioning. In comparison to the nuclear genome, the mutation rate of mtDNA is thought to be ~17-fold higher (Wallace et al. 1987). It was initially thought that the majority of mtDNA point mutations occurred following ROS-induced mtDNA damage (Loschen et al. 1971). However, extensive examination of mtDNA point mutation spectra revealed a pattern more compatible with mtDNA replication errors by the endogenously error prone Poly (Zheng et al. 2006). Whilst large-scale mtDNA deletions were originally postulated to arise during replication via a slipped-strand mechanism (Shoffner et al. 1989), others have argued that the spectrum of deletion breakpoints is more compatible with a mechanism whereby deletions are induced following mis-repair of mtDNA double-strand breaks (Krishnan et al. 2008).

The polyploid nature of mtDNA means that cells can exist in a state of homoplasmy (all mtDNA molecules are identical) or heteroplasmy (mixed population of mtDNA genotypes within a cell) (Taylor and Turnbull 2005). The majority of mtDNA mutations are functionally recessive, and it is only when a critical threshold of mutated mtDNA is reached and the residual wild-type mtDNA molecules are no longer able to compensate, that a biochemical defect becomes manifest (Sciacco et al. 1994). The threshold at which the biochemical defect is induced varies depending on the mtDNA mutation type and metabolic demand of the tissue and is clinically used as a biomarker in the diagnosis of mitochondrial disease (Lightowers et al. 1997). Adding further layers of complexity to mitochondrial genetics, mtDNA replication, unlike the nuclear genome, is uncoupled from the cell cycle (Bogenhagen and Clayton 1977). Owing to this semi-autonomous attribute and the polyploid nature of mtDNA, the level of mtDNA mutations within a cell is not stable. In fact, a single mutated mtDNA molecule within a cell can come to dominate the mtDNA population within a cell over time. This process is termed clonal expansion. The mechanism by which it occurs is still heavily debated and is likely to be tissue type dependent (discussed below).

Mitochondrial DNA Mutations in Ageing

Histochemical staining of cytochrome *c* oxidase (COX, complex IV of the ETC) provided the first association between increasing age and mitochondrial dysfunction. Early studies showed an age-related increase in the frequency of COX-deficient cells including cardiomyocytes, skeletal muscle fibres and neurons of the brain (Muller-Hocker 1989, 1990; Cottrell et al. 2001). The mosaic pattern of these COX-deficient cells was similar to the pattern observed in patients with primary mtDNA disease, but at a much lower frequency, indicating that mtDNA mutations may be the underlying cause, which was subsequently confirmed (Brierley et al. 1998; Bender et al. 2006). Similarly, in ageing mitotic tissues, COX deficiency has been reported in the intestine, liver and stomach (Taylor et al. 2003; Fellous et al. 2009; McDonald et al. 2008), establishing that age-related COX deficiency due to somatic mtDNA mutations was also a feature of ageing mitotic tissues maintained by adult stem cells. Interestingly, there is a clear unsolved disparity in type of mutations present in post-mitotic tissues versus mitotic tissues with large-scale deletions detected in aged-post-mitotic tissues, whilst mtDNA point mutations accumulate in aged-mitotic tissues.

MtDNA Deletions

MtDNA deletions encompass mtDNA molecules that have lost large segments of their genetic material, and commonly affect the major arc of mtDNA (Samuels et al. 2004). Cortopassi and Arnheim (1990) were first to identify somatic large-scale mtDNA deletion in ageing tissues, detecting the common 4977 nucleotide pair deletion, mtDNA⁴⁹⁷⁷, in aged brain and heart (Cortopassi and Arnheim 1990). Further groups have subsequently confirmed mtDNA deletions in the ageing brain, heart and skeletal muscle (Corral-Debrinski et al. 1992; Simonetti et al. 1992); however, the levels of mutation in these early reports were extremely low undermining a possible role for mtDNA mutations in ageing. Subsequently in 1998, Brierley et al. reported high levels of mtDNA deletions in individual skeletal muscle fibres with an associated biochemical defect in aged subjects (Brierley et al. 1998). This observation was complemented by Bender et al. (2006) who showed a significant accumulation of mtDNA deletions in aged substantia nigra neurons. Numerous studies have tried to identify the mechanism through which mtDNA deletions clonally expand in individual cells during ageing (Vincent et al. 2018; Krishnan et al. 2008; Campbell et al. 2014; Elson et al. 2001). This has been of particular interest as clonally expanded mtDNA deletions have been implicated in age-related conditions such as muscle fibre atrophy, heart disease and Parkinson's disease (Herbst et al. 2007; Bender et al. 2006; Mohamed et al. 2006; Bua et al. 2006). Early in silico predictions supported the theory that simple random genetic drift could explain clonal expansion of mtDNA mutations with age (Elson et al. 2001); however, these models do not control for diffusion of mtDNA along muscle fibres nor from neuronal synapses to the nodes of Ranvier. Subsequently theories

including a replicative advantage of mtDNA deleted molecules (Wallace 1989; Fukui and Moraes 2009) and feedback mechanisms contributing to mtDNA replication due to low levels of mitochondrial proteins have been postulated (de Grey 1997). More recently, in muscle, the perinuclear niche hypothesis has been described. The authors propose that mtDNA deletions causing respiratory chain deficiency induce retrograde stress signalling which induces mtDNA biogenesis in the perinuclear foci of muscle fibres, resulting in further replication of those deleted molecules. This hypothesis is supported by the finding of respiratory chain deficient regions of muscle fibres being localised to perinuclear regions (Vincent et al. 2018). In contrast, the mechanism of clonal expansion of mtDNA deletions in neurons is thought to be predominantly mediated through mitochondrial transportation and fission and fusion dynamics. This is supported by the observed transference of mitochondria to sites of high energy demand or for their degradation in the soma (Kang et al. 2008; Miller and Sheetz 2004; McWilliams et al. 2016). Moving forward studies investigating driving forces of mtDNA deletions in ageing must ensure tissue mosaicism and cytology of the different cell types are fully accounted for before inferences can be made.

MtDNA Point Mutations

Somatic mtDNA point mutations have been detected in numerous mitotic tissues maintained by adult stem cells (ASCs), with the first report being in ageing buccal epithelium (Nekhaeva et al. 2002). Subsequently age-related mtDNA point mutations have also been observed in the intestine, stomach, liver, oesophagus and prostate (Taylor et al. 2003; McDonald et al. 2008; Fellous et al. 2009; Blackwood et al. 2011). Combined histochemical and sequencing studies of single human colonic crypts by Taylor and colleagues revealed that the mtDNA mutations were occurring and clonally expanding within the stem cell compartment causing COX deficiency. This resulted in ribbons of COX deficiency from the base to the lumen of the crypts as each of the daughter cells and subsequent progenitors inherited the mtDNA genotype of the stem cell (Taylor et al. 2003; Greaves et al. 2006, 2012). As with the mtDNA deletions discussed earlier, the mtDNA point mutations detected in ageing mitotic tissues had occurred somatically and clonally expanded over time. Mathematical modelling studies support the mechanism of random genetic drift coupled with mitotic segregation at cell division to explain clonal expansion. Here cells undergoing division amplify their mtDNA pool including mtDNA mutations, and following segregation into daughter cells, mtDNA species can ultimately be lost or clonally expand. This has been shown in colonic crypt stem cells (Stamp et al. 2018), normal buccal epithelial cells and tumour cells (Coller et al. 2001).

Understanding the consequences of mtDNA mutation accumulation with age has extended beyond the impairment of oxidative phosphorylation. Indeed Nooteboom et al. (2010) showed that complex IV deficiency resulted in a decrease in the size of the proliferative compartment of the crypts, an increase in apoptosis and ultimately a decreased crypt cell population. What is more, age-related mitochondrial OXPHOS

dysfunction has been exploited as a dynamic lineage tracer for stem cell fate and homeostasis in the ageing colon (Nicholson et al. 2018; Stamp et al. 2018), and the advent of massively parallel single-cell sequencing has highlighted the utility of somatic mtDNA point mutations in lineage tracing in tens of thousands of cells (Kwok et al. 2022). Interestingly inherited mtDNA point mutations whilst stable in post-mitotic tissue with age, are lost with age in mitotic tissues including the colon and blood (Su et al. 2018; Kwok et al. 2022). This suggests that different selective pressures are at play in young versus ageing mitotic tissues.

Mouse Models of Mitochondrial Alterations with Ageing

Despite the plethora of evidence that mtDNA mutations occur and clonally expand in ageing human tissues, resulting in a mosaic pattern of respiratory chain deficiency, direct evidence of a causal role in ageing and the development of age-related disease has been lacking. Therefore, to test this hypothesis numerous groups have used transgenic mice as experimental models. The first mouse with tissue-specific age-related mitochondrial dysfunction to be developed was the *Tfam*^{-/-} mouse model. Tissue-specific knockout of the transcriptional activator *Tfam* in the pancreatic β-cells (Silva et al. 2000) and nigrostriatal dopamine neurons (Ekstrand et al. 2007) resulted in a decrease in mtDNA copy number, respiratory chain dysfunction and an associated onset of diabetes or Parkinsonism phenotype respectively. However, the effect was not universal, for example, skeletal muscle specific *Tfam* knockout did not result in an overt phenotype (Wredenberg et al. 2006), and led to some experts questioning the general utility of the *Tfam*^{-/-} model in the ageing field. The generation of the mutator mouse model with an erroneous mitochondrial polymerase gamma (*PolyA*) (Trifunovic et al. 2004; Kujoth et al. 2005) allowed direct investigation of the effect of mtDNA mutations in ageing. An aspartate to alanine substitution at residue 257 of the conserved proofreading domain of *PolyA* impairs the exonuclease activity of the enzyme resulting in an up to eightfold increase in the likelihood of point mutations occurring during replication of the mitochondrial genome. This results in a reduction in life span, and the development of kyphosis, anaemia, greying of hair, alopecia, osteoporosis, weight loss, muscle loss, all akin to a premature ageing phenotype. The *PolyA*^{mut/mut} model has not however been free of controversies within the ageing field. Initial reports documented an unanticipated absence of an increased ROS production by the *PolyA* mutation. This was not resolved until almost a decade later when Logan et al. (2014) applied a molecular probe to specifically targeting the mitochondria and showed increased levels of ROS in ageing *PolyA*^{mut/mut} tissues. Additional *ex vivo* and *in vivo* analyses of *PolyA*^{mut/mut} mice have subsequently been carried out to dissect the molecular mechanisms through which the accumulation of mtDNA mutations leads to an ageing phenotype. Results have suggested that apoptosis (Norddahl et al. 2011), inflammation (Logan et al. 2014) and stem cell dysfunction

(Ahlqvist et al. 2012) are the major players in the premature ageing phenotype of the *PolyA^{mut/mut}* mice.

Mitochondrial Function and Stem Cell Ageing

Tight control over the self-renewal, differentiation, and regeneration of long-lived adult stem cells (ASCs) is necessary to support homeostasis of almost all mammalian tissues. Notably the homeostasis of ASCs relies heavily on metabolic status, ROS signalling and the availability of specific cofactors and epigenetic modifiers, all of which have mitochondrial involvement. In a quiescent state ASCs are often associated with a glycolytic metabolic phenotype (Simsek et al. 2010; Takubo et al. 2013; Zheng et al. 2016) which upon differentiation switches to OXPHOS (Zheng et al. 2016; Wüst et al. 2018; O'Brien et al. 2015). Within the haematopoietic stem cell (HCS) niche, quiescence is maintained in response to low levels of oxygen via activation of hypoxia-inducible factor 1 α (HIF-1 α). HIF-1 α restricts the entry of pyruvate into the TCA cycle and subsequent OXPHOS in the mitochondria by its activation of pyruvate dehydrogenase kinase (PDHK) (Takubo et al. 2013). The predominance of glycolytic metabolism favours the generation of the cofactor acetyl-CoA and histone acetylation in HSCs maintaining pluripotency (Moussaieff et al. 2015), whereas OXPHOS supports the maintenance of α -ketoglutarate (α -KG) levels favouring demethylation of DNA and HSC differentiation (Ho et al. 2017). Perturbations in this balance can have major implications on stem cell fate and have been linked with the age-associated impairment of autophagy in murine HSCs (Ho et al. 2017), and impaired glucose uptake resulting from reduced expression of the glucose transporter 3 (GLUT3) in aged pluripotent stem cells (Zhang et al. 2017). Both of these mechanisms may promote aberrant differentiation at the expense of maintaining stemness.

The critical coenzyme NAD $^{+}$ is intricately linked with mitochondrial metabolism, participating as an essential electron transporter and as a critical catalyst for pol (ADP-ribose) polymerase (PARP), a nuclear repair enzyme, and the sirtuin family of NAD-dependent histone deacetylases. A delicate balance in the regulation of the NAD/NADH ratio is critical. Reductions in intracellular levels of NAD $^{+}$ have been reported with advancing age (Braudy et al. 2011; Massudi et al. 2012), and have been shown to recapitulate phenotypes of neural stem cell ageing (Stein and Imai 2014). These effects are thought to be mediated through sirtuins, which have been well documented as central metabolic nodes regulating cellular ageing and longevity. Three of seven sirtuins are thought to be housed within mitochondria, SIRT3–5, and their implication in stem cell regeneration and degeneration during ageing is ongoing. In HSCs, SIRT3 is downregulated with increasing age and as such, its promotion of antioxidant activity and ROS scavenging is impaired resulting in a reduction in HSC pool size and in self-renewal capacity. Reversal of these effects is observed following upregulation of SIRT3 (Brown et al. 2013). SIRT3 has since been shown to help maintain cell homeostasis and co-ordinate the mitochondrial unfolded protein

response (UPR^{mt}) that regulates antioxidant machinery and mitophagy in response to stress (Papa and Germain 2014). Meanwhile SIRT7 is also linked to another axis of the UPR^{mt} and has been shown to decrease in expression in ageing HSCs rendering them less capable of dealing with an age-related increased level of mitochondrial protein folding stress (Mohrin et al. 2015). A premature HSC ageing phenotype with increased apoptosis, a myeloid differentiation bias and reduced regenerative potential in SIRT7 deficient mice was subsequently rescued by SIRT7 overexpression and linked to the repression of nuclear respiratory factor 1 (NRF1). This reduces mitochondrial biogenesis and respiration alleviating protein folding stress (Mohrin et al. 2015). Activation of SIRT1, although its primary localisation is nuclear, is linked to the regulation of mitochondrial function and stem cell ageing via several mechanisms such as the promotion of mitochondrial biogenesis (Cantó et al. 2009), autophagy (Huang et al. 2015) and the upregulation of antioxidant gene expression (Hori et al. 2013).

Increased levels of ROS are also shown to lead to the activation of p38 MAPK, a member of the mitogen-activated protein kinase family, which subsequently upregulates $p16^{Ink4a}$ and $p19^{Arf}$ in the HSC population inducing senescence and thus limiting stem cell lifespan (Ito et al. 2006). In the intestine mitochondria ROS have been shown to activate p38 MAPK and drive intestinal crypt differentiation (Rodriguez-Colman et al. 2017). Subsequently He et al. (2020) implicated p38 MAPK activation in the age-related exhaustion of intestinal stem cells. In comparison to young animals, aged murine intestinal $Lgr5^+$ stem cells demonstrate an increased activation of the nutrient and growth factor sensing mammalian target of rapamycin complex 1 (MTORC1). They showed that $Lgr5^+$ specific deletion of an MTORC1 suppressor, tuberous sclerosis 1 ($Tsc1$), induced a premature intestinal ageing phenotype in young mice which was ameliorated with administration of the MTORC1 inhibitor rapamycin. In the absence of rapamycin, $Tsc1$ ablation led to mTORC1 activation, increased mitogen-activated protein kinase 6 (MKK6) expression, p38 MAPK activation and the enhancement of p53 expression. Subsequently this resulted in the exhaustion of intestinal stem cells in addition to reductions in villus size and density, which again could be rescued by p38 MAPK or p53 inhibition (He et al. 2020). These data further support mitochondria as regulators of stem cell homeostasis and ageing.

MtDNA Mutations and Stem Cell Ageing

Utilising the $PolyA^{\text{mut}/\text{mut}}$ mouse model to investigate how age-related mtDNA mutagenesis might be implicated in HSC ageing, Ahlqvist et al. (2015) showed that mtDNA mutagenesis and consequential ROS signalling in immature erythrocytes perturb erythrocyte maturation leading to anaemia in these mice. The maturation of erythrocytes involves their migration from the bone marrow into the peripheral blood, where they enucleate and mature further within the circulation with removal of their mitochondria and clearing of transferrin receptors. In comparison to wild-type animals, 11-month-old $PolyA^{\text{mut}/\text{mut}}$ mice showed an increased

frequency of reticulocytes, immature erythrocytes, with significantly elevated mitochondrial retention. The mitophagy marker NIX indicated that the active removal of mitochondria was also significantly reduced in *PolyA^{mut/mut}* reticulocytes. The transferrin receptor (TfR) was also found to be abnormally retained within *PolyA^{mut/mut}* reticulocytes, causing increased loading of free iron within these cells. As free iron is highly reactive with oxygen, ROS are generated in reticulocytes in which both TfR and mitochondria are retained. Cell surface proteins which are normally appropriated to the inner cell membrane as erythrocytes mature, remain on the surface due to oxidative damage to the enzymes that facilitate their segregation. These cell surface proteins label the erythrocytes for premature destruction by splenic macrophages, as indicated by significantly increased levels of iron loaded activated macrophages within the spleen of *PolyA^{mut/mut}* mice (Ahlgqvist et al. 2015).

Intestinal crypts of *PolyA^{mut/mut}* mice have been shown to rewire their metabolism in response to complex I deficiency, which promotes cellular biomass and antioxidant production (Smith et al. 2020). When *PolyA^{mut/mut}* mice were crossed with an inducible mouse model of intestinal cancer (*Lgr5-EGFP-IRES-creERT2; Apc^{flox/flox}*), transformed cells hijacked these growth promoting pathways, increasing the tumour growth rate and ultimately reducing the lifespan of these mice in comparison to controls (Smith et al. 2020). Following this, Stamp et al. (2021) showed that complex I deficiency in the *Lgr5*+ intestinal stem cells of *PolyA^{+/mut}* mice increases the rate of stem cycle re-entry. In humans several cancers including kidney, colorectal and thyroid cancer, mtDNA mutations have been documented to be enriched indicating oncogenic roles (Yuan et al. 2020). Taken together these studies suggest that mtDNA mutations may enhance the development of pathologies associated with advancing age such as cancer. The understanding of age-related mitochondrial changes at the stem cell level, particularly those that are targetable may therefore assist in the development of therapeutics with the aim of rejuvenating stem cell function and thus ameliorating the disorders their functional decline may promote.

Mitochondrial Regulation of the Ageing Immune System

The immune system encompasses a complex stress response network to fight and protect against disease aiding our survival. A dynamic balance between pro- and anti-inflammatory networks is crucial for a beneficial response. However, it is well documented that with age a low-grade, chronically activated innate immune system known as ‘inflamm-ageing’ and an ultimate decline in the adaptive immune system, termed immunosenescence can arise (Franceschi et al. 2000; Xia et al. 2016). This has been evidenced through meta-analysis highlighting an augmented presence of circulating inflammatory mediators e.g. IL-6 (Soysal et al. 2016), alongside a paradoxical dampened response to cytokine levels due to a chronic activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway in immune cells (Shen-Orr et al. 2016). The onset of chronic systemic inflammation with age has subsequently been linked to disease including

osteoporosis, Alzheimer's disease, heart disease, Parkinson's disease, cancer, type II diabetes, amongst others. Partially due to their prokaryotic origin, mitochondria are seen to play an active role in the immune system and are proposed to partially mediate the decline in immunity with age and related pathologies.

The innate immune system recognises a range of ligands with repetitive molecular signatures through pattern recognition receptors (PRRs) on the cell surface e.g. Toll-like receptors (TLRs), or intracellular compartments e.g. the cytosolic DNA sensors cGAS and STING. Ligands deemed as foreign to the innate immune system can be grouped into pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). Microorganism constituents encompass PAMPs which are sensed by the innate immune cells presenting PRRs on their surface. DAMPs comprise an array of endogenous cellular components which are released upon cell stress, senescence, apoptosis or necrosis. Mitochondria present a select array of potent DAMP agents, e.g. mtDNA, cardiolipin, n-formyl peptides e.g. fMet, TFAM, ATP, and ROS. Intracellularly, mitochondrial DAMPs can either activate the cytosolic DNA sensors cGAS and STING pathway (cGAS-STING) evolved for recognition of single-stranded viral DNA, or the inflammasome to induce an inflammatory response. Inflammasomes are an assembly of multiprotein platforms in the cytosol that promotes the activation of caspase-1 to induce cytokine production and cell death (Vanaja et al. 2015). A variety of inflammasome types exist, with the NLRP3 inflammasome being the most well studied and shown to be activated by oxidised mtDNA (Shimada et al. 2012).

The stimulation of the innate immune system by bacterial N-formylated proteins and bacteria DNA is initiated by the extracellular release of mtDNA and the N-formyl peptide which are sensed by TLR9, and formyl peptide receptors respectively. The significance of these mitochondrial DAMPs as circulating elicitors of the immune response is seen during trauma (Zhang et al. 2010) and heart failure (Oka et al. 2012). Moreover, circulating mtDNA levels are documented to increase with age, alongside a correlative increase in levels of pro-inflammatory cytokines TNF- α , IL-6, and IL-1ra (Pinti et al. 2014). Conversely however, a reduction in the circulating mtDNA copy number is observed in neurodegenerative disease (Pyle et al. 2016; Podlesniy et al. 2013). Pyle et al. (2016) showed that Parkinson's disease patients had a lower level of circulating mtDNA compared with age-matched controls, which coincides with reduced mtDNA levels also seen in the substantia nigra pars compacta tissue of these patients. Subsequently the application of circulating mtDNA as a biomarker in the aetiology of Parkinson's disease has been proposed, with recent advancements showing that therapeutic treatment can influence circulating mtDNA levels (Lowes et al. 2020). Furthermore circulating mtDNA has been suggested as a biomarker of neurocognitive impairment and neurodegeneration linked to HIV (Pérez-Santiago et al. 2017) and as a biomarker in many types of tumours including breast and ovarian (Kohler et al. 2009; Zachariah et al. 2008).

Another closely linked route of mitochondrial dysfunction driving chronic inflammation during ageing is through the senescence associated secretory phenotype (SASP). Senescent cells accumulate with age (Waaijer et al. 2012) and

contribute to a number of different age-related diseases including diabetes and osteoporosis, with their clearance being shown to alleviate these pathologies (Xu et al. 2015; Farr et al. 2017). Along with pro-inflammatory cytokines and growth factors the SASP produces mitochondrial ROS to fortify cell cycle arrest to signal to the immune system for their removal whilst inadvertently initiating senescence in neighbouring cells (Passos et al. 2010; Nelson et al. 2012). Mitochondrial dysfunction is a key feature of cellular senescence (Passos et al. 2007, 2010), which has been associated with the NLRP3 inflammasome via ROS signalling (Yin et al. 2017) and mediation of the SASP through the cGAS-STING pathway via recognition of cytoplasmic chromatin fragments including mtDNA (Dou et al. 2017; West et al. 2015). Recently, the *Poly^{mut/mut}* mice have been shown to exhibit a rewiring of the innate immune system with a hyperinflammatory innate immune status driven by mtDNA instability triggering the cGAS-STING pathway for IFN-I priming and repression of the anti-inflammatory transcription factor NRF2 (Lei et al. 2021). The ablation of IFN-1 in these mice improved the health span and consequently strengthened the evidence that age-related mtDNA dysfunction can induce a pro-inflammatory state in ageing.

Along with mitochondrial DAMPs contributing to the senescent phenotype, mitochondrial metabolites e.g. low NAD+/NADH (Lee et al. 2012) and mitochondrial quality control mechanisms (Manzella et al. 2018; Hoshino et al. 2013) have also been suggested to be involved in the regulation of senescence. Desdín-Micó et al. (2020) showed in mice that when *Tfam* was knocked out in T cells, this resulted in the generation of an inflammatome phenotype that caused systemic senescence and premature ageing. Supplementing these mice with nicotinamide riboside, the NAD+ precursor, could partially rescue the age deterioration seen in the *Tfam* deficient T cells. These data show that mitochondria may regulate senescence during ageing via numerous molecular mechanisms and suggest that targeting these pathways may help to ameliorate age-related morbidities.

Targeting Mitochondria in Ageing

Targeting mitochondrial dysfunction to improve the treatment of age-related disease and ultimately the health span of the older population is an area of ongoing research. A range of aspects of mitochondrial physiology including mitochondrial dynamics, metabolism, ROS production, mitochondrial biogenesis, and mitophagy have been explored as targets. Multiple studies have hypothesised that these mitochondrial functions may be regulated through lifestyle interventions, as well as through pharmaceutical compounds. Dietary restriction (Weindruch 1996) and exercise (Lee and Paffenbarger 2000) are the two most long-standing non-pharmacologic interventions that have provided evidence for improvement in the lifespan and health span of biological systems. Rejuvenation of the ageing process through dietary restriction and exercise are thought to centre on evolutionary conserved metabolic sensory pathways encompassing mTOR, AMPK and sirtuins (reviewed in (Stanfel

et al. 2009; Ruetenik and Barrientos 2015)). Studies to investigate the molecular mechanisms through which dietary restriction and exercise are able to mediate life and health span have suggested that enhancement of mitochondrial function is a major player. This is predominantly through the transcription co-activator peroxisome proliferator-activated receptor-gamma 1(PGC1 α) which controls mitochondrial biogenesis. However, many studies directly manipulating PGC1 α to enhance mitochondrial biogenesis have generated conflicting results. For example, in one study, PGC1 α was shown to induce the switching of muscle fibre types from fast-twitching glycolytic fibres to slow-twitching oxidative fibres with a high concentration of mitochondria (Lin et al. 2002). Conversely, in muscle-specific PGC1 α knockout mice, Rowe et al. (2012) demonstrated that PGC1 α was dispensable for exercise-induced mitochondrial biogenesis. Furthermore when Dillon et al. (2012) targeted PGC1 α to increase mitobiogenesis in the muscle of *Poly*^{mut/mut} mice, they observed improved heart and skeletal muscle heart function but also a paradoxical increase in mtDNA mutation burden.

Consistent with the MFRTA considerable efforts have been made to ameliorate oxidative stress by targeting ROS formation or increasing antioxidant defence systems with synthetic or naturally occurring compounds, in a bid to promote health span (Ishii et al. 2004; Parkes et al. 1998; Melov et al. 1999). Notably, the application of mitochondrial targeted antioxidant treatments has shown more consistent and beneficial evidence for their potential use in reducing oxidative stress in mitochondria than non-targeted in therapy (Jauslin et al. 2003; Kelso et al. 2001). However, many studies targeting oxidative stress have failed to show improved health benefits, with many showing no health benefits or on the contrary high ROS levels to be life promoting (Lee et al. 2010), and antioxidants to be disease-promoting (Myung et al. 2010; Bjelakovic et al. 2004). Subsequently ‘mitohormesis’ first coined by Ristow and Zarse (2010), conceptualised the theory that by inducing a mild level of mitochondrial stress, an increase in mitochondrial metabolism and increased stress resistance develop, conferring an adaptive response to oxidative stress and improving health span. Whilst at high levels such stressors can be ultimately damaging, low levels cause transient increases in ROS that activate genes involved in cellular stress response mechanisms. Thus, mitohormesis somewhat contradicts the MFRTA, and suggests a beneficial role of ROS in healthy ageing. Furthermore, Flockhart et al. (2021) have recently shown that excess exercise causes mitochondrial impairment stressing the existence of a delicate homeostatic balance regulating mitochondrial function.

Other avenues, including the selective clearance of dysfunctional mitochondria (Sterky Fredrik et al. 2011) and post-translational modification of mitochondria (Stram and Payne 2016), have also been explored as routes to improve mitochondrial function to lessen age-related dysfunction and associated pathologies. However, these studies further highlight the many uncertainties surrounding the mechanisms of mitochondrial physiology in ageing and age-related disease and stipulate further delineation of the molecular pathways involved in improving mitochondrial fitness and homeostasis for development of therapeutics in ageing and age-related disease.

Concluding Remarks

Ageing and age-related disease present an extremely intertwined network of processes and states that may or may not be reversible. It is clear that mtDNA mutagenesis and dysfunction is a prominent feature in the ageing process and in multiple age-related diseases; however, unravelling causal relationships and underlying mechanisms remains a difficult task particularly due to the complexities of mitochondrial genetics and their diverse cellular functions. There is growing evidence suggesting the potential both for therapeutically targeting mitochondria, and its use as a biomarker in disease. The use of new, highly sensitive single-cell technologies may aid in revealing the importance of mtDNA mutations and functional consequences in the aetiology of ageing and age-related disease, and the potential for the development of interventions. The implication that somatic mtDNA mutations which occur early in life may have the greatest impact in later life (Elson et al. 2001; Stamp et al. 2018) suggests that life-long lifestyle choices for beneficial mitochondrial health and fitness may lead to a better health span during ageing.

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Chapter 5

The Proteasome and Ageing



Ashok N. Hegde, Lindsey M. Duke, Logan E. Timm, and Hannah Nobles

Abstract The proteasome is a multi-subunit proteolytic complex that functions to degrade normal proteins for physiological regulation and to eliminate abnormal proteins for cellular protection. Generally, the proteasome targets substrate proteins that are marked by attachment of multiple ubiquitin molecules. In various types of cells in an organism, damage to proteins occurs both from internal sources such as reactive oxygen species and from external ones such as UV radiation from the sun. The proteasome functions to protect the cells by degrading damaged proteins. With ageing, however, the capacity of the proteasome to degrade damaged proteins is reduced as indicated by evidence gathered by many studies. Studies on ageing in muscle, skin, and brain show that with age catalytic activity of the proteasome is decreased and the expression of proteasome subunits is altered. Age-related accumulation of damaged or misfolded proteins causes further reduction of proteasome activity. Abnormal proteins also accumulate as a result of age-related neurodegenerative diseases. Deficits in proteasome activity might be responsible for accumulation of protein aggregates and thus contribute to the pathology. Results from several studies suggest a link between the proteasome and longevity. This chapter reviews the various ways in which the proteasome is associated with the ageing process and examines evidence gathered from investigations on cultured cells, model organisms, and humans.

Keywords Protein degradation · Proteolysis · Ubiquitin · Oxidative damage · Misfolded proteins · Longevity · Senescence · Neurodegeneration · Proteostasis

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Introduction

Ageing is a complex process to which many genetic and environmental factors contribute. It is thought that accumulated damage to the genome as well as the proteome causes age-related structural deterioration and functional decline at the cellular and organismal levels. A key contributor to the maintenance of a healthy proteome is a proteolytic complex called the proteasome which functions physiologically to degrade ubiquitin-tagged proteins. It also has an important role in degradation of abnormal proteins. At the cellular level, protein degradation is the yang to the yin of protein synthesis and the two systems together maintain the proteome. This exquisitely maintained balance of cellular proteins slowly becomes unstable with ageing and might occasionally collapse when an organism succumbs to disease. The functional connection of the proteasome with ageing is perhaps best understood in the context of physiology as well as pathology. Here we review the evidence for the role of the proteasome in normal ageing in different parts of the body as well as in age-related neurodegenerative diseases. We also examine the literature linking the proteasome to longevity and possible ways of activating the proteasome to promote healthy ageing.

The Proteasome: A Brief Overview

The proteasome consists of a 20S catalytic core and two 19S regulatory caps attached on either side of the cylindrical core, and is termed the 26S proteasome (Fig. 5.1). The 19S regulatory cap is also known as PA700 based on its molecular mass (700 KDa). The 20S proteasome can be modulated by alternative regulatory complexes called PA200, PA28, or 11S (Hegde 2004).

The catalytic core has two types of subunits α and β . There are seven α subunits ($\alpha_1\text{--}\alpha_7$) and seven β subunits ($\beta_1\text{--}\beta_7$) and these are arranged in four stacked rings with inner rings comprising two sets of β subunits and the outer rings consisting of one set of α subunits. The catalytic activity of the 20S is conferred by a threonine residue in the N-terminus of the β subunits β_1 , β_2 , and β_5 . Enzymatically the proteasome possesses three types of activities, namely chymotrypsin-like, trypsin-like, post-glutamyl peptidase. These activities cleave after hydrophobic, basic, and acidic residues, respectively (Fig. 5.1).

The 19S regulatory cap has a base and a lid. The base consists of six ATPase subunits (Rpt1–Rpt6) and two non-ATPase subunits (Rpn1, Rpn2, and Rpn13). The lid comprises 10 non-ATPase subunits (Rpn2, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn10, Rpn11, Rpn12, and Rpn15) (Tanaka 2009).

Alternative forms of the 20S proteasome are formed under certain conditions. In immune cells, the β_1 , β_2 , and β_5 of the catalytic core are replaced with LMP2 (β_{1i}), MECEL-1 (β_{1i}), and LMP7 (β_{5i}) which are typically induced by the cytokine interferon- γ . This type of proteasome has a slightly different catalytic activity in

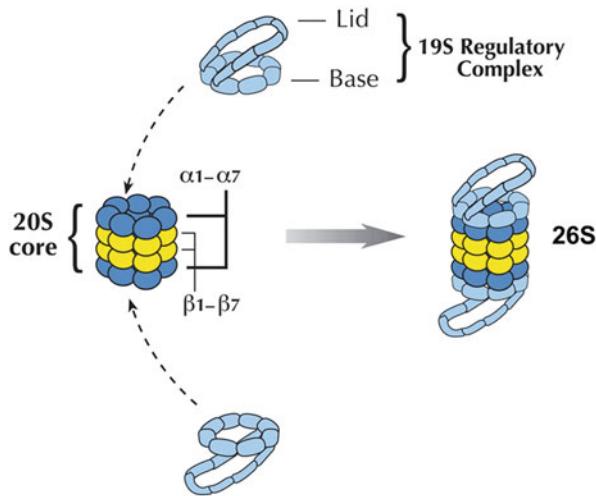


Fig. 5.1 Structure of the proteasome. The proteasome consists of the 20S core and the 19S cap. The 20S proteasome contains the catalytic sites and comprises four stacked rings. The inner two rings have seven β subunits (β_1 – β_7) and the outer rings have seven α subunits (α_1 – α_7). The 19S regulatory cap is made of base and a lid each with eight subunits. Two 19S regulatory caps are attached on either side of the 20S catalytic core and together they constitute the 26S proteasome. Adapted from Hegde (2004)

that the chymotrypsin-like activity of $\beta 5i$ is higher than that of $\beta 5$ and therefore the immunoproteasome cleaves peptides with C-terminal hydrophobic residues of antigenic peptides more efficiently. This alternative form of proteasome is called “the immunoproteasome.” In non-immune cells, the immunoproteasome is induced by proinflammatory cytokines (Murata et al. 2018).

The 26S proteasome degrades proteins marked with polyubiquitin chains in which ubiquitin molecules are linked to each other through their 48th lysine residues. The 20S proteasome can exist as part of the 26S proteasome as well as an independent proteolytic entity (Hegde 2004).

Cellular Senescence and the Proteasome

Cellular senescence was originally described as permanent replicative arrest. Research carried out in the past several years has broadened our understanding and currently cellular senescence is thought to be a response to a multitude of stressors. A variety of internal stimuli such as oxidative stress and mitochondrial dysfunction and external stimuli such as irradiation and exposure to toxic chemicals can accelerate cellular senescence. As cells age, accumulated protein damage can overburden the clearing systems such as the lysosome or the proteasome. Coupled

with this, overall proteolytic ability of cells might be reduced with ageing (Sabath et al. 2020).

In various model organisms, a link between the proteasome and ageing has been shown. For example, fission yeast has a long lifespan in quiescence upon nitrogen starvation. In this model, proteasome inactivation causes accumulation of reactive oxygen species (ROS) and mitochondrial dysfunction. Under these conditions, mitochondria are consumed by autophagy which is believed to be a defensive mechanism against harmful accumulation of ROS (Takeda and Yanagida 2010). A role for the proteasome in degrading oxidatively damaged proteins has also been shown in higher organisms. For example, in the mouse hippocampal HT22 cell line, Hsp70 promotes the action of the proteasome in degrading proteins damaged by treatment of the HT22 cells with hydrogen peroxide (Reeg et al. 2016).

Muscle Ageing and the Proteasome

With ageing, progressive loss of muscle function occurs often accompanied by loss of muscle mass. Experiments on mammalian model systems investigated the changes in the function of the muscle proteasome with ageing.

Studies on the LOU rat (an inbred strain derived from Wistar rats) focused on the three types of proteolytic functions of the 20S proteasome, namely chymotrypsin-like, trypsin-like, and peptidylglutamyl peptidase activities. The researchers investigated these activities in the gastrocnemius medialis muscle in rats of 4, 18, 24, 29, and 34 months of age. They found that the three proteolytic activities of the 20S proteasome increased up to 29 months and decreased in the 34-month-old rats (Bardag-Gorce et al. 1999).

Investigation on the role of the proteasome on muscle atrophy with ageing compared Type I or slow-twitch muscle fibers from F344BN rats. The study found a threefold increase in the 20S catalytic core of the proteasome with ageing but the catalytic activity of the 20S proteasome remained the same, leading to the conclusion that specific activity of the proteasome reduced with ageing (Husom et al. 2004).

A study on fast-twitch muscle of F344BN rats found a two-to-threelfold decrease in the content of the 20S proteasome as well as 75% reduction in two regulatory complexes PA700 and PA28. These authors also found an increase in two cytokine-inducible subunits of the immunoproteasome called LMP2 and LMP7. Degradation of a test substrate, oxidized calmodulin, was faster in the muscle from young animals compared to that in the muscle from aged animals (Ferrington et al. 2005).

Muscle mass and integrity is maintained in adults through regeneration from muscle stem cells (also called satellite cells). The proteasome appears to be required for function of muscle stem cells. When a component of the 19S proteasome called Rpt3 (which is one of the six ATPases in the base of the 19S regulatory cap), this study tested regeneration of tibialis anterior muscle in C57BL/6J mice after cardiotoxin-induced injury. When Rpt3 was knocked out specifically in satellite cells, muscle regeneration was impaired compared to control mice. This study also

showed that regeneration is accompanied by an increase in chymotrypsin-like and trypsin-like activities of the proteasome. Blockade of muscle regeneration in Rpt3 deficient mice is linked to stabilization of p53 which prevents cell proliferation and results in cell death. This investigation found that when p53 was knocked down in Rpt3-deficient myoblasts, proliferation of those cells was restored (Kitajima et al. 2018).

Skin Ageing and the Proteasome

Several investigations have explored the connection between skin ageing and the proteasome.

A study carried out on human dermal fibroblasts showed a significant decrease in all three catalytic activities (chymotrypsin-like, trypsin-like, and peptidylglutamyl peptidase) of the 20S proteasome in fibroblasts from 50-year-old individuals compared to those from 20-year-old persons. The same study also used immunoblotting to measure the levels of proteasome catalytic subunits as well as some 19S regulatory subunits. The experiments revealed a decrease in two β subunits ($\beta 1$ and $\beta 5$) and two 19S subunits (Rpt3, Rpn12) in fibroblasts from older individuals compared to those from younger ones. Additional experiments to measure the levels of mRNAs of proteasome subunits using quantitative real-time PCR (Q-PCR) showed a significant decrease in $\beta 1$, $\beta 5$, $\beta 7$ mRNAs in fibroblasts from older individuals relative to those from younger ones. The Q-PCR experiments also found that levels of Rpt1 mRNA were sixfold higher in fibroblasts from younger individuals compared to those from older people (Hwang et al. 2007).

Experiments on epidermal cells obtained from healthy donors undergoing plastic surgery procedures (mammary gland reductions) measured the content of carbonyl derivatives as a proxy for oxidized proteins. The epidermal samples came from young (17, 20, and 25 years old), middle-aged (39 and 42 years), and older (50, 60, and 67 years old) donors. The results showed an increase in oxidized proteins in older individuals compared to the young donors. Assays on other types of protein damage showed an increase in lipid peroxidation and glycation with advanced age. This study also found a decrease in chymotrypsin-like and peptidylglutamyl peptidase activities of the 20S proteasome. The researchers also tested the quantity of 20S proteasome by immunoblotting and found 10% and 60% reduction in some of the 20S subunits in keratinocytes from 50-year-old and 67-year-old donors, compared to the keratinocytes from a 17-year-old donor. In addition, enzyme-linked immunosorbent assay provided supporting evidence for reduction of proteasome content in epidermal cells of older individuals compared to the younger ones (Petropoulos et al. 2000).

One of the causes of skin ageing is excessive exposure to sunlight which is termed photo ageing. This type skin ageing mainly occurs because of the ultraviolet (UV) radiation coming from sunlight. UV-induced ageing is often studied using exposure of cultured skin fibroblasts to specific types of UV radiation. In a study

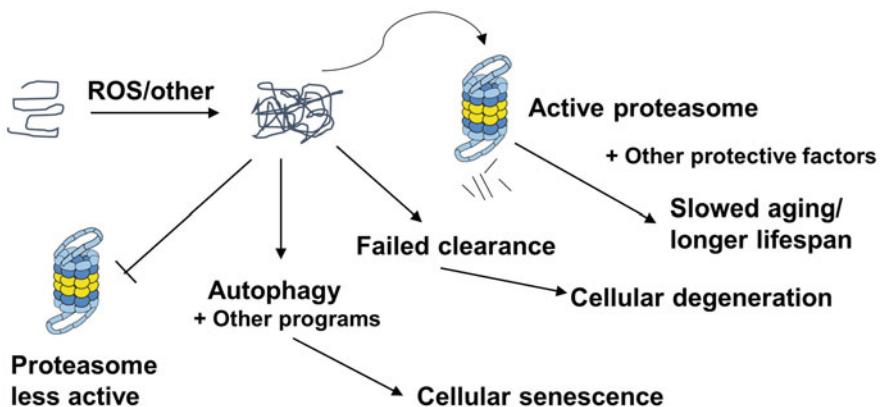


Fig. 5.2 Schematic diagram showing the role of the proteasome in ageing. When a soluble protein (top left) is exposed to reactive oxygen species (ROS) or other damaging agents (such as UV radiation), it forms aggregates. Functionally fully active proteasome will degrade the damaged/aggregated proteins which slows down ageing and increase the lifespan. If the proteasome is less active, it fails to degrade the aggregated proteins triggering autophagy and other cellular programs (such as development of secretory phenotype) which leads to cellular senescence. If autophagy or other cellular clearance mechanisms (such as lysosomal degradation) fail, it would cause cellular degeneration.

using exposure of human dermal fibroblasts to UVB (280–315 nm wavelength) irradiation, researchers found that irradiation decreased proteasome activity as measured by stabilization of a substrate fused to green fluorescent protein. The same study also found that when proteasome activity was inhibited, formation of autophagosomes increased. Autophagy appears to be essential for cellular senescence induced by UVB exposure. If autophagy is inhibited, dermal fibroblasts undergo apoptosis. Therefore, it is likely that early events upon UVB exposure such as formation of reactive oxygen species cause inactivation of the proteasome which in turn causes an increase in autophagy, thus triggering senescence (Fig. 5.2) (Cavinato et al. 2017).

A study looked into the relationship between mitochondria and the proteasome in dermal fibroblasts. Mitochondria are believed to play a key role in the ageing process because they produce reactive oxygen species which cause mutations, damage protein, lipids and other molecules in cells. The proteasome is linked to the ageing process because of its role in degradation of oxidized proteins. The experiments used dermal fibroblasts obtained from healthy young (23–29 years old), middle-aged, and old donors. This study used fibroblasts obtained from a biobank enriched for longevity. The fibroblasts derived from 90-year-old subjects were designated “old” and the fibroblasts from skin biopsies of offspring enriched for familial longevity and their partners (60 ± 8 years) were considered “middle-aged.” The results showed a decrease in proteasome activity in skin fibroblast of middle-aged donors compared to those from young donors, but proteasome activity did not decline further in fibroblasts from “old” donors. The experiments also found a significant decrease

in mitochondrial membrane potential and an increase in the production of reactive oxygen species. Additional studies indicated that proteasome inhibition caused a decrease in mitochondrial function (Koziel et al. 2011). A caveat for the observation on interdependence between activities of the proteasome and mitochondria is that the study used N-acetyl-L-leucyl-L-leucyl-L-norleucinal to inhibit the proteasome. This chemical is not a highly specific proteasome inhibitor and is known to inhibit other proteases such as calpain (Hughes et al. 1996).

Brain Ageing and the Proteasome

Research on various model systems has discovered a link between the proteasome and brain ageing.

Experiments carried out on Wistar rats and C57/BL6 mice showed a decrease at 15 months of age (compared to 6-week-old animals) in overall proteasome activity in several regions of the brain including cortex, cerebellum, globus pallidus, substantia nigra, and the cerebellum. In the frontal cortex chymotrypsin-like activity was 41% and 37% lower in aged rats and mice, respectively. The chymotrypsin-like activity was also reduced in all the brain regions tested except cerebellum. These authors also observed a significant decrease in trypsin-like activity in substantia nigra and the cerebellum and a substantial reduction in peptidylglutamyl peptidase activity in substantia nigra (Zeng et al. 2005).

Based on research in several model organisms and on human cells, a general view of brain ageing is that, with age, protein degradation slows down causing accumulation of misfolded proteins in neurons. This in turn is likely to cause further decline in proteolytic activity (Fig. 5.2). A study carried out using a short-lived vertebrate *Nothobranchius furzeri* (killifish) sought to make a quantitative assessment of protein homeostasis by combining transcriptomics with proteomics. These fish have a median life span of 3–7 months which allowed researchers to test the effect of ageing in a short period. The experiments included sexually mature young fish (5 weeks post-hatching), adult fish (12 weeks post-hatching), and old fish (39 weeks post-hatching). The researchers determined transcripts using RNA-Seq and amounts of proteins using an algorithm called iBAQ (intensity-based absolute quantification) (Schwanhäusser et al. 2011). The results showed progressive decrease in correlation between amounts of transcripts and proteins. One of the consequences of this decoupling between RNA transcripts and proteins is the deterioration of ribosome structure because of loss of stoichiometry which occurred because 13 ribosomal proteins increased in abundance with age whereas 14 of them decreased. Other experiments showed that ribosomal proteins are enriched in age-dependent protein aggregates.

These authors then tested effects of ageing on the proteasome. They observed a decrease in both transcripts and proteins of proteasome subunits which led to altered stoichiometry between the 19S subunits and 20S subunits. With age, there was an increase in 20S subunits relative to 26S subunits. The results also showed

downregulation of ubiquitin conjugating enzymes and ubiquitin ligases. Experiments to test whether partially blocking proteasome activity induces age-related phenotype used Bortezomib to achieve about 50% reduction in proteasome activity in the brains of killifish. Protein-abundance profile after this treatment was similar to that observed in the ageing phenotype including alterations in the large subunits of the cytosolic and mitochondrial ribosome. The investigations also tested whether a decrease in the proteasome in early life has an effect on the lifespan of killifish. When RNA-seq data from biopsies of 159 fish at 10 weeks and 20 weeks of age were tested in relation to lifespan, the results showed that a decrease in expression of proteasomal transcripts was correlated with an increased risk of mortality. Moreover, individual fish exhibiting largest reduction in transcripts encoding the subunits of the proteasome had shorter lifespan compared to those with largest upregulation of proteasomal transcripts (Kelmer Sacramento et al. 2020).

Proteasome and Age-Related Neurodegenerative Diseases

Activity of the proteasome is known to be impaired in Parkinson's disease (PD). A key protein linked to PD is α -synuclein. Even though some studies have shown that α -synuclein is a substrate for degradation by the proteasome (Bennett et al. 1999), some other studies have shown that aggregated α -synuclein impairs the activity of the proteasome. In PC12 cells stably expressing mutant α -synuclein, oligomers of α -synuclein bind to the 26S proteasome and decrease the chymotrypsin-like, trypsin-like, and peptidylglutamyl peptidase activities. Treatments that disrupt oligomerization of α -synuclein reduce its inhibitory effect on the activity of the proteasome. Furthermore, the experiments showed that the proteasome is capable of degrading α -synuclein oligomers (Emmanouilidou et al. 2010).

A link between the proteasome and PD pathology is also supported by investigation on cultured rat mesencephalic dopaminergic neurons. When the proteasome activity is inhibited in these neurons by application of a selective inhibitor lactacystin, proteins accumulate in cytoplasmic inclusion bodies that stain positively for α -synuclein and ubiquitin. In addition, with application of increasing concentration of lactacystin a proportionate increase in degeneration of dopaminergic neurons occurred (McNaught et al. 2002).

Degradation by the proteasome is linked to the pathology of Alzheimer's disease (AD) as well. Several studies have provided evidence for association of the proteasome with the two main pathological mechanisms of AD, namely, accumulation of amyloid β ($A\beta$) leading to plaque formation and hyperphosphorylation of tau resulting in neurofibrillary tangles.

With respect to the link between $A\beta$ and the proteasome, there are conflicting reports as to whether the $A\beta_{(1-42)}$ peptide inhibits the proteasome or it is a substrate for the proteasome. One of the early *in vitro* studies showed that $A\beta_{(1-40)}$ inhibits the chymotrypsin-like activity of the 20S proteasome. In cultured neurons, $A\beta_{(1-42)}$ inhibited the degradation of a test substrate fused to green fluorescent protein

(Oh et al. 2005). Other studies using mouse models of AD showed inhibition of activity of the proteasome in single transgenic mice (Tg2576) as well as triple transgenic mice (PS1_{M146V}, APP_{Swe}, and tau_{P301L}) (Almeida et al. 2006; Tseng et al. 2008). In contrast, some other investigations have found evidence to support the idea that the proteasome degrades the A β peptide. For example, *in vitro* studies showed that human 20S proteasome is capable of degrading A $\beta_{(1-42)}$ peptide. The same researchers also showed that oligomers of A $\beta_{(1-42)}$ inhibit the proteasome. They propose that monomeric A $\beta_{(1-42)}$ is a substrate for the proteasome and inhibition of the proteasome by A $\beta_{(1-42)}$ comes about by excessive accumulation of the oligomeric species of the A β peptide (Zhao and Yang 2010).

Tau is a substrate for ubiquitination and subsequent degradation by the proteasome. A ubiquitin ligase called CHIP (C-terminus of Hsc-70 Interacting Protein) attaches ubiquitin molecules to tau. This is facilitated by interaction of CHIP with Hsp70 (Dickey et al. 2008).

Proteasome and Longevity

Given the association of proteasome function with ageing, it is reasonable to hypothesize that proteasome function is linked to longevity. Some studies have tested this idea. For example, using the *C. elegans* model system, researchers tested the idea that forcing an organism to shift the metabolic resources away from maintenance of reproductive tissues to somatic ones is likely to extend the lifespan. They found that *glp-1* mutants which are germ-line deficient showed a sixfold increase in proteasome activity. These worms had increased lifespan and elevated expression of the proteasome subunit Rpn6, part of the lid of the 19S regulatory cap. Knockdown of Rpn6 using RNAi significantly reduced the lifespan of worms. In addition, overexpression of Rpn6 in wildtype worms conferred resistance to proteotoxic stress imposed by oxidative damage, heat shock, or UV damage. Overexpression of Rpn6 increased the lifespan of worms at 25 °C but not under 20 °C. Because 25 °C causes mild heat stress in worms, it appears that Rpn6 promotes the survival of worms under physiologically stressful conditions (Vilchez et al. 2012).

Evidence for a role of the proteasome in longevity has also come from investigations on the *Drosophila* model. When β 5, a subunit of the catalytic core of the proteasome is overexpressed in neurons, it prevented age-related decline in learning and memory as measured by aversion to certain natural odors paired with mild electrical shock. The flies overexpressing β 5 also had increased lifespan (Munkácsy et al. 2019).

In addition to the components of the proteasome that have been linked to longevity through experimentation, it is likely many other molecules that are part of the ubiquitin-proteasome pathway (UPP) have a role in determining longevity. For example, a study analyzing proteins in mammals that are targets for selection

when longevity evolves, found that genes encoding the components of the UPP are under selection in lineages where longevity increased (Li and de Magalhães 2013).

Other Components of the Ubiquitin-Proteasome Pathway Implicated in Ageing

The 26S proteasome degrades proteins marked by attachment of polyubiquitin chains. Therefore, a brief survey of molecules that participate in ubiquitin attachment and their role in ageing is in order. Specifically, we consider the role of the UPP in regulating insulin receptor signaling which is linked to ageing (Kenyon 2010). Insulin or insulin-like growth factor-1 (IGF-1) bind to the receptor and activate a signaling cascade which leads to inactivation of the forkhead box (FOXO) transcription factor, which reduces the lifespan. Any modulation of the signaling cascade from the insulin receptor that dampens the signaling and thus prevents inactivation of the FOXO will lead to transcription of longevity-promoting genes by FOXO.

Many components of insulin receptor signaling cascade are regulated by ubiquitin ligases. For example, the ubiquitin ligase CHIP targets the insulin receptor and marks it by monoubiquitination for endocytosis and lysosomal degradation, thus regulating the availability of the receptor on the plasma membrane. A kinase called AKT that is part of this signaling is also a substrate for CHIP which polyubiquitinates AKT for degradation by the proteasome (Su et al. 2011). In *C. elegans*, the FOXO transcription factor DAF-16 is a substrate for a ubiquitin ligase called RLE-1 and proteasome-mediated degradation. Loss of function of RLE-1 increases the lifespan of worms (Li et al. 2007).

Activating the Proteasome to Decelerate the Ageing Process

The evidence from several studies indicates that proteasome activity declines with ageing which suggests that activating the proteasome might work to slow down the ageing process (Fig. 5.2). Investigations on cell lines and model organisms have attempted to test this idea. Research on human embryonic fibroblast cell line WI3T/T and promyeloblast cell line HL60 showed that stable overexpression of β 5 subunit of the 20S proteasome caused an increase in all three catalytic activities. When treated with different oxidants, the cells overexpressing β 5 subunit showed higher survival rates compared to controls. In addition, the cells overexpressing β 5 subunit showed increased number of population doublings compared to controls cells, indicating that increased activity of the proteasome delayed senescence (Chondrogianni et al. 2005).

Investigation on the *C. elegans* model also employed manipulation of a core subunit of the proteasome called *pbs-5* (an ortholog of the human β 5 subunit). The

worms overexpressing *pbs-5* showed increased amounts of both α and β subunits of the 20S proteasome core. The increase in proteasome content was accompanied by an increase in chymotrypsin-like activity. Overexpression of *pbs-5* also led to a significant increase in the lifespan of worms (Chondrogianni et al. 2015).

In *Drosophila*, activation of the 26S proteasome through a gain-of-function mutation of Rpn11 (a subunit of the 19S lid) suppressed the age-related reduction in proteasome activity and decreased accumulation of polyubiquitinated proteins. Moreover, Rpn11 overexpression suppressed expanded polyglutamine-induced progressive neurodegeneration and prolonged the lifespan of flies (Tonoki et al. 2009).

Experiments on outbred mice showed that chronic intranasal administration (for 9 months) of recombinant human Hsp70 in “old” NMRI (Naval Medical Research Institute) mice (17 months of age) caused improvement in spatial memory, increase in proteasome activity in the brain and longer lifespan (Bobkova et al. 2015).

In this context, it is interesting to note studies on naked mole rat which have a long lifespan (~31 years). The liver tissue of these animals has higher proteasome activity compared to that of mice. The proteasome of these animals also is resistant to blockade of activity by proteasome inhibitors such as MG-132 and lactacystin. Furthermore, a proteasome-depleted cytosolic fraction from liver lysates of naked mole rat contains a “factor” that confers inhibitor-resistance to yeast, mouse, and human proteasomes. Additional analyses showed the cytosolic factor contained heat shock proteins Hsp72 and Hsp40. Given that the effect of the factor on increasing the chymotrypsin-like activity of the proteasome and imparting inhibitor-resistance could not be reconciled with any known properties of the heat shock proteins, whether additional components of this factor are responsible for these functions remains to be determined (Rodriguez et al. 2014).

Although there are no rigorous studies on humans to test augmentation of the proteasome and its possible beneficial effects on ageing, there is circumstantial evidence that enhanced proteasome activity is associated with reduction of damaged proteins. Research on the effects of zinc supplementation in older adults tested protein oxidation levels in lymphocytes from peripheral blood and proteasome activity. The results showed a reduction in protein oxidation levels and an increase in the chymotrypsin-like activity of the proteasome (Cabreiro et al. 2008). Support for a role of robust proteasome activity in healthy ageing also comes from investigations of proteasome activity in fibroblast cultures of healthy centenarians. The peptidylglutamyl peptidase activity of the proteasome was maintained at high levels in a centenarian and RNA expression of a proteasome subunit PA28 β (11S β) was comparable to that of younger individuals (8 and 28 years old) and more than that of a healthy older (80-year-old) one (Chondrogianni et al. 2000).

Conclusion and Future Directions

Based on the research on several different model systems, it appears that the catalytic activities of the 20S proteasome (chymotrypsin-like, trypsin-like, peptidylglutamyl peptidase) are reduced with ageing although in varying patterns. Sometimes alterations in the activities of the proteasome are accompanied by changes in expression levels of some 20S proteasome subunits as well as those of the 19S regulatory cap. Evidence is also slowly accumulating with respect to the causative role of the proteasome in longevity. With respect to ageing, future research would benefit from experimental approaches to test the mechanistic links between the proteasome and the “ageing phenotype” as well as cross-talk between the proteasome and other cellular clearance systems such as the lysosome and the autophagosome. In addition, it would be fruitful to develop strategies to study specific substrates of the 26S proteasome that are integral parts of the molecular mechanisms underlying the ageing process. In the coming years, we might expect advances in techniques to stimulate the proteasome using small molecules with a view to develop therapeutic approaches to enhance degradation of damaged or misfolded proteins.

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Chapter 6

Gap Junctions and Ageing



Michael J. Zeitz and James W. Smyth

Abstract Gap junctions, comprising connexin proteins, create conduits directly coupling the cytoplasms of adjacent cells. Expressed in essentially all tissues, dynamic gap junction structures enable the exchange of small molecules including ions and second messengers, and are central to maintenance of homeostasis and synchronized excitability. With such diverse and critical roles throughout the body, it is unsurprising that alterations to gap junction and/or connexin expression and function underlie a broad array of age-related pathologies. From neurological dysfunction to cardiac arrhythmia and bone loss, it is hard to identify a human disease state that does not involve reduced, or in some cases inappropriate, intercellular communication to affect organ function. With a complex life cycle encompassing several key regulatory steps, pathological gap junction remodeling during ageing can arise from alterations in gene expression, translation, intracellular trafficking, and posttranslational modification of connexins. Connexin proteins are now known to “moonlight” and perform a variety of non-junctional functions in the cell, independent of gap junctions. Furthermore, connexin “hemichannels” on the cell surface can communicate with the extracellular space without ever coupling to an adjacent cell to form a gap junction channel. This chapter will focus primarily on gap junctions in ageing, but such non-junctional connexin functions will be referred to where

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appropriate and the full spectrum of connexin biology should be noted as potentially causative/contributing to some findings in connexin knockout animals, for example.

Keywords Connexin · Gap junction · Ageing · Intercellular communication · Homeostasis

Introduction

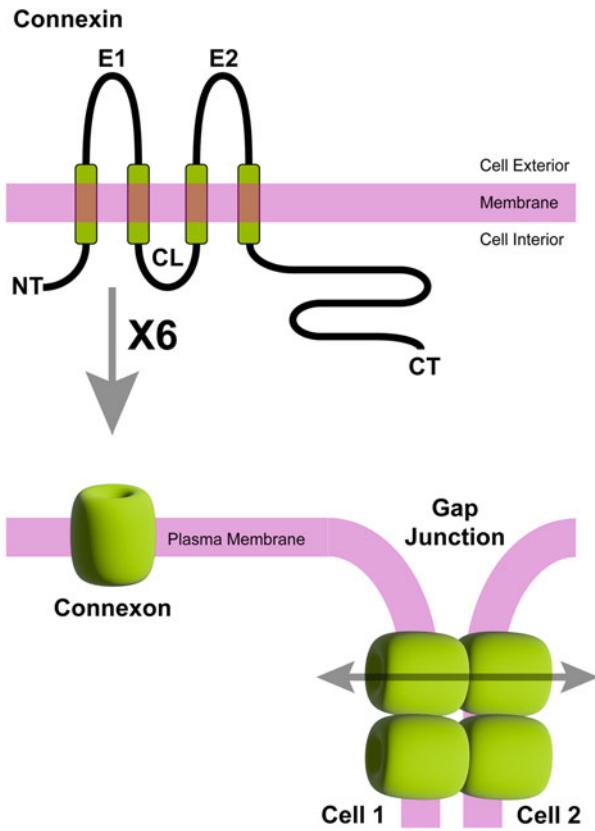
Gap Junctions and the Connexin Family

Intercellular communication is common to, and essential for, all multicellular life. A primary means by which cells communicate directly with each other is via gap junctions; densely packed, yet dynamic, arrays of intercellular channels comprising connexin proteins (Kumar and Gilula 1996; Sohl and Willecke 2004). Six connexin proteins oligomerize to form a connexon, or hemichannel, which is trafficked to the cell surface where it can communicate with the extracellular milieu or, when forming a gap junction, can couple with a connexon channel on an apposing cell (Fig. 6.1) (Nielsen et al. 2012). Gap junction channels coalesce into dense arrays of hundreds to thousands of channels termed gap junction plaques. Gap junctions enable the direct exchange of small molecules, typically estimated at 1 kDa or less, including ions and metabolites by coupling the cytoplasms of adjacent cells, effecting electrical and metabolic intercellular coupling. Occurring across all organs and tissues, and even within those made up of motile cell populations such as the immune system, gap junctions represent a common and rapid mechanism for cells to communicate. Cell-type/tissue specificity in gap junction coupling is achieved through differential expression of specific connexin proteins, of which 21 have been identified in humans and are named based on predicted molecular weight (Cruciani and Mikalsen 2006). Alterations in gap junction function and expression are associated with an array of human medical disorders, with disruptions in gap junction intercellular communication (GJIC) associated with, and underlying, many pathologies associated with ageing.

Throughout the evolution of multicellular organisms, a necessity for direct intercellular coupling to maintain homeostasis and perform complex coordinated actions between cells resulted in the gap junction. Invertebrates utilize innexin proteins which are essentially homologous to the vertebrate pannexin family. Connexins evolved as separate genes in vertebrates to form gap junctions, with the same protein topology as innexins/pannexins. Vertebrate pannexins, however, serve solely as channels communicating between the cell and extracellular environment (Goodenough and Paul 2009; Herve et al. 2007). Of the 21 human connexins, connexin43 (Cx43, gene name *GJA1*) is the most ubiquitously expressed and intensely studied. Much of our understanding regarding gap junction regulation is based upon Cx43 research, but it should be noted that many specific binding partners are connexin-specific in this highly active research space (Smyth and Shaw 2012).

Fig. 6.1 General connexin protein structure. All connexins encompass intracellular N- and C-terminal domains (NT and CT, respectively), two extracellular loops (E1 and E2), a cytoplasmic loop (CL), and four transmembrane domains (green rectangles). Six connexin protomers oligomerize at the Trans-Golgi-Network to form a connexon, or hemichannel, which is then transported to the cell surface.

Hemichannels can communicate with the extracellular space but when a connexon couples to a connexon of an apposing cell a gap junction channel is formed. Hundreds to thousands of these channels coalesce to form dense gap junction plaques effecting electrical and metabolic coupling through the formation conduits coupling the cytoplasms of adjacent cells



Connexins are transmembrane proteins encompassing intracellular N- and C-termini, four transmembrane domains, two extracellular loops, and one intracellular loop (Fig. 6.1). This general protein structure is conserved across all connexins, with the least degree of conservation occurring within the C-terminal tail and portions of the extracellular loops attributed to hemichannel-hemichannel binding (Cruciani and Mikalsen 2006; Sohl and Willecke 2004). Complexity of gap junction regulation and tissue-specific function is augmented through the ability of distinct connexins to complex with each other, whereby protomers within the same connexon may encompass a mix of connexins, forming what is termed “heteromeric” connexon hemichannels. Moreover, coupling between connexons on apposing cells may occur between distinct connexons, creating what are termed “heterotypic” gap junction channels (Das Sarma et al. 2001; Koval 2006). Importantly, only certain connexins will oligomerize together, and only certain connexins within connexons will couple as gap junctions based upon extracellular loop sequences. With differential expression of connexins across cell types, the potential for exquisite control of specific cell-cell coupling within diverse cellular populations, and fine-tuning of what molecules are conducted through gap junctions, is vast.

Early expression of specific connexins ensures correct electrical coupling in the heart during development, so it stands to reason that aberrant expression of connexins in stressed or ageing tissue can underlie either limited, or inappropriate, intercellular communication affecting normal physiology (Zhang et al. 2011). Indeed, such alterations in connexin function have been reported in aged tissues with major associated pathologies including dementia, cataracts, bone loss, and cardiac arrhythmogenesis.

Regulation of Gap Junctions at the Molecular Level

Gene Structure and Transcription

Regarding gene structure, most connexin genes simply comprise two exons separated by an intron of variable size with the entire protein coding sequence residing within the second exon. Alternative splicing and dynamic transcription start site usage contribute to variability in mRNA 5' untranslated regions (UTR), which are largely encoded in the first exon. Some connexin genes are slightly more complex, however, and include two or more 5'UTR exons (e.g., Cx45; *GJCI*) and others (Cx36; *GJD2* and Cx23; *GJEI*) include single introns interrupting coding regions (Condorelli et al. 1998; Jacob and Beyer 2001). Splice variants do not alter protein sequences however, as variability in mRNA content seems to exclusively occur within the 5'UTR, highlighting translational control as an important facet of connexin regulation (Anderson et al. 2005; Pfeifer et al. 2004; Zeitz et al. 2019).

Dynamic transcriptional regulation of connexins occurs during normal development as cell types differentiate and invest in tissue formation. Several transcription factors (TFs) are known to regulate transcription of connexin genes, with some acting on several connexins and some displaying unique specificity, reviewed in Oyamada et al. (2013). Likewise, a variety of signal transduction cascades have been demonstrated to induce or suppress connexin transcription by acting directly and indirectly on these TFs. The ubiquitous TFs acting on connexin genes include activator protein-1 (AP-1), TATA-binding protein (TBP), and Sp1/Sp3 (Oyamada et al. 2013). In addition to binding sites for these general TFs, promoters of connexin genes also contain sequences that complex with cell-specific TFs. For example, Nkx2.5 is well-studied for its role in cardiovascular development and regulation for the three major cardiac connexins Cx40, Cx43, and Cx45 (Dupays et al. 2005). Additionally, during development, the TF IRX-3 regulates connexin expression in the His-Purkinje network of heart, specifically through repression of Nkx2.5 promotion of Cx43 transcription (Zhang et al. 2011). While such regulation during development enables specific patterning of connexin expression within and across tissues, alterations in TF expression of function during ageing could therefore affect not just gap junction formation, but expression of specific connexins which may normally require restricted expression for normal organ function.

Ageing-associated epigenetic mechanisms affecting gene expression is an area of intense research, and connexins are most certainly subject to such dynamic regulation (Pagiatakis et al. 2021; Vinken 2016). Research into the role of histone acetylation in regulation of connexin gene expression is dominated by use of compounds such as histone deacetylase (HDAC) inhibitors. In general, HDAC inhibitors increase connexin expression, facilitating AP-1 and SP1 binding to elicit transcription, for example (Hernandez et al. 2006). Methylation within the promoters of connexin genes negatively regulates their expression, and can lead to pathological silencing of connexin expression during cancer progression (Chen et al. 2003; Piechocki et al. 1999). During ageing, acetylation and methylation are altered, but additional research is needed to understand specifically how this pertains to changes in connexin expression. MicroRNAs (miRNAs) also provide a mechanism for modulation of connexin expression and are expressed differentially during ageing (Kinser and Pincus 2020). The list of miRNAs known to directly or indirectly regulate connexin expression is constantly growing, with several such miRNAs identified as undergoing altered expression in disease states. Specifically, miR-206 has been demonstrated to negatively regulate Cx43 in skeletal muscle and osteoblasts, where modulation of cellular differentiation through targeting Cx43 occurs (Anderson et al. 2006; Inose et al. 2009). miR-1 also targets Cx43 in myoblast differentiation, but is upregulated in coronary artery disease and thought to contribute to arrhythmogenesis in the heart (Anderson et al. 2006; Yang et al. 2007). More recently, miR-1 has been demonstrated to regulate muscle function during ageing, highlighting the potential significant impact of such post-transcriptional regulation on gap junctions in ageing tissues (Schiffer et al. 2021).

Translation

Translational control is now well accepted to function as a dynamic process as evidenced through increasing reports demonstrating discordance between the transcriptome and proteome (Payne 2015). Protein translation is subject to regulation at the global cellular level through modulation of signaling pathways converging on the translational machinery, such as PI3k/AKT/mTOR and/or RNA binding proteins (RBPs) (Araujo et al. 2012). At the gene-specific level, inclusion/exclusion of specific *cis* elements within mRNA can influence the translational readout, as described for *GJA1* below. Internal ribosome entry sites (IRES) within 5'UTRs enable translation of mRNAs when cap-dependent protein synthesis is inhibited, such as during stress or cell division (James and Smyth 2018). Cx43 (*GJA1*) and Cx32 (*GJB1*) encoding mRNAs have been reported to harbor such IRES sequences in their 5'UTRs (Hudder and Werner 2000; Schiavi et al. 1999). Interestingly, mutations ablating the Cx32 5'UTR IRES result in the genetic nerve disorder Charcot–Marie–Tooth disease, confirming the importance of translational control on gap junction regulation (Hudder and Werner 2000).

Translation of connexins has garnered increased interest in recent years given the discovery that *GJA1*, and likely other connexin mRNAs, undergoes “internal” alternative translation initiation within the coding sequence to yield truncated isoforms that impact gap junction formation, amongst other functions (Salat-Canela et al. 2014; Smyth and Shaw 2013; Ul-Hussain et al. 2014). While up to 6 internally translated isoforms of Cx43 have been identified, the most robustly expressed and currently best understood, is GJA1-20k. The GJA1-20k isoform encompasses most of the fourth transmembrane domain and entire C-terminus of Cx43. As research progresses, GJA1-20k may well account for several of the “non-junctional” intercellular functions of Cx43 historically attributed to its C-terminus. GJA1-20k positively regulates formation of Cx43 gap junctions and importantly, internal translation of *GJA1* mRNA is subject to dynamic regulation whereby its expression is inhibited by growth factor signaling cascades including mTOR, ERK1/2, p38, and MnK1/2 (James et al. 2018; Salat-Canela et al. 2014; Smyth and Shaw 2013; Zeitz et al. 2019). GJA1-20k has also been found by others to perform functions independent of its role in Cx43 gap junction formation. These include stabilization of actin filaments to direct Cx43 trafficking, promotion of mitochondrial transport, and protecting Cx43 from degradation to prevent arrhythmias (Basheer et al. 2017; Fu et al. 2017; Xiao et al. 2020). Recently, GJA1-20k was observed to be upregulated in response to ischemic injury, and experimental overexpression prior to ischemic injury was found to therapeutically regulate cardiac mitochondrial function and biogenesis (Basheer et al. 2018). Increases in GJA1-20k levels at early time points in cells exposed to hypoxic conditions have been reported in other studies. This gives way, however, to a robust reduction in GJA1-20k expression during stress indicating the importance of considering the duration of the stressor (Zeitz et al. 2019). A direct role for GJA1-20k in the transcriptional regulation of N-cadherin to control cell migration was also recently observed (Kotini et al. 2018). These studies highlight the diverse biological activity of GJA1-20k and its importance in health and disease, with a common outcome that GJA1-20k promotes cardiac health. The signaling cascades controlling internal translation of *GJA1*, and therefore gap junction formation, are often reported as deregulated in ageing and age-related pathologies (He et al. 2020; Papadopoli et al. 2019; Zeitz et al. 2019; Zeitz and Smyth 2020). Although complex, it is important that we consider effects on mRNA translational readout, and not just transcription and/or posttranslational protein modifications elicited by these cascades.

Moving from cellular signaling cascades to connexin/gene-specific regulation of translation, as stated above, by altering 5’UTR composition and thus modulating the inclusion of *cis* regulatory elements, alternative promoter usage has widespread implications for proteome regulation (Araujo et al. 2012). Implementation of rapid amplification of cDNA ends under conditions where GJA1-20k translation was suppressed revealed the existence of distinct *Gja1* mRNA transcript variants (with varying 5’UTR length) whose unique translational regulation controls gap junction formation (Zeitz et al. 2019). Elevated stress signaling in aged mouse heart tissue, correlates with alternative *Gja1* promoter usage generating transcript isoforms that modulate Cx43 translation and suppression of GJA1-20k synthesis to reduce gap

junction formation (Zeitz et al. 2019). Therefore, targeting these pathways may represent a viable approach to preserving gap junction coupling during ageing through promotion of alternative translation.

Trafficking

Connexins predominantly adhere to the canonical membrane-protein vesicular transport pathway, being co-translationally translocated into the membrane of the rough ER before progressing through the Golgi apparatus, assembling into hexameric channels, and being transported to the cell surface via the dynamic microtubule and actin cytoskeletons (Smyth and Shaw 2012). Of relevance to ageing, studies in cardiac tissues have demonstrated that increased oxidative stress disrupts this process, through perturbation of microtubule-mediated delivery of connexons to the plasma membrane (Smyth et al. 2010). Cx43 protein has a half-life of just 1–5 h depending on cell/tissue type and so alterations in forward transport to the cell surface rapidly affect gap junction coupling with significant impact on cardiac function, for example (Beardslee et al. 1998; James et al. 2018; Smyth and Shaw 2012). GJA1-20k has been shown to facilitate Cx43 trafficking through both promotion of hemichannel formation at the Golgi apparatus, and stabilization of actin structures in cardiomyocytes to promote trafficking of *de novo* channels to the gap junction (Basheer et al. 2017; James et al. 2018). Together with altered TSS usage resulting in differential translation of *GJA1* mRNAs, these findings connect transcriptional events directly with the regulation of gap junction formation.

Posttranslational Modification of Connexins

Given their essential function and the constant necessity of intercellular communication across tissues, connexins are remarkably short-lived proteins (Beardslee et al. 1998). The evolutionary advantage to this may be to enable rapid shutdown of intercellular communication and limit spread of toxic metabolites. During ageing, tissues experience chronic alterations in signaling, with increased hypoxia and often inflammatory states contributing to direct and indirect remodeling of gap junctions. As discussed above, connexin protein expression can be impacted from transcription to translation, and gap junction formation is dependent upon dynamic trafficking (Smyth and Shaw 2012). Connexin proteins *en route* to the cell surface and within gap junctions are also subject to a variety of posttranslational modifications typically attributed to affecting channel function, gap junction formation and stability, and eventual degradation (Johnstone et al. 2012). Unlike similar channels, such as pannexins, connexins are not known to be glycosylated. Phosphorylation of the connexin protein c-terminus has been intensely studied, but additional modifications including nitrosylation and ubiquitin and ubiquitin-like (neddylation, sumoylation)

are being increasingly recognized (Kjenseth et al. 2012; Lillo et al. 2019; Pogoda et al. 2016).

Phosphorylation of connexins is complex, with most research focused historically on the Cx43 C-terminus. Taking Cx43 as a model gap junction channel, these studies have informed the importance of phosphorylation on gap junction assembly, conductance, stability, and internalization/degradation. Several kinases converge on the Cx43 C-terminus to elicit positive and negative effects on gap junction formation and function (Marquez-Rosado et al. 2012). Although a constantly evolving field, here we list the primary kinases and known impact of their acting upon the gap junction. PKA, driven by cAMP, is associated with promotion of gap junction assembly (Paulson et al. 2000). Once in the gap junction, phosphorylation of Cx43 Ser368 by PKC is associated with reduced channel opening probability, and occurs during stress and pathological gap junction remodeling (Lampe et al. 2000; Smyth et al. 2014). During stress/injury Akt phosphorylates Cx43 at Ser373, promoting Ser368 phosphorylation, and acting upstream of MAPK phosphorylation events that elicit gap junction internalization and degradation (Dunn and Lampe 2014; Smyth et al. 2014; Solan and Lampe 2014). Counter to this, Casein Kinase 1 can exert protective effects on gap junctions through phosphorylation of sites, which when phosphomimetically mutated, preserve cardiac gap junctions and protect against arrhythmia during stress (Remo et al. 2011). Connexin tyrosines are also subject to phosphorylation by Src where they also appear to be targeted during the cascade of phosphorylation eliciting gap junction degradation (Solan and Lampe 2020). The sheer number of sites dynamically phosphorylated on just one connexin speak to the depth of regulation to which gap junctions are subjected. From an energetic perspective, internalization of gap junctions is a major commitment by the cell. It stands to reason, therefore, that several “checkpoints” exist where communication can be reduced (i.e., through Cx43 Ser368 phosphorylation) prior to eliciting the endocytic and degradation pathways. Internalization of gap junctions has been reportedly controlled through ubiquitination and progression to the lysosome or autophagosome. Interestingly, gap junctions can be internalized in their entirety, with one cell phagocytosing part of its neighbor to form double-membrane “annular” gap junctions that are degraded in the autophagosome (Fong et al. 2014; Gilleron et al. 2009; Hesketh et al. 2009). Redox changes are of high relevance to ageing and altered gap junction and hemichannel functions. From cardiac systems to cataract formation, oxidative stress and its direct and indirect impacts upon gap junction function have been subject to intense study (Berthoud and Beyer 2009; Smyth et al. 2010). Overall, signaling pathways induced by chronic stressors, such as hypoxia, impact every step of the gap junction lifecycle and reduce intercellular communication. While evolutionarily this might be an attempt by the cell to limit the spread of damage, it can have severe deleterious effects on several major organ systems during ageing, the major of which are described below.

Heart

The heart is arguably one of the, if not the, most intensely studied organs in terms of gap junction biology. Every heartbeat depends upon the coordinated contraction of billions of individual cardiomyocytes, a feat achieved through the electrical intercellular coupling effected by gap junctions (Nielsen et al. 2012; Rohr 2004; Shaw and Rudy 1997). Cardiomyocytes are large highly polarized cells that mechanically and electrically couple to each other end-to-end via a complex junctional structure known as the intercalated disc (ID), where gap junctions are highly enriched. Other ID resident junctions include desmosomes and adherens junctions, and significant interplay/cross talk occurs between these and gap junctions with increasing evidence that these junctions are far removed from the discrete independent structures they were once thought to be based on historical pathology (Forbes and Sperelakis 1985; Vermij et al. 2017; Zhao et al. 2019). Cx43 gap junctions also facilitate enrichment of the cardiac voltage-gated sodium channel $\text{Na}_v1.5$ at the ID, specifically within the perinexus adjacent to gap junctions, where ephaptic electrical coupling of cells occurs in addition to the direct exchange of ions via gap junctions (Agullo-Pascual et al. 2014; George et al. 2016; Leo-Macias et al. 2016a, b; Veeraraghavan et al. 2015). The composition, amount, and size of gap junction plaques in the mammalian heart vary by region. This variability contributes to regulating specialized physiological functions such as cardiac conduction velocity and cell-specific electrical coupling. The three predominant connexin isoforms present throughout the cardiac conduction system (CCS) and working myocardium are Cx40, Cx43, and Cx45 (Severs et al. 2008). Specific localization of Cx43 gap junctions to the ID of the ventricular working myocardium is necessary for the anisotropic propagation of action potentials and coordinated contraction. It is well established that alterations in Cx43 expression and/or gap junction function underlie the arrhythmias of sudden cardiac death. In addition to loss of expression, relocalization of Cx43 away from the ID to lateral cardiomyocyte plasma membranes has been demonstrated to create a substrate for electrical reentry to initiate arrhythmias in stressed heart tissue (Peters et al. 1997; Severs et al. 2008).

Aside from the cardiomyocyte, the true muscle cells of the heart, the CCS comprises specialized myocytes that initiate and rapidly propagate action potentials throughout the myocardium. Proper CCS function is necessary to ensure coordinated contraction of the cardiac chambers beginning with the atria and then apex-to-base contraction of the ventricular myocardium. This exquisite orchestration of action potential propagation exploits specific connexins to utilize gap junctions with differing conductance. The cells of the sinoatrial (SA) node play the critical role of cardiac pacemaker and initiate each electrical signal that goes on to form a heartbeat. Gap junctions in the SA node are less numerous and of smaller sizes than the working myocardium. The predominant connexins expressed within the SA node include Cx45 and Cx40. Interestingly, alterations in Cx43 expression at SA node periphery may be linked to sick sinus syndrome in the elderly (Jones et al. 2004a). The SA node resides in the wall of the right atrium, and following initiation of the

action potential, the atria are the first region to receive this signal and are characterized by rapid conduction velocity with the principle connexins expressed being Cx40 large-conductance gap junction channels and Cx43 medium-conductance gap junction channels (Vozzi et al. 1999). The electrical signal then arrives at the atrioventricular (AV) node which serves to slow conduction originating from the atria to ensure completion of contraction and emptying of blood from the atria prior to ventricular depolarization. Many factors contribute to this slowing of conduction, including limited GJIC between myocytes due to smaller gap junction size and conductance together with reduced $\text{Na}_V1.5$ expression (Greener et al. 2011; Shimada et al. 2004). AV nodal cells express Cx45 and Cx40, with Cx30.2 reported in the mouse although its corresponding human isoform Cx31.9 has not been detected (Coppens et al. 1999; Kirchhoff et al. 1998; Kreuzberg et al. 2006, 2009). Specifically, the relatively slower conductance of Cx45 gap junctions in the AV node is suggested to contribute to slowing of conduction. Action potentials then progress through the CCS to the bundle of His from where the bundle branches arise and rapidly propagate signal down the septum towards the cardiac apex. The Purkinje fibers then spread the depolarization signal throughout the working myocardium of the ventricles, achieving apex-to-base contraction. A gradient of specific connexin expression through the bundle branches and Purkinje fibers ensures rapid impulse propagation and appropriate electrical coupling with the working myocardium. Specifically, Cx40 dominates initially, with Cx43 expression gradually increasing through the Purkinje fiber network allowing direct coupling with cardiomyocytes and subsequent activation. Given the essential role of gap junctions in every step of the CCS coordinating impulse propagation and contraction of the working myocardium, it is unsurprising that alterations in connexin expression and function affecting gap junctions during ageing underlie a variety of cardiac disease states.

The ageing heart is susceptible to pathological remodeling affecting multiple levels of organization ranging from whole heart size to molecular components (Bonda et al. 2015; Cheng et al. 2009; Cohn et al. 2000; Fannin et al. 2014). Reduced connexin expression at the mRNA and protein level has been reported in hearts of aged guinea pigs, mice, and humans, and it is now well established that conduction velocity slows with ageing (Jones et al. 2004a; Roberts-Thomson et al. 2009; Spach and Dolber 1986; Stein et al. 2008a). Indeed, alterations to gap junction expression and function are reported throughout essentially all structures of the heart during ageing. Age-related changes to SA node have been reported in guinea pigs SA nodes, where detection of Cx43 by immunostaining progressively declines from the center outward throughout ageing. Cx43 levels are observed lower by western blot and this correlates with slowed conduction velocity (Jones et al. 2004b; Peters et al. 2020; Saeed et al. 2018). Further into the CCS, “heart block” is the term used to describe a disruption in impulse propagation, often resulting in AV dissociation. Remarkably, it is estimated that 30% of individuals over 65 years of age harbor some form of heart block or conduction defect (Fleg and Kennedy 1982). Such loss in CCS function and ventricular conduction often involves induction of fibrotic mechanisms with gap junction remodeling occurring separately or as an indirect consequence as the tissue remodels.

Atrial fibrillation (AF) represents the most common cardiac arrhythmia, with more than 37 million people worldwide living with this disorder (Lippi et al. 2021). While fibrillation of the atria is not as directly lethal as ventricular fibrillation in terms of cardiac pumping function, the resulting pooling of blood creates a significant risk of clot formation and stroke. Ageing is a primary risk factor for AF, with clear involvement of gap junction biology demonstrated through studies revealing human atria have progressive reduction of Cx43. Cx43 has also been reported to be reduced in aged guinea pig atria at mRNA and protein levels (Nagibin et al. 2016). Activation of JNK signaling during ageing is believed to contribute to gap junction remodeling. Consistent with this, experimental JNK activation reduces gap junctional Cx43 in HL-1 myocytes and aged mice (Yan et al. 2018). An additional study also reported reduction of total Cx43 levels and increases in phosphorylated Cx43 as a result of an increase in activated JNK in the right atria of guinea pigs during ageing (Jones and Lancaster 2015). Patients with left atrial fibrillation have increased TGF- β 1, connective tissue growth factor (CTGF), RAC1, and N-cadherin, consistent with fibrotic tissue remodeling. While Cx43 is also upregulated, it is lateralized away from gap junctions. In mice, TGF- β activates RAC1 and CTGF and *in vitro* experiments confirm CTGF upregulates NCAD and induced aberrant Cx43 expression in primary neonatal cardiomyocytes (Adam et al. 2010).

Disruption of Cx43 localization to the ID of ventricular cardiomyocytes results in decreased gap junction intercellular communication (GJIC) and increased susceptibility to arrhythmias of sudden cardiac death (Gutstein et al. 2001). In the ventricle, ageing reduces Cx43 gap junction coupling and increases ventricular fibrosis, increasing arrhythmia risk in aged mice (Jansen et al. 2012; Stein et al. 2008b). Aged rat myocardium was also reported to express significantly less Cx43, as determined by immunofluorescence microscopy (Watanabe et al. 2004). Cx43 is also reduced in the LV myocardium and mitochondria of aged mice hearts, and this correlates with loss of cardioprotection during ischemic preconditioning (Billur et al. 2022; Boengler et al. 2007). Although *Gja1* mRNA levels were not changed, Cx43 protein levels were reduced in 24-month-old aged mouse hearts observed by western blot, implicating post translational mechanisms affecting Cx43 stability. Remodeling of intercalated disc was observed including lateralization of Cx43 by confocal microscopy. Interestingly, desmosomes were not affected despite losses in gap junctions at the ID which correlated with prolonged ventricular depolarization (Bonda et al. 2015). Alterations in GJA1-20k expression, and therefore gap junction formation, have also been reported in aged mouse hearts. As discussed above, here altered TSS usage downstream of p38 signaling was found to result in truncating *Gja1* mRNA 5'UTRs to limit internal translation and subsequent gap junction formation (Zeitz et al. 2019).

Brain

Intercellular communication in the brain occurs through chemical synapses which rely on the release and detection of neurotransmitters, and through electrical synapses encompassing gap junctions and adhesion proteins (Martin et al. 2020). While the number of electrical synapses decreases significantly during development, their maintenance in the adult brain is associated with complex roles, for example effecting neuronal synchrony and rhythmicity, and supporting cognitive functions, including learning and memory (Bennett and Zukin 2004; Connors 2017; Connors and Long 2004). The primary connexin expressed by neurons responsible for electrical synapse formation is Cx36 (*GJD9*), with additional connexins expressed in other brain cell types and the ocular system, described below. Just as in the heart, neuronal gap junctions have surprisingly short half-lives, which enables plasticity during normal cellular functions, but also results in rapid losses in direct intercellular coupling during stress.

Glia, the major non-neuronal cells of the brain, are increasingly gaining interest as targets for therapeutic intervention due to their key role in numerous aspects of brain physiology. Neuroglia cells, originally thought to passively support neurons, are now recognized to play much more critical roles in neuronal function including uptake of neurotransmitters to modulate synaptic transmission and providing immune support. Astrocytes, the most abundant glial cell type within the adult brain, are implicated in an array of neurodegenerative diseases, many of which are associated with ageing. Astrocytes express high levels of connexin proteins with Cx43 and Cx30 being the dominant isoforms and Cx43 and Cx30 double knockout mice display significant neuropathology (Lutz et al. 2009). Cx43 has long been observed to form gap junction channels in astrocytes (Giaume et al. 1991), but in addition to gap junctions, astrocytes express significant levels of non-junctional Cx43 hemichannels on their surface that can open to the extracellular space. These hemichannels have been demonstrated to release glutamate and glutamine to affect synaptic function with implications in neurodegenerative disease as elevated glutamate is associated with neuronal cell death (Cheung et al. 2022; Ye et al. 2003).

The role of astrocytes and Cx43 in Alzheimer's disease (AD), a disorder where age is well established as the primary risk factor, has seen increased attention. Transcriptome analysis of postmortem brain samples from patients identified *GJA1* as a key regulator of AD (Kajiwara et al. 2018). A hallmark of AD is the formation of extracellular amyloid- β (A β) plaques which are associated with areas of neuronal death. In both mouse models of AD and in tissue from human AD patients, Cx43 labeling was observed to be elevated proximal to A β plaques (Mei et al. 2010; Nagy et al. 1996). In cell culture experiments, treatment with Amyloid β -Protein Fragment 25–35 (A β _{25–35}) was observed to increase Cx43 hemichannel activity while reducing GJIC (Maulik et al. 2020). As mentioned above, such increased Cx43 hemichannel activity that triggers excessive glutamate release from astrocytes has been demonstrated to induce neurotoxicity (Orellana et al. 2011a, 2011b). Deletion of astroglial Cx43 improved synaptic function in a mouse model of AD, as did pharmacological

inhibition of hemichannel activity (Ren et al. 2018; Yi et al. 2017). Therefore, modulation of Cx43 function to promote gap junction formation and limit Cx43 hemichannel activity may represent a viable therapeutic approach for AD.

The Ocular System

Ocular lens epithelial cells initially express Cx43, but as they differentiate into fiber cells expression of Cx50 transiently increases and Cx46 eventually dominates. Alterations in these gap junctions are understood to contribute to cataract development during ageing both through mutation and loss of channel function. Cataract, a clouding of the lens, is the leading cause of blindness worldwide with ageing being the greatest risk factor. As stated above, connexins expressed in the lens include Cx43, Cx46, and Cx50. Cx50 is expressed throughout the cells of the lens. Cx43 is predominantly expressed in the lens epithelial cells and immature outer fiber cells, while Cx46 is expressed at greater levels as the differentiating fiber cells mature (Mathias et al. 2010). Fiber cell differentiation includes loss of cellular organelles which renders GJIC crucial for supporting fiber cell homeostasis. It is unsurprising that several studies have historically linked disruptions in connexin expression to cataract development and associated losses in Cx46 and Cx50 with ageing (Gong et al. 1997, 1998, 2021; Rong et al. 2002; White et al. 1998). Homozygous Cx46 knockout mice developed cataract by 2 months of age (Gong et al. 1997). Homozygous Cx50 knockout mice also develop cataract with an additional feature of reduced lens mass (Rong et al. 2002; White et al. 1998). Interestingly, a large number of germline mutations in human Cx46 and Cx50 have also been identified as causative in hereditary forms of cataract (Shi et al. 2022; White and Paul 1999). These results indicate the importance of both these connexins function to lens transparency. Examination of the lens in aged mice observed decreased Cx46 and Cx50 expression correlating with a reduction in gap junction coupling conductance (Gao et al. 2013). An initial hypothesis of the role of gap junctions in cataract suggested that, during ageing, increased oxidative stress leads to impaired GJIC, and this impairment could hinder the circulation of antioxidants leading to cataracts in the lens fiber cells, reviewed in Berthoud and Beyer (2009). A recent study examining mouse models of human congenital cataracts with mutations in Cx46 or Cx50 casts doubt on oxidative stress being an early event in cataract formation in these mice however (Jara et al. 2020). There are increasing data suggesting instead that impaired GJIC leads to accumulation of calcium and calcium precipitation to generate cataracts (Berthoud et al. 2019; Minogue et al. 2022). In addition to gap junctional mechanisms of cataract development, it should be noted that connexin hemichannels have been suggested to play a protective role against oxidative damage potentially through the uptake of glutathione and their dysregulation may also be a contributing factor in ageing-associated lens pathologies (Shi et al. 2018).

Bone

Bone is a dynamic structure that is continuously remodeled throughout an organism's lifespan. As age increases, remodeling shifts balance to an increase in bone degradation and a decrease in new bone formation (Demontiero et al. 2012). Remodeling is controlled by numerous factors including hormonal signaling and mechanical load (Robling et al. 2006; Siddiqui and Partridge 2016). This remodeling is accomplished through the coordinated work of multiple cell types including osteoclasts, and osteoblasts. Osteoclasts are large multinucleated cells that function to break down bone in a process termed resorption. Osteoblasts, on the other hand, function to synthesize bone matrix promoting new bone formation. Terminally differentiated osteoblasts, termed osteocytes, are encased in bone matrix in pockets called lacunae and communicate through cellular protrusions within canaliculi connected to neighboring cells forming a complex network that modulates bone formation and communicates through both GJIC and Cx43 hemichannel functions (Civitelli 2008; Marotti et al. 1992). Cx37, Cx45, and Cx46 are also detected, but Cx43 is the most abundantly expressed connexin in bone, and is expressed in all three of the aforementioned cell types (Civitelli 2008; Civitelli et al. 1993; Ilvesaro et al. 2000). Cx43 has been demonstrated to play a key role in osteocyte survival with mice lacking Cx43 displaying increased osteocyte apoptosis, leading to endocortical resorption and periosteal bone formation (Bivi et al. 2012; Plotkin et al. 2008). Just as in other tissues where GJIC is essential, Cx43 RNA and protein levels within bone have been observed to decrease with age (Davis et al. 2017). An additional study in rat osteoblastic cells saw no change in Cx43 expression with age, but did identify age-dependent effects on GJIC in response to parathyroid hormone (PTH) stimulation which normally increases cAMP and gap junction formation (Genetos et al. 2012). The authors associate this with perturbed G protein coupled receptor signaling in aged animals, which limits the cellular ability to respond to PTH and increase GJIC. Osteocyte apoptosis also increases with age and overexpression of Cx43 in osteocytes reduces apoptosis in aged mice and results in reduced endocortical bone resorption (Davis et al. 2018). Recently, it was found that Cx43 reduction in osteocytes leads to reduction of microRNA 21 (miR21), which is sufficient to induce apoptosis and disrupt a signaling cascade regulating osteoclast formation (Davis et al. 2017). Together, this data indicates a prominent role for Cx43 mediated osteocyte viability and suggests age-related reduction in Cx43 can disrupt the balance of bone remodeling. The relationship between hemichannel function controlling bone remodeling in addition to GJIC once again presents a complex mechanism that is an active area of research where changes in Cx43 expression affect far more than gap junctions in the aged bone microenvironment (Hua et al. 2021; Plotkin et al. 2015).

Other Tissues

The liver is rich in gap junctions and predominantly expresses Cx26 and Cx32. A rat model of natural ageing showed their expression at the protein level is reduced in hepatocytes during ageing. Interestingly mRNA levels do not fall early in ageing, signifying that altered connexin protein stability/regulation occurs prior to losses in gene expression (Moscati et al. 2020). Additional research here has focused on the influence liver gap junctions have on tissue regeneration, carcinogenesis, and/or the intersection with lifestyle factors such as alcoholism (Dagli et al. 2004; Naiki-Ito et al. 2012). Epithelial barriers in general are known to deteriorate during ageing, which clearly involve perturbed gap junction formation and function (Parrish 2017). Such compromised barrier function is a key component of ageing-associated lung disease, for example, but a direct causative role for gap junction loss has not been established whereby alterations to connexins may occur downstream of tight junction disruption. Alterations in connexins are involved in a variety of lung pathologies, however, and further research may connect ageing-related changes to development of pulmonary disease (Swartzendruber et al. 2020). Gap junctions also play important roles in the ear, and mutations in connexin genes are well established in both non-syndromic and syndromic deafness. Research interrogating these mutations, primarily in Cx26, has provided extensive insight into the role of gap junctions and hemichannels in effecting normal hearing (Beach et al. 2020; Martinez et al. 2009). Regarding age-related hearing loss, it has been proposed that, in addition to possible downregulation of connexin expression, oxidative stress could impair connexins to affect cochlear function (Martinez et al. 2009). In line with this, a recent study analyzing connexin expression and function in ageing mice demonstrated that older animals had smaller and fewer cochlear gap junctions, concomitant with reduced Cx26 and Cx30 protein levels (Tajima et al. 2020). Finally, gap junctions play key roles in several aspects of reproduction and fertility, with disruption in connexin expression and function occurring with age. Briefly, in terms of women's reproductive health, disruption of connexin expression and function impair fertility through oocyte deficiencies and GJIC is critical in the uterus for placental formation and implantation, for example (Kaushik et al. 2020; Winterhager and Kidder 2015). In males, both erectile dysfunction and reduced spermatogenesis, which occur during ageing, are associated with alterations to gap junctions in the penile erectile tissue and testes, respectively (Christ 2000; Melman and Christ 2001; Sridharan et al. 2007).

Concluding Remarks

As direct intercellular communication is critical across all organs and tissues for normal function, it is unsurprising that pathological gap junction remodeling is a hallmark of so many ageing-related maladies. Increased understanding of connexin

regulation however is creating hope that a better understanding of the gap junction life cycle will inform therapeutic interventions and strategies aimed at preserving intercellular communication, and therefore more normal physiological functions, during ageing.

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Chapter 7

Cellular Senescence and Ageing



Rebecca Reed and Satomi Miwa

Abstract Cellular senescence has become a subject of great interest within the ageing research field over the last 60 years, from the first observation *in vitro* by Leonard Hayflick and Paul Moorhead in 1961, to novel findings of phenotypic sub-types and senescence-like phenotype in post-mitotic cells. It has essential roles in wound healing, tumour suppression and the very first stages of human development, while causing widespread damage and dysfunction with age leading to a raft of age-related diseases. This chapter discusses these roles and their interlinking pathways, and how the observed accumulation of senescent cells with age has initiated a whole new field of ageing research, covering pathologies in the heart, liver, kidneys, muscles, brain and bone. This chapter will also examine how senescent cell accumulation presents in these different tissues, along with their roles in disease development. Finally, there is much focus on developing treatments for senescent cell accumulation in advanced age as a method of alleviating age-related disease. We will discuss here the various senolytic and senostatic treatment approaches and their successes and limitations, and the innovative new strategies being developed to address the differing effects of cellular senescence in ageing and disease.

Keywords Senescence · Senolytic · Senostatic · Telomeres · SASP · SA- β -Gal · Ageing · Age-related disease

What Is Cellular Senescence?

Cellular senescence is a state of irreversible growth arrest in which cells cease to proliferate but do not undergo apoptosis. Instead, these cells experience significant changes in morphology, metabolism and enzyme activity, as well as developing a

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pro-inflammatory phenotype with wide-ranging implications for the surrounding microenvironment. These phenotypic changes are responsible for both beneficial and detrimental roles of senescent cells in human development. While such cells are key to embryonic development, wound healing and tumour suppression in early life, they have also been associated with ageing and age-related disease. In each of these circumstances, senescent cells accumulate as a result of upregulated anti-apoptotic pathways and downregulated pro-apoptotic pathways, promoting cell survival and persistent cell cycle arrest.

One of the major determining factors of a senescent phenotype is the activation of a persistent DNA damage response (DDR). Double- and single-strand DNA breaks are detected by DNA damage kinases ataxia-telangiectasis mutated (ATM), ataxiatelangiectasis and rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) (Ciccia and Elledge 2010), which initiate a cascade of downstream signalling to recruit DNA repair proteins. Factors involved in this DNA repair process localise at the site of damage and can form foci identifiable by immunofluorescent staining and used in the detection of senescent cells (d'Adda di Fagagna et al. 2003; Jackson and Bartek 2009). Such factors include ATM (Dupre et al. 2006), p53 binding protein (53BP1) (Eliezer et al. 2009) and phosphorylated histone 2AX (yH2AX) (Burma et al. 2001). H2AX is also responsible for chromatin remodelling protein recruitment, which allows alterations to chromatin structure to permit DDR protein access to the site of damage (Nakamura et al. 2010).

ATM and the checkpoint kinase Chk2 also promote activation of the transcriptional regulator p53 (Turenne et al. 2001), a major regulator of both senescent and apoptotic pathways (Seluanov et al. 2001). p53 in turn stimulates activation of the cyclin-dependent kinase (CDK) inhibitor p21 (Jackson and Pereira-Smith 2006) leading to cell cycle arrest (Dutto et al. 2015) via retinoblastoma protein (pRb) hypophosphorylation and stabilised binding to E2F transcription factor 1 (E2F1) (Georgakilas et al. 2017). Chk2 is also responsible for the degradation of CDC25 phosphatase, which prevents G1-S phase transition and supports growth arrest (Mailand et al. 2000). An additional barrier to cell cycle progression is established by double strand break (DSB) activation of the CDK inhibitor p16, a positive regulator of the pRb pathway and able to induce cell cycle arrest by inhibiting CDK4/6 and reinforcing pRb inhibition by E2F1 (Beausejour et al. 2003).

The major beneficial role of cellular senescence is as a tumour suppressor mechanism. By preventing damaged and pre-malignant cells from dividing, cellular senescence acts as a barrier to tumour development. This is largely achieved by modifying levels of cell cycle regulators, primarily p53, p21 and p16, which induce a permanent halt to cell cycle progression. Secretion of SASP factors IL-6 and IL-8 can also interact with specific cell-surface receptors to trigger signalling pathways such as the DDR. This is discussed in further detail below.

During mammalian embryonic development, senescent cells have been found in the apical ectodermal ridge (AER) and neural roof plate. It is here that senescent cells are thought to influence growth and patterning through p21, p15 and SASP signalling. Indeed, studies have found that mice lacking p21 exhibit dysfunctional embryonic senescence (Storer et al. 2013). Notably, two fibroblast growth factor genes

(FGF4 and FGF8) involved in AER signalling for proliferation and pattern formation were also reduced in p21-deficient mice, suggesting that this aspect of AER function is compromised when senescence is prevented. Senescent markers have also been linked to placental formation and function. In particular, the known SASP components matrix metalloproteinases 2 and 9 MMP2 and MMP9 are important in achieving trophoblast invasion in early pregnancy, demonstrating a link between SASP and placental development. Moreover, activity of these MMPs is decreased in murine placentas with deficient senescent signalling (Gal et al. 2019). Other reports have detailed a p21-dependent senescence in the mesonephros and endolymphatic sac in the inner ear (Munoz-Espin et al. 2013).

Cellular senescence is also important to the process of wound healing, both by limiting proliferation of damaged tissue and recruiting immune cells to eliminate the accumulated senescent cells. This is demonstrated in liver fibrosis, where senescent hepatic stellate cells are known to accumulate and secrete greater levels of extracellular matrix (ECM) degrading enzymes and are the subject of enhanced immune surveillance. This promotes immune-mediated clearance of the senescent cells, alleviating fibrosis progression and liver scarring (Krizhanovsky et al. 2008). Similar findings have also been reported in the pancreas, in which accumulated senescent pancreatic stellate cells correlate with greater inflammation and fibrosis, which is limited by immune-mediated clearance of senescent cells (Fitzner et al. 2012). Furthermore, *in vivo* studies have demonstrated that cellular senescence is involved in tissue regeneration in both zebrafish (Da Silva-Alvarez et al. 2020) and salamanders (Yun et al. 2015).

In addition to mediating immune clearance of senescent cells, pro-inflammatory SASP factors are also involved in numerous other processes within wound healing. For example, platelet-derived growth factor AA (PDGF-AA) accelerates wound closure by promoting myofibroblast differentiation (Demaria et al. 2014), while cellular communication network factor 1 (CCN1) limits skin fibrosis by inducing fibroblast senescence via cell-surface receptor interaction in cutaneous wound healing (Jun and Lau 2010). These senescent cells exhibit a persistent DDR, p53 and p16 activation, and express antifibrotic genes. This, along with SASP-mediated clearance of the senescent cells, restricts fibrosis in cutaneous injury. The pro-inflammatory senescence-associated secretory phenotype (SASP) is responsible for triggering immune-mediated clearance of senescent cells by recruiting key immune cells including macrophages, B cells, T cells and NK cells (Coppe et al. 2008; Krizhanovsky et al. 2008; Storer et al. 2013). This clearance of senescent cells is key to both wound healing, by limiting scarring and fibrosis, and tumour suppression, by eliminating pre-malignant and damaged cancerous cells (Xue et al. 2007; Krizhanovsky et al. 2008; Ruscetti et al. 2018).

In contrast to these positive effects in early life and development, it is well established that senescent cells are also associated with the ageing process and age-related disease, discussed in greater detail in the section on Senescence and Age-Related Disease, below. Alongside this, the SASP has been shown to possess a paradoxical role in cancer development over time. While SASP factors are key to immune-mediated clearance of senescent cells, contributing to tumour suppression,

it has also been noted that the chronic inflammation generated by SASP production may contribute to diseases such as diabetes (Xu et al. 2015; Praticò et al. 2018), COPD (Birch et al. 2015), osteoarthritis (Livshits et al. 2009; Xu et al. 2017) and cancer (Bavik et al. 2006; Coppe et al. 2010; Ortiz-Montero et al. 2017). It has also been observed that accumulation of senescent cells can promote cancer development, and can be accompanied by SASP-mediated paracrine effects on cancer progression (Eggert et al. 2016; Lau et al. 2019) and treatment failure (Demaria et al. 2017). Notably, Demaria and colleagues reported that selective elimination of senescent cells by navitoclax treatment alleviated a number of chemotherapy-induced side effects, including cancer recurrence and bone marrow suppression. Similar observations of increased chemotherapy-induced fatigue in patients with greater senescent marker presence were reported in patients with breast cancer (Demaria et al. 2017).

History

Cellular senescence was first described in 1961 by Hayflick and Moorhead, who determined that normal proliferating fibroblasts in culture were limited to a finite number of divisions before halting cell cycle progression (Hayflick and Moorhead 1961). This is now referred to as the ‘Hayflick limit’ (Hayflick 1965). This form of senescence resulting from extended cell culture is termed replicative senescence, discussed in more detail in the section on Replicative Mechanisms, below. Other forms of senescent phenotype are triggered by different stimuli, but share a large proportion of common markers and characteristics are also described below. One central feature of all of these phenotypes is the choice between apoptosis and senescence induction. How this choice is made remains unclear, but there are many possible determining factors, such as damage severity, stimulus type and cell type. It is also possible that a shared mediator may be involved in this choice, as both apoptotic and senescent pathways are regulated by the p53 pathway (Kirschner et al. 2015).

The contrasting roles of senescence in embryonic development and early life versus ageing and disease have also caused much debate within the ageing research field. It is striking that cells initially so beneficial to human development and survival should have such detrimental effects later in life. One prominent hypothesis is the antagonistic pleiotropy theory of ageing, which proposes a preferential selection of genes with reproductive fitness advantages during early life, but which may come with negative consequences in later life as an unintended consequence. Though to date there is little evidence to support this theory.

Characteristics and Markers

Cellular senescence can result from numerous stress-inducing triggers including telomere attrition, persistent DNA damage, oncogene activation, mitochondrial dysfunction and oxidative stress. The specific senescent profiles produced by each

of these triggers is discussed in further detail below, but there are a number of common characteristics and markers that are observed in multiple models of senescence (Fig. 7.1).

Telomere Shortening

Telomeres are end chromosome structures formed by repetitive DNA sequences and capped with proteins that protect them from being mistakenly identified as damage or double strand breaks by DNA repair processes (Blackburn 1991; d'Adda di Fagagna et al. 2004). However, DNA polymerase activity during cell division cannot fully copy these telomeric regions, leading to progressive shortening of telomeres with each cell division. Eventually, a critical length is reached at which the protective telomeric caps are lost during replication, and the newly uncapped single-stranded telomere ends are recognised by DNA repair machinery as double strand breaks (DSBs) (d'Adda di Fagagna et al. 2003). This triggers a DNA damage

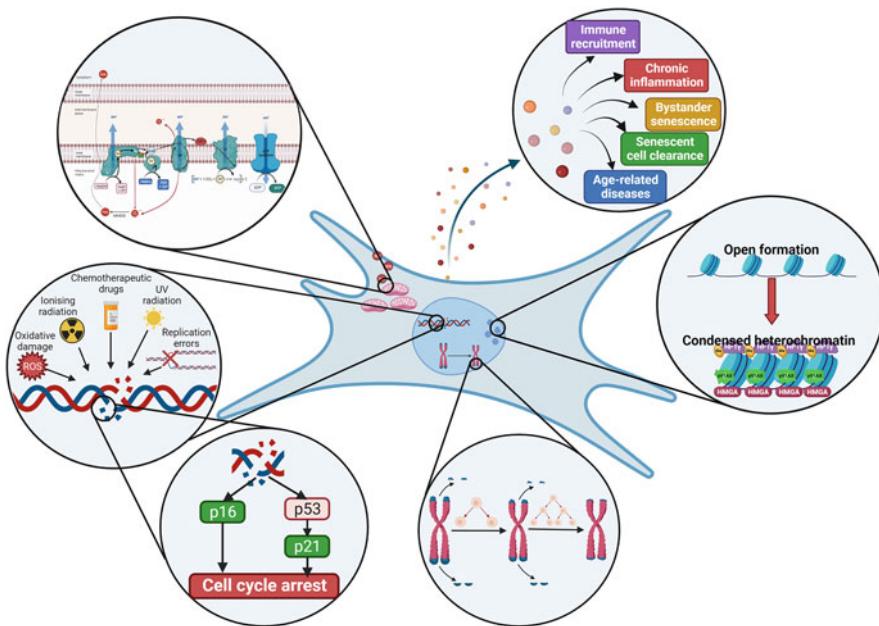


Fig. 7.1 The senescent phenotype: Cellular senescence can be triggered by a number of different stressors, but a selection of common markers have been identified which can be used to determine the presence of senescent cells. (a) elevated senescence-associated beta-galactosidase (SA- β -Gal) activity identified by blue histochemical stain at pH 6, (b) elevated reactive oxygen species (ROS) production by the mitochondria, (c) DNA damage caused by a range of stressors can be identified as foci of DNA repair machinery components, (d) telomere shortening, (e) senescence-associated heterochromatin foci (SAHF) and F) production of pro-inflammatory cytokines, chemokines and growth factors that form the senescence-associated secretory phenotype (SASP)

response (DDR) which attempts to repair the ‘damage’; however, telomeres are inaccessible to DNA repair machinery as components of the protective shelterin complex inhibit the non-homologous end joining repair pathway (Bae and Baumann 2007). This inability of DNA repair processes to fix the perceived damage leads to a persistent DDR (Galbiati et al. 2017), which is required for cellular senescence (Fumagalli et al. 2014).

Some DNA repair components translocate to the site of the damage and accumulate into identifiable foci. These foci are also present at the sites of non-telomeric DNA damage, and can be used as quantifiable markers of senescent cells (Takai et al. 2003). In the case of telomere attrition, these foci localise at the telomeres and are termed telomere-associated foci, or TAFs. It is also worth noting that a persistent DDR can also be triggered at telomeres following DNA damage, irrespective of telomere length, such as that resulting from ROS-induced oxidative damage (von Zglinicki 2002; Reichert and Stier 2017). The reduced efficiency of DNA repair at telomeres promotes a persistent DDR regardless of the initial cause of damage (Bae and Baumann 2007; Hewitt et al. 2012; Fumagalli et al. 2014).

The role for telomere shortening in cellular senescence is further supported by the observation that overexpression of telomerase prevented senescence in a study of telomerase-negative human retinal pigment epithelial cells and foreskin fibroblasts, along with extending the replicative lifespan of such cells (Bodnar et al. 1998).

DNA Damage

As discussed, a persistent DDR can be triggered by DNA damage at sites other than telomeres, culminating in permanent cell cycle arrest (Fumagalli et al. 2014). Often, a persistent DDR is triggered by double strand DNA breaks (DSBs), though single-strand breaks (SSBs) can also trigger a DDR. At these points of damage, key components of the DNA repair machinery can accumulate and provide identifiable markers of a persistent DDR and senescent cells. Such markers include phosphorylated histone 2AX (γ H2AX), mediator of DNA damage checkpoint protein 1 (MDC1), 53BP1 and ATM (Burma et al. 2001; Eliezer et al. 2009; Lavin et al. 2015). These foci of DNA repair machinery components are referred to as DNA damage foci (DDF), and when present at telomeric sequences, telomere-associated foci (TAF) which represent a robust marker of senescence (Hewitt et al. 2012). Work by Galbiati and colleagues provided evidence of prolonged checkpoint activation in senescence by demonstrating the presence of long-term unrepaired DSBs in persistent DDFs (Galbiati et al. 2017).

Notably, inhibition of the key DDR factors ATM, ATR and CHK1/2 enables senescent cells to re-enter the cell cycle, confirming the role of a persistent DDR in maintaining senescent growth arrest (d'Adda di Fagagna et al. 2003; Di Micco et al. 2006; Mallette and Ferbeyre 2007). The induction of senescence in response to severe DNA damage also demonstrates its key beneficial role in tumour suppression. By limiting the proliferation of cells which have accumulated excessive DNA damage, cellular senescence restricts the ability of pre-cancerous and cancerous cells to continue to multiply and impact on tissue function.

SA-**B**-Gal

A common marker of cellular senescence is increased β -galactosidase activity (Dimri et al. 1995) that is not observed in quiescent or immortalised cells. Though β -galactosidase activity has previously been observed in confluent quiescent cells, this was reversible, distinguishing senescent cells from these phenotypes (Yang and Hu 2005). Similarly, elevated β -galactosidase activity has been shown in immortalised cells, but was dependent on high confluence, and in cultures at low cell density β -Gal staining was not observed (Severino et al. 2000). This increase in β -galactosidase activity occurs in response to elevated lysosomal activity (Kurz et al. 2000) following induction of senescence (senescence-associated β -galactosidase, or SA- β -Gal). This reliable marker has also been observed *in vivo* (Dimri et al. 1995). However, while a common marker of senescence it is not unique to the phenotype, and can also be present in serum-starved or highly confluent cell cultures (Severino et al. 2000; Yang and Hu 2005). As such, the presence of SA- β -Gal activity is not sufficient alone to identify cellular senescence.

Cyclin-Dependent Kinase Inhibitors

Senescent cells also exhibit greater upregulation of cyclin-dependent kinase inhibitors. Specifically, p21 and p16 signalling is key to maintaining the senescent growth arrest, as inhibitors of cell cycle progression. Serrano and colleagues identified that mice lacking p16 were more prone to tumour formation (Serrano et al. 1996) and that oncogene-induced senescence involved elevated p16 expression (Serrano et al. 1997). p16 has also been used in a number of studies to identify and selectively eliminate senescent cells *in vivo* (Baker et al. 2011), which has led to improvements in age-related pathologies (Baker et al. 2016; Bussian et al. 2018; Patil et al. 2019; Ogrodnik et al. 2021). These two markers are generally robust identifiers of senescence; however, not all forms of senescence include elevated p16 levels (Muñoz-Espín et al. 2013; Storer et al. 2013), and some aspects of senescence are independent of p16 expression. Moreover, in senescent cells lacking p16, an inhibition of p53 activity can cause a dramatic increase in SASP production along with re-entry into the cell cycle, producing dangerous pro-inflammatory cells prone to malignant transformation (Beausejour et al. 2003; Coppe et al. 2008; Rodier et al. 2009). Therefore these CDK inhibitors cannot be used individually to confirm the presence of senescence (Coppe et al. 2010).

Morphology

One of the most noticeable markers of senescence is a change in cell morphology. Upon the induction of a senescent phenotype cells become significantly enlarged, with a flattened cell body and a greater cytoplasm: nucleus ratio *in vitro*. The nucleus

itself also becomes enlarged, and can be used as a marker of senescent cells both *in vitro* and *in vivo* (Mitsui and Schneider 1976; Yoon et al. 2016; Fielder et al. 2022). Some studies have even suggested that this increase in cell size may in fact drive the permanent growth arrest, as a result of a cell's inability to appropriately scale up nuclear and protein component synthesis (Neurohr et al. 2019).

Chromatin Alterations

Senescent cells also display significant changes to chromatin architecture, including loss of nuclear lamina allowing release of cytosolic chromatin fragments (CCFs) (Ivanov et al. 2013; Dou et al. 2017), the formation of heterochromatin foci (Narita et al. 2003), and altered DNA methylation (Hanzelmann et al. 2015).

Senescence-associated heterochromatin foci (SAHF) can be used as markers of senescent cells and can be identified as dense DAPI-positive foci within the nucleus. These condensed chromatin structures are associated with the silencing of E2F activity and dependent upon the Rb tumour suppressor pathway (Narita et al. 2003). Notably, SAHF do not develop in cells where growth arrest is reversible. However, some have noted that they appear to be specific to oncogene-induced senescence and senescence induced by stressors of DNA replication. SAHF are also thought to lessen persistent DDR signalling, as HDAC inhibitor-induced chromatin relaxation results in elevated DDR signalling and promotion of apoptosis (Di Micco et al. 2011).

CCFs have been found to trigger the cGAS-STING pathway (Chang et al. 2017; Dou et al. 2017). Treatment with HDAC inhibitors has also been shown to reduce CCF formation and subsequent SASP production (Vizioli et al. 2020).

Lack of DNA Replication/Cell Proliferation

Another major marker of cellular senescence is the lack of cell proliferation and DNA replication. These can be identified by immunostaining for the proliferation marker Ki67 (Lawless et al. 2010), though this is not unique to senescent cells.

SASP

The SASP is a major part of the senescent phenotype and responsible for much of both the beneficial and detrimental effects of senescent cell accumulation. The secretome is composed primarily of pro-inflammatory cytokines, chemokines, growth factors, matrix metalloproteinases and ECM-degrading proteins (Coppe et al. 2008, 2010). Most commonly, IL-6 and IL-8 are used as identifiers of senescent cells and are regularly used in cell culture models. These cytokines are also known to both reinforce and induce senescence via autocrine and paracrine mechanisms (Acosta et al. 2008; Nelson et al. 2012). However, it should be noted that some

forms of senescence do not possess the IL-1 arm of the SASP (Wiley et al. 2016) or exhibit an otherwise altered SASP not inclusive of these two markers, as in the case of p16 overexpression (Coppe et al. 2011). Moreover, SASP can also vary between cell type and senescence trigger. Therefore, while valuable markers of senescence, IL-6 and IL-8 are not sufficient on their own. Nevertheless, there are common SASP factors observed across most forms of senescence and are widely used together to monitor the presence of senescent cells and effectiveness of senolytic and senomorphic drug treatments (discussed below in: Senescence and age-related disease).

This diverse and variable pro-inflammatory phenotype is primarily controlled via the transcription regulator NF κ B, which regulates the activation of SASP genes (Chien et al. 2011; Ohanna et al. 2011). In multiple models of senescence activation of the cGAS-STING pathway is known to regulate SASP production via NF κ B, providing a link between CCF formation and SASP production (West et al. 2015). Though the relationship between cGAS-STING is not straightforward, as downregulation reduces SASP production *in vivo*, while activation has also been shown to lead to accumulation of nuclear DNA in the cytosol (Takahashi et al. 2018).

Numerous other components of the DDR signalling pathway influence SASP production, and studies have demonstrated that a persistent DDR is required in order for a full SASP to be established (Rodier et al. 2009). Indeed, p38MAPK is known to be both necessary and sufficient to induce a SASP via NF κ B activation (Freund et al. 2011). Moreover, inhibition of key DDR components ATM, Chk2 and p53 has been found to suppress the SASP, while heterochromatin reorganisation and SAHF formation has been linked to greater nuclear pore density in OIS, which can influence SASP gene expression (Boumendil et al. 2019). High mobility group box 1 and 2 (HMGB1/2) proteins have also been identified as regulators of the SASP, by inducing the expression of SASP genes in OIS (Huang et al. 2015; Aird et al. 2016) and signalling the immune system to senescent cells (Davalos et al. 2013). In contrast, cells which enter senescent growth arrest as a result of p21 or p16 overexpression do not produce a SASP (Rodier et al. 2009; Coppe et al. 2011), meaning that SASP production alone cannot be used as a universal marker of senescence.

Though multiple robust markers of cellular senescence have been identified, no one marker is unique to senescence, and the heterogeneity of the senescent phenotype between cell type, senescent stimuli and resulting phenotypic sub-type show that not all markers are present in all forms of senescence. Individual markers are therefore insufficient to identify senescence cells alone, and multiple markers must be used to determine the presence of cells in a senescent state (Gorgoulis et al. 2019).

Types and Mechanisms of Cellular Senescence

Replicative

The first description of cellular senescence in normal cultured human fibroblasts by Hayflick and Moorhead in 1961 pertained primarily to what is known as replicative

senescence; the circumstance in which cells have reached their replicative limit as a result of critically short telomeres leading to growth arrest. The role of telomere shortening in senescence has been confirmed by reports of senescence prevention and extended replicative lifespan in cells overexpressing telomerase (Bodnar et al. 1998). Following extended proliferation and the arrival at this critical telomere length (Harley et al. 1990)—and triggering a persistent DDR as discussed previously—these cells arrest in the G1 phase of the cell cycle (Stein and Dulic 1995). However, despite being unable to continue proliferating, such cells are also resistant to apoptotic cell death processes. This is thought to be a result of upregulated anti-apoptotic members of the BCL-2 family (Yosef et al. 2016). It should also be noted, however, that there are numerous determining factors that impact the response to extreme stress, including cell type, stimuli strength, and regulation of pro- and anti-apoptotic proteins (Rebbaa et al. 2003; Hampel et al. 2004).

Stress-Induced

Accumulation of DNA damage at sites other than telomeres and at telomeres that are not critically shortened can also trigger induction of a senescent phenotype (Di Leonardo et al. 1994). Such damage is most often caused by stimuli such as ionising radiation, oxidative stress (Parrinello et al. 2003), or genotoxic chemicals including chemotherapeutic drug treatments (Roninson 2003). Under these circumstances senescent cells have been known to accumulate in young animals, showing that senescence is not strictly an age-related phenotype (Le et al. 2010; Shao et al. 2014). This stress-induced senescence primarily involves elevated p16 expression and pRb signalling pathways in response to DNA damage (Parrinello et al. 2003). Upregulation of pro-inflammatory SASP factors has also been shown to induce senescent changes in otherwise healthy cells through a process known as bystander senescence, discussed in further detail later.

Oncogene-Induced

Oncogene-induced senescence (OIS) was first described by Serrano and colleagues in 1997, following the observation that oncogenic RAS protein induced premature cellular senescence characterised by elevated p53 and p16 activity. This was later supported by others who found a similar senescence induction in the presence of oncogenic BRAF (Michaloglou et al. 2005), E2F (Lazzerini Denchi et al. 2005), PTEN and p53 (Chen et al. 2005). It is also thought that OIS may be triggered by excessive mitogenic stimulation (Mathon et al. 2001), supported by the observation that cells relieved of mitogenic pressure by culture in serum-free medium do not undergo OIS (Woo and Poon 2004). It is important to note, however, that the extension of replicative capacity by telomerase does not promote cancerous transformation, demonstrating a key difference between immortalised and tumorigenic cells (Morales et al. 1999).

The presentation of this oncogene-induced senescent phenotype is largely similar to other forms of senescence, with chromatin alterations and formation of heterochromatin foci (Zhang et al. 2005), markers of a persistent DDR (Di Micco et al. 2006), and establishment of a SASP (Coppe et al. 2008). One notable difference, however, is the induction of a hyperproliferative phase caused by oncogene expression. This period of hyperproliferation is accompanied by altered DNA replication and triggers a persistent DDR, as in other senescent sub-types (Bartkova et al. 2006; Di Micco et al. 2006; Halazonetis et al. 2008).

A role for oncogene-induced ROS production in OIS establishment has been identified, in which excessive ROS generated by NADPH oxidases promote hyperproliferation and DNA damage (Ogrunc et al. 2014). Though this is not universal for all OIS triggers. For example, activation of the PI3K-AKT pathway induces a p53-dependent senescence, but lacks the hyperproliferation and DDR observed in other forms of OIS (Astle et al. 2012). OIS has also been found to exhibit a more amplified SASP compared to other forms of senescence (Coppe et al. 2008).

Bystander-Induced

It is also now well established that senescent cells can promote senescence induction in neighbouring healthy cells through SASP-mediated paracrine effects (Nelson et al. 2012). Among the known SASP factors, the transforming growth factor beta (TGF- β) family, vascular endothelial growth factor (VEGF) and chemokines CC motif chemokine ligand 2 (CCL2) and chemokine ligand 20 (CCL20) have all been found to trigger major senescent pathways including a persistent DDR following interaction with cell-surface receptors (Acosta et al. 2013). High levels of IGF β can also cause elevated ROS production and p53-dependent DDR (Moiseeva et al. 2006). *In vivo* studies have further demonstrated an increase in senescent cell accumulation and tissue ageing in a mouse model with shortened telomeres following extended exposure to interferon gamma (IFN γ) (Katlinskaya et al. 2016), supporting the role of SASP factors in senescence induction in animal models.

TGF- β signalling demonstrates a paradoxical effect in bystander senescence, as it can both cause p16-pRb signalling and SAHF formation (Vijayachandra et al. 2003; Zhang and Cohen 2004), and has also been shown to prevent the spread of bystander-induced senescence in liver, improving regeneration (Bird et al. 2018). The ability of some SASP factors to improve regenerative capacity over short exposure times has been demonstrated in mouse keratinocytes (Ritschka et al. 2017) and skeletal muscle (Chiche et al. 2017). Comparatively, following long-term exposure paracrine senescence is induced, suggesting that this relationship may be more complicated than first thought and may be dependent on numerous factors.

The ability of the SASP to promote further induction of senescence likely contributes to the accumulation of senescent cells and deterioration of tissue function. It is thought that dysfunctional mitochondria produce excessive amounts of toxic reactive oxygen species (ROS), which can trigger ROS-dependent NF κ B signalling and subsequently senescence in bystander cells (Nelson et al. 2018).

MiDAS

It has been well established that mitochondria are required for the development of a senescent phenotype (Correia-Melo et al. 2016) and that manipulation of mitochondrial function can impact senescence development (Wiley et al. 2016). Moreover, mitochondrial dysfunction has been shown to promote senescence, and vice versa (Correia-Melo et al. 2016; Wiley et al. 2016; Nelson et al. 2018). A major regulator of this relationship is the production of mitochondrial reactive oxygen species. Notable changes in mitochondrial morphology during senescence have also been described, including a hyperperfused network with increased mitochondrial mass (Yoon et al. 2006; Dalle Pezze et al. 2014).

Work by Wiley and colleagues demonstrated that mitochondrial dysfunction-induced senescence (MiDAS) exhibits a unique SASP relative to other forms of senescence, which lacks the pro-inflammatory IL-1 arm of the phenotype. This is accompanied by loss of LaminB1, a reduced NAD+/NADH ratio, activation of AMPK and p53, and—surprisingly—a reduction in NF κ B signalling. Inhibition of sirtuin 3 (SIRT3) specifically resulted in a senescent phenotype with a distinct secretome lacking IL-1 β , IL-6 and IL-8. This led to the hypothesis that a functional mitochondrial network is essential for a complete SASP to occur. Similar mitochondrial function-modifying interventions include mtDNA depletion, pharmacological inhibition of respiratory complexes, and heat shock protein family A member 9 (HSPA9) depletion, a key mitochondrial chaperone, although these are supraphysiological conditions. Each of these interventions also demonstrated a unique senescent phenotype lacking the IL-1 arm of the SASP.

MiDAS also appears to be independent of DNA damage and ROS production as the phenotype is reversible in the presence of pyruvate, but not antioxidants. It is thought that MiDAS can be driven by a decline in the NAD+/NADH ratio as a result of mitochondrial dysfunction. A low NAD+/NADH ratio then causes activation of AMPK and subsequently p53 activation. Notably, elevated p53 activity suppresses NF κ B signalling and is therefore a likely cause of a SASP lacking the IL-1 arm.

Table 7.1 is a summary of some of the well-established markers of senescence and their presence in specific sub-types of senescent phenotype. It should also be noted, however, that this list is not exhaustive and that these phenotypes demonstrate heterogeneity between different cell types, for example. Moreover, there remains some debate in the literature as to the presence or relevance of these markers (Mirzayans et al. 2012).

In Vivo Relevance of Senescent Cells

The well-known roles for senescent cells in human development and disease can be observed in a variety of tissues, and the development of a robust profile of senescent markers across different tissue types has been a valuable goal within the ageing field

Table 7.1 Overview of markers for different sub-types of senescent phenotype. Markers that have been observed at least *in vitro* are marked ‘yes’, markers that have not been observed in a given phenotype are marked ‘No’. The exception here is telomere shortening in stress-induced premature senescence as this form of senescence has been observed both with and without the presence of shortened telomeres. It is important to note that this table lists currently observed markers, and is not exhaustive

	Replicative	SIPS	MiDAS	OIS	Bystander
SA- β -Gal	YES	YES	YES	YES	YES
p21	YES	YES	YES	YES	YES
p16	YES	YES	YES	YES	YES
p53	YES	YES	YES	YES	YES
Nuclear enlargement	YES	YES	YES	YES	YES
ROS production	YES	YES	NO	YES	YES
IL-6	YES	YES	NO	YES	YES
IL-8	YES	YES	NO	YES	YES
IL-1 α	YES	YES	NO	YES	YES
IL-1 β	YES	YES	NO	YES	YES
SAHF	YES	YES	NO	YES	YES
DDF (γ H2AX)	YES	YES	NO	YES	YES
Telomere shortening	YES	SOMETIMES	NO	NO	NO
Reduced LaminB1	YES	YES	YES	YES	YES
Reduced nuclear HMGB1	YES	YES	YES	YES	YES

for many years. The heterogeneity of the phenotype makes this more difficult, but over the years senescent cells have been observed in many tissue types and organisms, providing the basis for an overarching picture of senescence *in vivo*. One major contributor to this work has been the establishment of key animal models, namely INK-ATTAC (Baker et al. 2011) and P16-3MR mice (Demaria et al. 2014). These models of premature ageing have allowed researchers to examine the progress and impact of senescent cell accumulation, as well as investigate potential therapeutics targeted specifically at senescent cells (discussed below, in Anti-senescence interventions). Importantly, Baker and colleagues demonstrated that selective removal of p16-expressing senescent cells produced significant improvement in numerous age-related phenotypes.

Another commonly used model of *in vivo* ageing is the BubR1 hypomorphic mouse model, in which mice express ~90% less of the BubR1 protein, responsible for ensuring chromosome segregation into daughter cells during mitosis. This leads to a phenotype of shortened lifespan, infertility, and cataracts, among others (Baker et al. 2004) and is associated with greater p16 expression (Baker et al. 2008a, b). Subsequent investigations of this rapid ageing model incorporated the *Cdkn2a^{P16}*-knockout mice, which produced BubR1 mice lacking p16. These mice consequently did not develop the ageing-related phenotype of poor muscle, eye and adipose tissue function (Baker et al. 2008a, b), demonstrating the requirement for p16 in the pro-ageing phenotype. This was further confirmed in a study of naturally aged

mice and the INK-ATTAC system, where an extended lifespan and healthspan was observed (Baker et al. 2011).

It is also worth noting that our understanding of which cells can senesce is also expanding, with the recognition of senescent markers (Table 7.2). In the past it was thought that cellular senescence was restricted only to cells capable of proliferation. That has now been called into question by the observation of senescent-like phenotypes in neurons, skeletal myofibers and cardiomyocytes, among others (Jurk et al. 2012; Anderson et al. 2019; da Silva et al. 2019; Matias et al. 2022). Though these findings are still in the early stages of exploration, it presents an exciting new avenue for our understanding of cellular senescence as a whole.

Cell Types and Tissues in Which Senescence Has Been Observed

Table 7.2 Senescent markers observed *in vivo* in different cell types and tissues

Cell type	Tissue	Organism	Markers	Reference
Neurons	Brain	Mouse	IL-6, SA-B-Gal, DNA damage, ROS	(Jurk et al. 2012)
Astrocytes	Brain	Human	p16, SASP, reduced LaminB1, p53, SA-β-Gal	(Bhat et al. 2012; Xu et al. 2021)
Adipocytes	Fat	Mouse	SA-B-Gal, SASP, p53	(Minamino et al. 2009)
Adipocytes	Fat	Human	SA-B-Gal, SASP, p53, p16	(Gustafson et al. 2019)
Osteocytes	Bone	Mouse	p16, SASP	(Farr et al. 2017)
Cardiomyocytes	Heart	Mouse	Telomeric DNA damage, p16, p21, SASP, SA-β-Gal, TAF, ROS	(Ock et al. 2016; Anderson et al. 2019)
Dermal fibroblasts	Skin	Human	SA-B-Gal	(Dimri et al. 1995)
Epidermal keratinocytes	Skin	Human	SA-B-Gal	(Dimri et al. 1995)
Melanocytes	Skin	Human	p16, reduced HMGB1, TAF, SASP	(Gray-Schopfer et al. 2006; Victorelli et al. 2019)
Pancreatic β-cells	Pancreas	Mouse, human	p16, cell enlargement, SA-β-Gal, γH2AX, SASP	(Helman et al. 2016; Thompson et al. 2019)
Myoblasts	Muscle	Human	p16	(Thornell et al. 2009; Yang et al. 2018)
Fibroblasts	Lung	Mouse, human	p16, SASP, γH2AX, TAF	(Schafer et al. 2017)
Hepatic stellate cells	Liver	Mouse	SA-β-Gal, p16, p21, p53, Hmga1	(Krizhanovsky et al. 2008)
Hepatocytes	Liver	Mouse	γH2AX, TAF, SA-β-Gal, p53, p21, p16	(Wang et al. 2014; da Silva et al. 2019)

Age-Dependent Changes in the Abundance

Senescent cells are known to accumulate with age (Dimri et al. 1995; Wang et al. 2009; Jurk et al. 2014). Indeed, ageing is associated with greater prevalence of DDR signalling in both proliferating and non-proliferating cells (Fumagalli et al. 2012; Rossiello et al. 2017). Moreover, the accumulation of senescent cells has been linked to impaired immune-mediated clearance (Ovadya et al. 2018) resulting from a weakened immune system with age.

In addition to a decline in tissue function, there has also been an observed reduction in regenerative capacity with greater senescent cell abundance (Krishnamurthy et al. 2006; Sousa-Victor et al. 2015). This is thought to result from an increase in senescent stem and progenitor cells, which subsequently are unavailable to provide new cells for tissue growth and regeneration. This is also true of muscle progenitor cells such as satellite cells, which have important roles in wound healing and regeneration and can become senescent with age, leading to a decline in wound healing capabilities (Zwetsloot et al. 2013; Sousa-Victor et al. 2015), as well as a paracrine SASP-mediated impairment of tissue regeneration by HSCs (Gnani et al. 2019).

Senescence in Age-Related Disease

The accumulation of senescent cells with age can contribute to overall tissue dysfunction (Janzen et al. 2006; Molofsky et al. 2006) and development of disease. This link between cellular senescence and disorders of ageing has received further support from recent genome-wide association studies (GWAS) which confirmed the INK4/ARF locus as a major genomic determinant of susceptibility to diseases such as cardiovascular disease, neurodegenerative disease and cancer (Jeck et al. 2012). Moreover, *in vivo* studies have demonstrated significant tissue dysfunction and shortened lifespan in healthy mice when administered a quantity of senescent cells via transplantation (Xu et al. 2017). The stresses associated with disease can also trigger greater conversion of cells to senescence, particularly as aged cells are less capable of combating stress and damage (Weyand and Goronzy 2016; Chen et al. 2020).

The presence of numerous senescent markers has been identified in age-related diseases across multiple tissues. For example, shortened telomere length has been linked to both metabolic and cardiac dysfunction (Benetos et al. 2013; D'Mello et al. 2015), while levels of key SASP factors within the blood are known to correlate with chronic disease in advanced age (Fabbri et al. 2015). Increased levels of senescent markers such as p16 have also been identified in heart failure (Chimenti et al. 2003; Schafer et al. 2017), osteoarthritis (Loeser 2009; Jeon et al. 2017) and diabetes (Minamino et al. 2009), and recent studies have even linked senescence within the central nervous system to Alzheimer's disease (Bhat et al. 2012; Zhang et al. 2019).

Tau-containing neurons have demonstrated a senescence-like expression profile in mice, while senescent markers including SA- β -Gal, DNA damage and p16 have been found in retinopathies (Ouba et al. 2016).

Studies of heart health in ageing have reported elevated cell death and hypertrophy of heart tissue with advanced age, as well as accumulation of p16^{INK4a}-positive cells (Chimenti et al. 2003). In a mouse model prone to atherosclerotic plaque formation, plaques exhibit higher levels of p16 and SA- β -Gal activity. Conversely, plaque formation has been effectively reduced in both p16-3MR and INK-ATTAC mice by removing p16-positive cells, which also subsequently suppressed the SASP (Childs et al. 2016). Inflammatory macrophages have also been found to express high levels of p16 in human atherosclerotic plaques (Holdt et al. 2011). Interestingly, some studies have found that senescent cell accumulation may both promote and prevent aspects of atherosclerosis. Increased expression of p16INK4a, ARF and p15INK4b has been associated with lower incidence of atherosclerotic vascular disease (Liu et al. 2009). However, while the anti-proliferative role of senescent cells is favourable for preventing disease development, the pro-inflammatory aspect promotes atherogenesis which is relieved by targeted clearance of p16^{INK4a}-expressing cells (Childs et al. 2016).

In osteoarthritis models, greater IL-6 secretion (Livshits et al. 2009), telomeric dysfunction (Fragkiadaki et al. 2020; Manoy et al. 2020) and p16 expression has been observed (Malaise et al. 2019), while selective clearance of senescent cells with senolytic treatment has shown success in relieving SASP secretion, slowing disease progression, and improving bone health (Peilin et al. 2019). p53 expression and increased oxidative stress have also been linked to insulin resistance in diabetes, and adipose tissue samples from patients with type 2 diabetes exhibit prominent markers of senescence, including SA- β -Gal activity and pro-inflammatory cytokine expression (Minamino et al. 2009).

The SASP has also been proposed as a major regulator in the development of age-related disease, as chronic inflammation is observed in many conditions such as atherosclerosis (Zhou et al. 2006) and cancer (Thangavel et al. 2011). Work by Xu et al. (2018) has demonstrated that young mice given senescent cells by transplantation resulted in chronic physical dysfunction. This dysfunction was then relieved by senolytic treatment. Increased levels of SASP factors such as IL-6 and tumour necrosis factor (TNF) receptor in the blood have also been identified as predictors of chronic disease with old age (Fabbri et al. 2015).

Anti-senescent Interventions

Given the wide-ranging roles of senescent cell accumulation in ageing and age-related diseases, there has been much research aimed at identifying treatments targeted at senescent cells specifically. Studies have demonstrated that clearance of senescent cells can both prolong lifespan in model organisms and alleviate age-related pathologies (Baker et al. 2011; Zhu et al. 2015; Roos et al. 2016). Two

broad categories of therapeutic intervention have emerged over the years; one aiming at complete degradation and clearance of senescent cells (senolytics) (Zhu et al. 2015) and one targeting the harmful aspects of the senescent phenotype (primarily SASP) while allowing the cells themselves to remain (senomorphics/ senostatics). Senolytics commonly target pathways which promote cell survival and protect against apoptosis. Many studies have investigated the use of senolytics and senostatics to alleviate the senescent burden *in vivo*, with varying degrees of success.

Pre-clinical Evidence

In avoiding apoptotic cell death, senescent cells upregulate several anti-apoptotic factors from within the BCL-2 family. Thus, numerous studies have attempted to overcome this apoptotic resistance by treating senescent cells with BCL-2 inhibitors. One of the most commonly tested senolytic treatments is the combination of the tyrosine kinase inhibitor dasatinib with PI3K inhibitor quercetin (D + Q).

D + Q treatment reduces the expression of anti-apoptotic BCL-xL, allowing clearance of senescent cells. This has resulted in improved health span in multiple mouse models of cellular senescence (Xu et al. 2018). Studies have shown that this treatment is able to improve vasomotor function in hypercholesterolaemia (Roos et al. 2016) and lung function in pulmonary fibrosis (Schafer et al. 2017). Recently, D + Q treatment has also been shown to lower SASP factor secretion and senescent cell burden in a mouse model of age-related intervertebral disc degeneration, improving cell viability and limiting degradation (Novais et al. 2021). In a mouse model of Alzheimer's disease, researchers have found that D + Q treatment is also able to selectively remove senescent cells from A β plaques, along with an associated reduction in neuroinflammation and improved cognitive deficits (Zhang et al. 2019).

Navitoclax is another well-established senolytic which inhibits anti-apoptotic members of the BCL-2 family, promoting the release of pro-apoptotic factors (Zhu et al. 2016). Navitoclax-induced clearance of senescent cells *in vivo* has been found to improve ageing-related pathologies of haematopoietic and skeletal muscle stem cells in both irradiated and naturally aged mice (Chang et al. 2016). It has also been shown to improve atherosclerosis pathology (Childs et al. 2016). However, its use in humans is limited by side effects (Vogler et al. 2011).

Other notable senolytics include ABT-737, another inhibitor of the anti-apoptotic BCL-2 family members (Oltersdorf et al. 2005, Ritschka et al. 2017), EF24 (Li et al. 2019), azithromycin (Ozsvari et al. 2018) and HDAC inhibitors (Di Micco et al. 2011; Samaraweera et al. 2017). HDAC inhibitors are able to promote senescent cell clearance by stimulating chromatin relaxation. This reduces the formation of SAHF which dampen DDR signalling. With reduced SAHF presence the resulting increase in DDR signalling promotes apoptotic cell death (Di Micco et al. 2011) and has been corroborated by studies of the senolytic HDAC inhibitor Panobinostat (Samaraweera et al. 2017).

Senolytics have now been successfully used to alleviate disease pathology in mouse models of numerous diseases, reviewed in detail by Kirkland and Tchkonia (2020).

Rapamycin has also been identified as an effective senostatic and targets the mechanistic target of rapamycin complex 1 (mTORC1) (Brown et al. 1994; Sabers et al. 1995). This inhibition of mTORC1 interferes with signalling pathways required for cell growth and proliferation (Chung et al. 1992). Studies have demonstrated that rapamycin treatment can extend health span, activate autophagy and improve CI activity (Carames et al. 2012; Miwa et al. 2014). Other reported effects include the reduction of elevated mitochondrial mass, suppression of p21 and pro-tumorigenic SASP factor expression, and reduced prevalence of DNA damage foci (Herranz et al. 2015; Correia-Melo et al. 2016).

In vivo studies have shown that rapamycin treatment reduces IL-1 β expression and alleviates cartilage degradation in experimental models of osteoarthritis (Carames et al. 2012). In rat models of type 2 diabetes, rapamycin promotes insulin sensitivity and reduces inflammation (Zhou and Ye 2018). Furthermore, rapamycin has shown success in preventing Alzheimer's disease pathology by protecting against tau-induced neurodegeneration and neuroinflammation (Siman et al. 2015) and improved cognitive and cerebrovascular function in apolipoprotein E ε4 transgenic mice (Lin et al. 2017).

Antisense oligonucleotides are also a new line of investigation in the field of senostatics, aimed at inhibiting DDR signalling. This has proven useful in mouse models of accelerated ageing, which have shown that telomeric ASOs effectively alleviated a number of senescent markers, improved tissue function and extended mouse lifespan (Aguado et al. 2019).

Metformin is a widely used anti-diabetic drug, but has more recently shown promise in the treatment of other age-related diseases. Treatment with metformin has been reported to extend both lifespan and health span in *in vivo* studies in mice and worms (Anisimov et al. 2008; De Haes et al. 2014). Studies investigating the repurposing of metformin now cover a wide range of age-related diseases including cancer (Heckman-Stoddard et al. 2017), cardiovascular disease (Han et al. 2019) and neurodegenerative diseases (Kickstein et al. 2010; Mor et al. 2020). Chronic treatment with low dose metformin is able to inhibit age-associated atherosclerotic plaque formation in ApoE $-/-$ mice (Karnewar et al. 2018). In models of myocardial infarction, metformin treatment has been shown to protect against hypertrophic and apoptotic remodelling *in vivo*, while *in vitro* it has demonstrated reduced hypertrophic and apoptotic responses to stress (Loi et al. 2019). Notably, studies have reported that metformin treatment can improve neuronal insulin signalling in a model of Alzheimer's disease, though this was accompanied by an increase in Aβ levels (Zhang et al. 2015). In cancer cell models, metformin inhibits cell proliferation via the ATM-AMPK-p53/p21CIP1 pathway, and promotes apoptotic cell death following irradiation (Storozhuk et al. 2013).

More recently, metformin has shown promise as a senostatic treatment in a wide range of models, with the added benefit of a known safety profile in humans

(Anisimov et al. 2008; Algire et al. 2012; Chen et al. 2016; Hu et al. 2020; Jiang et al. 2020; Hansel et al. 2021; Le Pelletier et al. 2021; Fielder et al. 2022).

Clinical Evidence

Evidence of beneficial effects of senolytic treatment is now being reported in humans. In the case of D + Q, clinical trials have shown that patients with idiopathic pulmonary fibrosis experienced improved physical function after 3 weeks of treatment (Justice et al. 2019). In another study of diabetic kidney disease, patients had reduced senescent cell burden and circulating pro-inflammatory cytokines within 11 days, along with decreased p16, p21 and SA- β -Gal activity (Hickson et al. 2019).

The use of mTOR inhibitors clinically is limited by known side effects (Pallet and Legendre 2013; Duran et al. 2014), though some trials have found success at low doses (Mannick et al. 2014). For example, mouse model studies have found that while acute treatment with rapamycin improves insulin sensitivity (Tremblay and Marette 2001; Krebs et al. 2007), longer treatment periods worsen hyperglycemia (Fraenkel et al. 2008) and glucose intolerance (Chang et al. 2009) in diabetes. It is also important to note that disruption of mTORC2 can exacerbate insulin resistance (Lamming et al. 2012). Rapamycin treatment has, however, been approved as a method of immunosuppression following the identification of a major role for mTOR in regulating SASP and tumour progression (Herranz et al. 2015; Thapa et al. 2017). However, there is debate as to the risk-benefit value of rapamycin treatment in some circumstances (Knoll et al. 2014; Hahn et al. 2019).

Metformin is a popular avenue of investigation for clinical senostatic use, given its well-known safety profile and current use in humans as a diabetic treatment. Clinical trials of metformin as an ageing-related therapy have found that the expression of DNA repair genes is regulated by metformin in muscle biopsies of older adults (Kulkarni et al. 2018). Trials in cancer therapeutics have also reported that metformin reduces serum markers of breast cancer risk in women who have undergone chemotherapy and radiotherapy treatment courses (Goodwin et al. 2008; Campagnoli et al. 2012). Review studies have also determined that coronary artery disease patients experience reduced cardiovascular mortality, cardiovascular events and all-cause mortality when given metformin (Han et al. 2019).

Future Directions

How to Better Target Senescent Cells?

While a number of senolytic and senostatic treatments have shown promise in clinical trials, and have even been approved for the treatment of some age-related diseases, many are limited by side effect profiles and require much optimisation of

dosage and treatment timings. Moreover, it has not yet been confirmed that long-term effects following senolytic treatment do not become toxic or detrimental to the organism. There is also little data on any differing effects of senolytic treatment when given in old age with an assumed greater presence of senescent cells, or potentially toxicity in young age, for example treating particular conditions associated with senescent cells. More study is required to truly appreciate the safety and effectiveness of senolytic and senostatic drug treatments in different cell types, senescent triggers, and treatment regimens.

Nevertheless, novel approaches to senescent-targeted treatments are being reported regularly. The use of senescent-specific fluorescent tracers are becoming an interesting method of tracking senescent cell burden and therapeutic response (Wang et al. 2019), improving the ability to observe and amend the effects of senolytic or senostatic treatment in real time, at least in pre-clinical studies. A number of new studies are targeting specific aspects of the senescent phenotype to prevent or reduce the impact of senescent cell accumulation. For example, Bernardes de Jesus and colleagues have shown that viral delivery of telomerase-encoding gene (*Tert*) to prevent critical telomere shortening alleviates a number of other senescent markers and promotes lifespan extension in mice (Bernardes de Jesus et al. 2012). Another approach targets the characteristic increase in SA- β -Gal activity of senescence by coating fluorophores or cytotoxic chemicals with galacto-oligosaccharides to direct the delivering nanoparticles to cells with higher SA- β -Gal activity (Gonzalez-Gualda et al. 2020).

Other novel methods of targeting senescent cells concentrate on improving immune-mediated cell clearance. Chimeric antigen receptor T (CAR T) cell therapy is an exciting route of investigation, and studies have recently confirmed that this strategy can relieve a number of senescence-associated disorders (Amor et al. 2020). An important consideration in these studies, however, is the requirement of specific marker recognition which may vary between cell type or senescent stimuli. Another immune-mediated strategy is to target the decoy receptor 2 (DCR2) which is heavily expressed by senescent cells and can be targeted by natural killer (NK) cells via perforin-mediated granule exocytosis. The removal of this receptor subsequently allows the death receptors 4 and 5 (DR4/5) to be targeted by cytotoxic cells for degradation (Sagiv and Krizhanovsky 2013). Furthermore, consistent senolysis such as by immunotherapy-based strategies might become problematic for example, when senescent cells are required for wound repair.

Important Considerations in Senolytics Treatments

A number of key considerations are emerging from the literature on senolytic treatments. Primarily, many senolytic drugs require precise optimisation of dosage, treatment timing and duration in order to avoid toxic side effects. More work is needed in order to identify the minimum effective intervention for therapies which result in senescent cell clearance, particularly when considering the function of the

targeted tissue. This is another important consideration to be aware of, as tissue such as muscle, liver or kidney which have major functional roles in human development and function may experience greater detrimental effect following senescent cell clearance if the senescent burden is significant. This should be taken into account when administering senolytics for particular disorders. For example, some mouse studies have demonstrated potential toxicity of senolysis in the liver and perivascular tissue resulting from the removal of senescent endothelial cells, adipocytes and macrophages, impacting dramatically on tissue function (Grosse et al. 2020).

It should also be noted that senolytic efficacy can vary between cell type, particularly if effective treatment relies on the recognition of specific cell markers. Similarly, not all senescent phenotypes are created equal, and there is much heterogeneity between phenotypes induced by different stimuli. In order to obtain a more universally effective treatment, targets would have to be identified that are common to the most forms of senescence. Alternatively, senescent treatment would require stimuli-specific phenotype targets for individual sub-types, such as MiDAS, replicative, OIS, or stress-induced premature senescence (SIPS).

Additionally, a factor to be aware of when deciding between senolytic versus senostatic treatment strategies is the potential benefits of “one time” senolytic treatment compared to continuous administration of senostatic treatment. Though senolytics may indeed require repeated doses, this is likely to be far less than the continuous inhibition of SASP and other senescent markers.

Concluding Remarks

Senescence is emerging as a more dynamic and heterogenous phenotype than first assumed, which must be considered when designing treatments targeted at the clearance or modification of senescent cells. As presented here, there are a number of distinct senescent sub-types with differing stimuli and phenotypic presentations. Though there are many overlapping similarities between these phenotypes, their unique features and regulators should not be discounted. Moreover, it is becoming clear that cellular senescence has cell type-specific roles throughout human development, as well as cell type-specific presentations, and thus their removal may not have universally beneficial effects. It will be important in future work to address these specificities of the senescent phenotype, and consider the many variables that may influence treatment efficacy. Development of methodologies to non-invasively assess senescent cell abundance *in vivo* will also be necessary. Importantly, senolysis in humans has not yet been proven safe or effective, and long-term effects have not been assessed in *in vivo* models. Much has now been revealed of the heterogeneous nature of cellular senescence, but there is much still to be discovered. The next stages of investigation may reveal a path to both a more detailed picture of individual senescent phenotypes and a more tailored approach to senolytic and senostatic treatment.

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Chapter 8

Therapeutic Opportunities Presented by Modulation of Cellular Senescence



Richard G. A. Faragher, Neda Heidari, and Elizabeth L. Ostler

Abstract Cellular senescence is a permanent state of growth arrest coupled with profound changes in phenotype that can be triggered by multiple extrinsic or intrinsic stimuli. Senescence is a process-level example of the evolution of ageing mechanisms through antagonistic pleiotropy and plays a primary role in tumour suppression, although evidence is mounting for its involvement in other fundamental physiological processes. Evidence from human premature ageing diseases and from transgenic mice in which it is possible to specifically delete senescent cells is consistent with a model in which the accumulation of senescent cells through the life course is responsible for later life chronic disease and impairment. The removal of senescent cells or their reversion to a phenotypically benign state is thus an important emerging goal of translational medicine.

Modern bioinformatic approaches based on text mining have compiled co-mentions of cell senescence and age-related diseases allowing an impartial ranking of the impairments most closely associated with this process. Following this schema, the evidence for the involvement of senescence in several highly ranked pathologies is reviewed, alongside potential methods for the ablation of senescent cells or their reversion to their primary phenotype with polyphenolics or inhibitors of p38 MAP kinase. Lastly, the potential for senescence to act as a barrier to the

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development of bioartificial organs designed to treat some of these conditions is discussed.

Keywords Senescence · Ageing · Liver · Kidney · Chondrocyte · Hepatocyte · Tubular epithelial cell · Fibroblast · Resveratrol · p38-MAP kinase · Senolytic · Senomorphic

What Is Cell Senescence?

Cell senescence is a state of permanent growth arrest coupled with a major shift in phenotype in cell populations that undergo mitosis under normal conditions both *in vitro* and *in vivo*. Senescence was first, and most famously, reported in human fibroblasts by Leonard Hayflick and Paul Moorhead. Their pioneering studies demonstrated that multiple strains of these cells would only divide for a finite number of passages in culture. Latent infectious agents, composition of the medium and depletion of key metabolites were all shown not to be responsible for this failure to grow. Hayflick also observed that fibroblast cultures initiated from embryos grew substantially better than those derived from adults. Combined with the demonstration that normal somatic cells displayed limited lifespans during serial transplantation experiments these observations, led Hayflick to formulate a *cellular theory of ageing* (Hayflick 1965, 1979). The original tenets of this hypothesis were that a finite lifespan is an intrinsic property of normal human cells, that cell growth *in vitro* is somehow related to human ageing and that cultured primary fibroblasts were a useful model system in which to study some aspects of *in vivo* ageing. Hayflick termed this failure to grow ‘senescence’ a term which, for good or ill, has stuck. This chapter will not treat the molecular pathways or phenotypic changes seen in senescence exhaustively since these areas are explored in detail elsewhere in this volume. It is sufficient to say that for the purposes of this Chapter that senescence can be triggered by multiple different pathways some of which require cell division (e.g. senescence induced by telomere shortening) whilst others do not; that the senescent state is maintained through the action of a variety of cyclin-dependent kinase inhibitors (typically p21^{waf} and/or p16^{INK4a}) and that the phenotype of senescent cells is very different from that of their growing counterparts and is often associated with the secretion of pro-inflammatory cytokines and other enzymes which together form the senescence-associated secretory phenotype or SASP. Instead, it will focus on evidence consistent with their *in vivo* importance in key tissues and the main ways in which the senescent state can be modulated or reversed both *in vivo* and *in vitro* to achieve clinical benefit.

Since Hayflick’s original discovery many different cell types have been shown to behave analogously to human fibroblast populations *in vitro*. Senescent cells have also been shown by a variety of methods to be present in many different tissues *in vivo*. This finally silenced the criticism, not uncommon amongst gerontologists

until the early years of this century, that senescence was simply a ‘tissue culture artefact’ unworthy of study. Rather, senescence appears to be a ubiquitous characteristic of cells derived from regenerative somatic tissue and a primary causal mechanism of ageing and age-related disease. The senescent state is distinct from necrosis, apoptosis and the multiple overlapping molecular states of transient growth arrest collectively called quiescence, although linkages to these appear to be mediated through the mechanistic target of rapamycin (mTOR) axis. Senescence limits the capacity for expansion of clones of cells and thus prevents them from accumulating or expanding pro-carcinogenic mutations, acting thereby as an *in vivo* barrier to carcinogenesis. It is thereby effectively a process-level form of the evolutionary mechanism termed ‘antagonistic pleiotropy’ which is one of the ways in which ageing originally evolved in the biosphere.

The Relationship Between Cell Senescence and Ageing

Somewhat analogously to Koch’s postulates, any candidate mechanism of ageing must satisfy three basic criteria. Firstly, the mechanism must be present *in vivo*; secondly, it must be capable of exerting a physiological effect (a requirement that excludes some chronological ageing changes such as the quasi-annual dentine lines seen in human teeth). Lastly, acceleration or deceleration of the rate at which the mechanism acts should also either accelerate or decelerate the rate of ageing.

For roughly the first 40 years following its discovery cell senescence struggled to meet the first of these criteria. This is because the primary techniques by which senescent cells were detectable at the time *in vitro* relied on either label exclusion or loss of clonogenic capacity. However, the overwhelming majority of the somatic cells in conditional renewal populations within mammalian bodies are quiescent—often for periods of years at a time. Simply put, the problem with demonstrating the presence of senescent cells *in vivo* was to distinguish between cells which would never divide again from those that could but had a vanishingly small probability of doing so on the day of the assay.

Nonetheless some progress was made. An inverse correlation between donor age and the maximum number of population doublings achieved *in vitro* was reported by Martin et al. (1970). This used fibroblast cultures derived from 100 subjects with an age range from foetal to 90 years and obtained a regression line with a slope of 0.20 PD per year of donor life (roughly equivalent to an average of one cell division per fibroblast every five years). Although on one level these data were consistent with cell turnover and the generation of senescent cells *in vivo* they showed a very high level of intraindividual variability in the proliferative lifespans of cultures derived from different donors of the same age and were thus somewhat inconclusive—whether judged in terms of attempts to establish the presence of increasing numbers of senescent cells over time *in vivo* or to gauge their physiological effects.

Retrospectively, the real importance of this study was to highlight the reflexive conflation by researchers of two distinct aspects of organismal ageing (*ageing as the*

passing of years in contrast to ageing as the decline of physiological function) which persists to this day. This was brought into sharp relief by Goldstein et al. (1978) who demonstrated that physiological and chronological age could be parsed by cultured dermal fibroblasts from subjects with an age-associated disease (diabetes or prediabetes) alongside those from carefully selected healthy ‘normal’ subjects. The former showed a replicative lifespan with a stronger inverse correlation with donor age than that observed by Martin et al. (1970) whilst the latter fibroblast cultures showed no correlation with donor age. This is the result that would be expected if senescent cells were causal agents of physiological decline (in contrast to a uniform decline across subjects irrespective of health status which would be more consistent with a chronological effect).

More important for the existence of senescent cells *in vivo* was the work of Schneider and Mitsui (1976) who studied the *in vitro* growth of fibroblasts derived from ‘young’ (21–36 years old) and ‘old’ (63–92 years old) donors. Statistically significant declines in fibroblast migration rate, *in vitro* lifespan, population growth rate and saturation density at confluence were shown to be. Colony forming efficiency was also reduced in cultures from old donors compared to those from young donors. However, none of these differences was as great as those found between early passage ‘young’ (<20 PD) and late passage ‘old’ (>40 PD) embryonic human fibroblasts. Crucially, these authors also demonstrated that differences in growth potential between fibroblast cultures from old and young donors could not be explained by simple differences in the ‘cellularity’ (number of cells per unit tissue) of old and young skin. Rather the differences *in vitro* were consistent with the presence of higher numbers of senescent cells in the older tissues at the time of biopsy. Although elegant and provocative, the link between these data and the presence of senescent cells *in vivo* was inferential and the inference was one that a significant number of gerontologists preferred not to draw.

A link between the direct visualisation of senescent cells *in vivo* and health status was provided by Wolf and co-authors (Li et al. 1997) who examined the effects of age and long-term caloric restriction on the accumulation of senescent cells in the murine lens (which has the advantage that cell proliferation is limited to a defined zone). Animals from 4 to 45 months of age were subject to one of two complementary procedures; either a 2-week infusion of 2 µg/g body weight per hour bromodeoxyuridine by osmotic minipump followed by histochemical detection of labelled cells in the proliferative zone and equator of the lens or simple sacrifice and isolation of lens epithelial cells which were then subjected to a colony size analysis of the type pioneered by Smith (Smith and Hayflick 1974). Compared to their young counterparts’, old animals showed a highly significant ($p < 0.001$) increase in the number of label-excluding cells present in the lens *in vivo*. These changes were mirrored *in vitro* where ~40% of lens cells from 33-month-old mice were senescent compared to ~20% of cells from their 6-month-old counterparts. The conclusion that the label-excluding lens cells *in vivo* are the same population as the senescent lens cells *in vitro* seems inescapable and in both arms of the study calorie restriction significantly reduced their accumulation. Although eclipsed (at least as judged by relative citations) by the discovery of senescence-associated beta-galactosidase

staining which occurred at roughly the same time, this work was the first to establish a direct link between the relative absence of senescent cells, a deceleration of the rate of ageing and the extension of healthy lifespan.

In contrast, extensive evidence emerged in the first decades following the discovery of cell senescence that pathological life shortening and cell senescence were related. Multiple research groups demonstrated that patients with the accelerated ageing disease Werner's syndrome (an autosomal recessive genetic disease associated with premature greying and loss of hair, diabetes and cataract formation as well as dermal atrophy, vascular calcification, arteriosclerosis atherosclerosis, osteoporosis and cancer) showed premature cell senescence. For example, Salk et al. (1981) studied a total of 20 Werner's Syndrome fibroblast strains derived from three patients and concluded that the average cumulative population doubling level reached by the strains was only 27% of that obtained using 10 non-Werner fibroblast strains (obtained from donors whose age ranged from newborn to 34 years). No overlap between the total replicative potentials of Werner's Syndrome and normal cultures was detected in the longest-lived Werner's Syndrome strain giving only 75% of the shortest-lived normal strain tested. This finding was strengthened by Tollefsbol and Cohen (1984) who compared the lifespans of all Werner's Syndrome cell strains then published in the literature with those of published normal controls, the authors concluded that 90% of Werner's Syndrome cultures had an *in vitro* lifespan of less than 20 population doublings. However, whilst these and other studies clearly established a link between the pathology of Werner's syndrome and cell senescence it was possible to ignore the broader implications of these data by treating the disease as a phenocopy of ageing with mechanisms distinct from, and irrelevant to, the question of whether cell senescence was of physiological importance for normal ageing.

Although researchers had previously demonstrated that increased activities for the lysosomal enzymes were part of the suite of cytological changes seen when fibroblasts became senescent (Cristofalo and Kabakjian 1975; Stanulis-Praeger 1987) Campisi and co-authors (Dimri et al. 1995) were the first to take these observations and turn them into a robust method for detecting senescent cells *in vivo*. Working with seven different strains of human fibroblasts they modified the standard catalytic histochemical assay for lysosomal β -galactosidase activity by shifting the pH of the reaction buffer away from the β -galactosidase optimum of pH 4 to pH 6. This rendered the enzyme less efficient and ensured that only cells with a high lysosomal mass were 'senescence-associated β -galactosidase' (SA β -gal) positive. Multiple variations of this technique now exist and when combined with other methods for the visualisation of senescent cells (reviewed in Faragher 2021) these provide unequivocal evidence for both their presence in a range of mammalian tissues and alterations in their frequency and distribution with different pathological states. This, combined with the evidence that it is possible both to improve health status through the removal of senescent cells and to compromise it through their transplantation has established cell senescence as a primary mammalian mechanism (or 'hallmark') of ageing.

With the provisional establishment of cell senescence as a driver of the pathogenesis of both ageing and age-related disease, Fraser et al. (2022) have recently been able to undertake a fascinating bioinformatic study. The authors text mined ~920,00 literature abstracts looking for co-mentions of age-related diseases ageing hallmarks, including cell senescence. Correcting for different intensities of study between different diseases they were then able to identify the top 30 ranked age-related diseases specifically associated with each ageing hallmark. As well as various cancers, cell senescence was especially strongly associated with osteoarthritis, chronic kidney disease, viral infection and immunodeficiency, hypertension, COPD, cirrhosis, liver failure and fatty liver disease amongst others. This is an important new approach that suggests these diseases are particularly strong candidates for therapeutic interventions intended to modulate cell senescence. Based in part on this new rationale three particularly promising potential areas for intervention are described below.

Therapeutic Opportunities

Osteoarthritis

Since chondrocytes are primarily responsible for the anabolic–catabolic balance necessary for the maintenance and function of cartilage a relationship between their senescence and the development of osteoarthritis (OA) seems innately plausible given the strong age association of the disease and the recognition that its pathophysiology is more complex than simple mechanical wear and tear. Human chondrocytes undergo senescence when cultured in vitro, usually at around 20–30 population doublings. They can be efficiently immortalised with telomerase alone consistent with a primary senescence mechanism that is dependent on p53 and short telomeres although the possibility of secondary mutations (e.g. in p16) occurring during the immortalisation process cannot as yet be definitively ruled out.

IL-1-induced senescence is well characterised in chondrocytes. This cytokine was initially identified in the late 1970s as a potent inducer of cartilage degradation and was seen as a prime candidate for a causal factor in the development of OA, although this has been questioned in recent years. Chondrocytes exposed to IL-1 β in vitro enter a senescent state that is associated with the downregulation of one of the major collagens required to maintain tissue function (COL2A1), increased expression of p16 and the development of a SASP comprising elevated MMPs (MMP1, MMP13, MMP3) and cytokines (IL6, IL8, IL-1 α , TGF- β). However, the extent to which this occurs in vivo remains an open question because of the debate around the amounts of IL1- β present in OA tissue. Thus, central to discussions concerning the likelihood of senescence playing a role in the development of OA is the frequency and distribution of senescent chondrocytes in normal and diseased tissue, along with the capacity of such cells to impact local physiology.

Harbo et al. (2012) characterised the distribution pattern of ultrashort telomeres and senescent cells in tibial cartilage derived from three older women with bilateral OA. This involved the use of QFISH for median telomere length in conjunction with immunostaining for senescence-associated heterochromatin foci (SAHF) whilst critically short telomeres were detected using the PCR-based STELA (Single Telomere End Length Assay) technique. The authors found that 70–80% of the chondrocytes in and around the OA lesions were SAHF positive and this correlated strongly with the number of ultrashort telomeres ($p > 0.0002$).

There was also a strong inverse correlation between these senescence markers and distance within the tissue from the lesion sites ($p > 0.000006$ and $p > 0.00004$ for SAHF and STELA signals respectively). Clearly senescent cells and dysfunctional cartilage co-associate. A more recent study (Si et al. 2020) used some of the best characterised markers for senescent cells *in vivo* (senescence-associated β -galactosidase activity alongside γ H2AX, p53, p21 and p16^{INK4a} immunoreactivity) to determine their frequency in early and mid-stage OA knee cartilage compared to controls from trauma amputees ($n = 5$ per group, group, mean donor ages 48–58 years). Every senescence marker was significantly elevated both in early OA compared to normal and in late OA compared to early. In the case of SA- β Gal and p16, probably the best markers of cells that have been senescent for extended periods, the percentages increased from ~20% to ~80% for the former and < 10% to >80% for the latter. Markers conventionally associated with the recent entry into senescence (γ H2AX, p53 and p21) were also very heavily upregulated in diseased tissue compared to normal controls. Given that a senescent cell percentage of ~10% is sufficient to produce significant physiological effects, this is a picture of catastrophic and ongoing failure.

Direct evidence that the accumulation of senescent cells causes OA, rather than the OA disease state causing senescence has been provided by the work of Kirkland and colleagues (Xu et al. 2017). These authors injected small numbers of chondrocytes from luciferase-expressing transgenic mice into the knee–joint area of their wild-type counterparts. These chondrocytes were either growth competent or rendered senescent by exposure to 10 Gy γ -radiation. The presence of the luciferase transgene allowed the transplanted cells to be followed by bioluminescence demonstrating that they survived for a sustained period. Mice that received senescent cells displayed leg pain, impaired performance on rotarod tests and reduced overall mobility alongside radiographic and histological changes strongly suggestive of OA in the knees of animals that received senescent, rather than growth competent, chondrocytes. Crucially, older animals required an infusion of fewer senescent cells before pathologies became evident, consistent with an elevated frequency of senescent cells already present in the tissue.

Further evidence that senescent cells directly cause OA has been provided by Jeon et al. (2017). These researchers used a transgenic mouse (p16-3MR) in which senescent cells express a fusion protein comprising synthetic Renilla luciferase and monomeric red-fluorescent protein domains, along with a truncated form of herpes simplex virus 1 thymidine kinase (HSV-TK). The fusion protein allows the frequency and distribution of senescent cells to be visualised whilst HSV-TK allows the

same cells to be selectively removed with oral ganciclovir. Following anterior cruciate ligament transection (ACLT), the authors observed the accumulation of senescent chondrocytes in the articular cartilage. Elimination of these cells increased cartilage development and attenuated the development of post-traumatic OA.

As such, cellular senescence is a key step in OA formation and ageing, with multiple pathways by which it can be modulated, halted or reversed (Coryell et al. 2021). Chondrocytes in osteoarthritis display many features of cellular senescence, such as secretion of pro-inflammatory cytokines and matrix remodelling enzymes as well as elevated senescence-associated β -galactosidase and critically short telomeres.

Kidney Dysfunction

The kidney nephron is the basic unit of mammalian nitrogenous excretion, forming hypertonic urine from isotonic renal fluid and recovering water and essential ions via a counter-current multiplier in the loop of Henle. A coherent body of evidence now demonstrates that senescent cells accumulate in multiple different cell types within the kidney with ageing and that it may even prove to be a primary site of senescent cell accumulation. Li et al. (2020) designed highly selective fluorescent probes for mammalian lysosomal β -galactosidase (based on a 2-(20-hydroxyphenyl) benzothiazole scaffold) and established *in vitro* that the activity of these probes closely mirrors that of X-gal. The researchers then used these probes to demonstrate that fluorescence levels in kidney tissue sections from C57BL/6 J mice were increased at least 12-fold in 23-month-old mice compared to their 1-month-old counterparts. Mouse kidney sections showed by far the greatest increase in β -galactosidase activity with age compared to multiple other organs (e.g. lung, heart, muscle, brain, spleen and liver) consistent with the extensive accumulation of senescent cells with age.

Sis et al. (2007) measured levels of senescent cells by p16^{ink4a} immunostaining in 19 normal live donor kidneys at transplantation (donors aged 36 ± 9.6 years) 35 biopsies with glomerular disease (donor age 47.9 ± 19.5 years) and 12 biopsies from patients with tubulointerstitial nephritis (donor age 53 ± 20 years). Senescent glomerular and interstitial cell percentages showed significant increases with disease (e.g. $2.5 \pm 2.8\%$ vs $17.1 \pm 12\%$ in the case of glomerular cells from normal and glomerular disease biopsies) and senescent tubular epithelial cells frequencies were also strikingly increased between disease and control kidneys ($0.2 \pm 0.4\%$ in normal kidneys vs $1.37 \pm 0.9\%$ in those with glomerular disease ($p < 0.001$).

The increased frequency of senescent cells in diseased kidneys clearly illustrates their potential to perturb normal function. However, the phenotype of any kidney cell type at senescence remains relatively poorly characterised compared to fibroblasts. Lu et al. (2016) studied differential protein expression (by liquid chromatography-mass spectrometry confirmed by Western Blotting) between cultures of primary human tubule epithelial cells at passage 3 (30% nuclei stained

positive for 5-ethynyl-2-deoxyuridine (EdU) incorporation but without a labelling time provided) and passage 6 (10% EdU positive under identical conditions, very high levels of senescent associated β galactosidase-positive cells and treated as the senescent population). A total of 72 proteins were found to be differentially expressed (either up or downregulated) between the senescent and growing populations the majority of which are directly or indirectly with cytoskeletal regulation and the epithelial to mesenchymal transition (EMT). This is an important potential association because tubulointerstitial renal fibrosis, progressive connective tissue deposition on the kidney parenchyma, is a disease-independent driver of renal dysfunction caused in part by the adoption of an EMT by tubular epithelial cells. The presence of a SASP in senescent tubular epithelial cells from both rodents and humans has also been reported (Liao et al. 2021; Yang et al. 2022; Castellano et al. 2019) and although the physiological relevance of some of these studies is questionable (e.g. the induction of a senescent-like state in a conditionally immortalised human tubular epithelial cell line) there is no reason to doubt that SASP could exist *in vivo* and exert deleterious effects. The SASP component Activin A is a profibrotic cytokine associated with the EMT transition in immortalised human tubular epithelial cells driven into senescence by exposure to high glucose and TGF β 1 (Bian et al. 2019). Since Activin A is expressed during embryonic kidney development, but not in maturity unless the kidney is damaged, this is an important observation and highlights the importance of pro-senescence stimuli not just within a tissue but caused by factors circulating within the kidney as a result of the circulatory milieu such as the elevated levels of activin A seen in obesity (Palmer et al. 2019). This need not be limited solely to circulating cytokines or other SASP components. The organic anion transporters (OAT1, OAT3) are heavily expressed in tubular epithelial cells and are responsible for the uptake of many anionic waste products as an initial step in the elimination of organic anions. Niwa and Shimizu (2012) demonstrated that indoxyl sulphate (derived from dietary tryptophan via conversion to indole in the gut) is taken up by tubular epithelial cells *in vitro* through OAT1 and OAT3. Once inside it elevates ROS generation and triggers a p53-dependent senescent state. In addition, indoxyl sulphate suppresses the expression of *klotho* which is primarily produced in the kidneys and which causes systemic pro-ageing effects as its levels fall across the life course (Buchanan et al. 2020).

Liver Dysfunction

Although a primary function of the kidney is the elimination of urea, the main site of its production is the mammalian liver. The organ performs hundreds of functions including the production of bile and plasma proteins such as albumin, drug detoxification, metabolic waste processing and immunoregulation. If liver failure occurs metabolites normally processed in the organ accumulate and promote hepatotoxicity and senescence.

Wang et al. (2014) measured hepatocyte senescence in the livers of young (2-month-old) and aged (18-month-old) normal mice. This was characterised by loss of proliferation capacity, increased expression of SA- β -gal activity, accumulation of γ -H2A.X-positive hepatocytes, and elevated levels of p21 and p16. The percentages of SA- β -gal-positive and γ -H2A.X-positive hepatocytes increased from $1.37 \pm 0.64\%$ and $1.73 \pm 0.23\%$, respectively in 2-month-old mice to $40 \pm 4.6\%$ and $42.67 \pm 3.51\%$ by 18 months of age. This was accompanied by significant increases in the protein levels of p21 and p16. These data indicate that the proportion of senescent hepatocytes in the liver of mice increased with age. The same authors also showed that human hepatocytes gradually undergo senescence with age. Elevated numbers of enlarged human hepatocytes were observed in healthy liver tissue from 55 to 65 year olds compared to adults aged 21–30 years. Also, approximately 69% and 72% of old human liver sections were stained positively with SA- β -gal and γ -H2A.X, respectively, but only $8.2 \pm 1.87\%$ and $8.6 \pm 1.61\%$ of liver tissues were stained positively with SA- β -gal and γ -H2A.X, respectively, in younger adults. Thus, senescent hepatocytes are present *in vivo* and increase with advancing age.

Senescence in non-parenchymal liver cells may also occur as a result of ageing and chronic inflammation-associated tissue damage. The existence of cholangiocyte senescence in a number of biliary diseases including primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC) and biliary atresia (BA) has been described. Tabibian et al. (2014) measured the pro-inflammatory activity of cholangiocytes and markers of cellular senescence in patient livers with PSC, PBC, hepatitis C and healthy controls to investigate whether cholangiocytes in the PSC liver display features of cellular senescence (including the SASP). Cholangiocytes in PSC livers showed increased expression of p16^{INK4a}, histone γ H2A.x foci and decreased levels of the proliferation marker Ki67. *In vivo* the level of p16^{INK4a} mRNA expression was significantly higher in PSC cholangiocytes compared to PBC (3.1-fold, $P < 0.01$), HCV and normal liver tissue samples (both >20-fold, $P < 0.01$). Also, the percentage of γ H2A.x foci-positive cholangiocytes was significantly greater compared to other three conditions ($P < 0.01$). Whilst cholangiocytes in the PSC liver were negative for Ki-67, consistent with senescence. In addition, four known SASP components, IL-6, IL-8, CCL2 and PAI-1, were significantly upregulated in PSC cholangiocytes compared to PBC, HCV and normal liver ($p < 0.01$).

A cholangiocytes coculture model was also used to assess whether senescent human cholangiocytes can induce senescence in bystander cholangiocytes by paracrine secretion. They demonstrated that bystander cholangiocytes had significantly increased expression of SA- β -gal when exposed to senescent cholangiocytes directly treated with H₂O₂ or LPS over 10 days ($p < 0.05$). In summary, cholangiocytes in PSC display features and markers which are consistent with senescence and the increased expression of SASP proteins by senescent cholangiocytes is consistent with the hypothesis that they can contribute to progressive inflammation and hepatobiliary injury in primary sclerosing cholangitis.

Liver progenitor cells (LPCs) are a stem cell population within the liver that express markers for both hepatocytes and cholangiocytes including α -fetoprotein (AFP), cytokeratin 19 (CK19) and epithelial cell adhesion molecule (EpCAM). They

are activated and differentiate into hepatocytes and cholangiocytes during extensive liver injury to regenerate the tissue. However, LPCs lose their proliferative capacity during ageing. A study by Cheng et al. (2017) showed that LPCs from old mice lost their activation and proliferation upon liver injury caused by excessive ROS and chemokine production from activated hepatic stellate cells during ageing. LPC activation and proliferation were measured in young (2 months old) and old mice (24 old months) fed with a diet (modified CDE) designed to induce liver damage. The expressions of LPC-related markers, such as EpCam, CD133 and AFP were only increased in the livers of young mice ($P < 0.05$) after the CDE diet. Old mice had significantly lower numbers of proliferative markers including ki67 and cyclin E1 after the CDE diet feeding ($P < 0.05$), suggesting that the activation and proliferation of LPCs in old-DDC mice were decreased compared to that of young mice. In addition, a significant increase in neutrophil infiltration, together with high levels of ROS (using MDA as a marker of oxidative stress) were observed in livers of old mice compared to young mice ($p < 0.01$). The results of this study also showed that the expressions of chemokines such as CXCL1 and CXCL7 from hepatic stellate cells were responsible for the migration of neutrophils in mice. The mRNA levels of CXCL1 and CXCL7 were upregulated in livers of old mice compared to young mice, especially CXCL7 ($p < 0.01$) which indicates that CXCL7 production from hepatic stellate cells could be an important factor inducing neutrophil infiltration leading to the negative regulation of LPC response. A significant number of H2AX-positive LPC cells in old mice confirmed the DNA damage in LPCs, suggesting that neutrophils inhibited LPC activation and proliferation through oxidative stress and induction of senescence in LPCs in old mice which resulted from chemokine production from activated hepatic stellate cells during ageing, and lead to impairment of liver regeneration. Based on these findings, decreased LPC activation and proliferation impaired liver regeneration during ageing as a result of induction of senescence in LPCs.

Therapeutic Strategies

The seminal development of the INK-ATTAC transgenic mouse by Baker and colleagues allowed researchers to systemically eliminate senescent cells *in vivo* for the first time. In this model, an FKBP-CASP8 cassette, including a green fluorescent protein reporter behind an IRES sequence, is driven by the Ink4a/Arf promoter, which is only active in senescent cells. Oral administration of AP20187 causes dimerisation of the caspase 8-FKBP fusion protein transcribed from this cassette which then in turn causes apoptosis. This and related model systems allow the contribution of cell senescence to both ageing and distinct pathologies to be tested. Initially, treatment with AP20187 was shown to substantially delay the onset of age-related changes in BubR1 progeroid mice compared to untreated controls and outbred cohorts of INK-ATTAC mice with their senescent cells removed to show an ~25% increase in life span. Further experiments of this type have shown the benefits

of senescent cell removal in rodents in multiple clinical scenarios clearly implying that similar benefits will accrue if senescent cells are removed in humans. However, it is worth sounding a note of caution. The evolutionary life histories of the two species are not simply different but radically so with regard to critical features (e.g. population type and degree of genetic drift), see Overall and Faragher (2019), that are likely to affect the evolution of ageing. Thus, there is the possibility that direct translation of clinical benefits into humans may prove to be more difficult or more limited than is naively supposed.

Removal of Senescent Cells

An obvious requirement in order to translate the findings from these rodent models into the clinic is to find compounds capable of killing senescent cells (senolytics). The BCI2 inhibitor navitoclax was shown to be senolytic in some types of senescent cells (e.g. rodent fibroblasts and human endothelia) but not others (e.g. human primary preadipocytes). Recently, navitoclax was shown to provide therapeutic benefits in rodent models of atherosclerosis, mimicking the effects of senescent cell deletion models and reducing the size, number and overall burden of atherosclerotic plaques. Dual treatment with the senolytic compounds dasatinib and quercetin mimics the results seen in transgenic mouse senescence clearance models and improves both vasomotor function and vascular stiffness in aged and atherosclerotic mice, although the mechanistic spectrum of action of these compounds is broad and caution needs to be applied in ascribing the improvements in health status seen to senolysis alone.

Dasatinib is a tyrosine kinase inhibitor initially approved for the treatment of chronic myelogenous leukaemia. Dasatinib and the natural polyphenolic antioxidant quercetin inhibit key transcriptional nodes used by senescent cells for survival. The combination of dasatinib and quercetin greatly reduced the senescent cell load under multiple conditions and other senolytics, for example those based on ferroptosis (Liao et al. 2022) are now in trials in animal models.

Blockade of the Senescent Phenotype

Quercetin also inhibits matrix metalloproteinase activity, a class of molecules that are both SASP components and canonical senescence markers (Freitas-Rodríguez et al. 2017). This illustrates the potential of an alternative route to dealing with the degenerative effects of senescent cells, the development of senomorphic compounds capable of blocking their deleterious phenotypic effects rather than killing them. However, the SASP also has the potential for beneficial effects, depending upon the biological context (Freund et al. 2011). Beneficial aspects can include a reinforcement of growth arrest (Acosta et al. 2008), a positive contribution to the clearance of

senescent cells by the immune system (Xue et al. 2007) and the suppression of fibrotic scar formation (Krizhanovsky et al. 2008). These need to be traded off against detrimental effects such as the promotion of malignant phenotypes in neighbouring cells and accelerated tumour growth (Coppe et al. 2008; Krtolica et al. 2001; Gonzalez et al. 2017). In addition to being pro-inflammatory, some SASP factors such as activin A and GDF15 have complex systemic with the potential to disrupt overall physiological homeostasis at sites distant from their production. Thus, it will be advantageous for clinicians to be able to modulate the phenotype of senescent cells as well as being able to clear them.

To that end, we (Alimbetov et al. 2016) used two next-generation p38 inhibitors (UR-13756 and BIRB 796) with markedly improved selectivity and specificity compared to SB203580 (Bain et al. 2007; Mihara et al. 2008) and demonstrated that both compounds are highly effective at blocking the SASP in senescent cells when used at concentrations known to inhibit p38 MAPK. This strengthens the evidence base that signalling through p38 MAPK is required for the SASP in human cells and the fact that BIRB 796 has already been used in Phase III clinical trials (Force et al. 2004) demonstrates that SASP-suppressive compounds targeting the p38 MAPK have a reasonable chance of entering human clinical use.

Controlled Reversal of Cell Senescence

However, in some circumstances suppression of the SASP alone may prove insufficient to provide clinical benefit and a broader rescue of the senescent phenotype may be required. A clue to how this might be achieved was provided by the work of Harries and co-authors who undertook a large-scale ($n = 698$ individual) transcriptomic analysis using the well-characterised InCHIANTI human cohort to examine alterations in gene expression associated with ageing. Relatively few individual transcripts (~2%) demonstrated large-scale reproducible age-related differences in expression. However, gene set enrichment analysis (which determines whether specific molecular or functional pathways are associated with a given trait) indicated that the pathways most likely to be disrupted by ageing were those involved in messenger RNA splicing and post-transcriptional processing. More difficult to untangle was the relationship between this restriction of splicing factor activity and cellular senescence. Accordingly, the same group (Holly et al. 2013) demonstrated that the senescence of primary human fibroblasts and human aortic endothelial cells is associated with restricted splicing factor usage leading to changes in the ratios of alternatively expressed isoforms of a number of genes relevant to ageing (including CDKN2A, VCAN1, GRP18, EFNA1, and VEGFA). However, distinguishing cause from consequence in this system was not possible.

To address causality, we (Latorre et al. 2017) treated multiple senescent fibroblast cell strains with the polyphenolic resveratrol and a series of novel compounds based on it including ((E)-N-(4-(3,5-dimethoxystyryl) phenyl)methanesulfonamide, (E)-N-(4-(3,5-dihydroxystyryl)phenyl)acetamide, (E)-5-(4-(3,5-dimethoxystyryl)

phenyl)-1H-tetrazole and (E)-5-(2-(3,5-dimethoxystyryl)phenyl)-1H-tetrazole). We predicated this study on prior evidence that resveratrol treatment altered splicing pattern levels in transformed human cells but its pleiotropic effects (as a radical scavenger, anti-inflammatory and activity of SIRT1 amongst others) render resveratrol itself a poor compound to probe for the mechanism of action by which this occurs. In contrast, the use of a panel of resveralogues allows these effects to be dissected (some compounds lacked any anti-inflammatory properties, others were not SIRT1 activators). In each case treatment of senescent fibroblasts with resveralogues reverted the pattern of splicing factor expression back to that seen in young cells and produced a reduction in the senescence-associated β -galactosidase-positive fraction (a widely used marker for the senescent state), an increase in the cycling fraction (as measured by pKi67 immunoreactivity) and increased cell number. None of the compounds were senolytic, indicating a genuine reactivation of previously senescent cells, and the differential SASP suppression capacities of the resveralogues excluded suppression of paracrine senescence as a primary cause of this effect. Telomere length in the treated fibroblasts also increased. This is potentially due to the non-canonical effects of at least two splicing factors. HnRNPD (known to enhance the promotor activity of the telomerase gene) and hnRNPA1 (required for telomere maintenance in multiple species) although other mechanisms cannot be ruled out.

An underrecognised issue when using natural products with pleiotropic effects, such as resveratrol, is the issue of the dose required to produce a given effect. Polyphenolics typically elicit responses that are both biphasic and cell type specific. For example, In the case of resveratrol, concentrations above 25–50 μ M typically trigger growth arrest, senescence, or apoptosis in multiple different cell types which may be useful in an anti-cancer context. In contrast concentrations below 10 μ M, which are much less frequently used *in vitro*, frequently enhance the growth of log phase cell cultures and rescue senescence. To better understand the structural features regulating these biphasic effects we evaluated 24 of our structurally related resveralogues for their capacity to activate SIRT1, as determined by an *ex vivo* SIRT1 assay, their toxicity and their effects on replicative senescence in MRC5 human fibroblasts (Birar et al. 2020). In this screen, minor modifications to resveratrol were found significantly to alter the biological activities of the resulting molecules. For example, replacement of the 3,5-dihydroxy substituents with 3,5-dimethoxy groups significantly enhances SIRT1 activity, and reduces toxicity. Regardless of modification, at doses of 100 μ M (frequently used *in vitro*) many of the compounds induce senescence in primary MRC5 cells in culture whilst at 10 μ M, most of the resveralogues significantly enhance growth consistent with the rescue of a subpopulation of cells within the culture from senescence. SIRT1 activation is not required for rescue to occur but enhances the size of the effect. Thus, a key challenge in this area is to define the pathways that are impacted by compounds such as resveratrol and to determine which of these are critical in reversing key aspects of the senescent cell phenotype.

Cell Senescence as a Problem in Artificial Organs

Although phenotypes classically associated with the onset of senescence (e.g. the expression of cyclin-dependent kinase inhibitors, the presence or absence of a SASP and telomere length) have been relatively heavily studied in multiple cell types the effects of the primary phenotypes of differentiated cells have been the subject of far less intensive scrutiny. Early studies of human keratinocytes demonstrated that senescence could be separated out from normal differentiation and stratification either through the emergence of spontaneous mutants (such as the non-differentiating keratinocyte or NdK phenotype) or through growth in low calcium medium (Norsgaard et al. 1996). Under the latter circumstances, differentiation into mature keratinocytes could be initiated in primary cultures at different population doubling levels by increasing the calcium concentration in the medium. Differentiation could also be induced in senescent (35 population doubling) cultures, but the process was significantly hours (as measured by the expression of human keratinocyte transglutaminase) slower over the first 48 h than in log phase cultures (0–20 population doublings). Thus, in keratinocytes, the impact of senescence on core cell type-specific functions is superficially minimal. However, in adrenocortical cells undergoing senescence *in vitro* sustained cell turnover induces a parallel loss of key tissue-specific enzyme expression (17a-hydroxylase) in response to stimulation with cAMP—suggesting a potential problem *in vivo* (Yang and Hornsby 1989).

Whilst it is possible for those primarily interested in organ function over time to dismiss some of these changes as irrelevant dedifferentiation phenomena associated with growth in tissue culture environments that are insufficiently reflective of conditions *in vivo* there are situations in which the maintenance of quasi-normal cell function *in vitro* are critically important. The clearest examples of these are the ongoing attempts to produce functional artificial organs incorporating living cells. The most advanced of these are the various types of bioartificial liver devices (BALs). These extracorporeal devices are designed to support patients in whom the liver is failing due to a wide variety of insults (including ageing, alcohol abuse and acute fulminant hepatic failure induced by paracetamol overdose). The core of these devices is typically a hollow-fibre tissue culture cartridge containing approximately 800 g of cultured HepG2 cells (although primary porcine hepatocytes are sometimes used). HepG2 cells are preferred although they have approximately 20% of the albumin synthesis and urea production capacity of primary hepatocytes they are easy to expand in culture sufficiently to generate sufficient biomass to populate the hollow-fibre system.

Although many of the bioengineering issues associated with BALs have been solved the devices have a very poor record in use and have failed to impact patient survival to any appreciable extent. A plausible reason for this may well be the induction of senescence by molecules such as creatinine, bilirubin, sodium chenodeoxycholate (C-Doca) and ammonia circulating in the patient's plasma at supernormal levels due to partial liver failure (and collectively known as liver toxins). Abrahamse et al. (2002) populated the bioreactor of their BAL systems

using primary hepatocytes isolated from the resected livers of pigs. In an elegant study, these BALS were then either used to treat the donor pig which had rendered anhepatic, for 24 h ($n = 5$) or the BAL was simply maintained recirculating in tissue culture as a control. Liver function was unaltered in the controls but key liver metabolic functions including ammonia clearance, 7-ethoxycoumarin O-deethylase activity and urea synthesis were markedly reduced after 24 h exposure of hepatocytes to autologous porcine plasma although cell viability was unaltered. The signature of induced senescence seems patent. With HepG2 cells, we have also found that treatment with a cocktail of liver toxins *in vitro* for 6 h induces a senescent state (as measured by loss of pKi67 reactivity and label exclusion) alongside a near complete loss of albumin synthesis and urea production which can be blocked by resveratrol. It would thus appear that senescence is a hitherto unrecognised barrier in the production of BALS and probably, given the data on Kidney Function given above, attempts to develop bioartificial kidneys. Modulation of cell senescence in this area is an unexpected new frontier in the biology of ageing and the effects of the senescent cell.

Conclusions

Thirty years ago it was possible for mainstream figures within gerontology to hold the position that the senescence of cells was simply a tissue culture artefact or, at best, a phenomenon that was unrelated to mammalian ageing. This view is no longer tenable. Rather, the salient issues are now the relative importance of cell senescence to other ageing mechanisms, particularly in the human context; the identification of the tissues in which it is likely to play a primary role, in which bioinformatic approaches are becoming increasingly important, and optimisation of the approaches by which the effects of senescence may best be ameliorated in those tissues. Recognition of the profound effects senescence has on core metabolic phenotypes of cells in the liver and kidney is becoming increasingly salient which in turn opens up the possibility that new generations of bioartificial organs may soon become a reality. Although much work remains to be done before the modification or ablation of senescent cells is routine clinical practice it is possible to regard the future with optimism and hold the expectation, rather than simply the hope, that such treatments will become available for at least some age-related diseases.

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Chapter 9

Ageing at Molecular Level: Role of MicroRNAs



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Abstract The progression of age triggers a vast number of diseases including cardiovascular, cancer, and neurodegenerative disorders. Regardless of our plentiful knowledge about age-related diseases, little is understood about molecular pathways that associate the ageing process with various diseases. Several cellular events like senescence, telomere dysfunction, alterations in protein processing, and regulation of gene expression are common between ageing and associated diseases. Accumulating information on the role of microRNAs (miRNAs) suggests targeting miRNAs can aid our understanding of the interplay between ageing and associated diseases. In the present chapter, we have attempted to explore the information available on the role of miRNAs in ageing of various tissues/organs and diseases and understand the molecular mechanism of ageing.

Keywords Ageing · miRNAs · Senescence · Biomarkers · Age-related disease

MicroRNAs: Introduction

MicroRNAs (miRNAs) are RNA molecules that fine-tune the levels of proteins in between transcription and translation processes. A substantial amount of evolutionarily conservation reported in sequences and functions of different classes of miRNAs indicates their role and involvement in both normal cellular

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development/physiology and pathophysiology of different diseases (Shu et al. 2018). Before the discovery of miRNAs, it was considered that gene expression is regulated primarily by binding of different proteins (like nuclear receptors) or their ligands on regulatory regions of DNA, however, the advent of miRNAs has opened a new window of regulation. MiRNAs modulate the number of mRNAs by degrading or suppressing the mRNAs, which have sites for binding of miRNAs in their 3'-UTR regions (Ha and Kim 2014). There have also been reports of miRNA interactions with other regions, namely, 5'-UTR, coding sequences, or gene promoters (Broughton et al. 2016). The development of real-time PCR technology has favored the detection of these small RNA molecules in very small quantities with higher sensitivity and also helped in the identification of role of miRNAs in human physiology.

A series of cleavage steps have been included in the canonical biogenesis of these tiny regulatory molecules. Initially, Drosha and Di George Critical Region 8 (DGCR8) cleave primary (pri)-miRNA in the nucleus to generate precursor (pre)-miRNA, which is then processed by Dicer in cytoplasm yielding double-stranded miRNA. One strand of this complex is integrated into the RNA-induced silencing complex (RISC), which aids in the suppression of miRNA-driven genes (Daugaard and Hansen 2017). Lin-4 was the first miRNA found in *C. elegans* in the year 1993 (Iswariya et al. 2019). Presently, over 2000 miRNAs are estimated to be expressed in humans, and more than 60% of genes encoding proteins possess a minimum of one conserved miRNA-binding site. Many miRNAs show high conservation across species.

Ever since the discovery of these miRNA molecules, extensive research has been conducted to gain an understanding of their role in multiple cellular pathways. According to emerging evidence, the expression of mature miRNAs seems to play a significant role in almost every known physiologic and pathologic process, such as proliferation, migration, differentiation, and apoptosis (Pandey et al. 2015; Jauhari et al. 2017; Jauhari and Yadav 2019). The expression of these mature miRNAs has been shown to be dysregulated in numerous diseases such as cancer, hepatitis, and cardiovascular disease (Xu et al. 2018). Further expression of these mature miRNAs has provided an opportunity to develop novel and sensitive biomarkers for a variety of diseases, which are usually detected late due to a lack of biomarkers (Pereira-da-Silva et al. 2018). The presence of mature miRNAs has also been detected in extracellular vesicles (EVs) like exosomes, which are derived from different tissues in both normal and pathological conditions. Detection of mature miRNAs in tissue-specific exosomes provides an opportunity to mirror the biochemical conditions of organs in different pathological conditions, which will result in the development of very specific and sensitive biomarkers. Extracellular miRNAs can also be transported to target cells, where they function as autocrine, endocrine, and/or paracrine regulators to govern cellular activity (Iftikhar and Carney 2016). Toll-like receptors have also been reported to contain binding sites for extracellular miRNAs (Fabbri 2018), which activate downstream signaling events and, in turn, potentially trigger biological responses including tumor growth and metastasis (Fabbri et al. 2012), and neurodegeneration (Lehmann et al. 2012). As a result,

miRNAs also have the capability to function as chemical messengers in cell–cell interaction.

The expression of mature miRNAs is linked with both age-related disorders and ageing per se (Kogure et al. 2017). In *Drosophila*, miR-184 and let-7 are increased in response to calorie restriction, whereas adult-specific upregulation of let-7 extends the lifespan (Gendron and Pletcher 2017). According to miRNA profiling carried out by Inukai and his team, the expression of 93 miRNAs has been found to change with advancing age in the brain of aged mice (Inukai et al. 2012). Another study reported that the miRNA cluster miR-183/96/182 is linked to age-linked memory loss, a feature that can be reversed by overexpressing these miRNAs in the hippocampus region (Jawaid et al. 2019). Furthermore, miR-302b-3p was upregulated in senescent fibroblasts and aged skin of mice, implying that it was causally linked to ageing because mimicking or inhibiting the miRNA enhanced or delayed the senescence of skin fibroblasts, respectively (Tan et al. 2020a, b). Similarly, miR-339 and miR-556 target Klotho, one of the proteins that help people live longer and whose levels decline as they get older (Mehi et al. 2014). Studies conducted in the last few years have also indicated a role of cell metabolism in age-associated diseases like Parkinson’s Disease (PD) (Traxler et al. 2021). Suppression of gene expression through an expression of miRNAs helps in regulating cellular metabolism, which is related to ageing and development (Cassidy et al. 2019). In addition to regulating their targets, miRNAs were reported to be controlled by numerous intrinsic factors, and circular RNAs (circRNAs) are one of them. CircRNAs interact with miRNAs to partly inhibit their activity, implying that they may have a role in gene and protein expression. Through various miRNA-binding sites in the circular sequence, one circRNA can regulate one or more miRNAs. The first functioning circRNA, Human CDR1as, has 74 binding sites for miR-7, 63 of which have been proven to be conserved in at least one other species. CDR1as is abundant in neural tissues, and knocking off CDR1as expression in mice or zebrafish hampers midbrain development by disrupting miR-7 (Memczak et al. 2013). Another circRNA, i.e. circular SRY has 16 binding sites and was shown to interact with miR-138 in HEK293 cells, suggesting that SRY function as a sponge for miR-138 (Hansen et al. 2013).

The area of miRNA-based biomarkers and therapy has garnered a lot of interest in recent years. The development of miRNA specificity and controlled delivery are two current challenges. Understanding how miRNAs control cellular senescence and organismal ageing will lay the groundwork for future investigations into the involvement of miRNAs in better modulating and managing ageing and age-related diseases. In the current chapter, we have attempted to understand the mechanism of ageing by focusing on molecular changes and biomarkers of ageing as well as the alteration in the miRNA profile of the brain, and other different tissues/organs during ageing, the relevance of miRNAs in dicer knockdown, stem cell development, and in various ageing diseases.

Ageing: Molecular Changes and Biomarkers

Ageing is a continuous multifactorial biological process that leads to progressive decrease in systemic physiology due to damage at molecular, cellular, and tissue levels. During ageing, several subcellular events inside the cell such as nucleus to chromosome structure and organization, regulation of transcription, and nuclear import/export, and all the way out to translation and protein quality control, autophagic organelle recycling, and cytoskeletal structure maintenance deteriorate. The proportion of people above 60 years is increasing worldwide, and they are predicted to double by the year 2050, i.e., from 12% in 2015 to 22% in 2050 (Officer et al. 2020). During the same period, the oldest-old (those aged 80 years and above) are expected to increase by three times. An increase in the proportion of ageing population raises several issues. Moreover, older people's health varies greatly, with few 80 years old individuals having the same physical and mental capability as many 20 years old, while others experience a declining trend in physical and mental capabilities at a much younger age (Officer et al. 2020). The contributing factors for this variation are complicated and unknown. As a result, there is a pressing demand to improve our knowledge of the underlying pathways of ageing, so that the continuous increase in percentage of aged people in the population is helpful to future societies. Few molecular changes are considered as "hallmarks of ageing" like Genomic instability, epigenetic modifications, telomere shortening, proteostasis impairment, mitochondrial failure, cellular senescence, deregulation of nutrient-sensing mechanisms, stem cell depletion, and changes in intercellular signalling pathways (López-Otín et al. 2013).

Genomic instability results from the accumulation of unrepaired DNA, which is caused either by increased DNA damage or failure of DNA repair (Vijg and Suh 2013). Endogenous risks such as DNA replication abnormalities, reactions involving spontaneous hydrolysis, UV light, reactive oxygen species (ROS), and irradiation constantly challenge the integrity and stability of DNA, however, presence of optimally functional DNA repair machinery keeps correcting the damages (Hoeijmakers 2009). The causative or effect relationship between genome instability and ageing is still debatable (Vijg and Suh 2013). Studies have shown the role of dietary restriction in delaying genome instability during ageing (Dempsey et al. 1993; Garcia et al. 2008). A complex vice versa relationship has been observed between genomic instability and regulation of the transcription of genes. Due to the multiple indirect impacts of DNA damage on gene transcription, the correlations identified between DNA damage, repair, and transcription are extremely complex (Khobta and Epe 2012). A meta-analysis carried out using data from 36 studies has identified an association between DNA damage and ageing in humans (Soares et al. 2014). Cells obtained from elderly humans and model organisms accumulate somatic mutations (Moskalev et al. 2013). DNA damage in other forms, like chromosomal abnormalities (aneuploidy) and variations in copy number, has also been associated with ageing (Fagioli et al. 2012). Studies have shown that transcription is gaining prominence as a significant endogenous regulator of DNA

damage in cells, which can also result in alterations of miRNA expression (Khobta and Epe 2012).

Telomeres, also known as the chromatin tips, are responsible for preventing chromosomal end shortening. These telomeres shorten with age and become more vulnerable to damage (McHugh and Gil 2018). Telomere-induced senescence of postmitotic cells has recently been recognized as a key trigger of ageing (von Zglinicki et al. 2021). Employing Mendelian-randomization examination of the UK Biobank data, a potential causal relation between short genetically determined telomere length (gTLs) and accelerated facial ageing was reported (Zhan and Hägg 2021). Defects in the telomerase complex lead to various age-related pathologies in humans, including premature greying of the hair, lung fibrosis, liver disorder, and aplastic anemia (Armanios and Blackburn 2012). However, human telomere shortening may be slowed or reversed with gene therapy encoding telomerase components (Ramunas et al. 2015).

Epigenetic changes (phenotypic alterations which are not induced by mutations in DNA sequence) occurring with ageing are substantially correlated with modifications of DNA and chromatin organization like reduction in global heterochromatin, generalized hypomethylation of DNA, and hypermethylation of CpG island, remodeling of nucleosome, changes in histone marks, etc. (Teumer et al. 2016). Employing various cell types and tissues, large-scale genome-wide DNA methylation-based epigenetic analyses have identified a correlation between healthy ageing and “epigenetic clock” (Horvath 2013). At gene level, age-related epigenetic markers include enhanced acetylation of H4K16 and trimethylation of H3K4 and H4K20, along with reduced methylation and trimethylation of H3K9 and H3K27, respectively (Han and Brunet 2012).

Proteostasis (homeostasis of cellular proteins) management becomes weak with age. Preserving the proteome’s integrity can help in delaying and reducing ageing-associated changes (Labbadia and Morimoto 2015). Two types of proteolytic pathways are implicated in the breakdown and clearance of unrequired proteins: the ubiquitin-proteasome system (UPS) and the autophagosomal–lysosomal pathway, both of which lose their effectiveness with increasing age (Press et al. 2019). DAF-2, an insulin-like growth factor 1 (IGF-1) receptor coding gene in *Caenorhabditis elegans* regulates longevity through proteostasis (Matilainen et al. 2013). Furthermore, the repression observed in the expression of ATP-dependent chaperones during ageing also indicates that protein misfolding and aggregation promote ageing (Brehme et al. 2014). Misfolded aggregates of proteins are observed in the brain of age-associated disorders, including Alzheimer’s disease (AD), Parkinson’s disease (PD), and amyotrophic lateral sclerosis (ALS).

Nutrient sensing and signaling have also been reported to govern ageing in eukaryotes ranging from yeast to humans. An interplay has been reported between nutrient sensing/caloric restriction and ageing, as well as the role of caloric restriction in extending longevity in murine and non-human primate models (Vermeij et al. 2016). The kinase target of rapamycin (TOR), AMP kinase (AMPK), sirtuins, and insulin/insulin-like growth factor (IGF-1) signaling are some of the nutrient-sensing pathways identified to have a role in longevity (Pan and Finkel 2017). Several

proteins of the mTOR pathway have also been shown to be deregulated in elderly humans (Papadopoli et al. 2019). AMPK signaling is also involved in the age-related reduction of hippocampal neurogenesis, which regulates cognitive impairment in humans (Wang et al. 2019a). Human genome-wide association study (GWAS) showed variants related to low serum IGF-1 have also been linked with a higher possibility of living beyond 90 years of age (Teumer et al. 2016). Overall, the evidence available in the literature suggests that higher anabolic signaling promotes ageing, while reduced nutrient signaling prolongs lifespan.

Dysfunction of mitochondria is another common feature of aged cells (Bratic and Larsson 2013). The decreased efficiency of oxidative phosphorylation in aged cells results in lower ATP production and insufficient supply of energy to the cells. The impaired mitochondrial function leads to an ageing phenotype, particularly in organs that necessitate a massive amount of energy, including the heart, muscles, brain, and liver. Studies have demonstrated that ageing alters the regulation of mPTP (mitochondrial permeability transition pore) opening in mitochondria obtained from several aged/senescent tissues (Picard et al. 2011; Shum et al. 2016). Reduction of mitochondrial stress (or activation of the mitohormetic response), extends lifespan in a wide range of animal models, which range from worms to mammals (Cox et al. 2018).

Cellular senescence is irreversible and kind of irreversible cell cycle arrest resulting due to telomere erosion, triggering of DNA damage response, oxidative stress, and increased transcription of oncogenes. The abnormal buildup of senescent cells with increasing age can have detrimental effects (McHugh and Gil 2018). Key players in this phase include p53, p16, and p21 (Lidzbarsky et al. 2018).

Stem cell exhaustion or unavailability of stem cells, when required by different tissues during ageing, is also considered one of the primary causes of increased ageing. Many of the physical problems associated with ageing, such as compromised tissue regenerative potential, frailty, and a weakened immune system, are caused due to exhaustion of stem cells (Ren et al. 2017). Adult stem cells are maintained virtually in all tissues and organs, including the forebrain, muscle, and bone (Conboy and Rando 2012). Aged Hematopoietic Stem Cells (HSCs) exhibit increased replicative stress upon cycling and decreased ribosomal biogenesis (Flach et al. 2014). Autophagy impairment has also been linked with a reduction in stem cell activity in current research on hematopoietic stem cells (HSCs) and muscle stem cells (MSCs) (Cao et al. 2015; García-Prat et al. 2017). These results indicated that MSCs and HSCs lose their regenerative potential as they age and that ageing stem cells have autophagy defects. Recent promising research shows that rejuvenation of stem cells may reverse the ageing phenotype and provide a thrilling avenue for the treatment of age-linked degenerative ailments (Wang and Xie 2014; Bengal et al. 2017).

However, apart from cell-autonomous changes, ageing also includes alterations in intercellular signaling pathways, whether endocrine, neuroendocrine, or neuronal (Laplante and Sabatini 2012; Zhang et al. 2013). Increased inflammatory response, also known as “inflammaging,” and disruption of cytokine expression are two common ageing-related changes in intercellular communication. Defective inflammatory responses are implicated in age-related diseases like obesity and diabetes

type 2 (Barzilai et al. 2012). Zhang and colleagues published a study suggesting that the hypothalamus modulates systemic ageing by combining NF- κ B-driven inflammatory reactions with GnRH-driven neuroendocrine responses (Zhang et al. 2013) (Table 9.1).

Biomarkers of Ageing

Because ageing is a multifaceted process that involves complex interactions between molecular and biological pathways, a single or easily defined group of biomarkers is unlikely to offer a reliable assessment of biological ageing. As a result, there is no standard diagnostic tool for detecting healthy ageing, and no single parameter has yet been identified as a reliable and sensitive ageing biomarker. Many researchers suggest that a combination of biomarkers should be taken into consideration to accurately envisage ageing-associated outcomes (Earls et al. 2019; Kudryashova et al. 2020). Ageing biomarkers can be developed using phenotypic data or laboratory observations. Among the phenotypic biomarkers of ageing, physical properties, and anthropometry are the most commonly adopted measurements. Walking speed, grip strength, standing balance, chair stand, BMI, waist size, and muscle mass are all recognized indicators of ageing determination (Wagner et al. 2016).

Telomere length is a commonly discussed ageing biomarker (Vaiserman and Krasnienkov 2020). Kahl & Allen et al. summarized various telomere length measurement methods, including imaging-based methods Telomere length Combing Assay (TCA), Quantitative-Fluorescence *in situ* Hybridization (Q-FISH, Flow-FISH), Terminal Restriction Fragment (TRF) as well as PCR-based techniques (Kahl et al. 2020). The degree of DNA damage, particularly, mitochondrial DNA damage, is a primary contributor to mitochondrial impairment and is another DNA-associated possible biomarker of ageing (Dodig et al. 2019). Exosomes, ectosomes, and apoptotic bodies (ApoBDs) are some of the circulating biomarkers based on extracellular vesicles (EVs) being investigated for predicting age-associated disorders (Kalluri and LeBleu 2020; Noren Hooten 2020). Furthermore, alterations in the skin microbiome's community constitution have also been linked to age (Kim et al. 2019), and more accurate data is gathered when the gut microbiota is considered (Askarova et al. 2020).

One of the most well-studied ageing biomarkers is alterations in methylation patterns of DNA, as assessed by the epigenetic clock (Bell et al. 2019). Noncoding RNAs, such as microRNAs (miRNA), are also employed to predict age-linked disorders and are frequently extracted from peripheral blood mononuclear cells (PBMCs) (Kumar et al. 2017). A few of the age-regulated miRNAs identified in several studies are miR-34a, miR-132, miR-9, miR-212, miR-145, miR-21, and miR-96 (Halper et al. 2015; Budzinska et al. 2016; Owczarz et al. 2017; Hadar et al. 2018).

Table 9.1 Summary of molecular biomarkers of ageing

	Molecular changes	Mechanism	References
1	Genomic instability	<ul style="list-style-type: none"> Increased DNA damage Failure of DNA repair Accumulation of somatic mutations Chromosomal aneuploidies Variations in chromosome copy number 	(Fagioli et al. 2012, Moskalev et al. 2013, Vijg and Suh 2013, Soares et al. 2014)
2	Telomere attrition	<ul style="list-style-type: none"> Shortening of telomeres Defects in the telomerase complex 	(Armanios and Blackburn 2012, Ramunas et al. 2015, McHugh and Gil 2018, von Zglinicki et al. 2021)
3	Epigenetic alterations	<ul style="list-style-type: none"> DNA modifications Alterations in chromatin organization Reduction in global heterochromatin Hypomethylation of DNA Hypermethylation of CpG islands Remodeling of nucleosome Alterations in histone marks Increased histone acetylation Deregulation of noncoding RNA patterns (viz., microRNA expression profiles) 	(Han and Brunet 2012, Horvath 2013, Teumer et al. 2016)
4	Loss of proteostasis	<ul style="list-style-type: none"> Accumulation of aggregated and misfolded proteins Repression of chaperones 	(Brehme et al. 2014, Labbadia and Morimoto 2015, Press et al. 2019)
5	Deregulated nutrient sensing and signaling	<ul style="list-style-type: none"> Dietary modifications Genetic alterations Pharmacological modifications Mutagenesis High anabolic signaling Defective transduction of various Signaling pathways viz., mTOR pathway, IGF-1 signaling pathway, and AMPK pathway 	(Teumer et al. 2016, Vermeij et al. 2016, Papadopoli et al. 2019, Wang et al. 2019a)
6	Mitochondrial dysfunction	<ul style="list-style-type: none"> Decreased oxidative phosphorylation Decreased ATP production Altered mitochondrial number Decreased copy number of mitochondrial DNA Altered protein levels in mitochondria Altered regulation of mitochondrial permeability transition pore (mPTP) 	(Picard et al. 2011, Bratic and Larsson 2013, Cox et al. 2018)

(continued)

Table 9.1 (continued)

	Molecular changes	Mechanism	References
7	Cellular senescence	<ul style="list-style-type: none"> • Cell cycle arrest • Telomere erosion • Activation of DNA damage signaling • Oxidative stress • Increased expression of oncogenes 	(McHugh and Gil 2018)
8	Stem cell exhaustion	<ul style="list-style-type: none"> • Unavailability of stem cells • Compromised tissue regenerative potential • Frailty • Weakened immune system • Increased replicative stress • Decreased ribosomal biogenesis • Impairment in autophagy 	(Han and Brunet 2012, Flach et al. 2014, Cao et al. 2015, García-Prat et al. 2017, Ren et al. 2017)
9	Miscellaneous	<ul style="list-style-type: none"> • Alterations in intercellular communication (neuronal, neuroendocrine, or endocrine) • Inflammaging • Disruption of cytokine expression 	(Barzilai et al. 2012, Laplante and Sabatini 2012, Zhang et al. 2013)

In the past few years, the detection and characterization of cellular senescence biomarkers as ageing markers has garnered considerable attention. Senescence-associated-galactosidase (SA-gal) (Hernandez-Segura et al. 2018) and p16INK4A (Vandenberk et al. 2011) are the most commonly used markers. Triggered and constitutive DNA damage response (DDR), reduced telomere length and malfunction, and senescence-associated secretory phenotype (SASP) are other senescent cell markers. The senescence-associated secretory phenotype (SASP) is characterized by the elevation of multiple inflammatory markers in serum or EDTA plasma of probands employing the readily available ELISA technique (Hernandez-Segura et al. 2018; Tanaka et al. 2018).

Ageing biomarkers are multilayered and multifunctional, with a bewildering array of factors, as one would expect considering the complexity of the ageing process. However, an artificial intelligence software capable of forecasting a person's life expectancy on the basis of quantitative data in a vast panel of ageing biomarkers would not be out of the realm of possibility.

Ageing and Dicer Gene Ablation Studies

Ageing is a progressive change in cellular, biochemical, physiological, and morphological statuses of the body, which can be measured by nine hallmark changes, i.e., instability in genomic DNA, epigenetic alterations, telomere shortening, altered nutrient sensing, proteostasis failure, mitochondrial malfunction, stem cell exhaustion, cellular senescence, and impaired intercellular communication. The Dicer is a large (200 kDa) protein from the RNase III family that recognizes double-stranded RNAs and degrades them under cellular conditions. It was discovered in *Drosophila melanogaster* but is well-conserved among all eukaryotic organisms. Dicer cuts as the scissor to generate mature miRNAs from precursor miRNAs (Piroozian et al. 2019). It is suggested that in absence of Dicer genes, no miRNA will mature in the functional form.

The DICER1 gene participates in the DNA Damage Response (DDR) signaling by processing dsRNA, which is required for repairing double-strand breaks in DNA. This process appears to be required to retard telomere shortening, which is a hallmark of ageing (Rossiello et al. 2017). Furthermore, DICER1 loss has been linked to epigenetic alterations in mammalian cells including DNA methylation, histone modification, and chromatin remodeling (Haussecker and Proudfoot 2005; Chitale and Richly 2017). Interestingly, all of the ageing hallmarks can be influenced in absence of Dicer gene, not only by impairment in miRNA maturation but also by altered noncanonical DICER activities (López-Otín et al. 2013).

DICER1 depletion in adipocytes has been demonstrated to over-activate the sensing signaling molecule mTORC1 (Reis et al. 2016). It also decreases mitochondrial counts, which are irregularly structured and linked to decreased oxidative metabolism following caloric restriction, which is known as one of the most effective interventions for delaying ageing and the beginning of age-associated disorders (Reis et al. 2016). Previously, our group has reported that knockdown of the dicer gene in PC12 cells impairs their differentiation capacity resulting in NGF-differentiated neurons with shorter neurites and a greater number of neurites in comparison to the control group (Pandey et al. 2015). The production of numerous nuclei, which is frequent in senescent cells, was also reported in dicer-silenced neurons (Pandey et al. 2015). Another study from our lab found that decreased dicer levels drive differentiating SH-SY5Y neuroblastoma cells to enter senescence (Jauhari et al. 2017). Dietary restriction has been reported in studies to prevent the age-related reduction of Dicer in murine white adipose tissue (WAT), thus reverting a global drop in miRNAs expression that arises with ageing (Mori et al. 2012).

In both humans and mice, Dicer gene levels in adipose tissue are reduced with obesity and lipodystrophy (Mori et al. 2014; Oliverio et al. 2016; Torriani et al. 2016). Adipose-specific Dicer knockout mice (Adicer KO) develop severe obesity- and age-related insulin resistance and a large proportion of these mice die prematurely, implying that Dicer downregulation in adipose tissue contributes to ageing and age-linked T2D (Mori et al. 2014; Oliverio et al. 2016; Reis et al. 2016). Dicer levels in *C. elegans* are also affected by ageing and dietary restriction, a phenomenon

that is also reported in mouse adipose tissue (Mori et al. 2012). Worms overexpressing Dicer in the intestine, which is analogous to adipose tissue in mammals, are stress-resistant, but worms with whole-body Dicer loss-of-function mutations die early (Mori et al. 2012). Furthermore, the downregulation Interleukin 8 (IL-8) gene in endothelial cells following the knockdown of DICER1 (Suárez et al. 2007) demonstrates the possible role of this multifaceted enzyme in another ageing feature, which is cellular communication.

As people age, the gene DICER is shown to be decreased in different tissues, including the heart, adipose tissue, and brain (Mori et al. 2012; Boon et al. 2013). Long-term cellular stress is assumed to be the source of age-linked reduction in Dicer activity (Kaneko et al. 2011; Emde and Hornstein 2014), resulting in decreased miRNA processing and lowering of mature miRNA levels (Mori et al. 2012; Dimmeler and Nicotera 2013). Expression profiling data of miRNAs revealed a consistent decrease in miRNA levels in the aged mouse brain (Inukai et al. 2012). A reduction in the level of Dicer gene has also been found in dopaminergic nerve cells from Parkinson's disease (PD) patients (Simunovic et al. 2010). In vivo research has demonstrated that Dicer deletion in dopaminergic neurons accelerates cell death and leads to increased degeneration of their axonal extensions present in the striatum, thus mimicking the cell death pattern seen in clinical PD. Dicer ablation of dopaminergic (DA) neurons in mice lead to progressive deficits in balance and motor coordination, eventually leading to the development of a PD-like phenotype (Chmielarz et al. 2017; Guo et al. 2020). The Dicer1 gene is disrupted in embryonic DA neurons, which disrupts the DA system's development (Kim et al. 2014). Furthermore, Dicer deficiency leads to a number of PD symptoms, which include resting tremors, posture-related and gait difficulties, and stiffness. Nevertheless, increased Dicer function in these mice through treatment with enoxacin—a medication that stimulates Dicer activity—proved to be neuroprotective, revealing the important involvement of Dicer in the onset and development of this age-related disease (Chmielarz et al. 2017).

Reduced expression of mature miRNAs triggered by a malfunction in miRNA biogenesis mechanisms is witnessed in motor neurons of patients with ALS, as well as in cells following exposure to endoplasmic reticulum (ER) or oxidative stress (Emde et al. 2015). Dicer expression in the hypothalamus also declines with age, resulting in the differentiation of Pomp-expressing progenitors into AgRP/NPY phenotypes and subsequent metabolic dysfunction, which could be regulated by miR-107/103 (Crozier et al. 2018). As a result, Dicer could be a promising candidate for therapy or management of symptoms associated with age-related pathologies.

The circadian pattern of Dicer was lost/downregulated as a consequence of ageing and diabetes, and these implications were clearly displayed in the circadian patterns of Dicer-controlled essential RNAs, miRNAs, and toxic Alu RNA (Yan et al. 2013). Dicer levels were reported to be decreased in the macular retinal pigment epithelium (RPE), which is an eye-specific pluripotent tissue that is impaired in Geographic atrophy (GA), an advanced form of age-linked macular degeneration (Kaneko et al. 2011; Meister 2011). Dicer knockdown resulted in

increased cell death in mice and human RPE cells, indicating that Dicer dysregulation is implicated in disease etiology (Kaneko et al. 2011). When compared to centenarians, octogenarians have a global decline in the expression of miRNAs and also reduced the production of miRNA biogenesis factors like DICER1 in the blood cells of humans (Serna et al. 2012; Borrás et al. 2017). There is also mounting evidence suggesting that the expression of miRNA and Dicer1 in the liver may change as mammals age (Bates et al. 2010). Age-linked oxidative stress and Dicer1 downregulation are found to be involved in the dysregulation of miRNAs in aged cerebral microvascular endothelial cells (CMVECs). The discovery that increased expression of Dicer1 in elderly CMVECs elevated the expression of age-sensitive miRNAs lends support to this theory. Moreover, Dicer1 knockdown resulted in miRNA downregulation, mimicking the ageing phenotype (Ungvari et al. 2013).

Owing to its diverse and pleiotropic functions, the precise evaluation of the numerous roles of DICER1 seems to be a difficult task. Moreover, the factors that regulate DICER1 gene levels in specific cells and at different phases of development remain unknown. Besides that, the levels of DICER1 mRNA and protein levels are not always found to correlate (Kurzynska-Kokorniak et al. 2015), which adds yet another layer of complication. Furthermore, post-translational modifications (PTMs) such as SUMOylation and phosphorylation appear to control DICER1 activity (Gross et al. 2014), and the protein molecules can shuttle between the nucleus and the cytoplasm, performing various functions in each compartment based on interactions with numerous cofactors. The most perceptible series of processes in which Dicer is likely to participate in the development of ageing-related pathologies are likely deregulation of miRNA expression. Despite the abundance of data already gathered in this area, there are still a number of unexplored possibilities for future research. The discovery of novel regulatory domains, single nucleotide polymorphism (SNPs), and mutations, and also the characterization of cell- and tissue-specific Dicer activities, will almost certainly characterize future research into biological functions of Dicer in ageing and ageing-related abnormalities. In-depth research of the biological pathways underlying ageing pathologies may identify potential diagnostic and therapeutic interventions, leading to introduction of more efficacious and novel Dicer-dependent diagnostic and treatment approaches, likely to result in a variety of clinical advantages and improved quality of life for patients.

miRNAs Identified for their Role in Ageing of Different Tissues/Organs

Substantial evidence has revealed the involvement of mature miRNA expression in apoptosis, senescence, and ageing of cells of different origins (Kinser and Pincus 2020). The common link between apoptosis, senescence, and ageing of cells is their acceleration in cellular stress conditions. In a way, all three processes are a method to go away from proliferation and stop the normal metabolism of cells. Based on this,

we can expect some similarities in molecular changes occurring in cells undergoing apoptosis, senescence, and ageing. Unlike apoptotic cells, senescent and ageing cells are viable and have the capacity to secrete soluble factors or extracellular vesicles like exosomes, which can be used to monitor them and understand their molecular mechanism of action. Studies have provided a substantial amount of evidence that both senescent and ageing cells express mature and functional miRNAs, which are involved in inhibiting the protein synthesis of genes not required by these cells (Lai et al. 2019). Furthermore, deregulated expression of mature miRNAs has been identified in all ageing-associated diseases like Parkinson's, Alzheimer's, diabetes, cardiovascular, cancers, and metabolic disorders (de Lucia et al. 2017; Sandiford et al. 2018; Juźwik et al. 2019; Saul and Kosinsky 2021).

Muscle Ageing and miRNAs

Gradual loss of skeletal muscle mass, strength, and functions are hallmarks of ageing in humans. Degeneration of muscles with age is termed sarcopenia. Sarcopenia is a syndrome characterized by impaired balance, falls, frailty, reduction in physical activity, and increased susceptibility of aged people to lifelong disability, metabolic diseases, and other ageing disorders (Kinser and Pincus 2020). Causes of sarcopenia are multifactorial like long-term persisting inflammation, hormonal imbalance, muscle fiber denervation, oxidative stress, abnormalities of muscle stem/progenitor cells, and cellular senescence (Hu et al. 2014). Drummond et al. were the first to demonstrate a change in miRNA profile during sarcopenia in humans (Drummond et al. 2008). They have concluded that ageing is linked with higher miRNA levels in basal skeletal muscle and also the expression level of miRNAs is dysregulated after anabolic stimulus (Drummond et al. 2008).

Hamrick et al. have performed global miRNA profiling using Taqman Array cards and identified alteration in the expression of 57 miRNAs with ageing in mice (Hamrick et al. 2010). Also, they have found significant upregulation in the expression miR-7, miR-468, miR-698, and miR-542 and a significant decrease in the expression of miR-181a, miR-124a, miR-221, miR-434, miR-382, and miR-455 in muscles of ageing mouse (Hamrick et al. 2010). Furthermore, they have also studied the effect of body weight regulating hormone leptin (secreted from fat cells and muscles) on muscle mass in ageing mice and identified dysregulation of 37 miRNAs, out of which three deregulated miRNAs in aged mice, namely, miR-685, miR-155, and miR-142-3p were reversed (Hamrick et al. 2010). The expression of miR-221 is altered during myogenesis and is involved in the transition of myoblasts to myocytes as well as in the attainment of a fully differentiated phenotype (Cardinali et al. 2009). In addition, a microarray profiling study carried out in human skeletal muscle has identified significant upregulation in eight miRNAs (let-7a, -b, -e, and -f, as well as miR-98, miR-25, miR-1268, and miR-195) and downregulation in ten miRNAs (miR-22, miR-24, miR-27b, miR-27a, miR-30d, miR-133b, miR-133a, miR-378, miR-223, and miR-378*) in muscles of aged individuals in comparison to young

counterparts. The same study has suggested that increased expression of members of Let-7 family may effectively downregulate genes linked with cellular proliferation and identified miR-7 family as a determinant of impeded cell activity, potentially contributing to reduced renewal and regeneration of human muscle cells during ageing (Drummond et al. 2011).

Another group of scientists studied the effect of resistance exercise on the expression level of miRNAs in skeletal muscle. They discovered that 26 miRNAs were identified to be differentially expressed in aged compared to young muscles. The Akt-mTOR signaling pathway was expected to be targeted by nine miRNAs from the miR-99/100 family, suggesting the family's involvement in the regulation of muscle mass during the ageing process (Zacharewicz et al. 2014). A set of miRNAs, such as miR-434-3p, miR-181a, miR-431, miR-126, and miR-29 has been identified to have a key role in the pathophysiology of sarcopenia, regulating pathways such as senescence, apoptosis, and IGF-1 signaling in aged muscle cells (Rivas et al. 2014). MiR-181a downregulation with age has been shown in studies to be an effective mechanism to combat muscle atrophy; interestingly, in primates, this downregulation is reversed by calorie restriction, which probably reflects delayed muscle ageing (Mercken et al. 2013). Sirt1, a direct target of miR-181a, has its protein expression level altered in skeletal muscle from aged mice; miR-181a: Sirt1 interactions govern myotube size in C2C12 myotubes in vitro (Soriano-Arroquia et al. 2016).

MiR-434-3p expression in mouse skeletal muscle has also been reported to decline with age (Hamrick et al. 2010; Jung et al. 2017; Pardo et al. 2017), and this dysregulation may contribute to muscle loss by increasing apoptosis via derepression of eukaryotic initiation factor 5A (Eif5a1), a pro-apoptotic gene (Pardo et al. 2017). Another study discovered that miR-431 is downregulated in myoblasts from older mice in comparison to younger mice (Lee et al. 2015). Smad4, a component of the SMAD transcriptional complex that has been shown to inhibit muscle cell regenerative capacity, is one of the miR-431 targets. Furthermore, studies of Hutchinson–Gilford using the progeria mouse model found that miR-29 is increased during progeroid and normal ageing (Ugalde et al. 2011; Hu et al. 2014) and that this rise in miR-29 levels triggers senescence and suppresses the proliferation of muscle cells in young skeletal muscle via inhibition of several signalling proteins, including Igf1, P85, and B-myb (Hu et al. 2014).

Bone Ageing and miRNAs

Osteoporosis, which is thought to be primarily age-related and is characterized by bone loss and vulnerability to fragility fractures have a higher incidence rate in aged peoples (He et al. 2013). It has been shown by several studies that miRNAs are key players in bone metabolism, alteration of which results in osteoporosis (Feng et al. 2018). Li et al. were the first to provide substantial data on the role of miRNAs (miR-2861) in the differentiation of osteoblast and osteoporosis through its impact

on osteoblasts and posttranscriptional inhibition of histone deacetylase 5 (HDAC5) (Li et al. 2009). Overexpression of a neural development regulating miRNAs viz., miR-34c has also been linked with age-dependent osteoporosis in mice (Bae et al. 2012). The involvement of miR-21 in bone diseases and bone ageing has been reported by several labs and the same has been thoroughly revived by Chen and colleagues (Chen et al. 2021). A downregulation in miR-21 expression was reported in bones of aged mice, which caused osteocyte death via upregulating levels of a pro-apoptotic gene known as PTEN (phosphatase and tensin homolog) (Davis et al. 2017b). In vivo studies have demonstrated that miR-21 promotes osteoblast development and bone formation and its expression downregulates in mesenchymal stem cells of bone marrow and people with osteoporosis (Yang et al. 2013). MiR-21 has been reported to protect against osteoporosis by inhibiting Reck, a negative regulator of osteogenesis-related matrix metalloproteinases (Zhao et al. 2015b). Further, knocking out miR-21 has been shown to protect against bone loss as well as bone resorption during both normal ageing and ovariectomy-induced osteoporosis by targeting Pcd4 gene (Hu et al. 2017).

A miR-183 cluster member (i.e., miR-183-5p) is found in a very high amount in extracellular vesicles (EVs) collected from aged mouse blood (Davis et al. 2017a). Further, when these EVs are taken up by bone marrow stromal cells (BMSCs) of young mice, miR-183-5p suppresses BMSC cellular proliferation and the formation of bones (Davis et al. 2017a). Osteoclastogenesis induced by receptor activator of nuclear factor- κ B ligand (RANKL) increased miR-183 expression in bone marrow-derived macrophages (BMMs) (Ke et al. 2015). A miRNA known for its involvement in the regulation of senescence, i.e., miR-31, has been shown to inhibit osteogenic differentiation by targeting frizzled Class Receptor 3 (Fzd3), a Wnt Family Member 5A (Wnt5a) (Weilner et al. 2016). The expression level of miR-31 increases with advancing age in human plasma and osteoporotic males compared to healthy counterparts (Weilner et al. 2016). The expression level of miR-214 also upregulates in osteoporotic human and mouse bone and suppresses osteoblast function and mineralization by targeting Atf-4 (Wang et al. 2013). Additionally, inhibiting miR-214 in osteoblasts from old ovariectomy-induced osteoporotic mice leads to improvement in bone mass and architecture, and also boosts new bone formation (Wang et al. 2013). In contrast, miR-214 overexpression in osteoclasts in vivo results in greater osteoclast activity and bone loss (Zhao et al. 2015a). In ovariectomized mice that are getting older, osteoclast-derived exosomal miR-214 has been shown to function as a key player in inhibiting osteoblast function and bone formation (Zhao et al. 2015a; Sun et al. 2016). In human BMSCs, miR-188 regulates the bone metabolism genes, i.e., Rictor and Hdac9, by triggering PPARY, an adipocyte differentiation transcription factor, which promotes differentiation of BMSC into adipocytes instead of osteoblasts (Li et al. 2015). When compared to wild-type mice, elderly miR-188 knockout mice showed higher osteoblastic bone production, lesser bone loss, and low-fat buildup in the bone marrow (Li et al. 2015). Moreover, there was no difference in young animals between the two backgrounds, implying that miR-188 functions specifically during the ageing process (Li et al. 2015). Like miR-188, miR-218 is a direct regulator of Rictor gene and is

predominantly expressed in older mouse BMSCs; however, instead of altering differentiation, miR-218 accelerates bone loss via decreasing osteoblast adhesion and survival, inhibiting the production of new bone (Lai et al. 2016).

Heart Ageing and miRNAs

Ageing also affects cardiac function substantially and with ageing the risk of developing cardiovascular diseases increases dramatically. Like other organs, cardiac ageing is also a complex process, and the advent of noncoding RNAs has brought unprecedented molecular insights into the process. Boon et al. (2013) discovered that miR-34a is involved in ageing-induced cardiac degeneration, a miRNA that is also known to regulate bone and neuronal ageing process. In cardiac ageing, expression of miR-34 is linked with decreased expression of its downstream target protein phosphatase Pnuts, an apoptosis inhibitor, and in vivo inhibition or genetic deletion of miR-34a decreases age-related cardiomyocyte cell death by regulating telomere attrition and DNA damage responses. These findings suggest that miR-34a could possess great therapeutic potential in the context of heart ageing (Boon et al. 2013). In adult mice, miR-34a antagonists improved post-MI cardiac function via modulating cell cycle and survival proteins such as Cyclin D1, Bcl2, and Sirt1 (Yang et al. 2015). In transgenic mice and the mouse cardiac fibroblast (MCF) cell model, the transgenic expression of miR-17-3p extended survival and retarded senescence by targeting Par-4, a pro-apoptotic protein, and promoting the CEBPB/FAK senescence-associated signalling pathway (Du et al. 2015). The expression level of miR-22 also increases with advancing age in cardiac tissues of both mice and humans (Jazbutyte et al. 2013; Gupta et al. 2016). Silencing of Mimecan gene (also known as osteoglycin) and overexpression of miR-22 contributed to a considerable increase in the number of senescent cells in cardiac fibroblasts, indicating that miR-22 has a pro-ageing effect (Jazbutyte et al. 2013). Furthermore, inhibiting miR-22 lowers hypertrophy and triggers myocardial autophagy in elderly rat cardiomyocytes, both are important for preserving cardiac activity as people age (Gupta et al. 2016).

In other research using mice with a heart failure-resistant (C57Bl6) and heart failure-prone (C57Bl6 129Sv) genetic background found that miR-19a and miR-18a were significantly inhibited in aged cardiomyocytes, with concomitantly elevated production of ECM proteins such as thrombospondin-1 (TSP-1) and connective tissue growth factor (CTGF) (van Almen et al. 2011). MiR-21 was more abundant in the cardiac tissues of elderly mice in comparison to young mice (Zhang et al. 2012), and its expression is greatly enhanced by TGF-1 β in the post-MI (myocardial infarction) zone (Yuan et al. 2017; Zhou et al. 2018). Patients with aortic stenosis-induced fibrosis and acute MI have both been successfully identified by measuring the level of circulating miR-21 (Villar et al. 2013) (Zhang et al. 2016c). It was recently discovered that miR-21 promotes doxorubicin (DOX)-induced senescence in cardiomyocytes, probably via targeting the phosphatase and Tensin homolog, and

that silencing of miR-21 has been shown to prevent this senescence (Bei et al. 2018). Expression of miR-29 has also been significantly upregulated in the ageing heart as it is reported in ageing brain. During ageing in the heart ventricle, miR-29a and SERPINH1 (a particular procollagen chaperone) have a direct negative correlation (Rusu-Nastase et al. 2021). The accumulation of oxidative stress in the body as we age has been reported to regulate the miR-29 family, providing a preventive strategy against cardiac fibrosis (Heid et al. 2017). miR-1468-3p was found to promote cardiac fibrosis and cell senescence by increasing transforming growth factor b1 (TGF-b1)-P38 signaling (Lin et al. 2020).

Skin Ageing and miRNAs

Undoubtedly, skin is the body's first line of defense in response to biotic and abiotic environmental stimuli (Choi and Lee 2015; Lämmermann et al. 2018). Symptoms and severity of ageing first become visible on the skin, which is the ultimate multitasking and vital organ and protective layer in mammals. However, it is difficult to define the amount or severity of ageing by the appearance of skin. Despite a continuous renewal process, the regenerative capacity of cutaneous layer declines with the increase in age. Eventually, a decrease in regenerative potential results in decreased removal of senescent cells, and their progressive buildup leads to physiological ageing of the skin (Mancini et al. 2014). There are many unanswered questions about healthy ageing and achieving longevity. Skin can act as an ideal health sensor as changes in its appearance signal the organism's ongoing pathological alterations (ElSharawy et al. 2012). By understanding dermal health and the effect of ageing at molecular levels, skin can be crucial in the management of ageing and its effect. The expression of mature miRNAs in governing the development and homeostasis of skin has been reported by several studies, which reveal the molecular mechanism of skin ageing.

Keratinocytes are the primary and outermost layer of the cells found in the epidermis. One of the first researches on senescent skin cells discovered a set of 126 senescence-associated miRNAs in senescent normal human keratinocytes (Shin et al. 2011). Among the 126 senescence-linked miRNAs, 9 (7%) have shown downregulation and 117 (93%) have shown upregulation (Shin et al. 2011). During replicative cellular senescence, miR-137 and miR-668 were increased in particular, which induced senescence in proliferating human keratinocytes via triggering the senescence markers p53 and p16INK4A proteins (Shin et al. 2011). Rivetti and colleagues have identified upregulation in the expression of miR-138, miR-181a/b, and miR-130b in keratinocytes undergoing replicative senescence (Rivetti di Val Cervo et al. 2012). They observed that these four miRNAs altered cell proliferation pathways by targeting the p63 and Sirtuin-1 (SIRT1) genes (Rivetti di Val Cervo et al. 2012). Earlier studies have shown that levels of Sirt1 proteins help to increase mammalian lifespan, while its knockdown leads to cellular senescence (Rivetti di Val Cervo et al. 2012; Teumer et al. 2016; Korman 2019). The involvement of

miR-130b in regulating p63 gene has been reported by several labs (Rivetti di Val Cervo et al. 2012; Dong et al. 2013). The P63 gene encodes a transcription factor belonging to the p53 family, which is vital for epithelial development and p63 proteins can also prevent cellular senescence and ageing (Mancini et al. 2014). Moreover, studies have demonstrated that levels of p63 proteins inhibit the expression of miRNAs involved in inducing senescence, namely miRNA-138, miR-130b, miRNA-181a, and miRNA-181b (Rivetti di Val Cervo et al. 2012).

Lena and colleagues reported upregulation of miR-191 during keratinocyte senescence, and overexpression of miR-192 inhibits growth and limits SA-beta-galactosidase activity by regulating the expression of CDK6 and SATB1 genes (Lena et al. 2012). The expression of miR-191 inhibits the transition from G1 to S phase by regulating the levels of the cyclin-dependent protein kinase 6 (CDK6) gene. This obstruction manifests as a cell cycle arrest which leads to the progression of senescence conditions. Likewise, miR-191 reduces the mRNA of SATB1 (special adenine- and thymine-rich binding protein 1), which is coupled with an upsurge in senescence-related markers (Lena et al. 2012). SATB1 functions as a docking site for enzymes involved in chromatin remodeling, as well as a direct recruiter of HDACs (corepressors) or HATs (coactivators) to promoters (Pavan Kumar et al. 2006). Another study has demonstrated that miR-191 also inhibits the proliferation of primary human fibroblasts via targeting CDK9, NOTCH2, and RPS6KA3 genes (Polioudakis et al. 2015). The 8-oxoguanine DNA glycosylase (OGG1) is the main enzyme that coordinates the elimination of 8-OH-dG lesions by the primary step involved in the repair process, and its expression level in keratinocytes drops dramatically with age (Tinaburri et al. 2018). In silico studies of 3'-UTR of the OGG1 gene indicated the existence of binding sites of miR-33a and miR-200a. Copies of miR-200a increased strongly in primary keratinocytes of elderly human donors, whereas miR-33a was downregulated (Tinaburri et al. 2018). Another study reported miR-200c as a key driver that restricts cell migration during wound healing in keratinocytes and possibly contributes to age-linked changes in wound repair (Aunin et al. 2017).

The age-associated differential regulation of miRNAs has also been found in Langerhans cells, which are immature skin-residential dendritic cells (DCs) that work in immune response against antigens (Gerasymchuk et al. 2020). Multiple miRNAs have been linked with the development of Langerhans cells (miR-142 and miR-22 through the interferon regulatory factor 8 (IRF8)), maturation, differentiation (miR-34a, miR-99b, miR-21, miR-551, and miR-223), and immunological function (miR-21, miR-142-3p, miR-10, miR-155, and miR-146a) (Zhou and Wu 2017). Ageing Langerhans cells are associated with increased expression of miR-709, miR-449, and miR-9 and decreased expression of miR-200c and miR-10a (Xu et al. 2012). Increased levels of miR-449 and miR-9 regulated ageing of Langerhans cells by downregulating the TGF-signaling pathway (Xu et al. 2012). The miR-17-92 cluster, which encodes six miRNAs, i.e., miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, and miR-92a-1 is expressed in Langerhans cells (Kuo et al. 2019). However, due to functional redundancy, the miR-17-92 cluster

plays an unclear role in antigen presentation, survival, maturation, and migration capability in Langerhans cells (Zhou et al. 2014; Zhang et al. 2016b).

Another study found that the level of miR-29a and miR-30 rises during fibroblast senescence, an example of replicative senescence (Martinez et al. 2011). MiR-29a and miR-30 trigger senescence by directly suppressing B-Myb, a transcription factor that governs cellular senescence via regulating the expression of a number of genes related to cell proliferation (Martinez et al. 2011). Moreover, microRNAs are also targeted as biomarkers of ageing in human dermal senescent fibroblasts. The 15 identified miRNAs, include, let-7d-5p, let-7e-5p, 34a-5p, miR-23a-3p, miR-122-5p, miR-125a-5p, miR-125b-5p, miR-125a-3p, miR-181a-5p, miR-222-3p, miR-221-3p, miR-503-5p, miR-574-5p, miR-574-3p, and miR-4454. MiR-221/222 was found to be present in both categories of cellular senescence: replicative and stress-induced (Markopoulos et al. 2017). Other research demonstrated that miR-200c-3p was found to be threefold more abundant in older skin fibroblasts (65–80 years) than in young ones (4–6 years). This increase in miR-200c expression disrupts the sirtuin1 (SIRT1)/forkhead box O1 (FOXO1)/endothelial nitric oxide synthase (eNOS) regulatory loop, which enhances ROS production and reduces nitric oxide (NO), contributing to endothelial dysfunction (Carlomosti et al. 2017). Furthermore, prolonged oxidative stress-induced senescence elevated miR-200c in human fibroblasts (Magenta et al. 2011). Senescence in dermal fibroblasts has been also linked to the downregulation of miRNAs such as miR-106 and miR-17-92 cluster (Hackl et al. 2010). MiR-17-92 regulates the transcription of genes that control cell cycle and tumor development, including BCL2L11 (BIM) (Cloonan et al. 2008), p63 (Manni et al. 2009), and p21 (Inomata et al. 2009). It was believed to counteract senescence by enhancing the proliferation process (Mancini et al. 2014).

Because of prolonged/repeated exposure to ultraviolet (UV) solar radiation, human skin prematurely ages, a process known as photoageing. UV rays can penetrate deeper into the skin, triggering a series of specific molecular reactions in both dermis and epidermis (Yaar and Gilchrest 2007). Dermal fibroblasts are more sensitive to UV radiation than epidermal keratinocytes in humans. UV-A radiation specifically interferes with the expression of collagen genes in human dermal fibroblasts by upregulating c-Jun expression (Quan et al. 2010). Studies have identified downregulation of miR-155 with concomitant upregulation of c-Jun upon exposure to UV-A irradiation (Song et al. 2012). The expression of miR-146a is reduced by exposure to both UV-A and/or UV-B radiation in primary cultured fibroblasts. However, its overexpression inhibits UV-A irradiation-induced proliferation by upregulating the expression of photoageing-linked genes (p21WAF-1 and p53) (Li et al. 2013). The 3'-UTR of the SMAD4 gene is specifically targeted by miR-146a but whether this is the critical target and how Smad4 promotes photoageing remains to be understood (Li et al. 2013). Microarray-based studies discovered several miRNAs implicated in premature cellular senescence induced by UV-B irradiation (Zhou et al. 2012). MiR-34c-5p, which targets the 3'-UTR of E2F3 gene, is upregulated in irradiated cells. E2F3 protein is required for proliferation, development, and cell cycle progression, and it also provides protection to dermal

fibroblasts against UV-B-induced premature senescence by regulating the senescence-linked genes p53 and p21WAF-1 (Zhou et al. 2013). MiR-101, which targets the 3'-UTR of EZH2 gene is also upregulated in response to UV-B exposure (Greussing et al. 2013). In UV-B-induced senescence of human dermal fibroblasts, the functional interaction of miR-101 and EZH2 has been demonstrated. However, upregulating miR-101 while simultaneously downregulating Ezh2 is insufficient to inhibit the UV-B-induced senescence phenotype, implying system redundancy (Greussing et al. 2013). The findings coming from studies on UV-induced senescence in the in vitro system clearly suggest that miRNAs have a role in skin ageing as well.

The findings reveal that the expression of ageing-related miRNAs influences the function of several genes in different tissues and may accelerate or inhibit ageing. However, little is known regarding the involvement of miRNA in organismal ageing in terms of longevity and, presumably, age-related diseases.

miRNAs in Neuronal or Brain Ageing

Brain ageing is one of the most fundamental biological processes, which controls the delicate physiological balance or homeostasis between health and disease. Alteration in protein homeostasis or proteostasis has been observed in a number of age-linked neurological ailments including Parkinson's and Alzheimer's as well as in normal ageing. Studies from our group and several other labs have revealed dramatic regulation of miRNA in developing neurons and brain (Eacker et al. 2011; Li et al. 2011a; Inukai et al. 2012; Yin et al. 2015; Jauhari et al. 2017, 2018a, b; Chen et al. 2019a). Our group has identified several miRNAs viz., miR-200, miR-34 families, miR-153, miR-21, miR-193-5p, miR-146a, miR-221/222, miR-199a-5, and miR-29b and employing two different neuronal differentiation cellular models (PC12 and SH-SY5Y) which govern many aspects of neural formation (Jauhari et al. 2017, 2018a, b). The set of miRNAs that are identified by more than one lab to play role in neural or brain ageing are miR-34 (Li et al. 2011b; Zovoilis et al. 2011; Liu et al. 2012b; Kou et al. 2016, 2017; Jauhari et al. 2018a, b; Kennerdell et al. 2018; Chen et al. 2019b; Srinivasan et al. 2022), miR-29b (Baumgart et al. 2012; Takahashi et al. 2012; Fenn et al. 2013; Nolan et al. 2014; Takeda and Tanabe 2016; Ripa et al. 2017; Napoli et al. 2020), and miR-181 (Khanna et al. 2011; Rao et al. 2013; Stein et al. 2022).

Recent research on hippocampal tissues from young and aged mice reported that 80 out of 269 miRNAs were differentially expressed between different age groups (Mohammed et al. 2016). Another study found that out of the 70 miRNAs that were upregulated during murine brain ageing, 27 miRNAs were predicted to have direct binding sites for mitochondrial electron transport chain (ETC) and F1Fo-ATPase, both of which play an important role in oxidative phosphorylation and whose expression declines with age, possibly explaining why respiration rates decrease with ageing (Li et al. 2011a).

Several brain ageing studies have identified miR-34, which is highly conserved in *Drosophila melanogaster*, *C. elegans*, mice, and humans. miR-34 is essential in order to maintain healthy brain tissue and function in Drosophila, and its levels in the brain increase with age (Liu et al. 2012b). Mir-34 mutants exhibit shorter lifespans, early-onset neuronal death, and a higher rate of increase in age-related transcripts in brain, suggesting that miR-34 facilitates brain ageing (Liu et al. 2012b). Our group has reported that differentiated and aged neuronal cells were the greatest source of miR-34 following expression profiling studies of miR-34 family in distinct developing cells, neuronal, and non-neuronal (Jauhari et al. 2018a, b). According to our research, the brain-enriched miR-34 family upregulates with maturation of neurons and brain ageing, and the collaborative regulation of miR-34a and P53 aids in the differentiation of neurons through arresting cells in the G1 phase of cell cycle (Jauhari et al. 2018a, b).

miR-34 has been reported to suppress polycomb repressive complex 2 (PRC2) via regulating the expression of chaperones and promoting healthy brain ageing in Drosophila (Kennerdell et al. 2018). Furthermore, miR-34 also influences proteostasis in the ageing fly brain by regulating components of the Polycomb Repressive Complex 2 (PRC2) complex and Lst8, a subunit of Tor Complex 1 (TORC1) (Srinivasan et al. 2022). The activation of the SIRT1/mTOR signaling pathway, which is regulated by miR-34a, has been reported to protect against D-gal-induced cognitive impairment (Chen et al. 2019b). Another study found that inhibiting autophagy in brain tissues via ageing-associated activation of astrocytes and triggering the mTOR signaling pathway, as well as upregulating miR-34a, caused cognitive abnormalities in D-gal-induced ageing rats (Kou et al. 2016). The same group has further demonstrated that swimming can slow down brain ageing by reducing the damage of miR-34a-mediated autophagy and aberrant mitochondrial dynamics (Kou et al. 2017). A member of the miR-34 family, miR-34c, has been found to increase in the mouse hippocampus in both ageing and AD models, as well as in people with AD (Zovoilis et al. 2011). Other research reported an age-related upregulation in miR-34a expression in mouse brain and blood, as well as repression of its target Sirt1 in both tissues. The relationship between the expression of miR-34a and SIRT1 in the blood could be investigated to develop valuable biomarkers for the noninvasive detection of brain ageing (Li et al. 2011b). Also, research revealed an age-dependent decrease in the expression level of miRNAs such as mmu-miR-34a, mmu-miR-181a-1*, and mmu-miR-30e along with an increase in Bcl-2 expression and a decrease in pro-apoptotic genes like Bax and cleaved caspases (Khanna et al. 2011).

MicroRNA-29 family members (miR-29a, miR-29b, and miR-29c) are increased with age in the central nervous system (CNS) of various vertebrate species, including fish (Baumgart et al. 2012), mice (Ugalde et al. 2011; Takahashi et al. 2012; Nolan et al. 2014), monkeys (Fenn et al. 2013), and humans (Somel et al. 2010; Fenn et al. 2013). Age-related overexpression of the miR-29 family in the mouse brain leads to microglia dysregulation and enhanced neuroinflammation, both of which are hallmarks of brain ageing. MiR-29a and miR-29b, two members of miR-29 family, were found to be overexpressed in the brains of aged BALB/c mice in comparison to

young animals, and these elevated levels were negatively correlated with the expression of Insulin-like growth factor-1 (IGF-1) and fractalkine ligand (CX3CL1), both of which are negative regulators of microglial activation (Fenn et al. 2013). Moreover, miR-29 appeared to be upregulated in klotho-deficient mice (Takahashi et al. 2012), a model of premature ageing, and the progeroid mouse model Zmpste24^{-/-} (Ugalde et al. 2011). The miR-29 expression was enhanced during ageing in the brain of the short-lived turquoise killifish, and it governs iron homeostasis by targeting iron-responsive element-binding protein 2 (Ireb2), which encodes an RNA-binding protein that helps promote intracellular iron trafficking in the brain. This age-dependent rise in the level of miR-29 is an adaptive regulatory mechanism that prevents the development of several ageing-associated phenotypes. Its anti-ageing effect is largely due to its ability to govern intracellular iron homeostasis and lower excessive exposure to iron in nerve cells (Ripa et al. 2017). miR-29a has been discovered as a regulator of the plasticity brakes in visual cortex, which promotes age-dependent stabilization of visual cortical connections (Napoli et al. 2020). Another research reported that the knockdown of miR-29 in brain had a gender-specific effect on both longevity and reproduction in mice (Takeda and Tanabe 2016).

Genome-wide screening of miRNAs discovered miR-144 as the only miRNA that was consistently upregulated in the cortex regions and cerebellum of ageing humans and Chimpanzees. Ataxin-1, a gene responsible for spinocerebellar ataxia type 1 (SCA1) and other polyglutamine diseases was identified to be the direct target of miR-144 (Persengiev et al. 2012). Numerous miRNAs are elevated in the hippocampus region of both long-living Ames dwarf mice and growth hormone receptor (GHR) gene-disrupted strains (Liang et al. 2011). MicroRNAs such as miR-669b, miR-470, and miR-681 were identified as the top three upregulated microRNAs, which functionally target IGF1R and AKT, two major upstream genes governing the phosphorylation of FoxO3a, hence dampening the IGF1 signaling pathway (Liang et al. 2011). The expression of miR-186 in the cortical neurons of the mouse brain decreased with age, whereas its overexpression in neuronal cells considerably reduced the level of A β by repressing BACE1 expression (Kim et al. 2016). In the hippocampus and prefrontal cortex of SAMP8 mice, miR-449a expression has been observed to decrease with age, and overexpression of miR-449a enhances neuronal process extension through targeting sodium channel voltage-gated beta 2 (SCN2B) in the brain (Tan et al. 2020b). The switching of let-7-to-miR-125 during the pupal-to-adult transition ensures chinmo suppression in adults, preserving neuronal integrity and extending life span via regulating ageing-associated processes (Chawla et al. 2016). Another miRNA, miR-1000, is highly expressed in the central nervous system (CNS) but diminishes with age and has a neuroprotective effect in old Drosophila as well. Mir-1000 mutants have significantly shorter lifespans, disorganized locomotion, and neurodegeneration begins considerably earlier than in wild-type counterparts. Although miR-1000 seems to be unique to Drosophila, its seed sequence is very similar to mammalian miR-137, the depletion of which leads to increased neuronal death, implying that it might play a similar function in regulating neurodegeneration (Verma et al. 2015).

The area of miRNA and ncRNA research has been developed rapidly. The discovery of the highest number of brain-specific miRNAs in the past few years has provided a new degree of understanding of neurological complications related to ageing. Nevertheless, further investigation is needed to completely comprehend the mechanisms of action of miRNAs in normal brain ageing and neurodegenerative ailments, so that miRNA expression could be explored as a novel point of entry for therapeutic interventions. The challenge now is to annotate the biological functions of the ever-increasing number of miRNAs, each of which has a long list of potential targets (Table 9.2).

The complexity of ageing triggers a vast number of major disorders including cancer, cardiovascular, and neurodegenerative diseases (North and Sinclair 2012). Regardless of much evidence behind ageing-induced ailments, the scientific literature still has huge research gaps about the molecular pathways that link ageing process with diseases. Several factors including senescence, telomere dysfunction, alterations in protein processing and gene expression occur in both ageing and diseases. Regulation of miRNAs emerged as an interplay between ageing and diseases. Studies are being pursued for the pathomechanism behind age-related diseases which are summarized in this chapter.

MiRNAs and Age-Related Cardiac Diseases

The predominance of cardiac diseases has become the leading cause of mortality throughout the globe. Cardiac ageing is accompanied by impairments in both cardiomyocytes and non-cardiomyocytes, and further results in deterioration of the cardiac system. With an increasing number of heart-related diseases, there is an urgent need to identify and generate therapy to lead to treatment. Advancement in medicine opens new avenues for molecular biology which introduced next-generation throughput sequencing, a renowned technique for identifying miRNAs as potential markers for disease. Starting with the process of heart development, regeneration to severe heart failure, and other dysfunctions, miRNAs have emerged as an essential and vital player.

It is widely known that ageing reduces the cardio-protective mechanism which ultimately increases the probability of heart failure. More than 90% of deaths, which happened due to heart failure occurs in people aged 70 years or more (Strait and Lakatta 2012). The cause of heart failure is cardiomyocyte dysfunction, whose treatment does not resolve with normal routine treatment procedures. At the cellular level, miR-133 is highly enriched in cardiomyocytes, and is known to express at lower levels in animal models with hypertrophy and patients suffering from hypertrophic cardiomyopathy (Sacks et al. 2018; Colpaert and Calore 2019). Studies summarizing the role of miRNAs in normal heart and cardiovascular diseases are increasing at a rapid rate (Nishiguchi et al. 2015; Romaine et al. 2015; Ultimo et al. 2018). Van Rooij and colleagues first reported the abnormal expression of miRNAs in the heart due to hypertrophy (Van Rooij et al. 2006; Van Rooij and Olson 2007).

Table 9.2 Role of miRNAs differentially expressed in the ageing of different tissues

miRNA	Species	mechanism	References
Skeletal muscle			
miR-221↓	Mouse	miR-221 overexpression delays the exit of differentiating myoblasts from cell cycle and decreases myogenin levels.	(Cardinali et al. 2009)
miR-181a↓	Mouse, monkey	miR-181a: <i>Sirt1</i> interactions negatively regulate the size of myotube. Increases inflammation in the elderly.	(Soriano-Arroquia et al. 2016) (Mercken et al. 2013)
Let-7 s (let-7b and let-7e) ↑	Human	Downregulates the cell cycle regulators such as CDC34, CDK6, and CDC25A leading to reduced cell proliferation potential, regular renewal, and regenerative capability of skeletal muscle cells.	(Drummond et al. 2011)
miR-126↓	Human	Regulates plasticity of skeletal muscle with ageing by modulating signal transduction responding to insulin growth factor 1 (IGF-1).	(Rivas et al. 2014)
miR-434↓	Mouse	Increases apoptosis via de-repression of Eif5a1, a pro-apoptotic gene.	(Pardo et al. 2017)
miR-431↓	Mouse	Targets Smad4, one of the TGF/Smad signaling pathway's downstream effectors, which is reported to suppress muscle growth, myogenesis, and age-linked regeneration of muscles.	(Lee et al. 2015)
miR-29↑	Mouse	Progressive buildup of DNA damage via reducing the levels of Ppm1d protein, a major regulatory phosphatase of DNA damage response (DDR) important for cell viability control. Reduces fibroblast growth and enhances cellular senescence via suppression of several signaling proteins, including P85, Igf1, and B-myb.	(Hu et al. 2014) (Ugalde et al. 2011)
Bone			
miR-2861↓	Human	Reduced osteoblast activity can be caused by a mutation in pre-miR-2861, possibly leading to osteoporosis via elevating histone deacetylase 5 (HDAC5) protein levels and reducing runt-associated transcription factor 2 (Runx2) expression in bone.	(Li et al. 2009)
miR-34c↑	Mouse	Targets numerous components of the notch pathway, such as <i>Jag1</i> , <i>Notch1</i> , and <i>Notch2</i> leading to age-related osteoporosis owing to the improper mineralization and proliferation of osteoblasts, as well as enhanced osteoclastogenesis.	(Bae et al. 2012)
miR-21↓	Mouse, human	Increases PTEN (phosphatase and tensin homolog) protein levels and lowers Akt activation, which results in apoptosis of osteocytes. Promotes development of osteoblast and formation of bone in vivo.	(Davis et al. 2017b) (Yang et al. 2013) (Zhao et al.

(continued)

Table 9.2 (continued)

miRNA	Species	mechanism	References
		Protects against osteoporosis via inhibiting Reck, a potent inhibitor of osteogenesis-related matrix metalloproteins. Regulates bone mass accumulation and contributes to osteoporosis development.	2015b (Hu et al. 2017)
miR-183↑	Mouse	Decreases proliferation of primary bone marrow stromal cells (BMSCs) and differentiation of osteogenic cells, promoted senescence, and reduced heme oxygenase-1 (HO-1/HMOX-1) protein levels. Targets HO-1 to modulate receptor activator of nuclear factor- κ B ligand (RANKL)-induced OC differentiation via targeting HO-1.	(Davis, Dukes et al. 2017) (Ke et al. 2015)
miR-31↑	Human	Targets Fzd3, a Wnt5a receptor, leading to inhibition of osteogenic differentiation.	(Weilner et al. 2016)
miR-214↑	Human, mouse	Inhibits differentiation of osteoblast as well as osteoblastic bone formation. Targets phosphatase and tensin homolog (Pten), thus promoting osteoclastogenesis via the PI3K/Akt pathway. Upregulates the expression levels of miR-214 and ephrinA2 in serum exosomes significantly inhibiting osteoblast activity. Serves as an intercellular messenger that inhibits osteoblastic bone growth by mediating osteoclast-to-osteoblast communication.	(Wang et al. 2013) (Zhao et al. 2015a) (Sun et al. 2016) (Li et al. 2016)
miR-188↑	Mouse, human	Reduces the formation of bone and enhanced bone marrow fat accretion via targeting RPTOR-independent companion of MTOR complex 2 (RICTOR) and histone deacetylase 9 (HDAC9).	(Li et al. 2015)
miR-218↑	Mouse	Reduces osteoblast bone surface survival and adhesion via directly targeting Rictor, a specific constituent of the mechanistic target of rapamycin complex 2 (mTORC2) that controls the cell viability and organization of cytoskeleton.	(Li et al. 2016)
Heart			
miR-34a↑	Mouse	Inhibits PP1 nuclear targeting subunit (<i>PNUTS</i>) (also called PPP1R10) which limits shortening of telomeres, DNA damage response (DDR), and apoptosis of cardiomyocytes. Regulates cell cycle progression and cellular death by modulating its targets, such as cyclin D1, Sirt1, and Bcl2.	(Boon et al. 2013) (Yang et al. 2015)
miR-17↓	Mouse	Suppresses cardiac senescence and apoptosis via targeting Par4/PAWR, a Wilms' tumor 1 (WT-1) interacting protein that functions as a transcriptional repressor.	(Du et al. 2015)

(continued)

Table 9.2 (continued)

miRNA	Species	mechanism	References
miR-22↑	Mouse, human	Triggers senescence via targeting mimecan (also known as osteoglycin). Inhibits cardiac autophagy.	(Jazbutyte et al. 2013) (Gupta et al. 2016)
miR-18a/ 19a↓	Mouse, human	Reduces cardiac fibrosis via regulating ECM proteins—Thrombospondin-1 (TSP-1) and connective tissue growth factor (CTGF).	(van Almen et al. 2011)
miR-21↑	Mouse, human	Promotes cardiac fibrosis via targeting Smad7, a crucial inhibitor of the transforming growth factor β (TGF- β) signaling. By targeting Jagged1, it mediates TGF- β -1-induced cardiac fibroblast transformation (CMT) and myocardial fibrosis. Regulates cardiac fibrosis via targeting extracellular matrix (ECM)- and TGF- β -signaling-related elements. Increases cardiomyocyte senescence via targeting phosphatase and tensin homolog (PTEN).	(Yuan et al. 2017) (Zhou et al. 2018) (Villar et al. 2013) (Bei et al. 2018)
miR-29↑	Mouse, fish	Targets a specific collagen chaperon, SERPINH1 which governs the transcription of collagen proteins and also regulates collagen maturation. Influences the transcription of genes probably by age-associated alterations in DNA methylation.	(Rusu-Nastase et al. 2021) (Heid et al. 2017)
miR-1468- 3p↑	Human	Enhances TGF- β 1-p38 signaling, which promotes heart fibrosis.	(Lin et al. 2020)
Skin			
miR-137↑	Human	Induces senescence in proliferating human keratinocytes via triggering markers of senescence, viz., p53 and p16 ^{INK4A}	(Shin et al. 2011)
miR-668↑	Human	Induces senescence in proliferating human keratinocytes via triggering the senescence markers such as p53 and p16 ^{INK4A}	(Shin et al. 2011)
miR-138↑	Human	Modulates the levels of p63 and Sirt1 in primary human keratinocytes, thus contributing to the induction of senescence.	(Rivetti di Val Cervo et al. 2012)
miR-181a/b ↑ miR-181a↑	Human	Modulates the levels of p63 and Sirt1 in primary human keratinocytes, thus contributing to the induction of senescence. Targets COL16A1 in skin fibroblast, whose reduction is linked to impaired remodeling of ECM of older skin.	(Rivetti di Val Cervo et al. 2012) (Mancini et al. 2012)
miR-130b↑	Human	Modulates the levels of p63 and Sirt1 in primary human keratinocytes, thus contributing to the induction of senescence.	(Rivetti di Val Cervo et al. 2012)
Mir-191↑	Human	Induces senescence by triggering cyclin-dependent kinase 6 (CDK6) and special AT-rich binding protein 1 (SATB1) in primary human keratinocytes.	(Lena et al. 2012)

(continued)

Table 9.2 (continued)

miRNA	Species	mechanism	References
miR-200a↑	Human	Reduces oxidative DNA repair activity by downregulating OGG1-2a and may cause cell cycle arrest through upregulation of p16 in keratinocyte ageing. It also fuels chronic inflammation via activating the nucleotide-binding domain and leucine-rich repeat pyrin domain containing 3 (NLRP3) pathway.	(Tinaburri et al. 2018)
miR-200c↑	Human, mouse	Significantly reduced the expression of sirtuin 1 (SIRT1), endothelial nitric oxide synthase (eNOS), and forkhead box O1 (FOXO1) in fibroblasts of old compared to young subjects.	(Carlomostti et al. 2017)
miR-449↑	Mouse	Downregulates TGF-β signaling pathway and blocks development in aged LCs.	(Xu et al. 2012)
miR-9↑	Mouse	Reduces LC development and homeostasis via targeting TGF-β signaling.	(Xu et al. 2012)
miR-152↑	Human	Reduces dermal fibroblast adhesion by targeting mesenchymal integrins α5 (ITGA5).	(Mancini et al. 2012)
miR-29a↑	Human	Induces senescence in foreskin fibroblasts by directly repressing B-Myb, a transcription factor that controls cell proliferation genes.	(Martinez et al. 2011)
miR-30↑	Human	Targets B-Myb in senescent cells.	(Martinez et al. 2011)
miR-17-92 cluster↓	Human	Increases transcript levels of the p21/CDKN1A, a cyclin-dependent kinases (CDKs) inhibitor in ageing of skin fibroblast.	(Hackl et al. 2010)
miR-106↓	Human	Induces the expression of p21/CDKN1A in skin fibroblast undergoing senescence.	(Hackl et al. 2010)
miR-155↓	Human	Governs c-Jun expression in the pathogenesis of UVA-induced photoageing in human skin fibroblasts.	(Song et al. 2012)
miR-146a↓	Human	Increases the expression of photoageing-linked genes (such as p21WAF-1 and p53).	(Li et al. 2013)
MiR-34c↑	Human	Targets E2F3 which inactivates p53 pathway and regulates cell cycle progression in UVB-induced premature senescence in skin fibroblasts.	(Zhou et al. 2013)
miR-101↑	Human	Inhibits histone methyltransferase Ezh2 to regulate UVB-induced senescence of human diploid fibroblasts (HDF).	(Greussing et al. 2013)
Brain			
miR-34↑	Fly	Increases median lifespan and alleviates neurodegeneration via targeting Eip74EF, a key ETS domain transcription factor implicated in steroid hormone pathways. Mitigates age-linked neurodegeneration via targeting Su(z)12 and Pcl, two members of Polycomb repressive complex 2 (PRC2). Influences proteostasis via regulating components of PRC2 complex and Lst8, a subunit of tor Complex 1 (TORC1).	(Liu, Landreh et al. 2012) (Kennerdell et al. 2018) (Srinivasan et al. 2022)

(continued)

Table 9.2 (continued)

miRNA	Species	mechanism	References
miR-34a↑	Rat, mouse	Regulates SIRT1-mTOR signaling in D-gal-induced brain ageing. Swimming delays brain ageing process through attenuating miR-34a-driven autophagy and aberrant mitochondrial dynamics. Targets silent information regulator 1 (SIRT1) and serves as a noninvasive biomarker of brain ageing.	(Kou et al. 2016) (Kou et al. 2017) (Li et al. 2011b)
miR-34c↑	Mouse	Plays a crucial role in the pathogenesis of cognitive decline via translational repression of SIRT-1.	(Zovoilis et al. 2011)
miR-34a↓	Mouse	Maintains elevated levels of Bcl-2, leading to the loss of pro-apoptotic signaling.	(Khanna et al. 2011)
miR-181a-1*↓	Mouse	Maintains Bcl-2 levels to remain high in brains of aged animals, leading to loss of pro-apoptotic signaling and improved survival of neurons.	(Khanna et al. 2011)
miR-30e↓	Mouse	Maintains elevated levels of Bcl-2, leading to loss of pro-apoptotic signaling.	(Khanna et al. 2011)
miR-29a/b↑	Mouse	Targets insulin-like growth factor-1 (IGF-1) and fractalkine ligand (CX3CL1), both of which are inhibitors of microglial activation.	(Fenn et al. 2013)
miR-29↑	Mouse, fish	Downregulates collagen type IV, which could weaken the basal membrane in ageing tissues. Prevents development of various ageing-related phenotypes via regulating iron homeostasis by targeting Ireb2.	(Takahashi et al. 2012) (Ripa et al. 2017)
miR-144↑	Human, chimp	Targets ataxin 1 (ATXN1), accountable for spinocerebellar ataxia type 1 (SCA1) and other polyglutamine disorders.	(Persengiev et al. 2012)
miR-470↑	Mouse	Controls IGF signaling pathway via governing expression of AKT and IGF1R, two upstream genes that affect phosphorylation of FoxO3a.	(Liang et al. 2011)
miR – 669b↑	Mouse	Controls IGF signaling pathway via targeting IGF1R and AKT.	(Liang et al. 2011)
miR --681↑	Mouse	Controls IGF signaling pathway via targeting IGF1R and AKT.	(Liang et al. 2011)
miR-186↑	Mouse	Binds to 3'-UTR of β-site APP-cleaving enzyme 1 (<i>Bace1</i>) mRNA in neuronal cells.	(Kim et al. 2016)
miR-449a↓	Mouse	Overexpression of miR-449a enhances neuronal process extension by targeting sodium channel voltage-gated beta 2 (SCN2B) in the brain.	(Tan et al. 2020b)
miR-1000↓	Fly	Controls the release of neurotransmitters from presynaptic terminals.	(Verma et al. 2015)

Studies have also identified numerous up- and downregulated miRNAs in a mouse model of cardiac hypertrophy (Martinelli et al. 2014). Interestingly, a few of them were also identified in end-stage failing human hearts, indicating the relevance of study to human pathology. Several studies also reported the dysregulation of miRNA expression in hypertrophic hearts. A report using cellular and animal models identified overexpression of miR-133 with well-preserved cardiac functions while suppressing shows hypertrophy (Abdellatif 2010). The role of miR-133 is also shown to regulate the cardiac action potential (Kuzmin et al. 2021). Along with miR-133, miR-1 was also downregulated in animal models with hypertrophic heart along with patients suffering from hypertrophic cardiomyopathy, while upregulation of miR-133 or miR-1 rescues cardiac hypertrophy (Gladka et al. 2012). Revealing miR-133 and miR-1 as key regulators of cardiac hypertrophy, the possibility of using them as therapeutic applications for heart disease is quite strong (Care et al. 2007). It is known that insulin-like growth factor-1 signaling is a crucial contributor to cardiac hypertrophy and arrhythmias. Remarkably, miR-1 targets insulin-like growth factor-1, which further suppresses pre-miR-1 transcript process (Elia et al. 2009). Another miRNA, miR-208, was found to be differently expressed in cardiomyocytes (Callis et al. 2009; Huang et al. 2021) but later, deep sequencing data from human hearts showed lower expression of miR-208 expression compared to other cardiac miRNAs, such as miR-1 and miR-133.

MiRNAs and Age-Related Neurodegenerative Diseases

With the rise in the number of elderly people, the burden of neurodegenerative disorders (NDs) is increasing. As per the WHO, neurological disorders will overtake cardiovascular diseases as the second leading cause of mortality by 2040. It is predicted that the occurrence of dementia cases will rise by 300% in developing countries and up to 100% in developed countries over the coming years (Ferri et al. 2005). For these NDs, a few drugs have been developed which only manage the disease progression, but are unable to prevent or completely cure them. A major reason behind this unusual situation for incompetent drugs is different mechanisms for each ND. Selected degeneration of neurons in the brain causes several common NDs. Neurodegeneration is a broad term, which mostly affects the structure and function of neurons in brain. The family of NDs includes many members (Parkinson's, Alzheimer's, Huntington's, and Amyotrophic lateral sclerosis), whose nature depends on the affected region and neuron types, which are quite specific to each disease. Several pathomechanisms are behind these NDs including mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress, neuroinflammation, synaptic loss, calcium deregulation, and many more are being studied. But the detailed pathomechanism behind these NDs is still unclear. MiRNAs are highly investigated and may play a crucial role in neuronal development and neuronal degeneration. These miRNA sequences have been found to be deregulated in the peripheral blood, cerebrospinal fluid, and brain regions in patients

suffering from any one ND. To better validate the situation, nowadays in vitro and in vivo models of neurodegeneration are widely used to identify the regulation of miRNAs. Thorough research is needed to develop these miRNAs as biomarkers for these NDs.

Alzheimer's Disease

Alzheimer's disease (AD) is ranked the first ND characterized by neuronal loss and inflammation, and represents the most frequent form of dementia found in elderly people, affecting learning, language, memory, and behavior (Bookheimer et al. 2000). Studies have established the role of mutations in genes like amyloid precursor protein (APP) and Presenilin in the early onset of AD in a few cases. The slow and continuous accumulation of abnormal protein aggregates in the form of plaques and tangles in brain over the period of 10–20 years results in cognitive and behavioral symptoms, which is a remarkable feature of AD (Hardy and Selkoe 2002). Amyloid- β (A β) is generated through the sequential cleavage performed by two proteolytic enzymes: amyloid precursor protein (APP) by β -APP-cleaving enzyme 1(BACE1) and the γ -secretase complex (Tamagno et al. 2008). The exact pathologic processes underlying the onset and progression of AD are presently unclear. Various clinical and research studies have shown that abnormal regulation of miRNA-dependent expression of genes is closely related to molecular processes that are responsible for A β production, neuroinflammatory tangles (NFT) formation, and neuronal cell death. The distinguished and prominent function of miRNAs in the onset and progression of AD has been confirmed in various studies (Delay et al. 2012; Femminella et al. 2015; Wang et al. 2019b). Lukiw and colleagues firstly provided evidence for the involvement of miRNAs in AD pathology (Lukiw and Pogue 2007). Regulation of individual miR-132 or the miR-132/212 cluster has also been demonstrated in AD pathogenesis in many studies. Deficiency of miR-132/212 has been investigated in mice, which impairs the normal Tau mechanisms and leads to its aggregation (Smith et al. 2015). In addition, the study also defined the mechanism behind the association of cluster miR-132/212 and AD pathology (Pichler et al. 2017). They revealed that loss of miR-132/212 increases the level of nitric oxide, followed by disruption of S-nitrosylation (SNO) balance, which induces tau pathology and may contribute to the pathogenesis of AD and other tauopathies (Pichler et al. 2017). Recently, studies were carried out on human cortical brain tissues, which showed the consistent downregulation of the miR-132/212 cluster and implicated its crucial role in AD pathology (El Fatimy et al. 2018).

Hébert and colleagues reported the crucial role of miR-29 in the development of AD (Hébert et al. 2008). Using neuronal cellular models, they observed a negative correlation between miR-29 expression and levels of BACE1 proteins, which resulted in abnormal production of A β . Studies have also tried to develop miR-29 family members as biomarkers of PD and AD (Müller et al. 2016). Members of miR-29 family are reported to be regulated in the brains of aged humans (Ugalde et al. 2011; Ripa et al. 2017). Studies provide insight into AD pathological

mechanisms and revealed the miR-34 family as a therapeutic target (Sarkar et al. 2016). Using a transgenic AD model and AD patients, inhibition of miR-34c upregulated VAMP2 expression, which rescued synaptic failure and also alleviated learning and memory impairments induced due to A β toxicity (Hu et al. 2015). Kim and colleagues identified the crucial role of miR-186 in AD pathogenesis. They reported that inhibition of miR-186 suppresses the expression of BACE1 in aged brain, which is a prominent risk in AD development (Kim et al. 2016). In SH-SY5Y cells, upregulation of a known inflammatory regulator miRNA (miR-146a) was reported which inhibits LRP2 expression followed by increasing cellular apoptosis and suggested its major role in regulating AKL/LRP2 pathway in AD (Zhang et al. 2016a). A recent study identified miR-485-3p overexpression in the tissues of brain, cerebrospinal fluid, as well as in plasma of patients with AD. Inhibition of miR-485-3P has been shown to reduce A β plaque buildup, tau pathology propagation, neuronal inflammation, and cognitive impairment, which are the characteristic features of AD (Koh et al. 2021). Recent research has demonstrated that miR-212-3p, which is one of the major studied miRNAs in neurodegeneration has a protective role in animal and cellular models of AD (Nong et al. 2022). NF- κ B (nuclear factor kappa B) signaling has been extensively studied in AD pathogenesis. The latest study has also shown the involvement of IKK β -mediated A β aggregation and neuron regeneration in APP/PS1 mice and identified the potential role of miR-155-5p as a probable candidate in AD treatment (Wang et al. 2022). Synaptic loss associated with cognition impairment in AD identified miRNA-455-5p/CPEB1 pathway mouse-based AD model, which suggests miR-455 as a promising therapeutic target for AD (Xiao et al. 2021). Using AD patients and mice, the miR-124/PTPN1 pathway was identified as a critical regulator of synaptic dysfunction as well as cognitive decline in AD, and can be considered as a potential novel therapeutic target for AD (Wang et al. 2018).

Parkinson's Disease

Parkinson's disease (PD) is a progressive NDD affecting a large number of individuals around the world and is characterized by rigidity, bradykinesia, tremor, and shaking followed by loss of dopaminergic neurons in substantia nigra pars compacta. Two important genes which are known to associate with PD are SNCA and LRRK2. SNCA (Alpha-synuclein) is localized in the presynaptic terminals in association with the plasma membrane and its expression is broadly present in the adult brain, specifically the hippocampus, neocortex, and substantia nigra (Bekris and Mata 2010). LRRK2 belongs to the leucine-rich repeat kinase family and is found primarily in the cytoplasm, but also associated with the mitochondrial outer membrane. It is largely expressed in the brain, with hippocampus and striatum having the highest levels of expression (Galter et al. 2006; Melrose et al. 2006). Despite the fact that PD is a multigenic and fatal disorder, numerous efforts have been undertaken in the direction of therapeutic approach using miRNAs, and the findings are encouraging, even if clinical implementation still appears to be a long

way off. Though PD is a multigenic and fatal disease, various efforts have been made in the direction of miRNA-based therapeutics, with promising results, albeit still far from clinical implementation. Neuroinflammation in case of neurodegenerative diseases acts as a marker for diagnosing the effects of neuronal damage. Recent research identified the crucial role and regulation of miR-155 in mediating the inflammatory response in PD (Thome et al. 2016). Studies have also demonstrated ectopic expression of miR-7, reduced level of SNCA significantly in cultured neuronal cells, and knocking down of miR-7 greatly enhanced SNCA expression (Doxakis 2010). Another miRNA, miR-153 has been predicted to target and bind to the 3'UTR of SNCA in between 462 and 468 bases, which is a conserved sequence among all the vertebrate species. MiR-153 is highly abundant in brain, and is reported to regulate the expression of SNCA negatively (Kim et al. 2013). Significant studies by Chaudhuri et al. (2016) reported the contribution of miR-7 in mitochondrial dysfunction which is one of the contributors to PD (Chaudhuri et al. 2016). They identified VDAC1 as a target of miR-7, and suggested it to be a novel biomarker that protects neuronal cells undergoing mitochondrial abnormality (Chaudhuri et al. 2016). The significant role of miRNAs has also been demonstrated by profiling the expression pattern in PD patients. By profiling the expression of miRNAs in PD patients, it has been identified that level of miR-133b has significantly deregulated (Kim et al. 2007). Moreover, it has also been shown that a feedback loop mechanism takes place between Pitx3 and miR-133b, which represents a link between miRNAs and neurodegenerative disorders (Kim et al. 2007). Nevertheless, miR-133b has been reported as a negative regulator of differentiation of dopaminergic neurons, as its ectopic overexpression in primary rat embryonic midbrain cultures resulted in a decreased number of dopaminergic neurons, whereas its knockdown has shown the opposite effect. Furthermore, another miRNA expression profiling study in PD brains has shown a significant downregulation in the expression of miR-34b and miR-34c in brain regions including frontal cortex, amygdala, cerebellum, and substantia nigra (Miñones-Moyano et al. 2011). Additionally, in vitro studies have also suggested the role of miR-34b and miR-34c in the pathogenesis of PD. Decreased expression of miR-34b or miR-34c was observed in differentiated SH-SY5Y cells resulting in cell viability reduction as well as cellular mitochondrial function abnormality (Miñones-Moyano et al. 2011). Later, studies have also revealed that downregulation of miR-34b and miR-34c increases α -synuclein expression in PD (Kabaria et al. 2015). Dopaminergic neuron degeneration along with microglia activation underlies pathomechanisms of PD. A recent study identified miR-132-3p in cellular, animal, and brain tissues of PD patients, which targets GLRX and mediates microglia activation (Gong et al. 2022). Renowned brain-specific miRNA, miR-29 plays a role in both ageing and PD pathogenesis (Bai et al. 2021). Cerebrospinal fluid of PD patients identified miR-409-3p regulates ATXN3 which rescues the dopaminergic neurons from cell death (Tan et al. 2021). Recent studies from our lab have discovered a direct role of NF- κ B-mediated upregulation of miR-146a in controlling mitophagy via Parkin gene suppression in an animal model of PD (Jauhari et al. 2020). Indeed, different models identified a class of miRNAs that are crucial regulators of PD. Further studies are required to target these miRNAs as therapeutic models.

Huntington's Disease

Huntington's disease (HD) is a neurological disease caused by an increase in CAG repeats beyond 36 at exon 1 of the huntingtin gene (HTT). The symptoms behind HD include mental disorders, psychopathological dysfunctions as well as motor and cognitive abnormalities. Long projections of neurons and synapses in the cortex and striatum region of the brain are also lost in this condition. One of the most prevalent pathological problems in HD has been transcription dysregulation caused by abnormalities in the actions of transcription factors. Several pathways are targeted and interacted by miRNAs during the development of HD and its progression. Numerous studies have been cited to date about the role and regulation of miRNAs in HD. Studies by Fu MH et al. (2015) revealed the therapeutic potential of miR-196a in HD using a bioinformatics approach (Fu et al. 2015), which was previously reported as a terminator of pathological phenotypes of HD in different models (Cheng et al. 2013). Another miRNA, miR-214, has been reported in an HD cell model which targets mitofusin2 (MFN2), where its upregulation affected mitochondrial morphology and disrupted the cell cycle. One of the brain-specific miRNAs, miR-124 has been investigated in lowering the progression of HD by inducing neurogenesis in the striatum of brain (Liu et al. 2015). Compiled studies of next-generation sequencing profiling carried out on human HD and control brain tissues identified various miRNAs linked to neurite outgrowth, neuronal differentiation, survival, and cell death (Müller 2014). In this study, miR-10b-5p and miR-30a-5p were identified to target BDNF that is known to associate with neuronal functions (Müller 2014). Furthermore, regulation of miR-10b-5p expression in HD pathogenicity was reported in context with age as well as the onset and extent of striatal association (Hoss et al. 2015). This miR-10b-5p can be considered a biomarker for the detection and progression of HD. MiR-34b has also been reported as a putative plasma-based biomarker for HD as miR-34b was significantly elevated in mutated huntingtin (mHTT) cultured neurons (Gaughwin et al. 2011). Bioinformatic analysis identified hsa-miR-10b-5p and hsa-miR-196a-5p which were significantly upregulated in HD, targeting BDNF or homeobox genes which are critical regulators in the pathogenesis of HD (Wang and Dong 2020). Specific microRNAs whose alterations reduced the phenotypic features induced by mutant HTT and suggested possible therapeutic targets for HD have been explored by employing animal models of Huntington's disease and HD patients (Dong and Cong 2021).

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is the most prevalent and severe type of NDD, which leads to motor neuron degeneration and causes death within 3–5 years of diagnosis. Mutations in over 20 genes linked to familial forms of ALS have revealed newer insights into the causes of motor neuron death. Loss of motor neurons, muscle

atrophy, and deterioration of target muscles are all symptoms of ALS, which results in paralysis. ALS occurs in two forms, either familial ALS (FALS) (which accounts for 10% of cases) or sporadic ALS (SALS) (which accounts for 90% of cases). Indeed, it affects a large number of people but due to the little understanding of mechanisms, there is presently no established cure. Regulation of miRNAs during the development of ALS is well accepted and recent studies provide inclusive support for the involvement of miRNAs in disease development. A report by Williams et al. (2009) identified the role of miR-206 in the development of ALS in a mouse model. They reported that mice, which were genetically deficient for miR-206 formed normal neuromuscular synapses throughout development, while miR-206 deficiency in the ALS mouse model accelerates the progression of disease (Williams et al. 2009). In mice, miRNA-206 slows the course of ALS and encourages the regeneration of neuromuscular synapses (Williams et al. 2009). Studies by Koval et al. (2013) discovered the regulation of miRNAs in the rodent model of ALS and reported significant alteration in 12 miRNAs. Out of the 12 identified miRNAs (miR-17, miR-20a, miR-19b, miR-24-2, miR-142-3p, miR-106, miR-142-5p, miR-146a, miR-146b, miR-155, miR-223, and miR-338-3p) six miRNAs were tested and confirmed in ALS tissues of humans. Specifically, miR-155 was upregulated twofold in humans as well as fivefolds in mice spinal cord samples. Moreover, injection of anti-miR-155 increased survival by 10 days and duration of disease by 15 days in SOD1G93A mice (Koval et al. 2013; de Andrade et al. 2016). Recent studies by De Andrade et al. (2016) identified 11 miRNAs (miR-424, miR-503, miR-886-3p, miR-542-5p, miR-34a, miR-886-5p, miR-146b-5p, miR-504, miR-21, miR-214, and miR-183) differentially expressed in the skeletal muscle of ALS patients, and only two miRNAs (miR-424 and miR-206) were significantly overexpressed in the plasma of ALS patients. The baseline expression of miR-424 and miR-206 corresponds with the progression of clinical symptoms over time, suggesting the potential of these two miRNAs to be used as prognostic markers for ALS. Freischmidt and colleagues identified miR-1234-3p and miR-1825 in sera of sporadic ALS patients and reported it as a signature role in ALS development (Freischmidt et al. 2015). Research is going on to identify novel miRNAs which will behave like prognostic as well as diagnostic biomarkers for these NDs.

Stem Cell Development and miRNAs

In the current era, the lifespan of humans is substantially longer in comparison to other primates with maximum age reaching up to 115 years (Finch 2010). Extended longevity has also offered several challenges for our body and has increased vulnerability and susceptibility to age-associated diseases like cancer and neurodegenerative diseases. Stem cells are a class of undifferentiated and pluripotent cells, which have multilineage differentiation capability. Ideally, stem cells are categorized into embryonic stem cells (ESCs), adult stem cells, and induced pluripotent stem cells (iPSCs). Adult stem cells are classified as mesenchymal stem cells (MSCs),

cardiac stem cells (CSCs), hematopoietic stem cells (HSCs), endothelial stem cells, neural stem cells (NSCs), and so on, depending on the tissue from which they originated. ESCs are pluripotent stem cells generated from the inner cell mass of a blastocyst in epiblast tissue, whereas iPSCs are derived by reprogramming of somatic cells (Hochedlinger and Plath 2009; Patel and Yang 2010; Nichols and Smith 2012). Stem cells have much potential in clinical therapy because of their pluripotency and ability to self-renew. Several studies illustrated the use of osteoblasts, cardiac cells, and neural cells differentiated from stem cells in repair of damaged organs through transplantation (Pittenger and Martin 2004; Oryan et al. 2017). The promise of a stem cell is its enormous potential to self-renew and generate several differentiated cells which are governed by dynamic interaction between extrinsic signaling, transcriptional, posttranscriptional, and epigenetic regulations. Studies carried out in the last decade strongly suggest the role of miRNA-mediated translation inhibition in the differentiation and self-renewal of stem cells (Park et al. 2007; Guo et al. 2011). Several studies have demonstrated the involvement of miRNAs in the proliferation and differentiation of germline stem cells, embryonic stem cells, and various somatic tissue stem cells (Zhao et al. 2010; Shenoy and Blelloch 2014). Each kind of stem cell possesses a distinct pattern of miRNA profiles. It is hypothesized that miRNAs will be able to contribute to stem cell therapy in clinics in the near future for regenerative medicine therapy.

Stem Cell Pluripotency and Role of miRNAs

MiRNAs contribute significantly to physiological processes as well as to the pathogenesis of several diseases. Studies have shown that miRNAs have great potential as therapeutic tools for many disorders, including myocardial injury, neurodegenerative, and muscle ailments (Junn and Mouradian 2012; Harada et al. 2014; Alexander and Kunkel 2015). Studies by Houbaviy et al. (2003) have first identified the expression of miRNAs in undifferentiated and differentiated embryonic stem cells in mice and proposed that miRNAs may play a role in the maintenance of the pluripotency and early mammalian cell development (Houbaviy et al. 2003). The involvement of miRNAs has been implicated in the regulation of stemness via targeting the 3'-UTR of pluripotency factors or also known as Yamanaka factor. The direct involvement of miR-145 in the regulation of OCT4 (also known as Pou5f1), KLF4, and SOX2 which control pluripotency of human ESCs, has been demonstrated in numerous studies (Xu et al. 2009; Li et al. 2017, 2018a, b). Studies from our lab have demonstrated that upregulation of miR-145 during neuronal differentiation or maturation is regulated by another miRNA (miR-29b) in a P53-dependent manner in SH-SY5Y, a human neuroblastoma (Jauhari et al. 2017, 2018a, b). Furthermore, miR-470, miR-296, and miR-134 mediate mouse ESC differentiation via targeting coding regions of transcription factors such as Oct4, Nanog, and Sox2 (Tay et al. 2008a). MiR-145 inhibits stem cell self-renewal and induces differentiation by directly repressing SOX2, OCT4, and KLF4 (Xu et al. 2009). OCT4, in turn, suppresses miR-145 expression in a regulatory feedback loop

(Xu et al. 2009). In mouse ES cells, miR-296, miR-134, and miR-470 target the coding region sequences of the Oct4, Nanog, and Sox2 genes (Tay et al. 2008b). Bmi-1, a Polycomb family member, governs stem cell self-renewal by an epigenetic process. Neural stem cell populations and breast cancer stem cells (BCSC) are both triggered by Bmi-1 (Liu et al. 2006; Godlewski et al. 2010). MiR-128 inhibits carcinogenesis and suppresses CSC self-renewal via targeting Bmi-1. Upregulation of Bmi-1 is linked with miR-128 downregulation in glioblastoma cells (Godlewski et al. 2010). In addition to miR-128, miR200, miR-183, and miR-203 regulate stem cells via targeting Bmi-1 (Shimono et al. 2009).

miRNAs and Cardiovascular Differentiation

It is well studied that miRNAs mediate cardiovascular differentiation and generate vascular smooth muscle cells (vSMCs), cardiomyocytes, as well as endothelial cells (ECs). The role of miR-499 has been established in the differentiation of human cardiomyocyte progenitor cells via targeting Sox6 (Sluijter et al. 2010). Moreover, miRNAs have been studied in regulating cardiovascular differentiation of iPSCs and ESCs. Several miRNAs like miR-6086, and miR-6087 have been identified to suppress endothelial markers and play a role in endothelial biogenesis (Yoo et al. 2012). In mouse iPSCs, miR-199b is being studied as a regulator of iPSC-derived vascular cell differentiation by modulating crucial angiogenic signaling pathways (Chen et al. 2015). It is established that miRNA-regulated cardiovascular differentiation of stem cells has remarkable therapeutic potential in regenerative medicine.

Neuronal Differentiation and miRNAs

Unlike most cells, neurons pull out from a proliferation cycle and differentiate into mature structures with neurites, which establish connections with several other neurites for communications. Several studies have been carried out on global miRNA profiling and identified dramatic regulation in the expression of various miRNAs in differentiated neurons in comparison to naïve cells (Jauhari et al. 2017, 2018a, b, 2020). The role of miR-124 was identified first in promoting neuronal differentiation of the subventricular zone (SVZ) in adult mammalian brain (Cheng et al. 2009; Åkerblom et al. 2012). Studies from our lab have shown a significant increase in the expression level of three miRNA families (miR-34, miR-200, and miR-221/222) using nerve growth factor differentiated PC12 cells as a model of neuronal differentiation (Pandey et al. 2015). The expressions of miR-7, miR-9, and miR-214 play a key role in neural lineage determination (Liu et al. 2012a). MiR-9, a brain-enriched miRNA, targets the nuclear receptor TLX, thus stimulating NSC differentiation (Zhao et al. 2009). Enough evidence has proven that miR-29a work

as an essential element in regulating differentiation, cell proliferation, as well as survival (Kole et al. 2011; Roshan et al. 2014; Swahari et al. 2021). A recent report identified the role of miR-29a in NPC differentiation and provided hope for the treatment of CNS damage (Gao et al. 2020). The NR2F2 gene (nuclear receptor subfamily 2, group F, member 2) is known to regulate neural differentiation in human ESCs. It has been shown that Oct4 and miR-302 work as a regulatory loop with NR2F2 during embryonic stem cell differentiation (Rosa and Brivanlou 2011).

Conclusion

MiRNAs offer intriguing possibilities as biological regulators of gene expression, affecting the ability of transcripts to produce new proteins. They may have several targets and the ability to alter biological processes through various molecular pathways. A compilation of studies spanning a wide range of species and endpoints provides compelling evidence that altered miRNA function is linked with ageing and age-associated diseases. Many miRNAs have shown differential expression with ageing across several tissues and organs in mammals, and some were shown to govern pathways related to age-linked diseases, such as sarcopenia, cognitive impairment, bone loss, skin ageing, and heart dysfunction. Also, restoring Dicer levels as well as diurnal rhythms of Dicer-controlled RNA and miRNA expression levels may pave the way for new therapeutic opportunities for ageing-related problems. The key role of miRNAs in stem cells has also been studied in a number of biological pathways, such as differentiation, proliferation, and self-renewal. There is no doubt that many more miRNAs that govern ageing will be discovered as new functions for miRNAs are recognized on a regular basis. Despite this, many fundamental issues about the role of miRNAs in ageing remain unanswered. At cell, tissue, and organism level, there seem to be differences between reports of overall upregulation and downregulation of miRNAs, and a combination of both throughout ageing. It will be highly critical to determine whether distinct miRNAs are triggered or inhibited in certain conditions. Numerous miRNAs target genes that enhance lifespan, while others target genes that shorten lifespan; hence, miRNAs as a group of regulatory molecules do not appear to have a specific effect on ageing. Individual miRNAs, on the other hand, help to speed up or slow down ageing under specific situations. Hence, a much more focused investigation into the involvement of miRNAs in ageing is clearly needed. The expression and function of miRNAs are tissue-specific, which adds to the complexity of understanding their role in ageing, especially in mammalian species. As a result, examining how miRNAs regulate ageing in various tissues will contribute to uncovering possible targets for epigenetic reprogramming and possibly renewal of aged cells and humans, as well as providing a deeper knowledge of ageing process itself.

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Chapter 10

CircRNA and Ageing



Ebrahim Mahmoudi and Murray J. Cairns

Abstract Circular RNAs (circRNAs) are closed-loop RNA transcripts formed by a noncanonical back splicing mechanism. circRNAs are expressed in various tissues and cell types in a temporospatially regulated manner and have diverse molecular functions including their ability to act as miRNA sponges, transcriptional and splicing regulators, protein traps, and even templates for polypeptide synthesis. Emerging evidence suggests that circRNAs are themselves dynamically regulated throughout development in various organisms, with a substantial accumulation during ageing. Their regulatory roles in cellular pathways associated with ageing and senescence, as well as their implications in ageing-related diseases, such as neurological disease, cancer, and cardiovascular disease, suggest that circRNAs are key molecular determinants of the ageing process. Their unique structure, expression specificity, and biological functions highlight a potential capacity for use as novel biomarkers for diagnosis, prognosis, and treatment outcomes in a variety of conditions including pathological ageing. CircRNA may also have potential as target for interventions that manipulate ageing and longevity. In this chapter, we discuss the most recent advances in circRNA changes in ageing and ageing-associated disease.

Keywords circRNA · miRNA · Ageing · ceRNA networks · Translation · Transcription · Gene regulation

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Introduction

Ageing is a gradual and continuous process associated with progressive changes in the biology, physiology, psychology, and behavior of an organism. These changes lead to a functional and structural decline in the body, and therefore an increasing vulnerability to disease or disability (MacNee et al. 2014; Partridge et al. 2018). At the biological level, ageing is associated with the dynamic impact of accumulating molecular and cellular damage caused by environmental exposure, intrinsic challenges, and impaired checks-and-balance systems (Rodríguez-Rodero et al. 2011). Studies in a variety of species have identified a set of “hallmarks” that provide a framework to better understand the physiology and pathology of the ageing process. These include genomic instability, telomere attrition, epigenetic modification, and loss of proteostasis. These processes can lead to impairment in cellular growth and communication, responses to cellular stress and the immune system, and changed mitochondrial function (López-Otín et al. 2013).

Epigenetic changes are modifications that impact gene expression through various mechanisms including DNA methylation, chromatin remodeling, and noncoding RNA (ncRNA) (Stamatovic et al. 2019). Both small and long ncRNAs, such miRNA and lncRNA, have been shown to be critical regulatory molecules that modify the rate of gene transcription and modulate their translation and stability through posttranscriptional mechanisms (Wei et al. 2017). An emerging class of lncRNA are covalently closed circles known as circular RNA (circRNA) (Memczak et al. 2013). While these highly abundant molecules were long thought to be nonsignificant by-products of mRNA splicing, the advent of high throughput RNA sequencing technology has enabled systematic identification of circRNA and the further observation that these intriguing molecules have unique properties that can support biological functions (Memczak et al. 2013; Rybak-Wolf et al. 2015). circRNA are formed by back-spliced transcripts produced by joining the exon termini using canonical spliceosome machinery. These transcript isoforms have been discovered in many organisms, from simple species such as fungi to more complex species such as plants and animals. circRNAs are conserved and display high levels of stability (Wang et al. 2014). In humans, they are present across the whole body but show enrichment in the brain (Rybak-Wolf et al. 2015). circRNA investigations have suggested there are several important biological functions carried out by these molecules. In this chapter, we describe the molecular biology of circRNA and discuss their regulatory roles in the ageing process and ageing-related diseases.

Biogenesis of circRNA

circRNA molecules are synthesized from pre-mRNA through a covalent joining of the 3' and 5' ends of exons, which is carried out by the canonical spliceosome. The main back-splicing mechanism is through intron pairing, where complementary

sequences within the introns are aligned to form loop structures that promotes circularization. This is mediated by inverted repetitive sequences known as Alu elements that are enriched within the introns flanking circularized exons (Jeck et al. 2013). More studies have shown back-splicing is also driven by RNA-binding proteins (RBPs) such as Quaking homolog, KH domain RNA binding protein (*QKI*) (Conn et al. 2015) and Muscleblind-like protein (*MBL*) (Ashwal-Fluss et al. 2014), as well as Fused in Sarcoma (FUS) (Errichelli et al. 2017) that bind to the specific motifs in introns leading to the formation of circular transcripts. In addition, nuclear factor 90 (NF90) and nuclear factor 110 (NF110) were shown to promote the formation of circRNA through stabilizing intronic RNA pairs (Li et al. 2017b). Back-splicing, however, is negatively regulated by antagonists such as Adenosine Deaminase Acting on RNA 1 (ADAR1) and ATP-dependent RNA helicase A (DHX9) that have been shown to suppress circRNA formation (Rybak-Wolf et al. 2015). These genes specifically bind to the duplexed RNAs formed by the pairing intron and destabilize the stem structure, resulting in suppression of back-splicing events. circRNA can also be generated through lariat formation. Lariats, which are typically small lasso-shaped RNA molecules, are formed during canonical splicing when RBPs splice two exons skipping over the exons between them. This generates two products: (1) a truncated linear product, and (2) a lariat which is then processed into a circRNA (Ebbesen et al. 2016).

Diversity and Types of circRNA

According to the circRNA database CSCD, more than one million unique circRNA species have been predicted in the human genome (Xia et al. 2018). CircRNA are found throughout the genome, with the protein-coding genes producing the largest proportion of circRNA species (Rybak-Wolf et al. 2015). The majority of circRNAs have a length of a few hundred nucleotides which mainly contain coding DNA sequence segments (Szabo and Salzman 2016). Analysis of circRNAs revealed a high level of alternative splicing occurring within these transcripts (Gao et al. 2016; Mahmoudi et al. 2021). CircRNA are categorized into three types; exonic (ecircRNA), exon–intron (ElcircRNA), intronic (ciRNA), and intergenic. These are unequally distributed in subcellular compartments, ElcircRNA and ciRNA are enriched in the nucleus while ecircRNA are transported to the cytoplasm (Zhang et al. 2019b). circRNAs, unlike other RNAs, are significantly more stable due to their covalently closed structure without 5' or 3' ends, that results in a high resistance to exonuclease-mediated degradation (Jeck and Sharpless 2014). It has been shown that many identified circRNA transcripts exist across multiple species and organs (Barrett and Salzman 2016), indicating conservation of these molecules and also suggesting important roles of circRNAs in a variety of organisms.

Expression Patterns of circRNA

RNA-seq with either rRNA-depleted total RNA, polyA(-) enriched RNA, or RNAase R treated RNA has been the main method of systematic detection and quantification of circRNA transcripts. circRNA account for an estimate of 0.2–1% of the transcriptome (Guo et al. 2014; Salzman et al. 2013). They are generally expressed at low levels, which is comparable with lincRNAs and of lower abundance than protein-coding transcripts. However, the abundance of individual circRNA vary from very low to very high, with hundreds of circRNAs found significantly more highly expressed relative to their corresponding linear transcript, and even some loci exclusively produced as circular isoforms (Ji et al. 2019).

CircRNAs are dynamically expressed in various cell types, tissues, and organs in a temporospatial manner (Mahmoudi and Cairns 2019). Tissue specificity of circRNAs was shown by Ji et al. to be considerably higher than for protein-coding genes (64% of circRNAs versus 15% of coding genes were highly tissue-specific). They further revealed that the observed pattern was regulated by RBPs that associate with circRNA formation, such as QKI, ADAR, and DHX9. Studies in the mammalian brain have shown that circRNA are more enriched in this organ compared to others (Rybäk-Wolf et al. 2015). In addition to the mature tissues, circRNA are highly expressed in stem cells. Initially, it was found that eleven circRNAs were specifically expressed in human embryonic stem cells (hESCs), which were also validated in induced pluripotent stem cells (iPSCs) (Cao et al. 2018). High-throughput profiling of circRNA in bone marrow stem cells (BMSCs) revealed thousands of circRNAs were expressed in these cells, some of which were regulated after differentiation (Zhang et al. 2019a). Transcriptome analysis in mice showed over 18,000 circRNAs were expressed in male/female germline stem cells (Li et al. 2017a). Moreover, circRNAs were highly expressed in mouse neuronal stem cells, 37 of these were regulated during the neuronal stem cell differentiation (Yang et al. 2018a).

Function of circRNA

circRNAs have now been established as regulatory molecules that exert their functions at different molecular levels including transcriptional, post-transcriptional and post-translational stages. This broad level of action along with their diversity in function make circRNA a distinct group with a versatile role that is not typical in other RNAs. In the subsections below, we review the current evidence for their function.

CircRNAs as Regulators of Transcription and Alternative Splicing

The current evidence suggests that circRNA play a *cis*-regulatory role on their parent coding genes. A primary study by Li et al. revealed that EIciRNAs interact with promoters through U1 snRNP and RNA Polymerase II (Pol II), promoting gene transcription (Li et al. 2015b). It was shown that circSMARCA5 can introduce a transcriptional pause at exon 15 of SMARCA5 by binding to the gene locus and forming an R-loop, which is a complex hybrid DNA–RNA structure between the gDNA and the circRNA, resulting in decreased expression of SMARCA5 transcript and production of a truncated nonfunctional protein (Xu et al. 2020). Similarly, *circSEP3*, derived from exon 6, can bind to its host DNA locus and form an R-loop, through which it causes transcriptional pausing and induces the expression of the cognate exon6-skipped alternative splicing variant of SEP3 (Conn et al. 2017). Zhang et al. have provided evidence to indicate ciRNAs, spliced from lariats, can accumulate at their synthesis sites and promote the expression of cognate genes through regulating elongating Pol II activity (Zhang et al. 2013). For example, ci-ankrd52, was shown to accumulate at its transcription sites and interacting with Pol II, resulting in positive regulation of *ankrd52* transcription (Zhang et al. 2013). Another study revealed that circMbl, spliced from the splicing factor MBL, competes with canonical splicing of the cognate gene through binding to MBL protein and modifying its abundance, which leads to altered mRNA splicing (Ashwal-Fluss et al. 2014).

miRNA Sponges

There is increasing evidence to suggest that circRNA contains binding sites for miRNA, and these binding sites can sequester miRNA and suppress their activity. This allows circRNA to compete with mRNA for miRNA binding, thus acting as competitive endogenous RNA (ceRNA). The most established exemplar is CDR1 Antisense RNA gene (CDR1as), which contains more than 70 conserved binding sites for miR-7. Reduced expression of CDR1as resulted in decreased levels of mRNA targets of miR-7, supporting the capacity of CDR1as as a functional inhibitor (Memczak et al. 2013; Hansen et al. 2013; Hansen et al. 2011). This function was conserved across tissues and species; expression of human CDR1as in zebrafish disrupted development of midbrain, which was similar to the phenotype observed for miR-7 downregulation (Memczak et al. 2013). A testis-specific circRNA, circSRY, harbors 16 target sites for miR-138 in mouse, and was experimentally confirmed to interact with the miRNA (Hansen et al. 2013). Some circRNAs might, however, contain target sites for multiple different miRNAs rather than a single molecule, resulting in inhibition of a group of miRNAs. For example, circHIPK3 can sponge nine miRNAs, including miR-124, it inhibits miR-124, and regulates human cell

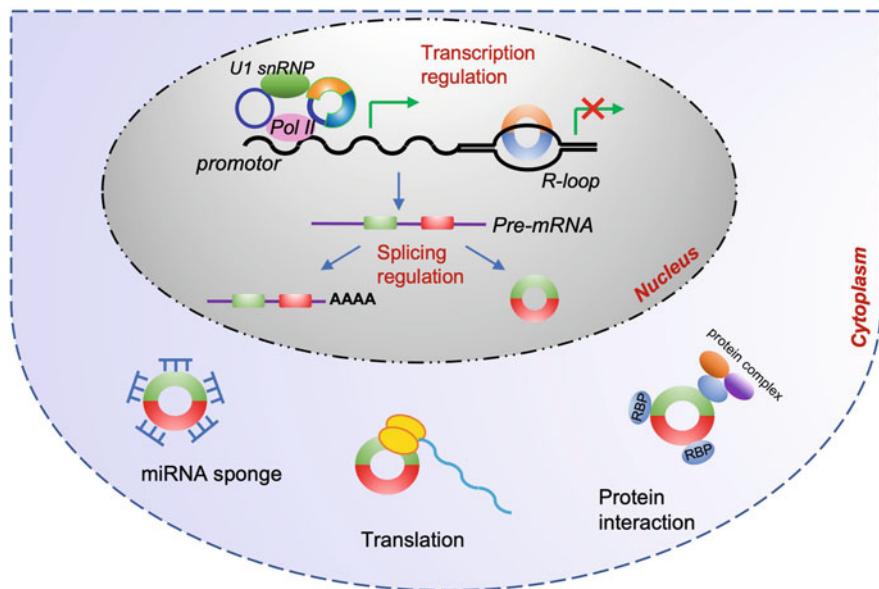


Fig. 10.1 A diagrammatic representation of the nuclear and cytoplasmic interaction of circRNA with miRNA

growth (Zheng et al. 2016). Similarly, circTCF25 was observed to sponge miR-103a-3p/miR-107, resulting in increased expression of several target genes associated with cell proliferation, migration, and invasion (Zhong et al. 2016). Depletion of circRNA-CER inhibited MMP13 expression through regulation of miR-136, leading to the degradation of chondrocyte ECM (Liu et al. 2016). Several investigations on circMTO1 indicated its roles in various cancer pathways through inhibition of its target miRNAs including miR-9, miR-223, miR-630, and miR-92 (Li et al. 2020; Zhang et al. 2019c). A recent report by Bronisz et al. indicated that *circ2082* expression leads to aberrant nuclear localization of DICER, a complex responsible for miRNA maturation, which in turn causes malfunctions in the post-transcriptional maturation of the glioblastoma miRNAome and thus tumorigenicity of glioblastoma cells *in vitro* and *in vivo* (Bronisz et al. 2020).

It has been suggested that the interaction of circRNA with miRNA may act beyond inhibitory activity; for example, it may also associate with sorting, localization, and storage of miRNA molecules (Hansen et al. 2011; van Rossum et al. 2016), see Fig. 10.1.

Modulator of Protein Activity and Expression

circRNAs are capable of modifying the activity of RNA-binding proteins (RBPs) and protein complexes by direct binding. For instance, circPABPN1 can target HuR, an RBP which is a regulator of protein expression, and suppress its binding to PABPN1 mRNA. This leads to decreased translation of PABPN1 (Abdelmohsen et al. 2017). It was shown that interaction of circCcnb1 with Ccnb1 and Cdk1 proteins inhibited the formation of the Ccnb1–Cdk1 complex, which leads to dysfunction of Ccnb1 and thus inhibition of tumor growth (Fang et al. 2019). Furthermore, circCcnb1 can regulate cell death through association with H2AX and wild-type p53, or otherwise forming a complex with H2AX and Bclaf1 (Fang et al. 2018a). circRNA are also implicated in assembly and activation of protein complex, such as in case of circACC1 which increases activation of the AMP-activated protein kinase (AMPK) complex during metabolic stress (Li et al. 2019). In addition, circRNA may contribute to the intracellular localization and transport of proteins; circAmotl1 was shown to associate with c-myc and enhance its binding affinity to a number of promoters, thus regulating the expression of their target genes (Bronisz et al. 2020). Finally, it was suggested that circRNA are capable of regulating protein expression. For example, circYap was shown to negatively modulate protein production of its cognate gene through inhibiting the assembly of translation initiation machinery, without impacting the mRNA levels (Wu et al. 2019). Similarly, circPABPN1 was indicated to compete with its corresponding mRNA for RBPs, through which it modulates translation rate of its target mRNA (Abdelmohsen et al. 2017).

Encoding Functional Proteins

Although circRNA have largely been considered as noncoding molecules, several reports have recently discovered that certain circRNA are, indeed, able to produce functional proteins. Pamudurti and colleagues provided evidence for a group of circRNAs able to be translated into proteins within *Drosophila* heads (Pamudurti et al. 2017). Particularly, circMbl protein was detected in mass spectrometry data which was found enriched in synaptosomes of fly heads. circSHPRH was also shown to produce a protein that was highly expressed in human brains and found dysregulated in glioblastoma (Zhang et al. 2018a). Similarly, circZNF609 was revealed to encode a protein in murine and human myoblasts, capable of modifying myoblast proliferation in response to stress (Legnini et al. 2017). Other examples include circFBXW7 and circLINC-PINT that were reported to serve as protein templates in brain tumors (Yang et al. 2018b; Zhang et al. 2018b), raising the possibility of additional coding circRNAs that are yet to be identified. As the identification of circRNA translation largely relies on back-spliced tags, it seems necessary to introduce novel approaches to systematically discover translated

circRNA. While the precise mechanism of their translation is not yet known, it has been suggested that the presence of some elements such as artificial internal ribosomal entry sites (IRES) and infinite open reading frames (iORF, where there is no stop codon on the circRNA) may promote their coding potential, possibly in a cap-independent manner.

circRNA in Ageing

Expression profiling studies have provided evidence that indicate circRNA abundance significantly increases throughout the lifespan in various organisms, and their accumulation has been demonstrated specifically in brain tissues, suggesting a biological role for circRNAs in the ageing process. These roles have been evident by following studies where circRNA were shown to associate with various ageing pathways whose impairment could lead to pathological conditions. As evidence, there have been many age-related diseases in human that are associated with circRNA expression, such as the case for Alzheimer's disease (AD), Parkinson's disease (PD), and diabetes.

circRNA Transcripts Accumulate During Ageing

A primary study by Gruner et al. profiled circRNA in the cortex, hippocampus, and heart of 1- and 22-month old mice, revealing a global increase of circRNA abundance in brain tissues of old mice as compared to the young ones (Gruner et al. 2016). However, the authors reported no change in aged heart circRNA, suggesting a tissue-specific age accumulation of circRNA. Investigation of circRNA in *C. elegans* during ageing, from the fourth larval stage through 10-day-old adults, displayed a significant accumulation of circRNA among the ageing time-points, with some transcripts observed to have over 40-fold change (Cortés-López et al. 2018). Notably, several of these circRNAs were produced from genes involved in the determination of lifespan (Cortés-López et al. 2018). A comprehensive profiling of circRNA was performed in *Drosophila* covering various developmental stages, tissues, and cultured cells. The results uncovered an increase in circRNA accumulation across embryonic development, enhanced accumulation in larval/pupal CNS compared to other tissues of these stages, and a predominant accumulation of circRNA in adult CNS relative to all other samples (Westholm et al. 2014). The study suggested the observed progressive accumulation may affect brain function, and also represent promising ageing biomarkers in the CNS. Similarly, transcriptome analysis of circRNA in whole body of ageing rat confirmed a substantial increase of circRNA abundance with age in the rat brain relative to other tissues (Mahmoudi and Cairns 2019; Zhou et al. 2018).

Mechanisms of circRNA Accumulation

The above-mentioned studies demonstrated that circRNA age accumulation is independent of their cognate linear isoforms, indicating they are not the consequences of higher transcription of their host genes. While the mechanisms underlying the circRNA accumulation are not clearly known, it has been suggested that the closed structure of circRNA transcripts that lack a free 3' and 5' end allows them to escape molecular digestion by cellular exonuclease (Jeck and Sharpless 2014). The resulting stability was shown to be significantly higher than the linear counterpart, with the half-life estimated to be over 48 hours (Jeck and Sharpless 2014). In addition, it has been proposed that changes in alternative splicing during ageing could also associate with enhanced back-splicing events (Cortés-López and Miura 2016). This contribution seems plausible, particularly given the recent reports implicating splicing factors FUS (Errichelli et al. 2017) and QKI (Conn et al. 2015) in biogenesis of circRNA.

Biological Roles of circRNA in Ageing

Characterization of circRNA functions in ageing have revealed biological significance of these molecules in the physiology of ageing and cellular senescence. For instance, circPVT1 expression can inhibit cellular senescence by sequestering miRNA let-7 and increasing its target genes, KRAS, IGF2BP1, and HMGA2, that are proliferative proteins (Panda et al. 2017). circFOXO3 was shown to sequester proteins, including ID-1, E2F1, FAK, and HIF-1a, which are implicated in cellular stress pathways and induced cardiac senescence (Du et al. 2017). It was found that circRNA-0077930 induced the senescence of vascular smooth muscle cells in human umbilical vein endothelial cell exosomes by suppressing miR-622 and inducing Kras, p21, p53, and p16 expressions (Wang et al. 2020b). A study of circRNA in muscle ageing and exercise in mice revealed that circBBS9 and its target gene pathways were altered by the advance of age but restored by aerobic exercise (Guo et al. 2020). Furthermore, overexpression of circBBS9 in differentiated C2C12 myotubes induced the genes involved in the predicted pathways and decreased muscle atrophic genes including Foxo3 and Atrogin, as well as elevated functional mitochondrial genes, Pgc1 α , Mfn1, and Atpase. The authors suggested that circBBS9 overexpression enhances muscle functionality gene programs and may associate with the muscle ageing process. Transcriptome analysis in young and prematurely senescent human fibroblast 2BS cells discovered a senescence-associated circRNA, circCCNB1. Knockdown of this circRNA inhibited CCNE2 expression through sequestering miR-449a activity, which led to the triggered senescence in young 2BS fibroblasts, suggesting circCCNB1-miR-449a-CCNE2 axis as a key in cellular senescence (Yu et al. 2019). Cheng et al. explored circRNA alterations in human granulosa cells (GCs) during maternal ageing and uncovered

age-related circRNA variations by showing 46 upregulated and 11 downregulated circRNAs in advanced ageing samples (Cheng et al. 2017). Following gonadotropin treatment adjustment, they particularly found circRNA_103827 and circRNA_104816 levels were positively associated with maternal age. Furthermore, enhanced expressions of these circRNAs were related to declining ovarian reserve and adverse reproductive outcomes. Another report in ageing human blood found that the conserved circRNAs circDEF6, circEP300, circFOXO3 and circFNDC3B, are implicated in parental longevity or hand grip strength (Haque et al. 2020b). Moreover, circFOXO3 and circEP300 demonstrated dysregulated expression in human senescent cells. In addition, circPlekhn1 expression level in spleen was shown to be positively associated with mouse lifespan (Haque et al. 2020b). An exciting *in vivo* study has recently revealed a critical role for a circRNA encoded by sulfateless gene, known as circSfl, in ageing (Weigelt et al. 2020). circSfl was shown to extend the lifespan of *Drosophila* in long-lived insulin mutant flies, where overexpression of CircSfl resulted in increased lifespan, particularly in muscles and the brain. Further functional experiments indicated circSfl is translated into a peptide that is sufficient to extend lifespan (Weigelt et al. 2020). This study also indicated that neuronal circRNA accumulation with age is malleable and decreased in long-lived insulin mutants.

circRNA in Age-Associated Disease

Since ageing is associated with a functional decline of cells, tissues, and organs, it results in the occurrence of various related diseases, such as neurological disorders, cardiovascular diseases, cancer, and others. As key regulators of cellular functions, circRNA have been shown to play roles in ageing-related pathways, and hence represent a potential contributor to development of age-associated diseases. The main mechanisms through which circRNA might contribute to this is through the circRNA–miRNA–mRNA regulatory axis and by circRNA–protein interactions.

CircRNA in Neurological Disorders

circRNAs are enriched in central nervous system (CNS), and also accumulate with the progression of age, suggesting that they play physiological and pathological roles in ageing CNS. Lukiw reported a dysregulation of CDR1as (ciRS-7) expression in hippocampal CA1 region of patients with sporadic Alzheimer's disease (Lukiw 2013). Following studies found that reduced expression of CDR1as leads to an increased level of miR-7 which in turn suppresses the level of UBE2A, a protein essential in the clearance of amyloid peptides in Alzheimer's disease (AD) brain (Zhao et al. 2016). CDR1as expression was also shown to promote APP and BACE1 degradation in a nuclear factor- κ B (NF- κ B)-dependent manner (Shi et al. 2017).

Interestingly, the CDR1as-miR-7 role has also been detected in pathology of Parkinson's Disease (PD), where high levels of CDR1as sequester miR-7, resulting in an increased level of α -synuclein protein, which is conducive to PD. Reduced abundance of circRNA was observed in PD SN (substantia nigra), and differential expression analysis revealed circSLC8A1 was upregulated in SN of PD patients with a potential to regulate miR-128 activity (Hanan et al. 2020). CircSLC8A1 expression was also induced in cultured cells exposed to oxidative stress but reduced in cells treated with neuroprotective antioxidants suggesting a link between circSLC8A1 and oxidative stress-related Parkinsonism.

Wu et al. uncovered a critical role for circTLK1 in ischemic stroke, where high expression of circTLK1 results in inhibition of miR-335-3p activity, and thus increasing ADP-ribose polymerase expression and neuronal injury. Similarly, circDLGAP4 was reduced in the plasma of acute ischemic stroke patients and in a mouse stroke model, causing a reduction in miR-143 targeted sponging and the corresponding reduction of HECT Domain E3 Ubiquitin Protein Ligase 1 (HECTD1) expression, which is a target of miR-143 (Bai et al. 2018). circRNA-0067835 levels were decreased in tissues and plasma from temporal lobe epilepsy (TLE) patients, and it was shown to modulate refractory epilepsy progression by acting as a sponge of miR-155 to increase FOXO3a transcript level (Gong et al. 2018). It was demonstrated that circEFCAB2 and circDROSHA can act as sponges for miR-48-5P and miR-1252-5P, respectively, to regulate the expression of target genes and impact TLE pathogenesis (Li et al. 2018). Dysregulation of many other circRNAs was reported in mesial temporal lobe epilepsy (MTLE), and two circRNAs, circ_Arhgap4 and circ_Nav3, were found to regulate miR-6328 and miR-10b-3p levels, respectively (Gomes-Duarte et al. 2021).

CircRNA in Cardiovascular Diseases

Several lines of evidence have revealed the contribution of circRNA in the development of cardiovascular diseases such as coronary heart disease (CHD), atherosclerosis, heart failure, and myocardial infarction (Tang et al. 2021). For example, dysregulation of a total of 171 and 624 circRNAs were reported in patients with CHD as compared to control groups (Wang et al. 2019b). Increased levels of circWDR77 and circRUSC2 cause disordered proliferation and migration of vascular smooth muscle cells by targeting miR-124/FGF-2 and miR-661/ SYK axes, respectively, which lead to the induced pathological process of atherosclerosis (Chen et al. 2017a; Sun et al. 2019). It was shown that upregulation of circSLC8A1 inhibits miR-133a to induce the target genes of the miRNA in cardiomyocytes, indicating its implication in cardiac hypertrophy (Lim et al. 2019). Wang et al showed circ-HRCR could suppress cardiac hypertrophy and heart failure by sequestering miR-223 (Wang et al. 2016). Downregulation of circ-Fndc3b was observed in post-myocardial infarction heart tissue, where circ-Fndc3b interaction with FUS regulates the level of VEGF, resulting in modulation of cardiac repair after

myocardial infarction (Garikipati et al. 2019). In addition, circ-Hipk3 interaction with Notch1 protein, which elevated the stability of N1ICD, and also with miR-133a led to improved cardiac regeneration after myocardial infarction in mice (Si et al. 2020).

CircRNA in Cancer

Ageing is a major risk factor for cancer development, and due to their shared mechanisms, such as genomic instability, telomere attrition, and epigenetic changes, cancer is also considered an ageing disease (Aunan et al. 2017). Accumulating evidence have indicated an essential role for circRNA in development of various cancers such as glioblastoma, lung cancer, hepatocellular carcinoma (HCC), prostate cancer, and others. For instance, CircNT5E was found to induce glioblastoma tumorigenesis by inhibiting the activity of miR-422a and controlling cell proliferation, migration, and invasion (Wang et al. 2018). In lung cancer, circITCH acts as tumor suppressor by enhancing ITCH levels through sequestering oncogenic miR-7 and miR-214 and thus inhibiting the Wnt/β-catenin pathway (Wan et al. 2016). A report by Chen et al. revealed that circRNA production was associated with disease progression in patients with prostate cancer, with 11.3% of highly abundant circRNAs found essential for cell proliferation (Chen et al. 2019). Functional characterization of individual circRNAs also showed circCSNK1G3 can drive prostate cancer cell proliferation via interacting with miR-181, and impact the expression of CBX7, CDK1, and CDC25A. A regulatory mechanism by circRNA-MTO1 was demonstrated to control cell viability and monastrol resistance in breast cancer; upregulation of circRNA-MTO1 inhibited cell viability, enhanced monastrol-induced cell cytotoxicity, and reversed monastrol resistance by sequestering TRAF4 from activating Eg5 translation, thereby suppressing the Eg5 protein level (Liu et al. 2018). Efforts in HCC have shown circRHOT1, as an upregulated circRNA in HCC tissues, enhances HCC progression by recruiting TIP60 to the NR2F6 promoter and initiating NR2F6 transcription (Wang et al. 2019a). Ma et al. found a dramatic downregulation of hsa_circ_0004872 in gastric cancer tissues, which was associated with tumor size and local lymph node metastasis (Ma et al. 2020). While hsa_circ_0004872 induced p21 and Smad4 expression via sponging miR-224, Smad4 could further modulate hsa_circ_0004872 expression by binding to the ADAR1 promoter and decreasing its transcription, resulting in forming a miR-224/Smad4/ADAR1/hsa_circ_0004872 negative regulatory circuit to control cancer progression (Ma et al. 2020).

CircRNA in Diabetes

Due to the elevated insulin resistance and impaired pancreatic islet function with ageing, older adults are at high risk of developing type 2 diabetes, and circRNAs have been reported to play a pathological role in this disease. The expression analysis of circRNAs in peripheral leucocytes of patients with type 2 diabetes mellitus (T2DM) and healthy controls identified 220 differentially expressed circRNAs (107 upregulated and 113 downregulated) (Fang et al. 2018b). Further investigation showed circANKRD36 level was positively associated with glucose and glycosylated hemoglobin, as well as with IL-6 expression, suggesting circANKRD36 is involved in T2DM possibly through interaction with IL-6 targeting miRNAs, including hsa-miR-3614-3p, hsa-miR-501-5p, and hsa-miR-498 (Fang et al. 2018b). A study by Haque and colleagues indicated four circRNAs, including circCIRBP, circZKSCAN, circRPH3AL, and circCAMSAP1, were associated with diabetes status; CircCIRBP was involved in insulin secretory index in human islets, and circCIRBP and circRPH3AL were linked to increased fatty acid in treated EndoC-βH1 cells (Haque et al. 2020a). Moreover, CircCAMSAP1 expression was correlated with T2D status in human peripheral blood. It was found that Hsa_circ_0054633 is differentially expressed in peripheral blood of T2DM patients, and also presented promising diagnostic capability for pre-diabetes and T2DM (Zhao et al. 2017b).

CircRNA as Biomarkers for Ageing

The emerging evidence on the implication of circRNA expression in ageing and their biological regulatory roles in ageing processes and related diseases suggests diagnostic and prognostic values for circRNA in normal and pathological ageing. Special features include consistent accumulation in various animals, high stability and conservation, tissue and stage specificity, as well as their presence in accessible tissues such as plasma (Zhang et al. 2017), serum (Gu et al. 2017), saliva (Bahn et al. 2015), and exosomes (Li et al. 2015a) support consideration of circRNA as reliable biomarkers. A recent attempt to investigate the circRNA capability of age prediction in human blood using multiple machine learning models suggested that circRNAs are promising for age prediction (Wang et al. 2020a). Using the blood of thirteen unrelated healthy individuals aged 20–62 years, a strong upregulation was observed during ageing in human blood, and seven circRNAs were further validated in a larger cohort of 50 healthy subjects and subsequently used for final prediction models. The results suggested that circRNA may have application as indicators of ageing in forensic samples due to their unique degradation-resistant molecular structure (Wang et al. 2020a). circRNAs can also be biomarkers for age-related diseases that advance with age. In such cases, circRNA can facilitate early diagnosis and also determine the stage and subtype of the disease, which would be useful in

selecting the treatment options and curation of disorder. They may also be beneficial in prediction of treatment outcomes or used as treatment targets, resulting in the better management of disease. In support of this, experimental evidence has suggested that circRNA's may serve as diagnostic and prognostic factors. For instance, Cdr1as expression is differentially expressed in AD and PD patients relative to healthy controls. A diagnostic analysis using blood circUBXN7 and circLMO1 distinguished patients with gastric cancer from healthy group (Han et al. 2020). In HCC, plasma circRNA panels including circHPCAL1, circRABGGTA, and circMTM displayed a high diagnostic accuracy in detecting patients with cancer from healthy and HCC precursor control patients (Yu et al. 2020). Weng et al. found Cdr1as a prognostic biomarker in colorectal cancer patients (Weng et al. 2017). Also, circZKSCAN1 expression was related to poor prognosis and clinical stage in lung cancer (Wang et al. 2019c) and to good prognosis in bladder cancer (Bi et al. 2019). It was identified that circCNOT2 is a prognostic biomarker of aromatase inhibitor therapy in advanced breast cancer (Smid et al. 2019). In coronary artery disease <https://www.sciencedirect.com/science/article/pii/S0735109718336167>, blood hsa_circ_0124644 could discriminate CAD patients from healthy controls (Zhao et al. 2017a).

Current methodologies also support accurate measurement of circRNA which is essential for biomarker application. High-throughput sequencing, particularly with RNAase R depletion, along with circRNA-specific prediction algorithms enable a precise quantification of circular transcripts. In addition, RT-qPCR platform using divergent primers can take advantage of back-spliced junction tags to specifically and accurately quantify circRNAs at the individual level. It is also possible to costume design microarray chips or qPCR panels to detect the transcript's level for a set of circRNAs of interest.

Perspectives and Conclusions

Emerging evidence that circular RNA transcripts increase with age in various animals, suggests they may have a conserved role in ageing and lifespan. However, their regulatory mechanism is very much an open question. We also do not know if circRNAs contribute causally to the ageing process, or if they have protective or pro-ageing effects. Understanding these impacts would allow perturbation of circRNA molecules in order to potentially slow down ageing process and increase lifespan. In support of this, it was shown that ectopic expression of circSfl resulted in extended lifespan of *Drosophila* in long-lived insulin-mutant flies (Weigelt et al. 2020).

Despite comprehensive analyses of circRNAs in various animal models of ageing, the circRNA transcriptome has not been explored in human ageing. This should be a high priority for the field as it would shed light on the contribution of circRNA to the genomic hallmarks of ageing. In addition, study of circRNA

alteration in ageing humans could also help identify ageing markers which would be beneficial in human forensic investigations.

Given the implication of circRNA expression in age-associated disease, it is plausible that they could serve as novel molecular markers for diagnosis, prognosis, therapeutic evaluation, and also as treatment agents. Several studies in cancer and heart disease have supported the idea that circRNA levels in circulating body fluids have predictive capabilities to distinguish patients from healthy individuals (Han et al. 2020; Yu et al. 2020; Weng et al. 2017; Wang et al. 2019c; Bi et al. 2019; Smid et al. 2019; Zhao et al. 2017a). Chen and colleagues showed that circPVT1 was an independent marker for overall survival and disease-free survival of patients with gastric cancer (Chen et al. 2017b). Specifically, cell- and stage-specificity of circRNA may also extend the clinical application of these molecules to identifying subtypes or phases of development of human disease. For example, circRNA expression was found associated with three subtypes of breast cancer (Nair et al. 2016). Considering the fast development of circRNA studies along with specific technical methods, as well as the promising outcomes so far, it is credible to anticipate that there will be more circRNA-based clinical approaches introduced in the future.

In conclusion, circRNAs appear to be key molecules in regulation of ageing physiology as well as in pathological conditions associated with ageing. They exert their functions through diverse regulatory mechanisms, particularly miRNA sponging. However, similar to other fields, circRNA investigations in ageing are still in their infancy, and the functional relevance of the majority of circRNAs is yet to be uncovered in ageing process.

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Chapter 11

Extracellular Vesicles and Cellular Ageing



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and Consuelo Borrás

Abstract Ageing is a complex process characterized by deteriorated performance at multiple levels, starting from cellular dysfunction to organ degeneration. Stem cell-based therapies aim to administrate stem cells that eventually migrate to the injured site to replenish the damaged tissue and recover tissue functionality. Stem cells can be easily obtained and cultured in vitro, and display several qualities such as self-renewal, differentiation, and immunomodulation that make them suitable candidates for stem cell-based therapies. Current animal studies and clinical trials are being performed to assess the safety and beneficial effects of stem cell engraftments for regenerative medicine in ageing and age-related diseases.

Since alterations in cell–cell communication have been associated with the development of pathophysiological processes, new research is focusing on the modulation of the microenvironment. Recent research has highlighted the important role of some microenvironment components that modulate cell–cell communication, thus spreading signals from damaged ageing cells to neighbor healthy cells, thereby promoting systemic ageing. Extracellular vesicles (EVs) are small-rounded vesicles released by almost every cell type. EVs cargo includes several bioactive molecules, such as lipids, proteins, and genetic material. Once internalized by target cells, their specific cargo can induce epigenetic modifications and alter the fate of the recipient cells. Also, EV’s content is dependent on the releasing cells, thus, EVs can be used as biomarkers for several diseases. Moreover, EVs have been proposed to be used as cell-free therapies that focus on their administration to slow or even reverse some hallmarks of physiological ageing. It is not surprising that EVs are also under study as next-generation therapies for age-related diseases.

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Intercellular Communication

Homeostasis and Intercellular Communication

Intercellular communication is a biological process necessary for the maintenance of homeostasis in the organism that relies on coordination between cells to complete their functions. It is crucial in the development, differentiation, and adaptation of cells to their environment (Armingol et al. 2021; Mittelbrunn and Sánchez-Madrid 2012; Song et al. 2019). Alterations in cell–cell communication are implicated in the development of pathophysiological processes in the organism such as tumorigenesis, metastasis, autoimmunity, senescence, and also in physiological processes such as ageing (Yang et al. 2021); concepts that we will develop in the chapter.

Intercellular communication generally requires the exchange of biochemical substances (ions, metabolites, hormones, cytokines, etc.) between them. This type of communication is called biochemical communication. There are two types of biochemical communication: direct and indirect (Fig. 11.1).

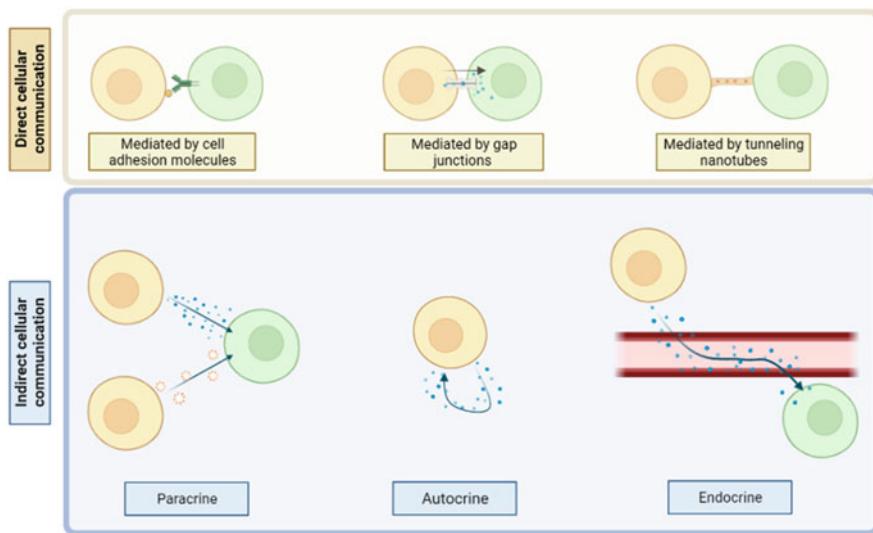


Fig. 11.1 The two types of biochemical communication. Direct cell communication is mediated by cell adhesion molecules, gap junctions, or by tunneling nanotubes. Indirect cellular communication includes paracrine, autocrine, and endocrine signaling. Effector cells (yellow) transmit signaling molecules to target cells (green). Created with [BioRender.com](#)

- Direct, contact-dependent, or juxtacrine communication occurs between neighboring cells near each other in which their membrane structures facilitate the exchange of molecules without these being secreted into the extracellular space. In turn, there are three direct biochemical communication pathways:
 - Communication mediated by cell adhesion molecules (selectins, integrins, cadherins, and immunoglobulins).
 - Communication mediated by gap junctions.
 - Communication mediated by tunneling nanotubes, which are thin projections of the plasma membrane.
- Indirect communication is characterized by the secretion of signaling molecules that are transported by flow or diffusion. These molecules are secreted freely and can either diffuse passively across the membrane (hydrophobic small molecules) or be transported into the cytoplasm of the target cell by endocytosis (hydrophilic molecules). However, there is another type of indirect communication in which the secretory cell releases extracellular vesicles containing the signaling molecules. Depending on the distance between the secretory cell and the target cell, indirect communication can be classified as follows:
 - Paracrine, in which communication only affects cells in the immediate neighborhood of the secretory cell.
 - Autocrine, in which the signals are directed to the cells that secrete them.
 - Endocrine, long-distance communication in which signaling molecules travel through the systemic circulation to reach the target cells (Armingol et al. 2021; Yang et al. 2021).

In addition to biochemical communication, there is also mechanical communication. Here, cells communicate with their surroundings by exerting mechanical forces on the extracellular matrix. These signals are involved in various processes such as cytoskeleton reorganization and cell migration (Van Helvert et al. 2018), matrix remodeling (Swartz et al. 2001), epigenetic modifications (Yang et al. 2021), or recruitment of immune cells to the tissue (Pakshir et al. 2019) among others.

Intercellular Communication and Ageing

Ageing involves changes in cell–cell communication, mainly due to increased cellular senescence. Senescence is implicated not only in ageing but also in age-related diseases such as cancer, arthritis, atherosclerosis, and Alzheimer's disease. The main characteristic of senescence is the stable arrest of the cell cycle; however, its influence on tissue homeostasis and the development of age-related pathologies is due to the Senescence-associated secretory phenotype (SASP). SASP can be considered a type of intercellular communication specific to senescent cells that are characterized by abundant secretion of molecules of diverse nature such as

cytokines, growth factors, chemokines, and matrix metalloproteinases. Recently, new components of the SASP such as extracellular vesicles, metabolites, and ions have been discovered (Cuollo et al. 2020; Fafian-Labora and O'Loghlen 2020; López-Otín et al. 2013).

It should be noted that the profile of secreted molecules of SASPs is diverse and depends on the tissue context. Sometimes SASP plays a beneficial role in tissue physiology as it is involved in the removal of senescent cells from tissue and is thus related to the maintenance of homeostasis and tissue regeneration. In addition, SASP is involved in the recruitment of innate and adaptive immune cells in the vicinity of tumor cells and premalignant lesions (Cuollo et al. 2020; Fafian-Labora and O'Loghlen 2020; Olivieri et al. 2018). SASP components involved in these functions are regulated by p53 and include nerve growth factor inducible (VGF), or insulin-like growth factor-binding protein (IGFB) (Lopes-Paciencia et al. 2019).

Despite this, ageing increases the number of senescent cells and alters the type of molecules secreted from the SASP and thus intercellular communication. In this situation, SASP plays a detrimental role in tissues and is associated with the development of age-related diseases (Cuollo et al. 2020; Olivieri et al. 2018). The components of SASP in ageing can be classified as pro-inflammatory, pro-oncogenic, paracrine senescent, and autocrine senescent.

SASP and Its Pro-inflammatory Components: Age-Related Inflammation

The C/EBP family of transcription factors and mainly the NF- κ B factor regulate the secretion of cytokines and pro-inflammatory factors such as Interleukin-1 β (IL-1 β), IL-6, IL-7, IL-8, chemokine “C-X-C motif” ligand 1 (CXCL1), or transforming growth factor β 1 (TGF β 1) among others, which contribute to the development of a chronic and systemic inflammation process known as “inflammageing” (Franceschi and Campisi 2014). This inflammageing situation contributes to the development of age-related diseases such as obesity, type 2 diabetes, atherosclerosis, and Alzheimer's disease. In addition, inflammageing and stress-mediated inflammatory responses are associated with activation of the NF- κ B pathway in the hypothalamus and induce a signaling pathway that results in reduced production of gonadotropin-releasing hormone (GnRH). Decreased GnRH has been shown to contribute to age-related physiological alterations including bone fragility, muscle weakness, skin atrophy, and reduced neurogenesis (Zhang et al. 2013).

Other studies, such as those of Pont and colleagues, link inflammation to ageing through the AUF1 factor (Pont et al. 2012). This factor is responsible for the degradation of the messenger RNA of pro-inflammatory cytokines, including tumor necrosis factor α (TNF α) and IL-1 β (Lu et al. 2006), and is therefore involved in halting the inflammatory response. In addition, AUF1 has also been found to be involved in the activation of the expression of the catalytic subunit of telomerase, which means that it contributes to maintaining telomere length. A reduction in the

expression of this factor, therefore, contributes to inflammeaging, increased cellular senescence, and premature ageing.

At the same time as the age-related chronic inflammation situation, the function of the immune system is increasingly impaired in ageing. This makes it more difficult to effectively eliminate pathogens and dysfunctional cells from tissues such as senescent cells. This leads to an accumulation of senescent cells in tissues resulting in an increase in pro-inflammatory cytokines and thus chronic inflammation.

SASP and Pro-oncogenic Components

The SASP in ageing is also formed by enzymes involved in the reorganization of the extracellular matrix such as the matrix metalloproteinases (MMPs). These enzymes, in addition to reorganizing the extracellular matrix, are responsible for processing chemokines in tissues. Therefore, the secretion of these proteases reduces the recruitment of macrophages that are responsible for eliminating senescent cells, thus increasing senescence in tissues (Fig. 11.2). In addition, this results in reduced

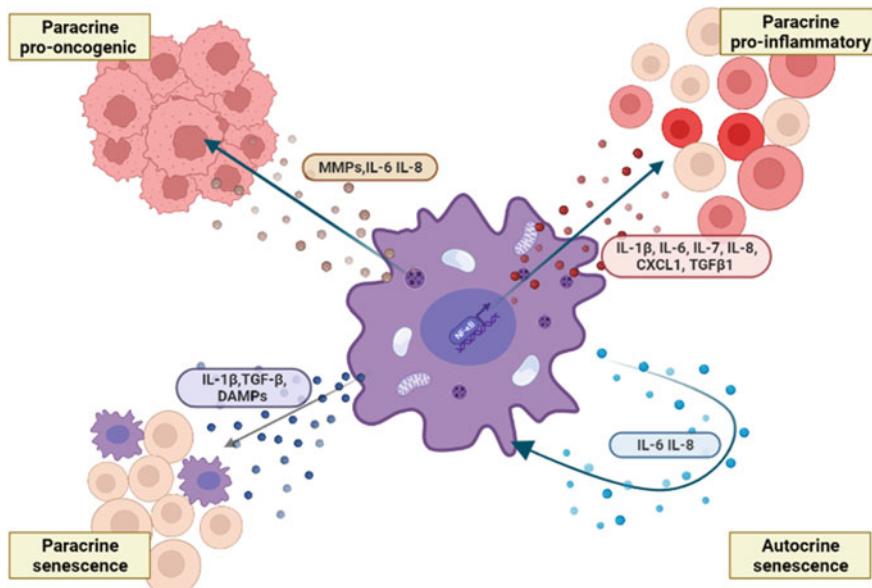


Fig. 11.2 Types of SASP components. SASP components that mediate autocrine senescence are interleukin-6 (IL-6) and -8 (IL-8). SASP components that promote paracrine senescence include interleukin-1 β (IL-1 β), transforming growth factor- β 1 (TGF- β 1), and damage-associated molecular patterns (DAMPs). SASP components can also spread pro-oncogenic effects through matrix metalloproteinases (MMPs), IL-6, and IL-8; as well as pro-inflammatory effects through IL-1 β , IL-6, IL-7, IL-8, chemokine “C-X-C motif” ligand 1 (CXCL1), and TGF- β 1. Created with BioRender.com

immune surveillance which, together with the immunosenescence characteristic of ageing, results in the body's inability to detect cells on the verge of malignant transformation (Lopes-Paciencia et al. 2019; López-Otín et al. 2013). IL-6 and IL-8 have also been found to have protumorigenic effects on cells in the vicinity of the senescent cell. That is, paracrine protumorigenic activity (Kuilman et al. 2008; Pietras et al. 2013). Consequently, SASP can also lead, in addition to chronic inflammation, to the development of tumors.

SASP and Paracrine Senescence

Acosta et al. (2013) demonstrated that there is a paracrine transmission of senescence from senescent cells with SASP to neighboring normal. However, this can spread locally with limitations, as the authors obtained results indicating that controls may exist to prevent the uncontrolled spread of senescence through an organ or tissue. The main components of SASP capable of triggering paracrine senescence are IL-1 β and TGF- β (Pont et al. 2012). Senescent cells can also secrete damage-associated molecular patterns (DAMPs) capable of inducing paracrine senescence (Lopes-Paciencia et al. 2019).

SASP and Autocrine Senescence

The factors IL-6 and IL-8, which are part of the SASP, are capable of forcing cell cycle arrest of the senescent cell that secretes them, accentuating the senescence of this cell (Lopes-Paciencia et al. 2019; Pietras et al. 2013).

In short, ageing is a process that not only affects cells but also their communication. It is worth noting that there are currently therapeutic strategies that act in this sense, senomorphics. These are drugs that neutralize the detrimental effects of cell communication in ageing, i.e., they reduce SASP without killing senescent cells as senolytics do. Senomorphics, therefore, offers an opportunity to modulate and mitigate the deleterious effects of ageing at the level of cell communication. Despite this, intercellular communication is very complex, highlighting the need for further research in this field to improve the prospects for patients suffering from these pathologies.

Stem Cells for Regenerative Medicine

Introduction to Stem Cells

A stem cell is presented as one that divides asymmetrically to produce a copy of itself and a second cell that is on its path to differentiate (Martinez-Agosto et al.

2007). The definition of a stem cell inevitably requires an assessment of its potential to give rise to several differentiated progenies. Therefore, according to their potency, stem cells can be divided into totipotent, pluripotent, multipotent, and unipotent. Stem cells can also be classified according to their origin into two groups: embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs are pluripotent cells that can give rise to cells from the three germ layers: endoderm, mesoderm, and ectoderm (Amit et al. 2000; Itskovitz-Eldor et al. 2000; Reubinoff et al. 2000; Schuldiner et al. 2000). Adult stem cells (ASCs) can give rise to mature cell types within the same germ layer: ectodermal cells will give rise to skin and neurons; mesodermal cells will generate cardiac, muscle, blood, and bone cells; and endodermal cells will produce visceral cells, such as pancreatic, lung, kidney, or thyroid cells.

In the adult organism, ASCs reside in niches. Several niches have been identified in almost all adult tissues: lungs, skin, adipose tissue, vasculature, heart, central nervous system, liver, pancreas, kidneys, gastrointestinal tract, endometrium, muscle, bone marrow, oral tissues, etc. (Mas-Bargues et al. 2019). All of them being potential sources of stem cells. Thus, ASCs can be easily obtained for regenerative therapies.

Several strategies have been employed to induce the conversion of differentiated cells into an embryonic state. All these approaches led to the generation of a new cell type, commonly referred to as “induced pluripotent stem cells” or iPSCs. The iPSCs are reprogrammed from differentiated somatic cells by going back to an undifferentiated state similar to ESCs. Both ESCs and iPSCs are defined as pluripotent and can differentiate into three germ layers (endoderm, mesoderm, and ectoderm) as well as self-renew. Thus, iPSCs can also be used for regenerative therapies.

The usage of ASCs and iPSCs in cell-based therapies has advantages and disadvantages. The ideal stem cell for transplantation should be obtained from an easy-access source, with high and fast expansion rates in vitro, long-term survival capacity, immunologically inert for a successful host integration, and able to differentiate in situ to replace the damaged cells (Kim and Park 2017). Although iPSCs have a higher replicative lifespan and differentiation potential, this feature has been associated with an increased formation of teratomas following iPSCs administration (Deng et al. 2018; Iida et al. 2017), whereas ASCs are non-tumorigenic when transplanted in vivo (Itakura et al. 2017). Another key point is the ability to attach to the target site, which has been directly related to their growth and differentiation (Lam and Longaker 2012). In this context, ASCs displayed better migration and engraftment than iPSCs. Indeed, ASCs are the preferred cells for cell-based therapies.

Importance of Stem Cell Culture Conditions for Clinical Applications

Any regenerative therapy using ASCs or iPSCs needs a very high number of cells which can only be achieved by a long-term in vitro culture. During this expansion

stage, stem cells can enter a senescence state, where they cease to proliferate and lose the ability to differentiate (Alves-Paiva et al. 2022). For this reason, *in vitro* culture conditions must be considered to obtain good-quality stem cells that can be used in cell therapies. Currently, stem cell expansion is mostly achieved via the conventional platform and protocol: tissue culture plastic (TCP) and 10% fetal bovine serum (FBS) in 21% O₂/5% CO₂ incubators. This combination has been a gold standard in obtaining millions of cells, but clinical studies require a new protocol for SCs expansion. Recently, new culture systems, reduced oxygen tensions, and media preconditioning have proven to improve stem cell proliferation and differentiation potentials toward a better outcome for regenerative therapies.

Stem cell culture systems can be two-dimensional (2D) or three-dimensional (3D) (Abdollahi 2021). 2D systems include common polystyrene flasks, whereas 3D systems mainly consist of bioreactors, such as two-chamber or hollow-fiber bioreactors, in which stem cells can be grown in higher densities (Gobin et al. 2021). Furthermore, 3D systems can capture physiological aspects that are missing in conventional 2D systems, such as tissue architecture, extracellular matrix composition, and heterotypic cell–cell interactions (Ader and Tanaka 2014). Indeed, a study suggested a decellularized extracellular matrix as an improved cell expansion setting for ASCs derived from bone marrow aspirates (Van et al. 2019). Another recent study reported that a gravity-controlled environment could enhance the proliferation and differentiation of ASCs (Nakaji-Hirabayashi et al. 2022). Moreover, there are several bioengineering strategies to create 3D constructs from iPSCs (Varzideh et al. 2022).

Most conventional *in vitro* cell cultures are performed under ambient oxygen concentration (20–21% pO₂), which is often referred to as “normoxia.” In contrast, *in vivo* ASCs are not exposed to such a high concentration of oxygen. ASCs are developed in environments with low oxygen tension, that ranges between 0 and 1% pO₂ in the bone marrow (Chow et al. 2001; Harrison et al. 2002), to 13–18% pO₂ in blood and lungs (Steurer et al. 1997). Keeley and Mann (2019) published a comprehensive review of the specific oxygen concentration for each tissue of the human body and concluded that an average range of 3–6% pO₂ would be considered as the physiological normoxia in which cells should be cultured *in vitro*. Indeed, culturing ASCs derived from dental pulps at 3% pO₂ proved to maintain their proliferation rate, differentiation capacity, and stemness potential compared to those cultured at 21% pO₂ (El Alami et al. 2014; Mas-Bargues et al. 2017, 2019). Moreover, it has been reported that low oxygen tension improves ASCs adhesion, proliferation, and differentiation in 3D scaffolds (Vina-Almunia et al. 2017). It is interesting to note that cultivating ASCs under physiological normoxia before transplantation improves tissue regenerative potential (Chen et al. 2022; Rosova et al. 2008).

Media preconditioning consists in activating cytoprotective pathways by either exposing cells to a sublethal environment (Hu et al. 2008), or by transfecting cells with survival genes (Zhang et al. 2008), or by conditioning cells with pharmacological molecules to activate specific cellular survival pathways. Among these pharmacological agents, oxytocin, celasterol, or melatonin have been commonly used (Noiseux et al. 2012; Touani et al. 2021; Zhao et al. 2015). As an example,

in vitro studies have demonstrated that melatonin exerts antioxidant and antiapoptotic effects on MSCs, thereby improving the outcome of stem cell transplantation (Lee et al. 2019; Zhang et al. 2017a). Melatonin in transplanted mice promoted neural stem cell proliferation and differentiation into oligodendrocytes and astrocytes while reducing oxidative stress (Mendivil-Perez et al. 2017).

Stem Cells in Clinical Trials

Stem Cell Properties Supporting Their Clinical Application

Among ASCs, mesenchymal stem cells (MSCs) are the most used in clinical trials. The therapeutic potential of MSCs includes their ability to differentiate into various cell lineages, modulate the immune response, and migrate to the exact site of injury.

Several in vitro and in vivo studies have reported that MSCs can differentiate into bone, cartilage, fat, muscle, tendon, and bone marrow cells (Okolicsanyi et al. 2015; Zheng et al. 2013). MSCs' fate is regulated by specific signals from their microenvironment, which includes biological molecules and biomechanical forces. These factors play a key role in determining MSCs differentiation and thus, their contribution to the repair process (Han et al. 2019). Indeed, MSCs-based therapies must ensure that the host microenvironment meets the requirements for the specific type of cell. It has been reported that the host environment led to the development of cell types other than those needed for the therapy (Ponticiello et al. 2000).

MSCs can modulate the immune system; MSCs can interact with both the innate and adaptive immune systems, leading to the modulation of several effector functions. Indeed, both in vitro and in vivo studies have reported that MSCs can interact directly with lymphocytes, dendritic cells, macrophages, and natural killer cells to prevent an excessive immune response (Han et al. 2012; Uccelli et al. 2008). This is of utmost importance regarding graft-versus-host-disease (GVHD), a syndrome that is characterized by systemic inflammation. For instance, donor-derived MSCs have been shown to induce long-term allograft acceptance in a rat model of heart transplantation (Popp et al. 2008). Although to prevent GVHD, the best solution is to use autologous stem cell transplants.

The homing ability of MSCs relies on their ability to migrate to the target tissue. The mechanism involves several signaling molecules and the corresponding receptors on the MSCs cell surface. Some of these signaling molecules have been identified: chemokines, adhesion molecules, and matrix metalloproteinases (MMPs) (Ries et al. 2007; Sohni and Verfaillie 2013). MSCs are known to express C-X-C chemokine receptor type 4 (CXCR4) and receptor tyrosine kinase (RTK) which are involved in MSC migration (Neuss et al. 2004; Shi et al. 2007). Notably, homing-related molecules can be upregulated by inflammatory cytokines such as tumor necrosis factor (TNF) and interleukins, such as IL-1 (Ren et al. 2010). Thus, this means that different inflammation statuses in different patients might lead to different MSCs engraftments and efficiency (Wei et al. 2013). If MSCs are

intravenously administered, they must adhere to the vascular endothelial wall and cross the endothelial and muscular layers (Schmidt et al. 2006). This transendothelial migration of MSCs is a three-step process: adhesion, rolling, and crossing endothelial cells, which seems to be mediated by the very late antigen-4/vascular cell adhesion molecule-1 (VLA-4/VCAM-1) (Ruster et al. 2006).

Notably, tracking studies on stem cell delivery in mice have been performed using gene reporters and noninvasive techniques (PET, bioluminescence, or fluorescence). The reported data suggests that MSCs successfully reach different organs depending on the previously induced pathology (Belmar-Lopez et al. 2022).

Stem Cell-Based Therapies: Clinical Trials

Accumulating evidence supports the increasing use of stem cells in clinical trials, suggesting their translation from the laboratory bench to the patient's bedside. According to the clinicaltrials.gov database, as of today (April 28th, 2022), there are over 9000 studies employing stem cells for regenerative medicine. Figure 11.3 highlights the use of MSCs for a wide diversity of pathological conditions.

Another important parameter to describe the use of stem cells in clinical trials is the phase of the investigation. As shown in Fig. 11.4, most studies are on phase 2, which consists of the analysis of the safety and effectiveness of the treatment. However, this phase is the most limiting since less than 25% of the studies progress to phase 3, and less than 10% reach phase 4, which are FDA approved drugs. Thus,

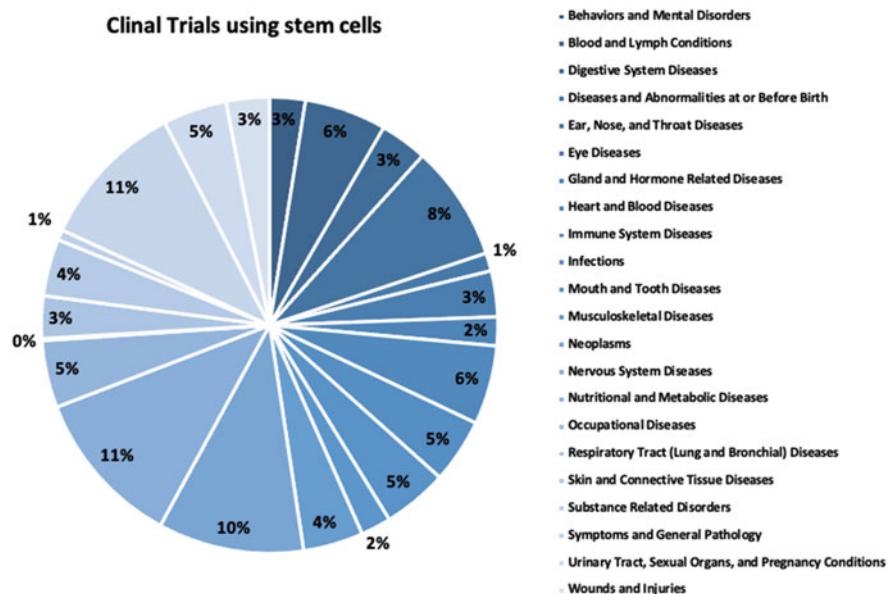


Fig. 11.3 Clinical trials using stem cells classified by disease condition

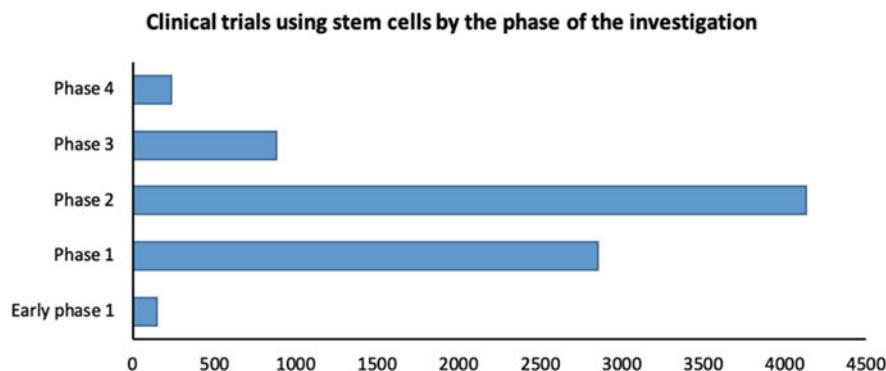


Fig. 11.4 Clinical trials using stem cells classified by the phase of the investigation

this figure reflects that the therapeutic effectiveness of stem cell therapies needs to be carefully addressed and should also focus on long-term effects, which have been poorly addressed.

Cardiovascular Diseases

Several MSC-based clinical trials have addressed cardiovascular diseases, ischemic heart diseases, and congestive heart failure. Myocardial infarction causes a loss of cardiomyocytes, and these dead cardiac cells will become replaced by fibroblasts to form scar tissue, leading to contractile dysfunction. MSC-based therapy aims at regenerating the damaged myocardium (Mathiasen et al. 2009).

In the study performed by Chen et al. (2004), myocardial infarction patients were divided into two groups. One group received an intracoronary injection of bone marrow (BM)-MSCs whereas the other group received saline. Six months after the beginning of the treatment, the group that received BM-MSCs displayed increased movement over the infarcted area, and left ventricular ejection was also improved in comparison with the control group.

The study of Katriotis et al. (2005) enrolled patients who had suffered an anteroseptal myocardial infarction, either recently or a long time ago. All patients had previously been subjected to angioplasty and stent implantation in the left anterior descending artery. BM-MSCs were administered through the balloon of the catheter. They observed improved end-diastolic and end-systolic diameter and volume, fraction shortening, and ejection fraction. However, these positive effects were only observable in patients who recently suffered a myocardial infarction.

Mathiasen and colleagues conducted a phase II trial assessing the intramyocardial delivery of autologous MSCs in patients with chronic ischemic heart failure. A total of 15 injections were administered to the ischemic region of the myocardium. After 12 months, the damaged myocardial tissue was regenerated improving the functional

capacity of the injured hearts (Mathiasen et al. 2012) ([ClinicalTrials.gov](#): NCT00644410).

Similar results were obtained by Friis et al., where the authors evaluated the effectiveness of the intramyocardial injection of autologous MSCs in patients suffering from stable coronary artery disease (CAD) and refractory angina. The patients that received the cell-based therapy displayed enhanced left ventricular function and exercise capacity (Friis et al. 2011) ([ClinicalTrials.gov](#): NCT00260338).

Neurological Disorders

Most MSC-based clinical trials involving the treatment of neurological diseases targeted Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and spinal cord injury (SCI).

Previous experimentation has reported that transplanting MSC into the brain is followed by the re-establishment of neural capacity (improved dopaminergic activity) through the mechanism of differentiation (Lindvall et al. 2012). Similarly, studies performed in humans have shown that transplanted intracerebral stem cells acquire local astroglial morphology and enhance the activity of local dopaminergic neurons (Niclis et al. 2017). Moreover, there is an ongoing clinical trial project named TRANSEURO investigating the benefits of grafting allogeneic dopaminergic neuroblasts derived from fetal ventral mesencephalic tissue into PD patients to recuperate the impaired activity dopaminergic neurons (Barker and Consortium 2019).

The study performed by Bachoud-Levi et al. analyzed the use of intrastratal grafts of human fetal striatal cells. Their results reported improved daily cognitive and motor functions in three of five total patients as demonstrated by increased metabolic activity at the graft site (Bachoud-Levi et al. 2000). However, another study showed that a decade after the first transplantation in HD patients, the grafts yielded no survival in the caudate region (Cicchetti et al. 2009).

Karussis and colleagues studied the long-term effect of autologous BM-MSC in patients with intractable MS or progressive ALS. BM-MSCs were injected either intrathecally or intravenously, and none of them revealed any side effects in the 6–25 months of follow-up. Magnetic resonance imaging displayed the dissemination of BM-MSCs from the injection site to the meninges and spinal cord parenchyma. Thus, this data suggested safety and clinical stabilization and even improvement in some patients (Karussis et al. 2006).

Mendonça et al. conducted a phase I trial involving patients with chronic traumatic SCI. BM-MSCs were locally injected at the injury site. The treatment was safe and feasible and all patients displayed improvements in tactile sensitivity and gained lower limb motor function (Mendonça et al. 2014) ([ClinicalTrials.gov](#): NCT01325103).

Taken together, stem cell therapy seems to have a potential neuroprotective effect. However, neurological disorders are often related to an advanced age. Ageing

could diminish their therapeutic effects on these diseases, potentially leading to graft failure and other risks (Nguyen et al. 2019).

Bone and Cartilage Diseases

The ability of MSCs to differentiate into osteocytes and chondrocytes both in vitro and in vivo makes them suitable candidates for the treatment of bone and cartilage diseases, such as osteogenesis imperfecta (OI) and osteoarthritis (OA) (Liu et al. 2014).

Horwitz et al. (2002) demonstrated the feasibility of combined allogenic BM-MSC transplantation for children with severe OI. The authors showed that injected BM-MSCs in bone marrow could migrate to the bone, and differentiate into osteoblasts to improve bone structure. The clinical trial performed by Le Blanc et al. (2005) assessed in utero MSC allogenic transplantation in a female fetus with severe OI. After birth, the infant showed no immunoreactivity against the donor and only three fractures appeared in the first two years of age with normal psychomotor development and correct growth tendencies.

Orozco et al. administered autologous BM-MSCs through intra-articular injection in patients with chronic knee pain and radiological evidence of OA. One year after the beginning of the treatment, patients showed a rapid and progressive improvement of functional movement, along with a decrease in poor cartilage area and an enhancement in cartilage quality (Orozco et al. 2013) ([ClinicalTrials.gov](#): NCT01183728). Similar results were obtained by Wong et al. (2013) in patients undergoing high tibial osteotomy and microfractures in knees with various cartilage defects. Their results showed improvement in repaired cartilage tissue after two years of postoperative outcomes.

Despite these optimistic results, the clinical application of MSCs in OA and OI remains in its infancy, and its effects need to be further addressed.

Other Pathologies

Several new clinical observations reported the efficacy of infused MSC in ameliorating tissue damage and/or improving function after injury to other organs, such as lungs, kidneys, liver, and eyes.

The study performed by Weiss et al. (2021) evaluated the time course of lung function, exercise performance, and exacerbation frequency following four monthly infusions of allogeneic BM-MSCs versus placebo. Their results reported that COPD patients treated with allogeneic BM-MSCs demonstrated significant improvements from baseline in forced expiratory volume in one second, forced vital capacity, and six-minute walk distance at 120 days after the first infusion. These improvements persisted variably over the 2-year observational period ([ClinicalTrials.gov](#): NCT00683722).

A randomized study enrolled patients with liver cirrhosis, divided into a control group (receiving conventional treatment) and an umbilical cord-MSC (UC-MSC)-treated group (receiving three injections in 4-week intervals). The reported results showed an increased overall survival rate in the UC-MSC-treated group than in the control group. Moreover, UC-MSC treatment improved liver function, as demonstrated by the levels of serum albumin, prothrombin activity, cholinesterase, and total bilirubin (Shi et al. 2021) ([ClinicalTrials.gov](#): NCT01220492).

A study analyzed patients with chronic kidney disease (CKD) due to different etiologies such as hypertension, or nephrotic syndrome. These patients received an intravenous infusion of autologous cultured MSCs. Follow-up visits of all patients did not reveal any cell-related adverse events. However, changes in glomerular filtration rate and serum creatinine were not statistically significant. This study showed the safety and tolerability of a single-dose infusion of autologous MSCs in patients with CKD but without beneficial results (Makhlough et al. 2018) ([ClinicalTrials.gov](#): NCT02195323).

A phase I clinical trial enrolled 14 participants who were treated with a single-dose intravitreal BM-MSC injection. During the 12-month follow-up period, they found significant improvements in the best-corrected visual acuity (BCVA) compared to the control group, although they returned to the baseline at 12 months. The visual field (VF) and central subfield thickness (CST) remain unaltered, indicating no remarkable disease progression. Importantly, the authors noticed mild and transient adverse events after one year of the beginning of the treatment and observed one severe but manageable adverse effect in one patient in year 3 (Tuekprakhon et al. 2021) ([ClinicalTrials.gov](#): NCT01531348).

Taken together, MSC-based therapies seem to have beneficial effects, but long-term side effects have also been reported.

Paracrine Effect of Stem Cells

A paradigm shift emerged suggesting that the beneficial effects of stem cells might not be restricted to cell replenishment alone, but also to their paracrine effect (Baraniak and McDevitt 2010).

The paracrine effect exerted by MSCs was hypothesized to support the observation that the number of implanted MSCs detected in target tissue was too low to explain tissue recovery or wound healing (Wang et al. 2011).

Stem cells secrete different kinds of factors that modulate the molecular composition of the environment to modulate responses from the resident cells' dynamics (Galderisi and Giordano 2014). Stem cells actively contribute to their environment by secreting cytokines, growth factors, and extracellular matrix (ECM) molecules that act either on themselves (autocrine actions) or on neighboring cells (paracrine actions).

All these factors promote cell protection, proliferation, and migration. Indeed, these cells secrete proangiogenic factors, antifibrotic factors, factors responsible for ECM homeostasis, as well as anti-inflammatory, antiapoptotic, antioxidant, and immunosuppressive factors. Some of the identified factors involved in immune system signaling are interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor- β (TGF- β). Other factors have been characterized as ECM remodelers, among them fibronectin, periostin, collagen, decorin, and matrix metalloproteinases (MMPs). Also, growth factors have been described, including vascular endothelial growth factor (VEGF), colony-stimulating factor (CSF), bone morphogenetic protein 2 (BMP-2), basic fibroblast growth factor (bFGF), and insulin-like growth factors (Baraniak and McDevitt 2010; Gnechi et al. 2008; Linero and Chaparro 2014; Ratajczak et al. 2012; Squillaro et al. 2016).

In addition to soluble factors, stem cells also release small vesicles, spherical membrane fragments shed from the cell surface or secreted from the endosomal compartment (Ratajczak et al. 2006b). These vesicles also have an important role in improving the function of damaged organs as discussed in the following sections.

Taken together, the characteristics of stem cells, including differentiation, immunomodulation, homing and paracrine effects, make them suitable for cell-based therapies (Fig. 11.5).

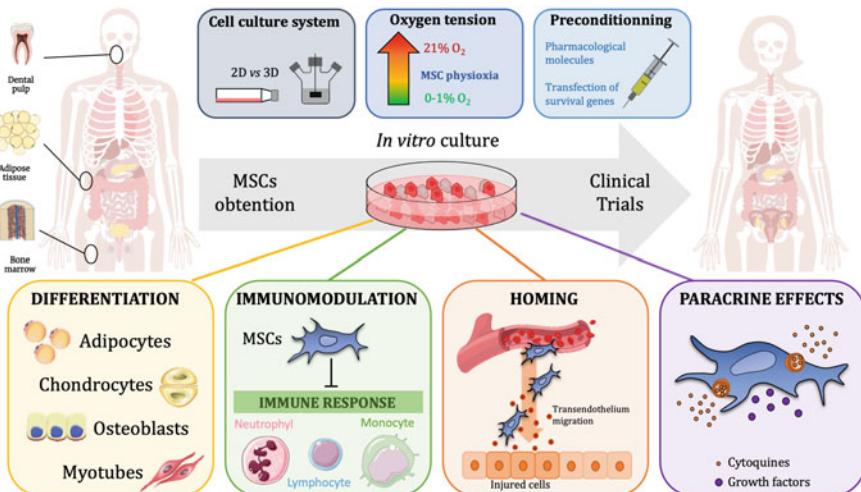


Fig. 11.5 Stem cell characteristics and culture conditions for clinical trials. After mesenchymal stem cell (MSC) obtention, an in vitro culture phase is modulated by the cell culture system, the oxygen tension used, and the media preconditioning. During the in vitro phase, the main characteristics of MSC (differentiation, immunomodulation, homing, and paracrine effects) can be checked before their use in clinical trials

Extracellular Vesicles as Tools for Intercellular Communication

EVs: Definition and Classification

Extracellular vesicles (EVs) are key effectors of intercellular communication. They can be defined as particles naturally released from a cell to the extracellular space, which are bound by a lipid bilayer and lacks a functional nucleus, hence cannot replicate (Théry et al. 2018). EVs serve as vehicles for intercellular transfer of cytosolic proteins, lipids, and nucleic acids, allowing for higher biostability and targeting capacity than other non-capsulated secretome components (Zaborowski et al. 2015). Although they are of utmost importance to maintain homeostasis in multicellular organisms, unicellular organisms such as bacteria are also capable of secreting EVs, named *outer membrane vesicles* (Yanez-Mo et al. 2015).

Traditionally, EVs have been classified according to their biogenetic pathway (Raposo and Stoorvogel 2013). Due to the difficulty in confirming their origin, a designation based on physical characteristics, such as size, is currently preferred. EVs size can range from 10 to 20 nm in diameter, although most research focuses on EVs of 100 nm and above (Margolis and Sadovsky 2019). According to the International Society for Intercellular Vesicles, EVs under 200 nm in diameter should be considered small EVs, while those over 200 nm are medium or large EVs. Alternative classifications may be based on properties such as density, biochemical composition or type, and culture condition of the cells of origin (Théry et al. 2018).

A Brief History of EVs

The first observations of EVs were reported by Chargaff and West (1946), who described them as procoagulant platelet-derived particles that were sedimented after plasma centrifugation. They were referred to as “platelet dust” in 1967 by Peter Wolf, who published the first electron microscopy images of these particles (Wolf 1967). Crawford termed those “microparticles” and showed that their cargo included lipids, RNA, and contractile proteins (Crawford 1971). Throughout the decade, EVs were isolated from a variety of sources, ranging from human tumor cell cultures to other body fluids (Yanez-Mo et al. 2015). In the early 1980s, parallel works from Johnstone and Stahl named these particles “exosomes” and proposed they served as a major route for the externalization of damaged proteins (Johnstone et al. 1991). A decade later, Raposo et al. (1996) found that these exosomes, which they isolated from Epstein Barr virus-infected B cells, were able to present antigens and induce a T-cell response. Their work suggested that exosomes’ biological functions may go far beyond cellular waste disposal (Couch et al. 2021). This hypothesis was confirmed in 2006 by Ratzjack et al., who evidenced that EVs mediate horizontal RNA

transfer capable of reprogramming gene expression of recipient cells (Ratajczak et al. 2006a). Ever since EVs have acquired renewed interest as vehicles of cell-to-cell communication.

EVs Biogenesis

There are currently three known EV biogenetic pathways, giving rise to (i) exosomes, (ii) microvesicles, and (iii) apoptotic bodies (Fig. 11.6). Exosomes, which range between 40 and 120 nm in diameter, are produced by endocytic budding of the cell membrane and the formation of an intra-luminal vesicle (ILV) inside the cell. The ILV is loaded with a particular cargo in an endosomal complex required for transport (ECRT)-dependent or independent pathway and matures to form a multivesicular body (MVB). The MVB is transferred alongside microtubules to fuse with the plasma membrane and exosomes are secreted into the extracellular space. Microvesicles, also called ectosomes, measure between 100 nm and 1 μm and are formed by outward budding and fission of the plasma membrane. Apoptotic

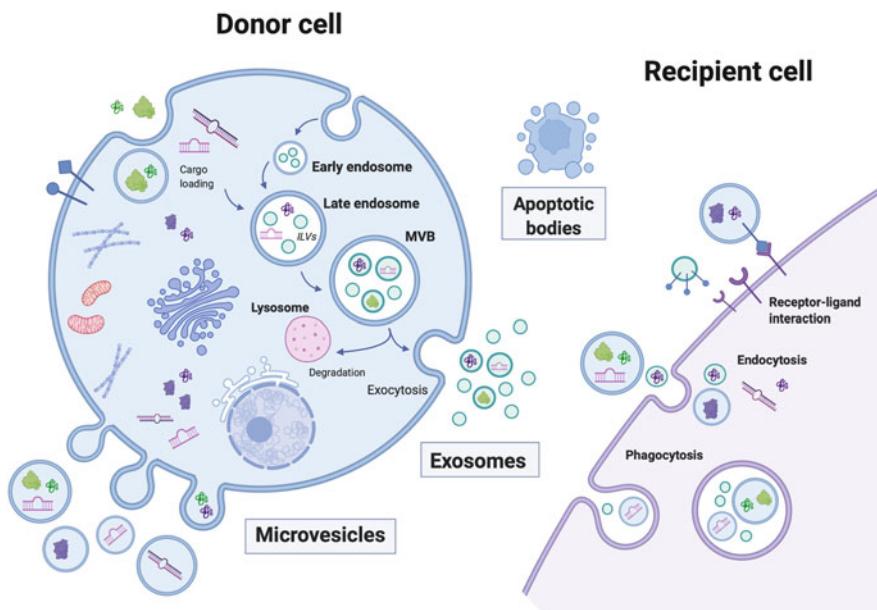


Fig. 11.6 Extracellular vesicles biogenesis. Extracellular vesicles (EVs) mainly include exosomes, microvesicles, and apoptotic bodies. Exosomes are derived from multivesicular bodies (MVB) inside the cells that fuse with the plasma membrane. Microvesicles are formed by outward budding and fission of the plasma membrane. Apoptotic bodies are released by blebbing of cells undergoing apoptosis. Recipient cells can incorporate EV cargo through endocytosis or phagocytosis. Created with BioRender.com

bodies, from 50 nm to 2 μm in diameter, are released by blebbing of cells undergoing apoptosis (Doyle and Wang 2019; Yanez-Mo et al. 2015; Zaborowski et al. 2015).

EV Composition

EVs' cargo can include proteins, lipids, genetic materials, and small-molecules metabolites (amino acids, ATP, amides, sugars, cytokines, etc.) (Hade et al. 2021).

EV biogenesis can be used to understand their proteome (Doyle and Wang 2019). EVs' formation and transportation are regulated by ESCRT proteins, thus, these proteins and their accessory proteins (Alix, TSG101, HSC70, and HSP90) are expected to be found in EVs (Morita et al. 2007). A wide range of proteins have been confirmed to be integrated into or attached to the membranes of EVs, or are present in their intra-luminal space. CD63, CD9, and CD81 are transmembrane proteins of the tetraspanin family and are commonly found in EV membranes (van der Koog et al. 2022). On the other hand, heat shock proteins (Hsp70 and Hsp90), lysosomal-associated membrane proteins (Lamp2a and Lamp2b), cytoskeletal proteins (actin, tubulin, and cofilin), integrins, and proteoglycans are found within EVs (Elsharkasy et al. 2020). Also, functional proteins might be characteristic of EVs isolated from specific cell sources. For instance, EVs derived from antigen-presenting cells (APCs), such as B-lymphocytes, dendritic cells, microglia, and macrophages, are enriched with major histocompatibility complex (MHC) proteins (Raposo et al. 1996).

EVs have a lipid bilayer and lipidomic analyses have shown that EVs contain lipid species that are present in the plasma membrane, although EVs are enriched in glycosphingolipids, sphingomyelins, phosphatidylserines, phosphatidylcholines, and cholesterol compared to the cellular plasmatic membrane (van der Koog et al. 2022). EVs are rounded vesicles, thus the lipid bilayer has a highly curved structure. This is reflected in the presence of lipid species that allow the curvature in the outer membrane, such as lipids with one fatty acid chain (Haraszti et al. 2016). EVs mainly contain monounsaturated fatty acids, polyunsaturated fatty acids, and saturated fatty acids, though the lipid composition depends on the parent cell (Skotland et al. 2019, 2020). Interestingly, EVs can transport several bioactive lipids and lipid metabolism-related enzymes, such as arachidonic acid, leukotrienes prostaglandins, phosphatidic acid, docosahexaenoic acid, and lysophosphatidylcholine (Subra et al. 2007).

The genetic cargo of EVs includes both DNA and RNA. RNAs have been extensively studied and several analyses have reported that EVs include various biotypes that represent a selected portion of the RNA content of the parent cell (O'Brien et al. 2020). Among them, small and long noncoding RNAs, including small nuclear RNAs, small nucleolar RNAs, ribosomal RNAs, PIWI-interacting RNAs, transfer RNAs, mitochondrial RNAs, Y RNAs, and vault RNAs (Chakrabortty et al. 2015; Lener et al. 2015; Xiang et al. 2013). Also, mRNA, small interfering RNAs, microRNAs, antisense oligonucleotides, guide RNAs,

self-amplifying RNAs, and circular RNAs have been described (Fanale et al. 2018; Geng et al. 2020; Li et al. 2014).

The RNA within EVs reflects the type and the physiological/pathological state of the parent cell. However, EVs' RNA content differs from the cellular RNA content, in terms of both RNA types and their relative concentrations, thus suggesting that some RNA species are selectively incorporated into extracellular vesicles (Baglio et al. 2015; Guduric-Fuchs et al. 2012; Mittelbrunn et al. 2011).

EV Internalization and Function

EVs can enter a recipient cell through many routes, including macropinocytosis, lipid-raft-mediated uptake, phagocytosis, and membrane fusion (Mathieu et al. 2019; Mulcahy et al. 2014), as well as tunneling nanotubes mediating transfer (Rustom et al. 2004). The main route of EV uptake is likely via clathrin/caveolin-mediated endocytosis (Mulcahy et al. 2014).

It is generally accepted that functional effects on recipient cells, besides direct signaling from the plasma membrane, might require EV internalization followed by cargo release into the recipient cell. Overall, so far little is known about the intracellular processing of EVs' cargo and how delivered instructions are interpreted by the recipient cell (van Niel et al. 2022).

Indeed, the evidence supports the conclusion that uptake does not equate to functionality. When assessing the functional delivery of cargo, it became evident that some cargo was retained in the endosomal compartment. The authors showed that some of the EVs content remained within the cell in a "non-functional" manner. Almost 17% of the internalized signal was retained in the recipient cell for 24 h after removing the external source of the signal (O'Brien et al. 2022). Similarly, other studies suggest that EVs, or at least the dyes or labels that are used to visualize them, eventually end up in lysosomes. These results suggest a possible mechanism of retaining cargo in endosomes of the recipient cell before degradation or possible re-release.

At the same time, there is overwhelming evidence that EVs transfer their cargo into the cytosol of recipient cells and induce epigenetic modifications, which indicates that EVs are capable of avoiding the endolysosomal pathway. It is still unknown whether the fate of EVs internalized cargo depends on intrinsic differences between EV subtypes or the fate is dictated by the characteristics of the recipient cell (van Niel et al. 2022).

Most therapeutic uses for EVs require the uptake of encapsulated content into the cytoplasm (Gurunathan et al. 2019). Although the functional delivery of EV content is often the desired outcome, it should be taken into account that endosomal uptake often results in lysosomal degradation with the purpose of recycling or eliminating molecules from the recipient cell (Tian et al. 2010).

Isolation Methods

EVs can be isolated from the supernatant of cultured cells and biological fluids. There are a plethora of different methods to isolate EVs, however, these have proven to produce different EV populations and purities. Moreover, some isolation and purification methods undergo cellular stress that affects EV function (Mas-Bargues and Borrás 2021).

EVs can be isolated based on their density, size, immunoaffinity, and solubility or aggregation properties. The most used method is based on the particles' density to separate them by ultracentrifugation at 100,000 g for 1–2 h (Crescitelli et al. 2013; Gudbergsson et al. 2016). Several protocols isolate EVs according to their size, such as ultrafiltration and size exclusion chromatography (SEC) (Varderidou-Minasian and Lorenowicz 2020; Xu et al. 2016). Both of them are a time and cost-effective alternative to the gold standard ultracentrifugation. Immunoaffinity-based techniques rely on the use of an antibody (such as tetraspanins CD9, CD63, or CD81) to interact with the molecules on the EVs' outer surface. These antibodies are attached to well plates, chromatography columns, magnetic beads, or microfluidic platforms, thereby allowing EV isolation (Doyle and Wang 2019; Li et al. 2017; Sidhom et al. 2020). Polymer-induced precipitation is another used method for EV isolation based on solubility or aggregation properties. Highly hydrophilic polymers (such as polyethylene glycol (PEG)) interact with water molecules surrounding the EVs to create a hydrophobic microenvironment, resulting in EV precipitation (Weng et al. 2016). This technique is inexpensive and allows an EV recovery of 90% and a high yield, but entails sample contamination with protein aggregates and nucleic acids.

Extracellular Vesicles in Regenerative Medicine

Regenerative Medicine: From a Cell-Based to a Cell-free Approach

Although stem cell-based regenerative medicine has yielded encouraging results, we are currently witnessing a transition to a cell-free approach, and notably to an EV-based approach (Gomzikova and Rizvanov 2017; Moghadasi et al. 2021). The reason for this is twofold: EVs might mirror most of the beneficial effects of cell therapy while facing considerably fewer safety concerns.

On the one hand, it has recently been questioned whether the benefits of cell therapy are explained by their structural role; that is, by the capacity of stem cells (SCs) to engraft in damaged tissues and repair them through differentiation. Contrarily, the paracrine hypothesis postulates that these regenerative effects might also, and perhaps more importantly, be attributed to the factors that SCs secrete to the microenvironment, including EVs. This last idea has received extensive support to

date. First, some studies found that injected SCs have a limited engrafting capacity *in vivo*, which is aggravated by their short half-life. Indeed, when injected intravenously, most of them are trapped and damaged in the narrow lung capillaries (Eggenhofer et al. 2012; Kurtz 2008). Second, the beneficial effects of SC transplantation have been shown to extend to tissues where SCs do not settle, as was shown by hematopoietic SC transplantation in renal damage (Duffield et al. 2005) and myocardial infarction (Jackson et al. 2001; Takahashi et al. 2006), or muscular SCs on a murine progeria model (Lavasanian et al. 2012). As shown by this last study, young healthy SCs are capable of rescuing proliferation defects in aged SCs; this suggests that tissue repair processes could be carried out by the recipient's SCs after reactivation by donor SCs, in line with the paracrine hypothesis (Lavasanian et al. 2012).

On the other hand, EV-based therapies offer remarkable benefits over cell-based therapies (Prockop et al. 2010). The most relevant risks of cell-based approaches are oncogenic transformation potential and undesired differentiation (Breitbach et al. 2007; Miura et al. 2006; Røsland et al. 2009), which do not occur with EVs since they lack a functional nucleus. Other safety issues include emboli formation with intravascular delivery (Furlani et al. 2009), pulmonary first-pass effect, and immune rejection (Moghadasi et al. 2021). Being nano-sized, biocompatible, and barely immunogenic, EVs overcome these drawbacks. Finally, their stability to freezing cycles, as well as their targeting capability and organotropism, make EVs an excellent candidate for both, cells and non-capsulated secretome components (Moghadasi et al. 2021).

EVs in Regenerative Medicine: Sources and Delivery Routes

Several sources have been described for EVs' isolation in regenerative medicine, both natural and artificial. Natural sources include SCs and plasma from young individuals or even centenarians (Yin et al. 2021). Among all types of SCs, mesenchymal SCs (MSCs) are the most used due to their many advantages, such as their presence in a wide range of tissues, their effective isolation methods, and their capacity to grow in adherent cultures. EVs are frequently obtained from bone marrow MSCs (BM-MSCs), adipose-derived MSCs (AD-MSCs), dental pulp MSCs (DP-MSCs), and umbilical cord MSCs (uc-MSCs), among others (Álvarez-Viejo 2020). Artificial sources include induced pluripotent SCs (iPSCs), as well as EVs from natural SCs subject to media preconditioning and coated EVs (Yin et al. 2021). While media preconditioning can alter EVs' content, coating only affects their surface, thus improving EVs' features such as skin absorption (Zhang et al. 2020). Regarding delivery routes, EVs are commonly delivered through direct intravenous or intraperitoneal injection, although the topical application is also being tested. Alternative routes are hydrogel injection, which allows for slower absorption, or coating of bioactive scaffolds made of electrospun fibers (de Jong et al. 2014).

Role of EVs in the Ageing Process

As fundamental vehicles of intercellular communication, EVs are a double-edged sword throughout the ageing process. EVs from young organisms, healthy tissues, and SCs may work as beneficial signaling molecules and stimulate cellular repair. On the contrary, EVs from old or injured tissues, such as SASP-related EVs, could spread cell senescence and promote chronic inflammation. In vivo, the circulating EV pool is likely to represent a continuum from “healthy” to “unhealthy” EVs, reflecting the status of the different microenvironments. Over time, the balance could tip in favor of ageing promoting EVs, which positively reinforce organ damage and lead to age-related diseases in the long term (Lananna and Imai 2021). Hence, two possible gerotherapeutic strategies emerge. First, target cells that secrete “unhealthy” EVs; this is the endpoint of senolytic and senomorphic drugs (Childs et al. 2017). Second, to replace those with “healthy” EVs, as is the purpose of EV-based regenerative medicine. The latter strategy is the subject of this chapter.

EVs as Gerotherapeutics: Lifespan and Healthspan

Several studies have sought to understand the role of circulating EVs in lifespan and healthspan in mammals, and whether they could be extended through an EV-based approach. The first evidence was provided by Zhang et al., who proved that ageing speed is largely controlled by the hypothalamus through EV-contained miRNAs (Zhang et al. 2017b). In their work, they showed that hypothalamic neural SCs (NSCs) were lost with age in a murine model, and implantation of young NSCs extended mice longevity. Interestingly, treatment with NSC-EVs achieved similar improvements in healthspan, measured through motor coordination, endurance, sociality, and memory tests. Subsequent studies have supported this hypothesis, achieving a healthspan extension by reducing hypothalamic NSCs senescence (Xiao et al. 2020).

Recently, Yoshida et al. identified another EV component involved in ageing and longevity regulation in mammals: extracellular nicotinamide phosphoribosyltransferase (eNAMPT), a nicotinamide adenine dinucleotide (NAD^+) biosynthetic enzyme (Yoshida et al. 2019). It has been established that NAD^+ bioavailability declines with age, causing a variety of age-related changes (Verdin 2015). Some studies show that eNAMPT secreted by adipose tissue—enhanced by certain stimuli, like fasting, through a SIRT1-mediated pathway—reaches the hypothalamus and locally increases NAD^+ , improving physical performance in mice (Yoon et al. 2015). Recent works prove that EV-contained eNAMPT decreases over time, therefore treatment with eNAMPT-containing EVs isolated from plasma of young mice can increase the lifespan and healthspan of old mice (Yoshida et al. 2019).

EVs as Gerotherapeutics: Hallmarks of Ageing

Many studies have questioned whether EVs from different sources can ameliorate different hallmarks of ageing, with promising results. Of all the hallmarks, the most frequently studied concerning EVs is cellular senescence. Recent work from Fafián-Labora et al. found that small EVs from young fibroblasts could ameliorate senescence-related biomarkers in fibroblasts from old or Hutchinson-Gilford progeria syndrome donors, as well as mice. They propose glutathione-S-transferase mu 2 (GSTM2), an antioxidant EV-contained enzyme whose activity declines with age, is responsible for the observed changes, in line with the decreased reactive oxygen species (ROS) levels and lipid peroxidation after treatment (Fafián-Labora et al. 2020). Another work with an iPSC-EVs treatment on senescent MSCs was able to decrease senescent phenotype by delivery of different antioxidant enzymes, peroxiredoxins 1 and 2 (Liu et al. 2019). Further studies in UVB-radiated fibroblasts support the senotherapeutic potential of EVs, although the underlying mechanism has not been elucidated yet (Deng et al. 2020; Oh et al. 2018). Recently, Dorronsoro et al., injected old mice intraperitoneally with MSC-EVs from young mice and measured senescence markers in different organs. They observed a decrease in most markers, including senescence-associated β -galactosidase, p16, p21, and pro-inflammatory cytokines IL-1 β and IL-6. Consequently, they proposed MSC-EVs might work as senomorphics; that is, they might downregulate the expression of SASP-related factors, hence impairing the spread of this senescent state to adjacent cells (Dorronsoro et al. 2021).

Regarding other hallmarks, evidence is less abundant but still optimistic. MSC-EVs may be able to attenuate stem cell exhaustion, as shown by in vitro studies on BM-MSC-EVs and DP-MSC-EVs (Dorronsoro et al. 2021; Mas-Bargues et al. 2020). In this last study, treated cells recovered their stemness as evidenced by pluripotency factor (OCT4, SOX2, KLF4, cMYC) overexpression and the shift toward a highly glycolytic and low oxidative metabolism (Mas-Bargues et al. 2020). In some recent studies, treatment with EVs from young serum and MSCs has increased the expression of telomere-related genes, which could attenuate telomere shortening (Lee et al. 2018; Sonoda et al. 2020). The first study also found that mTOR and IGF-1 levels were generally lower after treatment, therefore, they might play a role in alleviating deregulated nutrient sensing (Lee et al. 2018). Furthermore, research on cardiovascular and neurodegenerative diseases shows that the therapeutic potential of MSC-EVs point to an increase in autophagy as the underlying mechanism; for this reason, it has been suggested that MSC-EVs may also help preserve proteostasis (Chen et al. 2020; He et al. 2020; Liu et al. 2017).

EVs and Age-related Diseases

Nowadays, more than 20% of the total burden of disease is due to people aged 60 years and over, with special emphasis on cardiovascular disease (30%), cancer

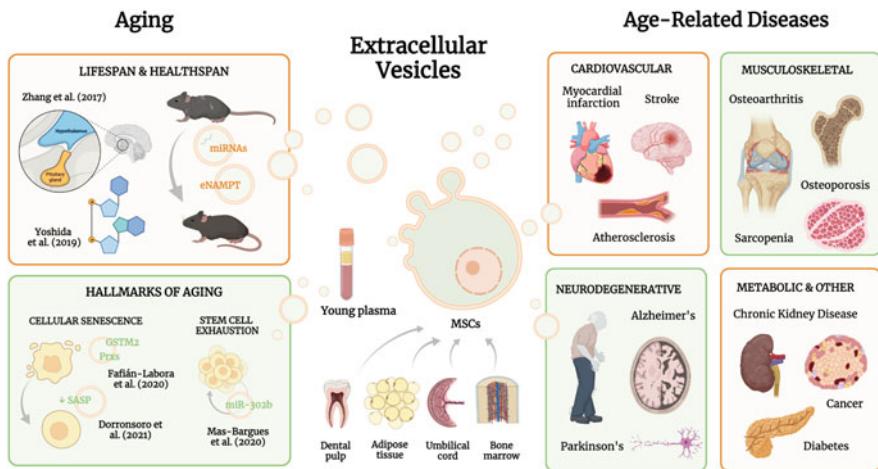


Fig. 11.7 Extracellular vesicles in ageing and age-related diseases. EVs can be isolated from plasma or MSC obtained from dental pulp, adipose tissue, umbilical cord, or bone marrow. EVs can modulate ageing and age-related diseases. Regarding the former, EVs' content (miRNAs and extracellular nicotinamide phosphoribosyltransferase (eNAMPT)) could be involved in longevity regulation, and EVs from young donors could ameliorate some hallmarks of ageing such as cellular senescence and stem cell exhaustion. Regarding the latter, EVs-based therapies are being used for cardiovascular, musculoskeletal, neurodegenerative, and metabolic diseases. Created with BioRender.com

(15%), and pulmonary, musculoskeletal, and neurodegenerative diseases (Prince et al. 2015). Given the promising results of EVs-based therapy to slow or even reverse some hallmarks of physiological ageing, it is not surprising that they are currently under study as next-generation therapies for age-related diseases (Fig. 11.7).

Cardiovascular Diseases

The cardioprotective properties of MSC-EVs have been investigated in a variety of age-related diseases, such as myocardial infarction (MI), ischemic stroke, and atherosclerosis. Recovery of cardiac function after MI has been in the spotlight since the origins of stem cell therapy (Jackson et al. 2001), and this interest has continued to rise with cell-free approaches, given its long-term morbidity as a cause of heart failure. Several studies have shown that MSC-EVs notably contribute to myocardial repair after MI, reducing infarct size and ultimately improving functional parameters such as stroke volume and cardiac output (Bian et al. 2014; Teng et al. 2015). Not only MSCs but different cell types, such as cardiomyocyte progenitor cells, hematopoietic stem cells (HSCs), and iPSCs have been used for EV isolation with similar results (Rezaie et al. 2019). Nevertheless, the underlying mechanism of these effects remains elusive. Since one of the main processes driving the recovery of

ischemic myocardium is angiogenesis, it has been proposed that MSC-EVs could induce capillary development through the delivery of miR-210 (Bian et al. 2014; Teng et al. 2015; Wang et al. 2017a). Another mechanism under study is the modulation of the pro-inflammatory microenvironment (Teng et al. 2015), which could be achieved by activating the S1P/SK1/S1PR1 pathway and M2 macrophage polarization, in close relation to EV-contained miR-182 (Deng et al. 2019). Lastly, MSC-EVs could prevent mycardiocyte apoptosis and cardiac fibrosis through miR-221, which downregulates PUMA (p53 upregulated modulator of apoptosis). Ex vivo assays, where cardiac stem cells were pretreated with MSC-EVs, have also shown promising results (Zhang et al. 2016). Regarding ischemic stroke, evidence suggests that both neural plasticity and revascularization promoted by MSC-EVs may accelerate functional recovery in animal models (Xin et al. 2013a, b).

Since atherosclerosis is the disease underlying the aforementioned events, it has become an emerging therapeutic target. MSC-EVs' contribution to the stabilization of atherosclerotic plaques is two-sided. First, they can inhibit endothelial cell (EC) apoptosis and promote proliferation by activating Nrf2, which protects ECs against oxidative stress (Chen et al. 2021). Secondly, the let-7c miRNA family carried by EVs downregulates the NF-κB inflammation pathway. This anti-inflammatory effect is enhanced through the shifting of macrophage phenotype from M1 to M2 (Li et al. 2019; Ma et al. 2021).

Neurodegenerative Diseases

In the central nervous system (CNS), EVs are studied with particular interest because they solve a difficulty of conventional drugs: the crossing of the blood–brain barrier (BBB). Being small-sized and highly lipophilic, they reach deep into the CNS, where they exert regenerative and immunomodulatory effects (Guy and Offen 2020). Recent studies on Alzheimer's Disease (AD) mice models show that MSC-EVs improved cognition and alleviated memory deficits, which was consistent with the observed decrease in Amyloid- β (A β) plaques in the brain. MSC-EVs decreased astrocyte and microglia activation by tipping the balance from pro-inflammatory IL-1 and TNF-a to anti-inflammatory cytokines like IL-10. This led to the discovery of miR-21, an EV-contained miRNA capable of downregulating the STAT3–NF-κB axis responsible for the amplification of neural damage in AD (Cui et al. 2018). Similar results have been obtained with intraventricular injection of MSCs-EVs, where a marked increase of synaptogenesis in the hippocampus was attributed to miR-146a (Nakano et al. 2020). In another study, neural recovery from A β -induced damage and ROS decrease was achieved by MSC-EVs through the transfer of enzymatically active catalase (Bodart-Santos et al. 2019). Likewise, blood marrow and umbilical cord MSC-EVs have shown therapeutic effects on Parkinson's disease rat models by stimulating neuronal differentiation in the *substantia nigra* (Mendes-Pinheiro et al. 2019), as well as inducing autophagy and decreasing apoptosis, thereby upregulating dopamine levels in the striatum and improving motor function (Chen et al. 2020).

Musculoskeletal Diseases

Current studies have unveiled the potential of MSC-EVs in musculoskeletal disorders, which are responsible for the exponential increase in frailty among the elderly. Osteoarthritis (OA) stands as the most prevalent disease in this group, several strategies have been pursued to restore cartilage and bone homeostasis. On the one side, MSC-EVs from adipose tissue were able to promote chondrocyte survival, decrease apoptosis, and improve autophagy; these effects were due to EV-contained miR-100-5p, which downregulates the mTOR pathway (Wu et al. 2019). Moreover, ADSC-EVs effectively tackled senescence features in OA osteoblasts (Tofiño-Vian et al. 2017). On the other side, MSC-EVs promoted repair of extracellular matrix (ECM) by increasing collagen type II synthesis and downregulating ECM-degrading enzymes, such as ADAMTS5 and metalloproteinases MMP1 and MMP13 (Cosenza et al. 2017; Jin et al. 2020; Wang et al. 2017b). The functional translation of these effects was remarkable, with recovery from gait abnormalities in murine models (Cosenza et al. 2017).

Positive OA osteoblasts results have driven interest in MSC-EVs to another major disease, osteoporosis. In vitro experiments showed that MSC-EVs obtained from human iPSC could increase protein expression of osteoblast-related genes and proliferation rates (Qi et al. 2016). In vivo models of osteoporosis in ovariectomized rats support these osteogenic and angiogenic effects of EVs, administered by two different routes: direct intraosseous injection or implantation of an EVs-releasing scaffold (Qi et al. 2016). MSC-EVs may as well have regenerative effects on the intervertebral disc via antioxidant and anti-inflammatory properties (Xia et al. 2019), thus constituting an attractive option for herniated disc prevention.

Sarcopenia is a condition that affects mostly elderly people to a greater or lesser extent and is particularly aggravated by episodes of illness or hospital admission. Although little has been published on age-related sarcopenia, there is growing evidence that MSC-EVs could be of value in preventing drug-induced muscle wasting (Cho et al. 2021; Li et al. 2021). A recent study found increased myotube diameter both in vitro and in vivo after treatment and an increase in muscle weight and strength, which may be explained by EV-contained miR-486-5p and its inhibitory effect on FoxO1 (Li et al. 2021). Similar work with tonsil MSC-EVs identified miR-145-5p as the responsible component for increased myogenic differentiation and total muscle mass in mice (Cho et al. 2021).

Other Diseases

One of the most important causes of morbimortality among the elderly is metabolic diseases, in particular, type 2 diabetes mellitus (T2DM), which is intimately related to obesity, non-fatty liver disease, and metabolic syndrome. T2DM is caused by increased peripheral resistance to insulin action, which drives an increased hepatic glucose output and, ultimately, impaired insulin secretion. In one study, uc-MSC-EVs proved useful in promoting hepatic glycogen storage and reducing

gluconeogenesis, via AMPK signaling and autophagy induction, both in hepatocyte culture and in a T2DM rat model (He et al. 2020). Likewise, they reduced glucose levels and improved insulin sensitivity, promoting the expression of GLUT-4 receptors in muscle (Sun et al. 2018). Even though pancreatic damage does not play a role until the late stages of the disease, results in this regard are also encouraging and leave the door open for future studies in T1DM (Sun et al. 2018) ([ClinicalTrials.gov](#): NCT02138331).

Chronic kidney disease (CKD) is a prevalent disease that greatly compromises the quality of life in the elderly, because the only available treatments, dialysis, and renal transplant, are potentially risky and have high costs. MSC-EVs have successfully been used to increase renal function parameters, such as glomerular filtration rate or urea and creatinine levels, in advanced stages of CKD in a recent clinical trial (Nassar et al. 2016). Interestingly, stimulation of MSCs with melatonin, a proposed renoprotective hormone, may enhance EVs' regenerative potential in a murine model of CKD.

Cancer accounts for one of the highest burdens in the elderly (Prince et al. 2015). MSC-EVs have not received so much attention in cancer therapy, given that many of the biological processes they stimulate, such as cell proliferation, angiogenesis, and immunosuppression, could favor tumor growth and extension (Xunian and Kalluri 2020). Nonetheless, a clinical trials are currently in progress using modified MSC-EVs as drug carriers for cancer therapy, such as colon cancer ([ClinicalTrials.gov](#): NCT01294072) and pancreatic cancer ([ClinicalTrials.gov](#): NCT03608631).

In conclusion, preclinical data provide evidence of the efficacy and safety of MSC-EVs-based therapies against a variety of age-related diseases. A number of these studies have already reached the stage of clinical trials, as is the case in ischemic stroke ([ClinicalTrials.gov](#): NCT03384433), AD ([ClinicalTrials.gov](#): NCT04388982), OA ([ClinicalTrials.gov](#): NCT04223622), CKD (Nassar et al. 2016), and cancer. While EV studies are an evolving field in preclinical ageing research, some factors still hinder their translation to the clinical setting. Among them, we could underscore the lack of established cell culture conditions, the absence of scalable EV isolation protocols, more thorough investigations on the optimal therapeutic dose, administration routes and schedule, and reliable assays to evaluate the efficacy of EV-based therapies.

Concluding Remarks

Stem cells play a key role in maintaining tissue homeostasis, both after injury and during ageing. Stem cells have unique characteristics, such as differentiation, homing, and immunomodulation which make them suitable candidates for regenerative medicine. Stem cell-based therapies are currently aiming to improve damaged tissue functionality with promising results. However, long-term issues have been associated with stem cell therapies, such as poor engraftment at the injury site. This led to the hypothesis that stem cell-derived effects were due to their ability to modulate the

microenvironment. Indeed, paracrine effects proved to be as effective as stem cell transplants. The study of the released molecules evolved into non-cell-based therapies; the golden star being mediated by extracellular vesicles. EVs' content includes bioactive molecules, such as lipids, proteins, and genetic material that modulate the fate of the target cell. EVs-based therapy could slow down or even reverse some hallmarks of physiological ageing. Moreover, EVs are being used in many clinical trials for several age-related diseases, both as biomarkers and treatments.

Overall, these studies need to be developed, and more research should be performed to address several questions, such as EVs' in vivo biodistribution and fate, the different processing of EVs cargo by target cells, or the relative potency of EVs in directing cell–cell communication compared to other secretome components. These and many other questions are key questions and challenges that remain to be addressed.

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Chapter 12

Fibrinogen, Coagulation, and Ageing



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Abstract The World Health Organization estimates that the world's population over 60 years of age will nearly double in the next 30 years. This change imposes increasing demands on health and social services with increased disease burden in older people, hereafter defined as people aged 60 years or more. An older population will have a greater incidence of cardiovascular disease partly due to higher levels of blood fibrinogen, increased levels of some coagulation factors, and increased platelet activity. These factors lead to a hypercoagulable state which can alter haemostasis, causing an imbalance in appropriate coagulation, which plays a crucial role in the development of cardiovascular diseases. These changes in haemostasis are not only affected by age but also by gender and the effects of hormones, or lack thereof in menopause for older females, ethnicity, other comorbidities, medication interactions, and overall health as we age. Another confounding factor is how we measure fibrinogen and coagulation through laboratory and point-of-care testing and how our decision-making on disease and treatment (including anticoagulation) is managed. It is known throughout life that in normal healthy individuals the levels of fibrinogen and coagulation factors change, however, reference intervals to guide diagnosis and management are based on only two life stages, paediatric, and adult ranges. There are no specific diagnostic guidelines based on reference intervals for an older population. How ageing relates to alterations in haemostasis and the impact of the

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disease will be discussed in this chapter. Along with the effect of anticoagulation, laboratory testing of fibrinogen and coagulation, future directions, and implications will be presented.

Keywords Fibrinogen · Coagulation · Older age · Platelets · Anticoagulation · Laboratory monitoring · Point-of-care testing · Reference intervals

Introduction

The healthy life expectancy reached 70.9 years and the average life expectancy was 62.5 years for males in 2019, and for females were 75.9 years and 64.9 years, respectively (Geneva World Health Organization 2021). The World Health Organization (WHO) estimates that the world's population over 60 years will nearly double from 12% to 22% between 2015 and 2050 (World Health Organization 2021). These changes are not limited to wealthy countries as by 2050, 80% of older people will be living in low- and middle-income countries. Australia's population has also been ageing faster with people aged 65 years or over doubling over the last half century (AIHW 2021). Thus, these demographic changes impose increasing demands on health and social services. For example, older adults consume more than 60% of the transfused blood (Tinegate et al. 2016; Simon et al. 2021). It has been estimated that 23% of the total global disease burden disorders are in older people aged 60 years or more (Prince et al. 2015). Just under a third of the disease burden in older adults is due to cardiovascular diseases, followed by malignant neoplasms, chronic respiratory diseases, musculoskeletal diseases, and neurological and mental disorders.

Haemostasis imbalance plays a crucial role in the development of cardiovascular diseases. Haemostasis involves the balance of coagulation and fibrinolysis and is associated with both arterial and venous thrombotic events. Ageing is associated with increased levels of some coagulation factors and a decrease in natural anticoagulant factors leading to a hypercoagulable state in the elderly (Amin et al. 2012). An increment of plasma fibrinogen level by 10 mg/dL (equivalent to 0.1 g/L) for each decade can be expected in healthy participants (Mari et al. 2008a, 2008b). It has been known for a few decades now through multiple studies that older people tend to have higher levels of blood fibrinogen, with concentrations increasing around 0.1–0.2 g/L each decade (Tybjaerg-Hansen et al. 1997; Siegbahn and Ruusuvuara 1988; Connelly et al. 1992).

In addition, it is important to recognise the gender differences in older age. The social, Environmental, and economic gender differences vary by country with the proportion of women in the labour force being lower, fewer with full-time contributing employment, and a lower socio-economic and health and well-being status (Carmel 2019). Along with this, age-related hormonal changes such as menopause influence the gender differences in haemostatic parameters (Bucciarelli and Mannucci 2002; Lippi et al. 2014).

This chapter will review the age-related alterations in haemostasis, the impact of disease on haemostasis, anticoagulation, and laboratory testing. Throughout this chapter older adults will refer to anyone aged 60 years or more.

Haemostasis

Normal functioning haemostasis reduces the risk of thrombosis and haemorrhage and is the balanced response to vascular injury to prevent bleeding through procoagulant pathways and the mechanisms that inhibit clotting away from the vascular injury. The physiological process of haemostasis in response to vascular injury begins with local vascular contraction, reduction of blood flow to the injury site and platelet plug formation, together this is known as primary haemostasis. The natural thrombogenic components in the body that tips the balance towards coagulation in primary haemostasis include exposed endothelium, tissue factor, and collagen from the damaged vessel wall along with circulating platelets. Secondary haemostasis is the clotting coagulation process that occurs in the plasma and involves the interaction between clotting factors and inhibitors to form a stable blood clot from the activation of fibrinogen to fibrin through the cascade of events that include circulating elements such as prothrombin, fibrinogen, and von Willebrand factor (Palta et al. 2014). When the vessel wall integrity has been repaired the process of removing the clot occurs which is termed fibrinolysis. If the normal physiological process of preventing bleeding and excessive clotting is not met patients are at increased risk for either thrombosis or haemorrhage. A schematic of the physiological coagulation cascade and the factors involved in secondary haemostasis are outlined in Fig. 12.1.

Haemostatic Changes with Age

The parameters of fibrinogen and coagulation may change as a part of normal ageing or they may be influenced by several disorders that occur with ageing. Common comorbidities that occur with ageing may account for the increased risk for both thrombosis and haemorrhage (Kwaan et al. 2014). Predominantly this includes changes to fibrinogen, FVIII, and von Willebrand Factor, which are all acute phase reactants and D-dimers and Antithrombin (Favaloro et al. 2014). As life expectancy increases some age-related changes occur with haemostasis that is likely driven by chronic inflammation. This effect occurs through various organ systems and pathways with cardiovascular and neurological diseases having the most frequently observed haemostasis alterations (Teer et al. 2019).

The ageing process activates leucocytes, specifically lymphocytes and monocytes. These leucocytes produce cytokines that induce inflammatory responses, increase inflammatory proteins and, in turn, express tissue factors in their

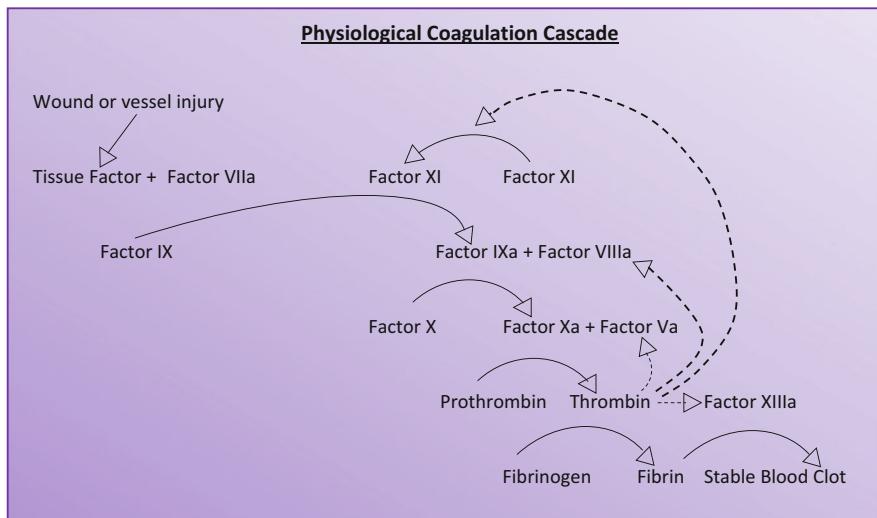


Fig. 12.1 Secondary haemostasis as outlined through the coagulation cascade after vessel injury.
Adapted from Pathology Tests Explained—Test Index 2021

cytoplasmic membranes initiating coagulation (Jabbour et al. 2009). Fibrinogen, the procoagulant protein in haemostatic reactions leads to blood coagulation and can interfere with plasma viscosity and is one of the most studied proteins in haemostatic changes throughout life (de Magalhães et al. 2020). The increase in coagulation occurs both during natural ageing and infection, as well as cellular and tissue stress. Thus, it is considered a biomarker of risk in thrombotic disease.

Older patients with increased markers of thrombin generation (D-dimer and prothrombin fragment 1 + 2) are at increased risk for cognitive decline and deterioration in their ability to perform activities of daily living. This is likely attributable to the increased risk of cerebral ischemic damage associated with prothrombotic states (Stott et al. 2010).

Chronic HIV infection shares the states of inflammation and hypercoagulability that are also seen with age-related changes. Despite control of HIV infection, patients persist with a subclinical inflammatory state due to changes in the immune system by latent infections such as cytomegalovirus and microbial translocation (Paiardini and Müller-Trutwin 2013). Chronic infection of HIV promotes changes in haemostasis and coagulation in consequence of persistent systemic immune activation, micro- and macro-vascular diseases, and, potentially, impaired hepatic synthesis of coagulation factors (de Magalhães et al. 2020).

Whilst chronic infection can impair the synthesis of coagulation factors in the liver and lead to bleeding complications, patients with liver disease can also experience thrombosis (Qamar et al. 2018). The risk for both bleeding and thrombosis stems from alterations in platelet activity, thrombin generation and fibrinolysis which favour both pro- and anticoagulable states (Wadsworth et al. 2021). Therefore, elderly patients with hepatic impairment are at increased risk of bleeding

complications and thrombotic events. Along with liver complications patients with chronic kidney disease have an increased risk for thromboembolic and bleeding events. Multifactorial haemostasis disorders are typical of patients with end-stage renal disease on chronic haemodialysis (Pluta et al. 2020). In 2016, over 300,000 patients with end-stage renal disease in the United States suffered from non-valvular atrial fibrillation, with a prevalence as high as 20% in patients on haemodialysis (Saran et al. 2019).

The cardiovascular system has the most frequently observed haemostasis alterations associated with increased age. Patients with prosthetic heart valves are at an increased risk for thrombotic events (Wadsworth et al. 2021). Patients with ischemic or non-ischaemic cardiomyopathy may develop left ventricular thrombus. Valvular heart disease and atrial fibrillation commonly coexist, and each contributes independently to thromboembolic events and mortality (Renda et al. 2017). Valvular atrial fibrillation can occur from a mechanical prosthetic heart valve or moderate to severe mitral stenosis, conditions that substantially increase thromboembolic risk and are associated as comorbidities with increased age (Chen et al. 2020).

Common comorbidities associated with ageing such as cancer increase the risk of venous and arterial thromboembolism and bleeding events (Mosarla et al. 2019). A sedentary lifestyle associated with ageing can also increase the risk of a thrombotic or bleeding event and lifestyle choices that may lead to obesity are a risk factor for venous thromboembolism, atrial fibrillation, and cardiovascular disease. With the increasing prevalence of obesity and the accompanying treatment for cardiovascular risks, anticoagulation in patients with obesity is increasingly a topic of concern (Wadsworth et al. 2021). In particular, drug interactions to treat comorbidities including human immunodeficiency virus, organ transplantation, infection, malignancy, epilepsy, arrhythmia, renal disease, or hepatic disease, increase the likelihood of direct oral anticoagulants (DOAC) drug interactions due to concomitant medications or pharmacokinetic changes (Chen et al. 2020).

Platelet Function

Through their aggregation, platelets release constituents from their granules, which are necessary for thrombus formation. Platelet activation leads to a change in the expression of surface glycoproteins (GP). During the stimulation and activation phase of platelets, P-selectin translocates from alpha granules and Weibel-Palade bodies of endothelial cells to the cell membrane (Stenberg 1985). On the platelet surface membrane, GPIIb-IIIa undergoes an activation-dependent conformational change which allows it to bind to fibrinogen (Shattil et al. 1985). Binding of thrombospondin to GPIV is elevated, and the von Willebrand factor binding site on the GPIb-IX complex is downregulated in thrombin-activated platelets (Kestin 1993). Recognition of these alterations that occur on the membrane of platelets has been applied to the analysis of platelet activation by specific antibodies (Gryglewski 1988). It is believed that procoagulant mechanisms are not the single reason behind

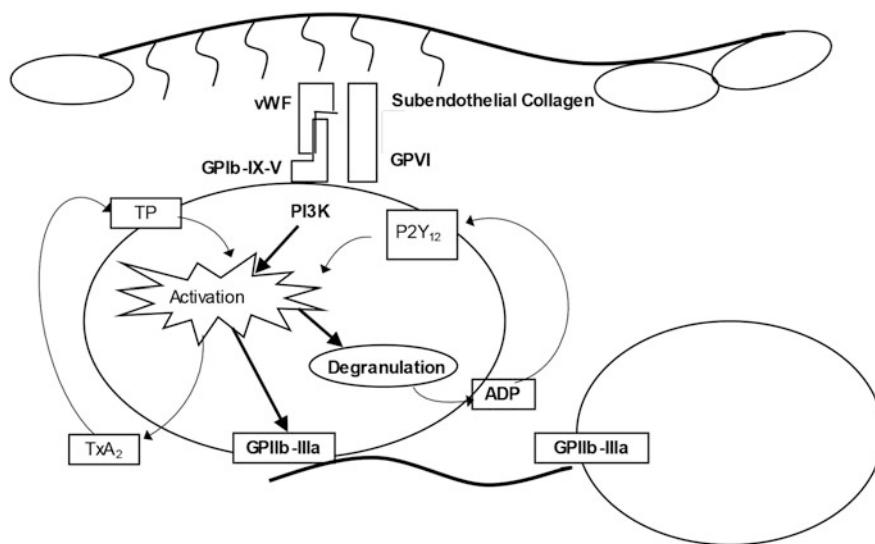


Fig. 12.2 Platelet receptors for vWF (GP1b-IX-V), fibrinogen (GP11b-111a), ADP (P2Y12), and thromboxane (thromboxane prostanoid)

increased platelet action. Loss of the preventive effect of antiaggregatory actions can lead to hyperactivity of platelets as well.

Key *in vivo* activating factors that stimulate platelets are thromboxane A₂, ADP, collagen, and thrombin. Adrenaline and serotonin are also weak stimulants of platelets (Rand et al. 2003). There are different receptors for the above-mentioned agonists and for other mediators such as von Willebrand factor (VWF) and fibrinogen (Rand et al. 2003). Some platelet receptors and their corresponding agonists are shown in Fig. 12.2.

Stimulation of platelet aggregation is initiated by the production and release of TXA₂, exocytosis of dense granules secreting ADP, serotonin, and thrombin (Rand et al. 2003). Release of P-selectin from α -granules is initiated in response to platelet activation and is expressed on the plasma membrane (Rand et al. 2003). Platelet pro-aggregators perform in an interactive way, so platelet activation is highly affected when one of the mechanisms of platelet aggregation is impaired (Rand et al. 2003). Platelet activation leads to a change in the level of expressed surface glycoproteins (GP). These GPs include integrins and non-integrins, which play an important role in platelet adhesion and aggregation and work as receptors for platelet agonists (Ashby et al. 1990). Upon the stimulation and activation phase of platelets, P-selectin is translocated from alpha granules in platelets and Weibel-Palade bodies of endothelial cells to the cell membrane (Stenberg 1985). On the platelet surface membrane, GPIIb-IIIa undergoes an activation-dependent conformational change which allows it to bind to fibrinogen (Shattil et al. 1985). In addition, the binding of thrombospondin to GPIV is elevated, and the von Willebrand factor binding site on

GPIb-IX complex is downregulated in thrombin-activated platelets (Kestin 1993; Coller et al. 1983). Recognition of these alterations that occur on the membrane of platelets, by special antibodies, is used for accurate analysis of the activation of platelets in the blood (Gryglewski 1988).

Anti-aggregators of Platelets

The most important antiaggregant includes prostaglandin I₂ (PGI₂) and nitric oxide (NO), which are released by the normal vascular endothelium and regulate the action of proaggregants to prevent the formation of thrombi in the intact blood vessels (Gryglewski 1988). Unlike antiaggregants and proaggregants, which apply their actions by attaching to specific receptors on the plasma membrane of platelets, NO crosses the membrane and stimulates guanylate cyclase, hence inhibiting platelet activation (Makhoul et al. 2018). The released signals from the stimulated receptor are transferred inside the platelet by a group of signal transduction processes, each including GTP binding proteins (G-protein) (Ashby et al. 1990). G-proteins represent many groups of variable cellular proteins that take part in different cellular processes, including regulation of signal transduction pathways (Zachariou et al. 2012). The outcome of activation of the proaggregatory signal transduction processes is the stimulation of other systems, such as phospholipase C (PLC) activated hydrolysis of inositol phospholipids and unlocking of ion channels. In contrast, the activator systems of the antiaggregatory processes initiate adenylate and guanylate cyclase, hence inhibiting platelet activation (Makhoul et al. 2018). Promotion of these activator systems results in different biological responses due to many changes in phosphorylation conditions, enzymatic actions, and physical characteristics of the main proteins of platelet (Gryglewski 1988).

Platelet–Endothelial–White Blood Cell Immuno-pathological Interactions

Platelet adherence and aggregation on the vascular endothelium can be prevented by anti-aggregators, such as NO and PGI₂ (Gryglewski 1988; Vinik et al. 2001) as shown in Fig. 12.3.

There are many components, like plasma thrombin and bradykinin, platelet-released serotonin, platelet-derived growth factor, interleukin-1, and ADP, which enhance the endothelial production of these anti-aggregators in the presence of aggregating platelets (Vane et al. 1990). Furthermore, the purpose of this inhibitory effect of NO and PGI₂ is to bind the platelet plug to the site of vascular damage. PGI₂ binds to a specific platelet receptor, which is linked to G-protein to activate adenylate cyclase. An adenylate cyclase inhibitory G-protein is associated with an α_2 -adrenergic receptor, that relates to epinephrine (Klaff et al. 1981). In addition, NO

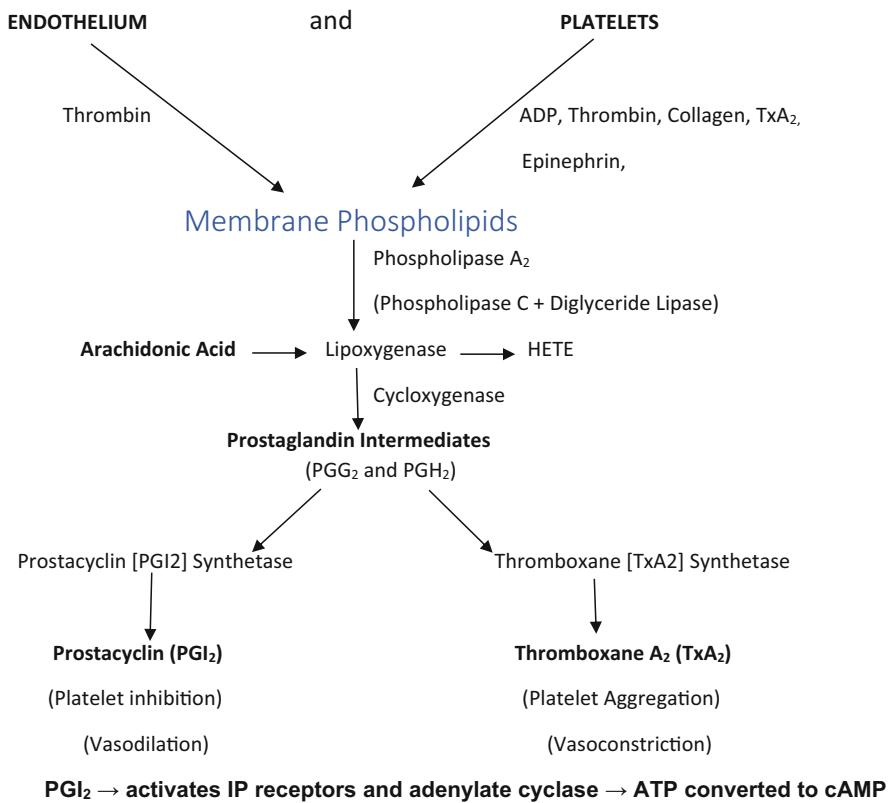


Fig. 12.3 Platelet–endothelium interaction

disperses through the plasma membrane of the platelet and stimulates guanylate cyclase (Mitchell et al. 2008). Consequently, phosphorylation by cAMP-dependant and cGMP-dependant protein kinases and consequent suppression of platelet proteins, which are fundamental for the process of aggregation, represents the outcome of these preventive mechanisms (Gryglewski 1988; Lazarowski et al. 1990).

Platelet Activity and Cardiovascular Disease

Increased platelet activity is an important predictive marker for cardiovascular disease (CVD) (Christie et al. 2008). Thrombotic effects of platelets can be initiated by vascular wall endothelial damage or injury by atheromatous plaque. The platelets adhere to damaged endothelium, which in turn, will change their shape and undergo degranulation and activation. Activation of the platelets leads to the conjugation of fibrinogen to the platelets through their GP IIb/IIIa receptors (Santhakumar et al.

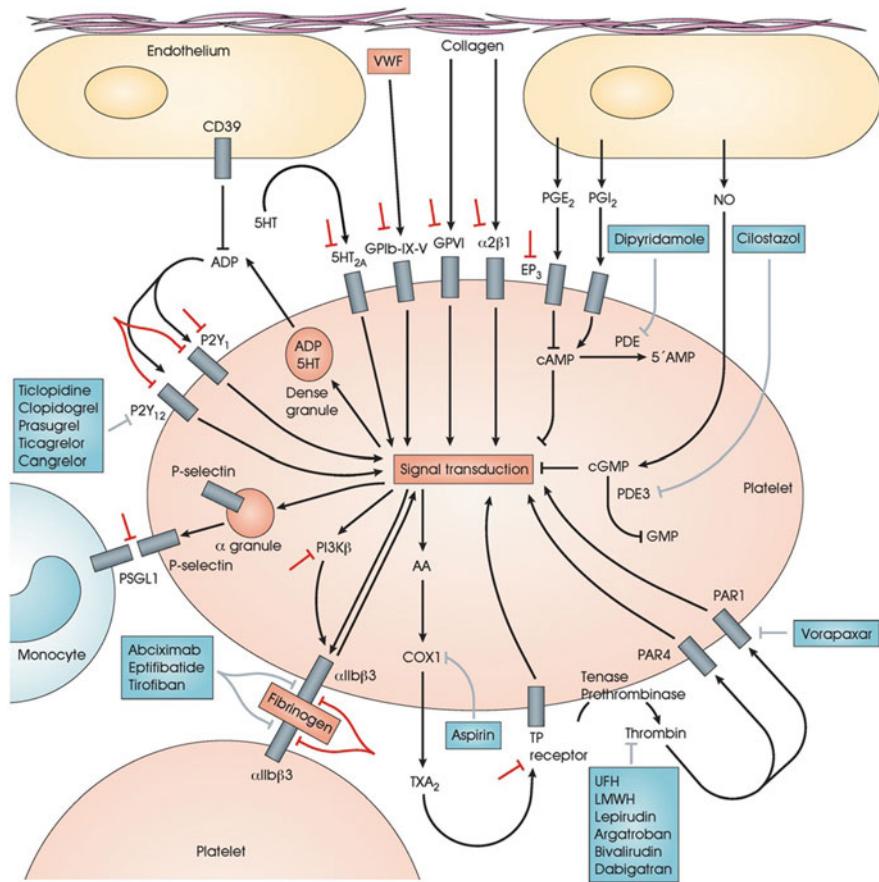


Fig. 12.4 Platelet function and molecular targets of antiplatelet agents. Gremmel et al. (2016). (Open access)

2014). Many antiplatelet medications have been used to mitigate platelet activation and aggregation and consequently lower thrombotic and CVD risks (Smith et al. 2019).

Antiplatelet therapies interfere with platelet adhesion and aggregation and are often used as an alternative to or complement anticoagulants. They act by blocking various platelet receptors. Aspirin and non-steroidal anti-inflammatory drugs, Dipyridamole, and Sulphinpyrazone are the COX1 inhibitors. ADP receptor inhibitors include Toclopidine hydrochloride, Clopidogrel bisulphate, and Prasugrel inhibit platelet ADP P2Y12 receptor. Other common antiplatelet therapy includes the GPIIb-IIIa receptor agonists such as Tirofiban, Abciximab, and Eptifibatide. Figure 12.4 shows the platelet receptors which are blocked by these antiplatelet drugs.

One of the most commonly used antiplatelet drug is Aspirin which is available over the counter without a prescription. Older people have been shown to be at risk

of cardiovascular diseases and diabetes and Aspirin is given to these people regularly for the prevention of a cardiovascular event particularly patients with a history of CVD. However, there is limited evidence showing that there are certain specific changes in platelets with ageing.

Platelet Changes with Age

Ageing is a complex physiological change that is related to increased oxidative stress that may change platelet activity thus leading to cardiovascular disorders. Platelet count shows a well-documented decrease during ageing. The platelet count remains relatively stable during middle age (25–60 years old) but falls in old age (60–70+). The effect of age on platelet function is slightly less clear. A progressive increase in platelet responsiveness to different agonists has been shown during middle age (25–65 years of age) both in men and women, but data is limited for older people. Very little evidence exists on the changes in platelet function in old age (i.e. greater than 75 years old). This is partly because of an assumption that the data from middle-aged subjects can be extrapolated into older age groups, but mainly because of the difficulty of studying platelet function in older subjects in whom the onset of chronic disease and the increased prescription of a range of medications make it hard to separate the effects of ageing on platelet function from other confounders. The Framingham Heart Study concluded that platelet aggregability decreases with increasing age in healthy individuals (O'Donnell et al. 2001). They indicated that the effect of age on platelet function is not linear but, like the effect of age on platelet count, changes at different stages of ageing. Despite little knowledge and evidence of age-related platelet activity, there is evidence showing biochemical changes in the aged population compared to middle age or younger people. Oxidative stress has also been shown to increase platelets during ageing. This increase results in the ability of NO to inhibit platelet activation, which may explain some of the phenotypic variations seen in platelets during ageing. The changes seen in platelets during ageing are likely to result from alterations in platelet production and a reaction to the environmental changes within the blood or vasculature such as age-related changes in the bone marrow. The level of haematopoietic tissue within the bone marrow and its cellularity remains relatively stable during middle age but drops in people over 80 years, with a marked increase in apoptotic cells within the bone marrow (Ogawa et al. 2000). This change in haematopoietic tissue probably also impacts platelet function. The evidence in the literature shows the fall in platelet count starts in humans over 60 years old, whereas haematopoietic tissue changes occur later. This discrepancy could be due to different age groups of subjects in various studies. It is also known that plasma composition and endothelial function change with age which could be instrumental in having an impact on platelet function.

It has been demonstrated that endothelial dysfunction increases with age, leading to reduced nitric oxide (NO) bioavailability and altered prostaglandin profiles (Qian et al. 2012; Walsh et al. 2009; Yavuz et al. 2008). NO and PGI2 being potent

inhibitors of platelet function, their gradual decrease may lead, in middle age, to increased platelet activity. Reduced platelet inhibition also leads to thrombocytopenia at the point where deterioration in vascular health prompts a significant number of platelets to become sufficiently activated to bind to sites of impaired endothelial function or vascular damage or be removed from the circulation in the liver or spleen.

There seems to be a combination of multiple age-related changes including the bone marrow anatomy, megakaryopoiesis, platelet production, and a change in the environment of platelets in circulation. All these play a part in regulating the modification of platelets during ageing. However, there is a lack of data to provide evidence of specific ages when platelet numbers decrease or platelet function increases. Nonetheless, there are changes related to middle age and old age such as mRNA and microRNA expression changes, increased oxidative stress, and modification of platelet receptors. These changes affect both bleeding and thrombosis and particularly impact thrombotic disease therefore, the use of anti-platelet drugs is much more prevalent in the elderly population. Further research in the older population as per age definition by WHO will improve the understanding of the changes in platelet activity and the mechanisms driving these changes leading to geriatric diseases due to physiological effects of ageing on platelets and cell signalling more generally. This will enable the development of anti-platelet therapies, particularly for the older population. The following section will describe the laboratory investigation to detect the risk of bleeding events and thrombotic disease.

Laboratory Monitoring: Diagnostics, Tests, and Assays

Haemostatic markers that predict and detect the risk for both thrombosis and haemorrhage are measurable in clinical pathology laboratories and are evaluated against reference intervals based on a normal healthy adult population. It is documented that age-related changes occur in both the procoagulant and the anticoagulant factors (Favaloro et al. 2014), however, age-adjusted normal reference intervals for an older population cease to exist. To understand how these haemostatic markers evaluate a result for a patient, a description of common assays for fibrinogen and coagulation is outlined.

Laboratory Principles of Tests and Assays for Fibrinogen and Coagulation

Fibrinogen, also known as Factor I, is a coagulation factor that is essential for blood clot formation. It is produced by the liver and released into the circulation along with over 20 other clotting factors. Normally, when a blood vessel wall is injured the

coagulation cascade activates these factors and soluble circulating fibrinogen converts to insoluble fibrinogen threads forming a mesh-like network. Platelets are entrapped in the fibrin mesh forming a stable blood clot. This acts as a barrier preventing additional blood loss, remaining in place until the injured vessel wall has been repaired.

Increases in the levels or activity of any of the procoagulant proteins can lead to faster or greater clot formation, and potentially to an increased prothrombotic risk (Favaloro et al. 2014). It is well known that the plasma concentrations of many coagulation factors increase in healthy humans in parallel with the physiological processes of ageing (Franchini 2006; Mari et al. 2008a, 2008b). This also includes the important procoagulant protein fibrinogen. Plasma fibrinogen levels are a recognised risk factor for thrombotic disorders when in excess and this has been shown to increase with advancing age (Kannel et al. 1987; Stone and Thorp 1985; Wilhelmsen et al. 1984).

Coagulation tests are based on what happens in the test setting (in vitro) and thus do not exactly reflect what is happening in the body (in vivo). Nevertheless, the tests can be used to evaluate specific components of the haemostasis system (Pathology Tests Explained- Fibrinogen 2021).

To understand coagulation testing and the pathways taken, it is important to know the physiologic coagulation cascade that occurs in the body in vivo (previously shown in Fig. 12.1) and the coagulation testing cascade (Fig. 12.5) that occurs *in vitro* when coagulation testing is performed in the laboratory to determine whether

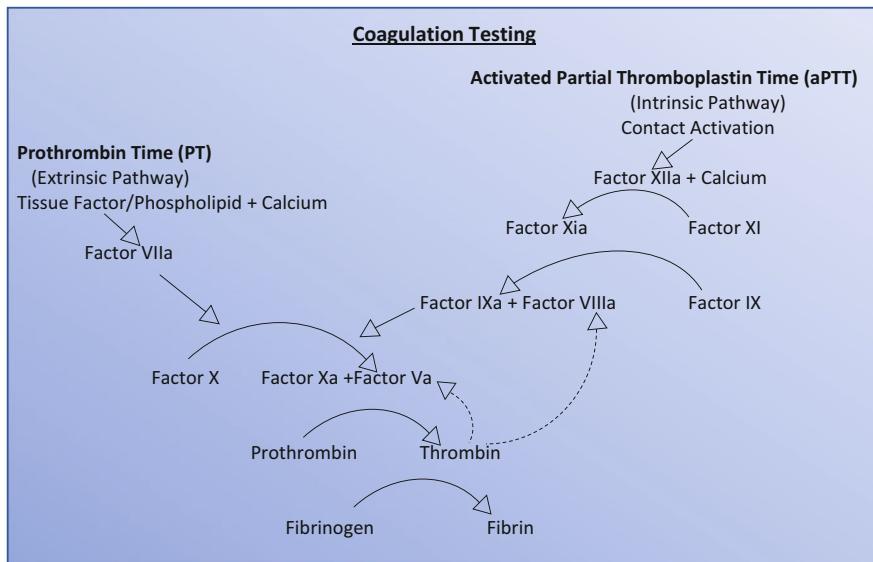


Fig. 12.5 Coagulation testing as described by Prothrombin Time (PT) outlining the extrinsic pathway and activated Partial Thromboplastin Time (aPTT) outlining the intrinsic pathway. Adapted from Pathology Tests Explained- Test Index 2021

PT Result	aPTT Result	Possible cause
Prolonged	Normal	Liver disease, decreased vit K, decreased or defective factor VII, use of Rivaroxaban
Normal	Prolonged	Decreased or defective factor VIII, IX, or XI or lupus anticoagulant present or use of Dabigatran
Prolonged	Prolonged	Decreased or defective factor I, II, V or X, von Willebrand disease, liver disease, disseminated intravascular coagulation, use of Rivaroxaban
Normal	Normal	Decreased platelet function, thrombocytopenia, factor XIII deficiency, mild deficiencies in other factors, mild form of von Willebrand disease

Fig. 12.6 Interpretation of PT and aPTT in patients with a bleeding syndrome (adapted from Pathology Tests Explained- Test Index 2021)

one or more of the coagulation factors is missing, deficient, or not functioning properly or the patient is taking warfarin-related anticoagulants.

The activated partial thromboplastin time (aPTT) measures those protein factors that are part of the cascade referred to as the intrinsic pathway: XII, XI, IX, VIII, X, V, II, and fibrinogen as well as prekallikrein (PK) and high molecular weight kininogen (HK) (Pathology Tests Explained- Coagulation Cascade 2021). The prothrombin time (PT test) measures the factors that make up the extrinsic pathway: VII, X, V, II, and fibrinogen. It may be standardised as the International Normalised Ratio (INR) which is a ratio of the patient's PT versus a normal sample. The INR result is particularly important if a patient is taking a warfarin-related anticoagulant drug (or has a bleeding disorder) to determine the drug's effectiveness by how much it prolongs the PT or increases the INR. Of note, the INR test is not helpful with monitoring the 'new' anticoagulants such as dabigatran, rivaroxaban, or apixaban. Figure 12.6 illustrates the possible condition and interpretation of the PT and aPTT results for a patient when results are normal or prolonged.

For both the intrinsic and extrinsic pathways there are common factors across both otherwise known as the common pathway. At the end of the common pathway soluble fibrinogen (factor I) has been changed into insoluble fibrin threads which form a mesh-like structure (Pathology Tests Explained- Coagulation Cascade 2021). The fibrin mesh along with aggregated platelets forms a stable blood clot.

A fibrinogen activity test evaluates the function of fibrinogen by measuring the time it takes for a fibrin clot to form following the addition of thrombin to plasma. A fibrinogen activity test is used as part of an investigation of a possible bleeding disorder or inappropriate blood clot formation. Low levels indicate reduced production (Liver disease, inherited deficiency) or increased consumption (DIC, Fibrinolysis). Levels are elevated in an acute phase response which can be associated with chronic inflammation in the elderly.

Fibrinogen is usually requested with PT, aPTT, platelets, fibrin degradation products, and D-dimer to assist diagnosis for bleeding or thrombotic events. However, age-related changes in microcirculation and blood coagulation, ultimately contribute to generating a hypercoagulable state with fibrinolysis markers such as D-dimer concentration gradually increasing with age (Favaloro et al. 2014) therefore interpretation of results should be mindful in an older population. Fibrinogen may be used as a follow-up to an abnormal prothrombin time (PT) or activated partial prothrombin time (aPTT) and/or an episode of prolonged or unexplained bleeding (Pathology Tests Explained- Fibrinogen 2021). Occasionally, fibrinogen can be used to help monitor the status of a progressive disease (such as liver disease) over time.

Sometimes fibrinogen is requested with other cardiac risk markers such as C-reactive protein high sensitivity (hsCRP), to help determine a patient's overall risk of developing cardiovascular disease. This use of measuring fibrinogen levels has not gained widespread acceptance though, because there are no direct treatments for elevated levels. A fibrinogen antigen test can be ordered as a follow-up test to determine if a decreased activity is caused by insufficient fibrinogen or dysfunctional fibrinogen. This test measures the quantity of fibrinogen. It uses a fibrinogen antibody to bind to fibrinogen in a blood sample (Pathology Tests Explained- Fibrinogen 2021).

The reference values for fibrinogen are 1.5–4.0 g/L, with some variation between methods (The Royal College of Pathologists Australasia 2021). Fibrinogen concentrations may rise sharply in any condition that causes inflammation, tissue damage, or stress, such as surgery or inflammatory illnesses which may increase with age. Elevated concentrations of fibrinogen are not specific. Usually, these elevations are temporary; returning to normal after the underlying condition has been resolved (Pathology Tests Explained- Fibrinogen 2021). However, reference intervals that determine whether concentrations of fibrinogen are normal may not be age specific. Not only do age-related mechanisms contribute to fibrinogen concentrations, in addition to an increase of procoagulant proteins, hormonal decline with age also increases fibrinogen and other clotting factors (Wilkerson and Sane 2002; Lippi et al. 2014).

Other reasons for altered fibrinogen results include blood transfusions within the past month, drugs that may cause decreased levels, including anabolic steroids, androgens, phenobarbital, fibrinolytic drugs (streptokinase, urokinase, tPA), and sodium valproate. Moderate elevations in fibrinogen may be seen sometimes with pregnancy, cigarette smoking, and with oral contraceptives or oestrogen use (Lippi et al. 2014).

Anticoagulation Therapeutics Amongst Older Adults

Heparin

Older patients are more likely to have a higher incidence of joint disease and undergo subsequent knee arthroplasty, total or revision hip replacement (Jiang et al. 2019).

These medical procedures incur a risk of developing deep vein thrombosis which may lead to a pulmonary embolism. Subsequently, patients are prescribed anticoagulation therapeutics to minimise these risks (Warwick et al. 2007). Low molecular weight heparin (LMWH) or unfractionated heparin are commonly used for the prevention and treatment of deep vein thrombus. More recently heparin has been shown to inhibit the ability and activity of SARS-CoV-2 to replicate in host cells and may have a potential role in inhibiting SARS-CoV-2 infection (Li et al. 2021).

Heparin is a naturally occurring anticoagulant and for medical purposes was initially extracted from bovine and porcine tissue that was originally purified from the liver, consequently the therapeutic was named ‘heparin’ (Gray et al. 2008). Although these drugs have a desirable anticoagulation effect, they have limitations and reported adverse reactions (Pierce et al. 2015; Lieberman and Hsu 2005). Limitations for LMWH in older adults occur when they are discharged from the hospital after surgery, mostly due to the route of administration of the drug which is commonly by subcutaneous injection. For an older patient, the administration method of subcutaneous injection can be complicated and can result in poor medication compliance. Because blood coagulation function needs to be regularly monitored whilst taking LMWH, not only is compliance an issue but also accurate and regular testing.

Warfarin

Patients with atrial fibrillation have a fivefold higher risk of stroke, which increases with age (Wolf et al. 1991). Warfarin is a vitamin K antagonist and was one of the first anticoagulants to be used to be highly effective in preventing stroke in atrial fibrillation (Hart et al. 1999). Warfarin’s mechanism of action is to reduce the total amount of clotting factors in the circulation by inhibiting the C1 subunit of vitamin K epoxide reductase enzyme complex (Rost et al. 2004). This results in the liver’s inability to produce vitamin K-depending clotting factors II, VII, IX, X, and the endogenous anticoagulants proteins C and S.

However, warfarin dosing in patients for the prevention of stroke and those with recurrent venous thrombosis or prosthetic heart valves is affected for several reasons, including genetic variance in hepatic clearance, vitamin K handling, age, drug–drug interactions, diet, alcohol consumption, and smoking (Flockhart et al. 2008). Foods, beverages, and herbal supplementations that contain vitamin K, can affect how warfarin works. Patients taking warfarin are advised to eat consistent amounts of vitamin K-containing food on a week-to-week basis. This includes a reduction in substantial amounts of leafy, green vegetables, or certain vegetable oils that contain substantial amounts of vitamin K. Herbals or supplementations that contain coenzyme Q10 (Ubidecarenone) such as Echinacea, garlic, Ginkgo biloba, ginseng, goldenseal, and St. John’s wort all interfere with the mechanism of action of warfarin which is attributable to adverse events such as a bleeding episode. It is because of the

highly variable responses between individuals that patients require careful monitoring of warfarin. This is particularly important in the elderly who require an age-adjusted protocol with a recommendation to decrease the dose for patients over 80 years old (Roberts et al. 2003).

Nonetheless, even with careful monitoring, warfarin dosing is associated with challenges of achieving and maintaining levels within the narrow therapeutic range that can lead to adverse events. Bleeding, particularly in the setting of over-anticoagulation, is a major concern. Regular monitoring of the anticoagulant effect is required through traditional laboratory monitoring through the international normalised ratio (INR) although assessing the response of warfarin is complicated by a delay of 2–3 days before the INR reflects any changes in warfarin dose.

Direct Oral Anticoagulants (DOAC)

Oral anticoagulants have a major role in the prevention and management of cardiovascular disease and the associated complications. Two commonly used anticoagulants are aspirin and warfarin. Aspirin is widely available and acts by impairing platelet function permanently through inactivating cyclooxygenase (Gremmel et al. 2016).

Warfarin is a commonly prescribed vitamin K antagonist (VKA) for treating and preventing thrombosis. However, warfarin has the disadvantage of a slow onset as it takes several days to lower the levels of vitamin K factors, conversely, it also has a slow offset with a half-life of 36–42 hours (Shameem and Ansell 2013). The slow offset is a challenge especially when warfarin-treated patients require emergency surgery. In addition, dietary interactions affect warfarin anticoagulation, frequent laboratory monitoring is required (refer to testing section), and the risk of bleeding complications because warfarin acts on multiple sites.

A small range of direct oral anticoagulants (DOAC) also known as novel/non-vitamin K oral anticoagulants (NOAC) or target-specific oral anticoagulants (TSOAC) have been introduced to overcome the warfarin-associated disadvantages. These agents target specific single coagulation enzymes—dabigatran inhibits thrombin, whilst apixaban, betrixaban, edoxaban, and rivaroxaban inhibit FXa (Lippi et al. 2019; Lin et al. 2020). Whilst DOAC provide the advantage of a fixed dose administration, rapid onset of action, fewer documented food and drug interactions, no need for routine coagulation monitoring and improved patient satisfaction, the risk of bleeding remains (Kacioglu et al. 2021). DOAC have been demonstrated to be at least as effective as VKA for the treatment of venous thromboembolism and stroke prevention in atrial fibrillation (Chan et al. 2016). DOAC in elderly patients is increasing, but concerns have been raised especially for patients with impaired renal function (Grandone et al. 2020). As ageing is associated with a decrease in glomerular function this leads to compromised elimination of DOAC as well as any other medication the patient may be taking. Consequently, drug accumulation may occur, hence the recommendation is to manage the DOAC in elderly patients with extreme

caution (Grandone et al. 2020). Another recent concern refers to the use of fixed-dose DOAC for adult patients (Sebaaly and Kelley 2020; Mocini et al. 2021). The prevalence of atrial fibrillation (AF) is known to increase with age, and obesity is associated with AF. This has led the Italian National Association of Hospital Cardiologists (ANMCO) to argue that pharmacokinetic data in extreme obesity indicate that a patient with a high body mass index (BMI) may be undertreated with the current fixed dose protocol (Mocini et al. 2021).

The advantages of DOAC need to be considered with the challenges of reversing the anticoagulant effects of DOAC, in situations of bleeding or the need for emergent surgery. Dabigatran can be reversed using idarucizumab (Pollack Jr et al. 2015), and apixaban and rivaroxaban using andexanet alfa, however, these agents are expensive, and availability is limited (Mujer et al. 2020). It is also difficult to assess the effectiveness of any reversal, as routine coagulation assays such as prothrombin time (PT) and activated partial thromboplastin time (aPTT) lack the sensitivity to do this.

Currently, evidence for the use of warfarin over DOACs for anticoagulation is strongest for patients with prosthetic valves, antiphospholipid syndrome, or a high risk of gastrointestinal bleeding. For several clinical situations, including mitral stenosis, obesity, altered gastrointestinal anatomy, pulmonary arterial hypertension, renal or hepatic impairment, and left ventricular thrombus, evidence is lacking but may eventually support the use of DOACs (Wadsworth et al. 2021).

Point-of-Care Testing—International Normalised Ratio (INR)

Patients prescribed oral anticoagulation therapy with warfarin or other vitamin K antagonists must be monitored for over-anticoagulation which could result in bleeding or haemorrhage, and under-anticoagulation resulting in a thrombotic event. The therapeutic range is monitored by the international normalised ratio (INR). The standard method for monitoring INR is done in the laboratory using a venesection blood sample by a qualified scientist. However, point-of-care (POC) testing defined as testing at or near where a patient is located, has the benefits of convenience for the patient when self-managed by a portable device. The patient or clinician can self-adjust the dose of the anticoagulant medication based on the results, using a pre-determined algorithm or protocol. This results in receiving faster test results, improving clinical outcomes, and reducing staff and laboratory resources. Point-of-care testing of the INR can be done in other locations such as pharmacies which may be more convenient and flexible for patients rather than a visit to a clinician or hospital (Tideman et al. 2015).

Evidence suggests that patients who self-monitor using point-of-care testing may have better outcomes and reduced risk of thromboembolic events, but an essential prerequisite is the ability of the patient to use the testing devices correctly, competently, and safely (Heneghan et al. 2012; Christensen et al. 2007; Garcia-Alamino

et al. 2010). One factor that contributes to worse adherence is older age. Literature describes the controversy over the influence of cognitive disorders, physical, and functional disabilities that arise in old age that impact adherence to anticoagulation therapy. Comorbidities associated with an older population including stroke, transient ischaemic attack, and dementia are all associated with poorer adherence to testing and compliance with taking the correct dose (Gumbinger et al. 2015; Horstmann et al. 2015; Jankowska-Polańska et al. 2016).

Point-of-Care Testing—ROTEM and TEG

Point-of-care haemostasis assays such as thromboelastography (TEG) and rotational thromboelastometry (ROTEM) are gaining popularity and wider application. Both the TEG and ROTEM are viscoelastic haemostatic assays that measure the viscoelastic properties of whole blood clot formation, and there are comparisons of these platforms (Ganter and Hofer 2008; Carll and Wool 2020). The use of whole blood provides the advantage firstly of faster turn around time (as early as 10 minutes after the assay begins) by avoiding the need for plasma separation. Secondly, the viscoelastic information encompasses not only coagulation protein but also their interaction with platelets, beginning with the initiation of the clot right through to clot fibrinolysis. More recent versions of both platforms are very user friendly, do not require specialised training and have a relatively small footprint allowing their location in the ward, operation theatres, or laboratory. Conversely, it may be difficult to ensure rigorous quality control of POCT instruments located outside a laboratory (Srivastava and Kelleher 2013).

The newer TEG6s (Haemonetics) measure clot strength (as shown in Fig. 12.7) using the resonance method, in a microfluidic system contained in either a global

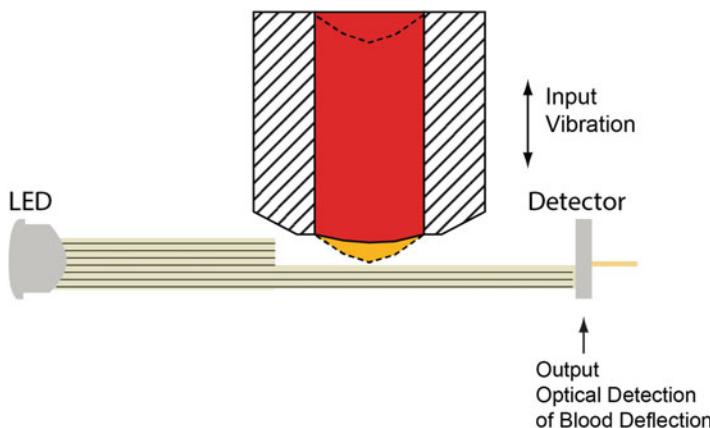


Fig. 12.7 TEG6s resonance detection (Haemonetics Corporation 2015)

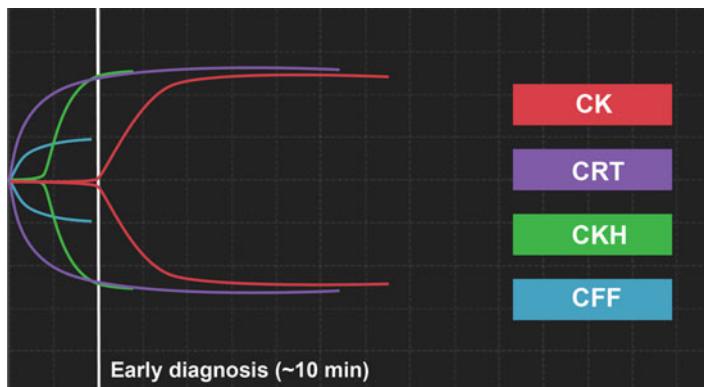


Fig. 12.8 TEG6s viscoelastic changes (Haemonetics Corporation 2015)

haemostasis cartridge or platelet mapping cartridge (Haemonetics Corporation 2015). Although the previous TEG-5000 measured haemostasis via the rotation of a pin immersed in a rotating cup both the TEG6s and TEG 5000 measure the same viscoelastic properties (Lloyd-Donald et al. 2019).

TEG6s cartridges have four channels, hence four different assays are conducted simultaneously. For example, as shown in Fig. 12.8, the Global Haemostasis cartridge contains channels for kaolin (CK), functional fibrinogen (CFF), rapid TEG (CRT), and heparinase (CKH). Graphical representations of the viscoelastic changes of all four channels (CK, CRT, CKH, and CFF) are generated as soon as the assay starts, hence experienced users are often able to interpret results from 10 minutes after the beginning of testing.

In a prospective comparison of first-time cardiac surgery patients aged over 80 years against those aged under 60 years, Boldt and colleagues found significantly different baseline TEG results (Boldt et al. 2002). With patients undergoing elective orthopaedic surgery, ageing was weakly associated with changes in TEG variability (Ng 2004). The Zheng et al. study of elderly patients aged 65 years or more, concluded that currently adopted cut-off values for TEG indices are poorly and modestly predictive of haemorrhage and obstruction (Zheng et al. 2013). A more recent TEG6s study reported that some CK-K and CKH-K results on healthy blood donors aged 60–78 years fell outside the manufacturer's reference range (Donkin and Fung 2020). Together these findings suggest that the adult reference range for various TEG results may not always be suitable for older patients, hence the results need to be interpreted carefully.

The whole blood sample on the ROTEM delta is static in a cup with a pin suspended in it, and the signal from the pin is transmitted via an optical detector system (Ganter and Hofer 2008). ROTEM has been used to guide blood management in various clinical and surgical situations such as cardiac surgery (Ortmann et al. 2015; Pearse et al. 2015). The newer ROTEM sigma like the TEG6s uses cartridges and has been reported to have a strong correlation with the ROTEM delta (Schenk et al. 2019). There are very few papers on the influence of age on ROTEM

results. A multi-centre study reported slightly enhanced clot formation in older participants (Lang et al. 2005). A three-way comparison of healthy young, healthy elderly, and patients with coronary artery disease (CAD), found significantly higher EXTEM clot formation time (CFT) and mean clot formation (MCF) in the healthy elderly and CAD patients relative to young volunteers (Feuring et al. 2011). There was no significant difference between the healthy elderly and CAD patients.

There are a few papers describing the utility of TEG and ROTEM to assess DOAC anticoagulation. In a recent case study, serial TEG testing proved useful for dabigatran reversal in a patient with acute kidney injury (Gilbert et al. 2021). Modification of ROTEM appears to be able to detect DOAC activity (Vedovati et al. 2020). In a different study, the CT and MCF of EXTEM and INTEM demonstrated a strong and significant correlation with the plasma dabigatran concentration in an AF cohort (Sokol et al. 2021). Though limited in number, the published data suggests that the general adult reference intervals for TEG and ROTEM results may not represent the haemostasis in healthy older adults. This highlights the need for studies to develop age-specific TEG and ROTEM reference intervals to support the effective interpretation of results for the world's rapidly growing older population who will require effective anticoagulation, bleeding, and blood management.

Implications and Future Directions

The Demands on Blood Supply for an Ageing Population

About 79% of blood in high-income countries is used by older adults for supportive care, for example cardiovascular, haematology, solid malignancies, and massive trauma (Tinegate et al. 2016; Geneva World Health Org. World health statistics 2021; Simon et al. 2021). As the majority of blood donors are younger adults these figures predict future challenges to securing an adequate blood supply from younger adults to meet the blood needs of older adults. Various strategies may be applied to effectively manage blood use in older patients.

Haemostatic management of older patients on anticoagulants, anti-platelets, or bleeding as discussed previously can be enhanced using reference intervals specific to older adults. A systematic review and meta-analysis concluded that liberal transfusion strategies might be more beneficial for older patients (Simon et al. 2017). To explain this Simon et al. (2019) proposed an oxygen delivery capacity model comparing young, middle-aged, and older adults (Simon et al. 2019). As ageing is associated with changes in the cardiovascular and pulmonary systems, this reduces the maximal oxygen delivery capacity of older adults, especially under metabolic stress. As a result, older adults are more susceptible to tissue hypoxia than their younger counterparts. For example, the model predicts that an older adult with a haemoglobin (Hb) of 100 g/L has a similar peak oxygen delivery capacity to a young adult with a Hb of 70 g/L. Patient blood management (PBM) guidelines such

as those developed by the Australian National Blood Authority (National Blood Authority 2021) have been developed to assist and guide healthcare professionals in making clinical decisions about blood management. These guidelines aim to optimise red cell mass, minimise blood loss and manage anaemia (Isbister 2013). Unfortunately, even though older adults are the largest age cohort of blood users, there is no PBM guideline dedicated to older adults.

Reference Intervals Specific to Older Adults

Laboratory results have an important role in the evidence-based management of haemostasis, and effective interpretation of these results is reliant on relevant reference intervals. There are different age-specific reference intervals for paediatric patients but for most adult reference intervals there are no age-specific ranges despite the rapidly growing older demographics. Ageing associated changes have been reported in the haemostatic system (Wilkerson and Sane 2002; Franchini 2006; Mari et al. 2008a, 2008b; Buccarelli and Mannucci 2002). A study of PT, aPTT, and TEG 6 s global haemostasis on healthy blood donors aged over 60 years revealed that some TEG6s and aPTT results did not fall within the recommended reference intervals (Donkin and Fung 2020). Another study on health participants reported ‘platelet exhaustion’ with ageing, as they found that circulating platelets were more activated but lost their full aggregatory potential (Kuhnla et al. 2019). In its 2020 Recommendations for Hemostasis Critical Values, Tests, and Reporting, the International Council for Standardization in Haematology specified that age and clinical conditions may affect critical value thresholds (Gosselin et al. 2020). Hence, there is growing evidence for age-specific haemostasis reference intervals.

COVID-19 and Vaccinations, the Prevalence of Thrombotic Events and Implications for an Older Population with and without Comorbidities

The risk for severe illness with COVID-19 increases with age, with older adults at highest risk. People 65 and older who received both doses of either Pfizer or Moderna vaccines showed a 94% reduced risk of COVID-19-related hospitalisation (CDC 2021). Older adults are more likely to get very sick, particularly those aged 85 years and older which means that older adults with COVID-19 might need hospitalisation, intensive care, or a ventilator to help them breathe, or they might even die. **The risk increases for people in their 50s and increases in their 60s, 70s, and 80s.** Other factors and comorbidities can also put these people at risk of getting severely ill with COVID-19.

A high prevalence of increased thrombotic events and prothrombotic pathophysiology has been observed through the infection of COVID-19. Both microvascular and macrovascular thrombosis and embolism have been shown to develop in COVID-19 patients in multiple organ systems (Loo et al. 2021; Chaudhary et al. 2021). A particularly high proportion of thromboembolic complications have been reported to arise in haemodialysis circuits and in young, otherwise healthy patients with no underlying predispositions to hypercoagulation despite the administration. It is also becoming obvious that there is an increased risk of complications from COVID-19 with increasing age (and thus increased benefit of vaccination), and the potentially lower, but not zero, risk of thrombosis with thrombocytopenia syndrome (TTS) with increasing age (ATAGI 2021a, 2021b, 2021c).

COVID-19 represents a coagulopathy distinct from disseminated intravascular coagulation (DIC). Numerous pathogenetic mechanisms may contribute to the hypercoagulable state as commonly observed in COVID-19 infection. DIC is the inappropriate activation of the tissue factor pathway of the coagulation cascade, leading to the dissemination and deposition of platelet-fibrin thrombi in the microvasculature. As this causes the rate of consumption of platelets and other procoagulants, the patient gains a tendency to bleed. It has been demonstrated that the coagulation changes mimic but are not identical to DIC (Levi and Toshiaki 2021). It would be reasonable to suppose that the hypercoagulation that can be observed in cases of COVID-19 infection are due to the consequences of the overall inflammatory response to infection and innate immune innervation. A range of signalling pathways are activated in an infected state which results in the release of pro-inflammatory cytokines, which is an auto-promoting process involving multi-organ dysfunction as the immunological response causes significant damage to tissues. Whilst the general principles of inflammation and increased coagulability in critically ill patients are understood and reported, the precise physiological mechanistic steps that occur to lead from COVID-19 infection to hypercoagulation are not well understood. A plethora of proinflammatory cytokines and general inflammatory markers are shown to be elevated in COVID-19 patients such as C-reactive protein and erythrocyte sedimentation rate as well as ferritin and Von Willebrand factor and Factor VIII. All of these have been reported by different groups to have pro-thrombotic effects in other clinical scenarios, but their pro-thrombotic relationship to COVID-19 has not been determined. COVID-19 hospitalised patients with a clinically prolonged activated partial thromboplastin time (aPTT) were found to have a high level of lupus anticoagulant (91% of tested patients) when compared to patients with an elevated aPTT that were not infected with COVID-19 (Bowles et al. 2020). The clinical relevance of the elevation in lupus anticoagulant is yet to be established, but this may be a contributor to thrombosis in COVID-19.

Currently, there is insufficient literature to identify and describe the role, significance, and appropriate use of anticoagulant therapies on a large clinical scale in the mitigation of hypercoagulation in COVID-19-afflicted patients. The enormous prevalence of this virus and the high rate of mortality resulting from thrombosis following its infection should sufficiently motivate clinical studies in this area to ascertain

the true mechanisms of action and therefore develop appropriate therapies. There is currently no targeted strategy for treating the hypercoagulative state observed in COVID-19 infection and the current guidelines suggest thromboembolism prophylaxis for all patients in the absence of significant contraindications. Universal therapeutic anticoagulation must be employed with great caution and monitoring as there is a high likelihood of significant uncontrolled bleeding events with an unrecognised benefit.

Whilst there is a significant health burden associated with thrombosis resulting from COVID-19 infection, there is also a small number of cases reported that has linked COVID-19 vaccination with thrombotic events. COVID-19 vaccines utilise DNA and RNA technology, respectively. One of the newly developed and implemented methods for vaccination is the introduction into tissues of a plasmid containing the DNA sequence which encodes the antigen for which an immune response should be developed. This relies on the production of the target antigen by the host's cellular machinery. There is not a significant difference between the modes of action of effectiveness between DNA and mRNA vaccines. mRNA is a more fragile molecular structure, therefore production may be considered more difficult, but can also be considered generally safer than DNA vaccines, as there is no potential for interruption in the host DNA sequence. The predominant COVID-19 vaccines that rely on mRNA technology are Comirnaty (tozinameran) by Pfizer developed by the German biotechnology company BioNTech in collaboration with American company Pfizer and Spikevax code named mRNA-1273 developed by American company Moderna. The predominant COVID-19 vaccines that rely on DNA technology are Covishield and Vaxzevria code named AZD1222 developed by Oxford University UK and British-Swedish company AstraZeneca and Janssen COVID-19 vaccine also called Johnson and Johnson COVID_29 vaccine developed by Janssen vaccines in Netherland and Belgian Janssen Pharmaceuticals. For those aged 60 years and above, the individual benefits of receiving a COVID-19 vaccine are greater than in younger people. The risks of severe outcomes with COVID-19 increase with age and are particularly high in older unvaccinated individuals. The benefit of vaccination in preventing COVID-19 with COVID-19 Vaccine AstraZeneca outweighs the risk of thrombosis with thrombocytopenia syndrome (TTS) in this age group and underpins its ongoing use in this age group (ATAGI 2021b). A very rare side effect involving blood clotting and low blood platelet count may occur after the AstraZeneca vaccine. However, this condition is very rare and it is less likely in older adults than in younger adults. For older persons, the benefits of being protected from severe COVID-19 through vaccination greatly outweigh the risk of harm from this condition (Australian Government Guidelines 2021).

Conclusion

Ageing is associated with changes to various aspects of the haemostatic system. These changes have significant clinical implications in preventing thrombotic events, managing anticoagulants, anti-platelets, and bleeding events. To support

more effective haemostatic management of the growing older demographic around the globe, age-specific reference intervals that include clear values for those aged 60+ years are needed.

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Chapter 13

An Insight into Platelets at Older Age: Cellular and Clinical Perspectives



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Abstract Higher access to medical care, advanced diagnostic tools, and overall public health improvements have favored increased humans lifespan. With a growing proportion of older adults, the associated costs to care for ageing-associated conditions will continue to grow. This chapter highlights recent cellular and clinical evidence of platelets at an older age, from the hyperreactive phenotype associated with thrombosis to the well-known hallmarks of ageing identifiable in platelets and their potential functional implications on platelets at an older age. Therefore, it is imperative to understand platelets' molecular and cellular mechanisms during ageing in health and disease. New knowledge will favor the development of new ways to prevent some of the age-associated complications where platelets are key players.

Keywords Ageing · Megakaryocytes · Platelets · Platelet hyperreactivity · Chronic degenerative diseases · Hallmarks · Thrombosis

Introduction

Ageing is defined as a time-dependent, accumulative, and irreversible decline of molecular, biochemical, and cellular functions (Kyriazis 2020; López-Otín et al. 2013). Ageing is influenced by intrinsic (genetics, epigenetics, sex, age) and extrinsic (lifestyle and environmental) factors (Passarino et al. 2006; Passarino et al. 2016; Bellizzi et al. 2012; Alves et al. 2018) that have a direct effect on the development and progression of chronic degenerative diseases. According to demographic population studies, by 2030, the proportion of older adults (over 60 years old) will reach 16.5% and 21.5% by 2050 (Un 2013). This demographic shift is likely the result of

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an increased lifespan due to better access to medical care and management of age-associated conditions (Meyer et al. 2020).

The impact of ageing on the hemostatic system is subtle but substantial. This can range from abrupt development of life-threatening thrombotic or hemorrhagic events to experiencing slight but significant functional alterations of its main components: the coagulation system, the fibrinolytic system, or the cellular element, platelets. Platelets are the essential cellular element of the hemostatic system in charge of preserving vascular integrity and halting bleeding, therefore maintaining hemostasis. This process initially requires tight coordination between platelets and coagulation factors to form a thrombus to restore vascular integrity efficiently. Meanwhile, the fibrinolytic component ensures that the growing thrombus does not enlarge beyond the required size to restore hemostasis. When hemostasis is disrupted (i.e., inflammation, infection, cardiovascular disease, diabetes, or low platelet counts), the thrombus enlarges and obstructs blood flow, causing thrombosis.

Ageing is a major independent risk factor for thrombosis (Iyer and Dayal 2019; Puurunen et al. 2018; Raskob et al. 2014). The impact of ageing on platelets has been primarily associated with developing a hyperreactive platelet phenotype. Platelet hyperreactivity is likely a key player in the significantly high incidence of thrombosis at an older age (Iyer and Dayal 2019; Puurunen et al. 2018). Remarkably, the principal causes of death among the elderly have remained unmodified over 19 years from 1990 to 2019 (Fig. 13.1). However, despite their diverse etiologies, platelet hyperreactivity and thrombosis are the most common pathophysiological mechanisms underlying morbidity and mortality among the leading causes of death during ageing (Raskob et al. 2014).

Qualitative Changes of Platelets at an Older Age

Platelets are the smallest, anucleate circulating blood cells with an 8–10 days lifespan (Lebois and Josefsson 2016). Megakaryocytes are platelet precursors whose function is significantly influenced by systemic and local cues in their bone marrow niche (Haas et al. 2015; Baaten et al. 2017; Cognasse et al. 2019). The RNA and organelles provided by megakaryocytes, to a large extent, define their functional profile. Despite lacking a nucleus, transcriptome analysis of platelet RNA is a common technique employed to gain an insight into the functional programming conferred to platelets by megakaryocytes under health and disease conditions.

Platelets contain RNAs, mitochondria, alpha, dense granules, and lysosomes acquired from megakaryocytes during thrombopoiesis. A small proportion of the RNA found in platelets has been estimated to be either obtained by platelets throughout their lifespan in circulation due to cellular crosstalk with immune or endothelial cells. During thrombopoiesis, megakaryocytes synthesize (for the most part) hundreds of bioactive molecules that are incorporated into alpha or dense granules, as well as lysosomes. A small number of plasma proteins like fibrinogen, VEGF, or factor V can instead be endocytosed by megakaryocytes and sorted into



Fig. 13.1 Principal causes of death among elderly between 1990 and 2019. The leading causes of death have remained almost the same over time. A high incidence of thrombosis is associated with the diseases highlighted in purple

platelet granules. Among many bioactive molecules, dense granules contain calcium, serotonin, histamine, and ADP, which modulate blood flow and amplify platelet activation (Maynard et al. 2010; Maynard et al. 2007).

Platelets are essential for maintaining hemostasis and, under pathological conditions, are critical participants in the pathophysiology of thrombosis (Xia et al. 2018; Laffont et al. 2013).

Platelets are unique cells that can go from a resting state to full activation and aggregation in a matter of minutes. Platelets are the responsible cells for reaching primary hemostasis. Briefly, at times of vascular damage (i.e., trauma), platelets adhere to the damaged site through many surface receptors such as GpVI that bind to collagen or GpIb, which can bind primarily to von Willebrand Factor. These interactions initiate activation of the alpha IIb/Beta 3 receptor that binds soluble fibrinogen and promotes platelet aggregation through platelet–fibrinogen–platelet interactions. Platelet aggregates then incorporate soluble coagulation factors to form larger clots. In parallel to this process, platelets release the contents of alpha and dense granules as many of these bioactive molecules will enhance or amplify clot formation. In summary, primary hemostasis requires tight coordination between platelets (cells), coagulation factors (i.e., fibrinogen and thrombin), and platelet granules to stop vascular damage and bleeding.

Platelet hyperreactivity has been defined as a low threshold for activation by low doses of agonists. Some of the mechanisms implicated include disruption of the redox balance and inflammation. We have recently described that platelet hyperreactivity in older mice (>18 months of age) is mainly due to chronic TNF- α . Platelets from old mice exhibit higher activation of the alpha IIb/Beta 3 integrin after stimulating the thrombin and collagen pathways. Transcriptome analysis of megakaryocytes from young and old mice revealed alterations in metabolic pathways that underly platelet hyperreactivity in mice. Moreover, the use of an anti-IL-1 β inhibitor in patients with established cardiovascular disease (CVD), elevated markers of inflammation, and a mean age of 61 years led to a significant reduction in cardiovascular events. While platelet activation was not determined in this study, it is possible to speculate that anti-IL-1 β treatment decreased platelet hyperreactivity and contributed to the positive outcome of this study. Thus, ageing-associated inflammation (inflammageing) is a crucial promoter of platelet hyperreactivity.

Interestingly, dense granules increase proportionally according to age and correlate with frailty. Moreover, dense granules and platelet size are elevated in patients with schizophrenia, suggesting a potential role for platelet granules in the physiopathology of some neurological disorders (Kessler et al. 1995). Similarly, alpha granules fuse with the plasma membrane to release their contents upon activation. Alpha granules contain chemokines (that recruit and modulate immune cells), tissue factors, coagulation factors, fibrinogen, and von Willebrand Factor (VWF). Higher levels of alpha granule-specific proteins and platelet–leukocyte aggregates are found in the plasma of older individuals compared to younger adults. Such findings suggest higher activation of platelets in older individuals during circulation, therefore, a lower threshold for activation. This ongoing activation would lead to platelet degranulation and the formation of platelet–leukocyte aggregates (Mohebali et al. 2014).

In addition, platelets are important mediators of inflammation, angiogenesis, wound repair, tissue remodeling, and host defense (Cognasse et al. 2019; Ed Rainger et al. 2015; Lindemann et al. 2001; Singh et al. 2022; Pourkarim et al. 2022; Kisucka et al. 2006). Non-hemostatic roles of platelets are dependent on some degree of

platelet activation. For example, higher amounts of exposed phosphatidylserine (PS) increase the prothrombotic potential of platelets as coagulation factors require PS to assemble and amplify coagulation. Similarly, low-grade degranulation of platelets has been documented in humans and mice with diseases where platelets are characteristically hyperreactive and are constantly undergoing some degree of activation in circulation without necessarily triggering thrombosis. This is supported by the identification of granule proteins like P-selectin on the surface of platelets and the elevated levels of granule proteins and metabolites like serotonin (dense granule) and platelet factor 4 (PF4) circulating in the plasma (Melki et al. 2021; Yamanishi et al. 1985). Markers of degranulation such as P-selectin on the surface of platelets or in circulation (soluble P-selectin) as well as higher levels of platelet-specific proteins like PF4, RANTES, or serotonin have been identified in older individuals. These findings suggest that at an older age, platelets have a lower threshold for activation in circulation during ageing, resulting in low-grade platelet degranulation. For that reason, the primary focus of the research surrounding platelet biology during ageing focuses on identifying the mechanisms that regulate platelet hyperreactivity and thrombosis.

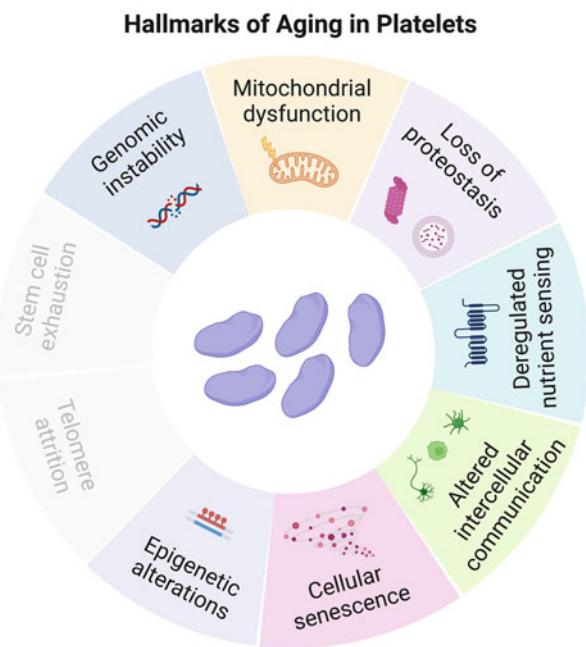
Hallmarks of Ageing

Ageing is a well-conserved phenomenon in metazoans, and transcriptomic profiling has proven to be a high throughput assay to identify the dysregulated pathways between young and old humans and rodents. In this regard, extensive evidence has led to the characterization of a series of signatures or hallmarks of ageing (López-Otín et al. 2013): mitochondrial dysfunction, loss of proteostasis, deregulated nutrient sensing, altered intercellular communication, epigenetic alterations, telomere attrition, genomic instability, stem cell exhaustion, and cellular senescence. However, since platelets lack a nucleus, not all these hallmarks, such as telomere attrition and stem cell exhaustion, apply (Fig. 13.2). Moreover, some of these hallmarks have not been studied in platelets from elderly subjects (Fig. 13.3).

Genomic Instability and Epigenetic Alterations

Genomic instability and epigenetic alterations have been related to ageing (López-Otín et al. 2013). Despite platelets lacking a nucleus, they do possess mitochondria. Therefore, platelets have mitochondrial DNA (mtDNA), which encodes 13 subunits of the oxidative phosphorylation system, rRNAs, and tRNAs. During the ageing process, mtDNA is prone to acquiring higher de novo mutations than the genomic DNA (Wei Soong et al. 1992; Corral-Debrinski et al. 1992). mtDNA is synthesized by polymerase gamma that has a high-fidelity function (Anderson et al. 2020). Its exonuclease domain is sensitive to oxidation. Consequently, its exonuclease activity

Fig. 13.2 Hallmarks of ageing in platelets. Platelets lack a nucleus and thus only exhibit some of the hallmarks of ageing. Such as genomic instability, mitochondrial dysfunction, loss of proteostasis, deregulated nutrient sensing, altered intercellular communication, cellular senescence, and epigenetic alterations. Created with BioRender.com



is affected by high levels of ROS (Anderson et al. 2020). Hence, the frequency and mutated nucleotides in mtDNA are significantly higher in older mice (10 months) than in younger mice (1 month). These observations suggest that these types of mutations increase and accumulate during the ageing process (Arbeithuber et al. 2020). There is no direct evidence of a higher frequency of mtDNA mutation in platelet of older individuals. However, if that was the case, we could speculate that a higher frequency of mutated mtDNA in platelets may have a functional impact on platelets.

Besides the above evidence, mtDNA is sensitive to epigenetic modifications (Fig. 13.3). This is illustrated in platelets from patients with CVD. These platelets exhibit hypermethylation of the Mitochondrially Encoded Cytochrome C Oxidase I genes (MT-CO1, 2, and 3) and also of the Mitochondrially encoded tRNA leucine 1 (MT-TL1) gene (Baccarelli and Byun 2015). Similarly, in a different study, hypermethylation of those same mitochondrial genes was a reliable predictor for CVD incidence in obese patients (Corsi et al. 2020). Moreover, evidence of epigenetic plasticity in platelets has been described in a recent publication (Bordoni et al. 2020). This evidence suggests that epigenetic changes in mtDNA of platelets are related to the progression of ageing chronic degenerative diseases. Therefore, the development and validation of assays to evaluate epigenetic modifications of mtDNA in platelets may be an alternative to identify individuals at significant risk for developing CVD at an older age.

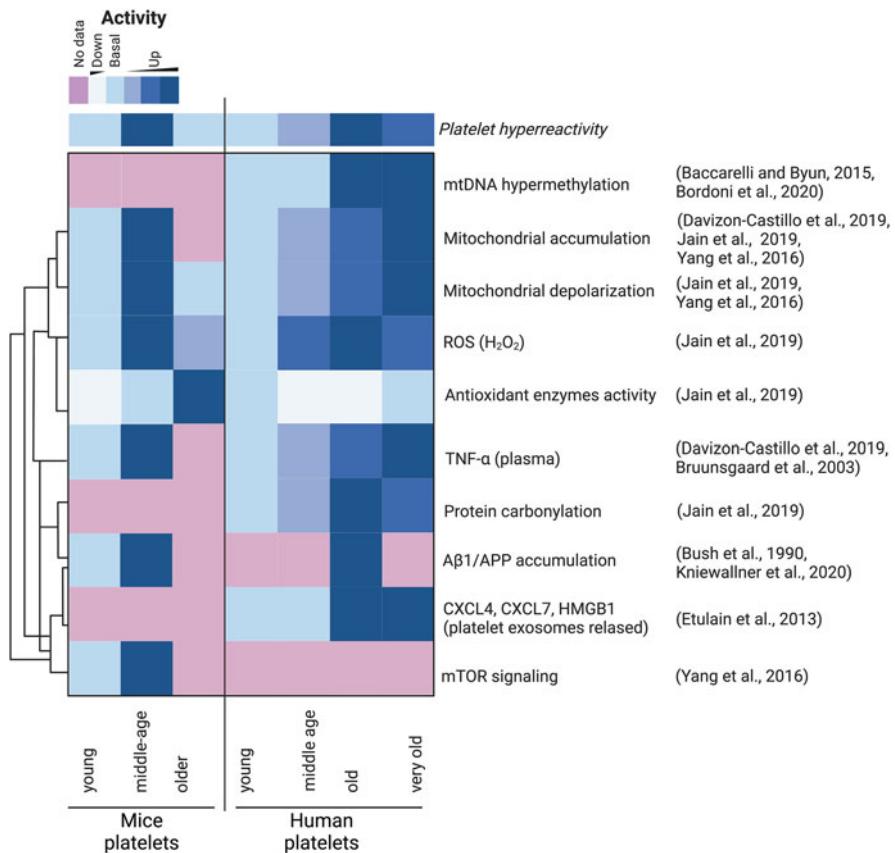


Fig. 13.3 Features of human and mouse platelets at an older age. The heatmap shows the relative levels of some of the most prevalent features of platelets during ageing. The map tree shows the associations among the hallmarks of ageing (Baccarelli and Byun 2015; Bordoni et al. 2020; Davizon-Castillo et al. 2019; Jain et al. 2019; Yang et al. 2016; Brunsgaard et al. 2003; Bush et al. 1990; Kniewallner et al. 2020; Etulain et al. 2013) Created with BioRender.com

Loss of Proteostasis

As per their etymological origin, proteins are the primary component of cells. Proteins are regulated at several hierachal processes such as transcription, translation, posttranslational modifications, and clearance by the autophagic and ubiquitin-proteasome systems. Ageing and chronic degenerative diseases are characterized by loss of proteostasis. This is manifested by improperly expressed proteins and by the accumulation of misfolded or damaged proteins.

Platelets lack a nucleus and thus are incapable of executing the transcriptional process de novo. However, during platelet formation (thrombopoiesis),

megakaryocytes load platelets with plenty of RNAs. mRNA (Supernat et al. 2021), miRNA (Landry et al. 2009), and lncRNAs (Yan et al. 2020) are present in platelets. For this reason, transcriptome analysis of platelets is a valuable tool used to identify relevant pathways modulating platelet function in disease states (Davizon-Castillo et al. 2020). Evidence suggests that RNAs change over the lifespan of platelets. Their decay is directly proportional to the increment of circRNA (Alhasan et al. 2016) and the decrease in protein translation rate (Angénieux et al. 2016).

Moreover, the plethora of RNAs in platelets allows them to selectively modulate translation under normal and pathological stimuli (Schwartz et al. 2006; Denis et al. 2005). For example, Bcl-3 protein is only synthesized and detected in activated platelets to participate in clot retraction (Weyrich et al. 1998). Similarly, tissue factor (TF) mRNA present in platelets is spliced and subsequently translated upon activation. Expressed TF by activated platelets will then enhance the activation of coagulation during clot formation (Schwartz et al. 2006). Also, platelets are important mediators of inflammation. Upon an inflammatory stimulus such as lipopolysaccharide, platelets trigger the splicing, translation, maturation, and secretion of potent mediators of inflammation such as IL-1 β (Brown and McIntyre, 2011) and cyclooxygenase-2 (COX2) (Shashkin et al. 2008) therefore amplifying inflammation in their surrounding environment.

Our understanding of the impact of COVID-19 on megakaryocytes and platelets is a rapidly evolving area. Platelet hyperreactivity is characteristic of patients with severe COVID-19 infection (Jakobs et al. 2022; Zaid et al. 2022; Barrett et al. 2021). Among the different mechanisms of platelet hyperreactivity in COVID-19 infection, dysregulation of the GpIb-IX-V /von Willebrand Factor (VWF) pathway has been implicated (Ji et al. 2022). Elevated exposure of P-selectin on platelets of patients with COVID-19 has also been found to increase the interaction of platelets and leukocytes. This platelet–leukocyte crosstalk leads to Neutrophil Extracellular Traps (NETs), which subsequently exacerbate tissue damage and promote thrombosis (Middleton et al. 2020; Ng et al. 2021; Skendros et al. 2020). Altogether, it is also plausible that several of the hallmarks of ageing underlying megakaryocytes and platelets of older individuals and patients with chronic inflammatory comorbidities (i.e., diabetes, obesity, and CVD) are exacerbated by COVID-19 infection. Such disruptions by COVID-19 disease would likely increase platelet hyperreactivity and thrombosis one of the leading causes of elevated morbidity and mortality of COVID-19, particularly for individuals with pre-existing comorbidities and older age (Zhang et al. 2022).

Non-coding RNAs have a role in platelet translation and horizontal gene regulation. Analysis of the differential expression of noncoding RNA in platelets under health or disease states has been employed as a biomarker for disease risk and drug responsiveness (Shi et al. 2013; Marketou et al. 2019). This type of analysis has proven helpful in studying age-associated diseases like hypertension or coronary artery disease. For example, miR-22 and miR-223 levels are reduced in platelets from hypertensive patients (Marketou et al. 2019). Furthermore, low miR-223 levels have been correlated to low responsiveness to clopidogrel in patients with coronary heart disease and platelet hyperreactivity (Shi et al. 2013).

Posttranslational modifications are important ways of regulating protein activity and fate. For example, reactive oxygen species (ROS) can irreversibly oxidize proteins by direct oxidation of amino acids (primary carboxylation) or by a second carbonylation reaction (oxidized glycans or lipids oxidize proteins). These types of modifications can lead to improper protein folding, promote unfolding, protein loss of function, changes in metabolism, and as a result, a decrement in cellular viability. For example, platelets from diabetic patients have high amounts of carbonylated mitochondrial proteins correlated with a low ATP production (Avila et al. 2012). Moreover, proteins from human platelets are carbonylated in an age-dependent manner (Fig. 13.3) (Jain et al. 2019) and directly proportional to ROS levels (Jain et al. 2019). The proteasome degrades ubiquitin-tagged proteins by either the 26S proteasome or, in the case of non-ubiquitin proteins, by the 20S proteasome. The 20S proteasome is responsible for recognizing and degrading carbonylated proteins. However, protein oxidation can lead to the exposure of the protein hydrophobic domain, which induces aggregates inhibiting the proteasome (Andersson et al. 2013; Terman and Sandberg 2002; Sitte 2000). Protein aggregates may contain a complex mixture of damaged proteins, lipids, and carbohydrates, such as lipofuscin (Terman and Sandberg 2002), which can generate ROS, triggering a vicious oxidative cycle. Moreover, the electron transport chain (ETC) and proteins are prone to carbonylation and hydroxynonenal (HNE) adduction (Choksi et al. 2004) (such as ETC and VTAC). These modifications give rise to mitochondrial dysfunction, opening the mitochondrial permeability pore, inducing the generation of more ROS, and releasing the cytochrome C (Choksi et al. 2004). Besides proteasome activity inhibition by protein aggregates, proteasome activity declines with age (Saez and Vilchez 2014).

The proteasomal activity of platelets during ageing has not been investigated; however, its role in platelet formation (thrombopoiesis) lifespan has. The conditional deletion of PSMC1 (a subunit of the 26S proteasome) in mice induced thrombocytopenia accompanied by a lethal postnatal phenotype (Shi et al. 2014). Moreover, inhibition of the proteasome leads to the accumulation of the proapoptotic protein Bax triggering phosphatidylserine (PS) exposure and increasing the clearance of platelets. Altogether, proteasome inhibition shortens the lifespan of platelets (Nayak et al. 2013). Moreover, pharmacological proteasome inhibition by MG132 and bortezomib decreases platelet activation (Gupta et al. 2014; Shi et al. 2014). Whether the mild inhibition of platelet activation is solely by altering platelet proteostasis or due to disruption of other metabolic routes in platelets remains to be evaluated.

Another regulator of protein turnover is the autophagic process. Autophagy is a housekeeping program that allows the degradation of damaged and aggregated proteins and damaged organelles (such as mitochondria, endoplasmic reticulum, and peroxisomes), RNA, and lipids (Dikic and Elazar 2018). Autophagy is always active at low basal levels; however, it can be triggered by different types of stress such as low levels of amino acids, nutrients, growth factors, ROS, DNA damage, and UV radiation (Dikic and Elazar 2018). Mammalian cells possess three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (Parzych and Klionsky 2014). However, until now, macroautophagy (hereafter referred to as autophagy) has been the most studied type of autophagy investigated

in megakaryocytes and platelets. It has been determined that autophagy is essential for megakaryopoiesis, thrombopoiesis, and platelet activation. Genetic ablation of the autophagic process in mice causes thrombocytopenia due to alterations in thrombopoiesis (Cao et al. 2015). Moreover, as the autophagic process is also critical for full platelet activation, its inhibition also results in a deficient platelet activation (Feng et al. 2014). Therefore, autophagy is critical for platelet generation (thrombopoiesis) and adequate platelet activation in mice.

In humans, platelets from patients with immune thrombocytopenic purpura (ITP) (characterized by a reduced platelet count due to immune-mediated clearance of platelets) exhibit activation of mammalian Target of Rapamycin (mTOR), a negative regulator of autophagy (Wang et al. 2019). These findings suggest that platelets from patients with ITP have decreased autophagy; thus, their function might be inadequate if their autophagic flux is disrupted. Moreover, Sirolimus, an inhibitor of mTOR, has been shown to improve platelet counts in patients with refractory (minimal to no clinical response to standard treatments) ITP (Wang et al. 2019). While Sirolimus is primarily employed as an immunomodulator (to decrease platelet clearance), it is plausible to speculate that Sirolimus induces autophagy in megakaryocytes and platelets, which in turn, ameliorates thrombocytopenia by improving thrombopoiesis (platelet formation) and platelet function (Feng et al. 2020). In summary, evidence suggests that autophagy is necessary for adequate megakaryocyte and platelet function. However, the autophagic flux status of platelets during ageing and its impact on platelet hyperreactivity and thrombosis remains to be elucidated.

Mitochondrial Dysfunction

Excessive generation of ROS is considered one of the main consequences of mitochondria dysfunction (López-Otín et al. 2013). Endogenous and exogenous; ROS, particularly H₂O₂, enhance the activation of murine and human platelets (Dayal et al. 2013; Pratico et al. 1992; Canoso et al. 1974; Levine et al. 1976). Exposure of platelets to H₂O₂ activates the production of thromboxane A2 (TxA2), which lowers the activation threshold of platelets by low doses of platelet agonists. Thus, in a way, H₂O₂ exerts a priming effect over platelets (Pratico et al. 1992; Praticò et al. 1991). Platelets from older (>60 years old) patients with acute coronary syndrome, therefore, with activated platelets, exhibit an inversely proportional relation between decreased glutathione peroxidase (GPx (antioxidant potential) and amounts of circulating ROS (Holley et al. 2016). Moreover, elevated markers of oxidative stress and mitochondrial dysfunction are directly correlated with platelet hyperreactivity and frailty in older adults (Arauna et al. 2020). Therefore, a growing body of evidence suggests that assessment for frailty in older adults could be a helpful tool to identify older individuals at risk for thrombotic events that might benefit from interventions to reduce ageing-associated thrombosis.

Our own and other teams have found that platelets from old mice accumulate mitochondria in a TNF- α -dependent way (Fig. 13.3), likely due to disrupted autophagic flux. While not directly investigated, it is plausible to hypothesize that a significant fraction of the mitochondria accumulated in platelets from older mice is dysfunctional, are therefore prone to generate higher amounts of endogenous ROS (Davison-Castillo et al. 2019). In a different study, investigators found that N-acetylcysteine (NAC), a potent ROS scavenger, ameliorates platelet hyperreactivity in older mice (Yang et al. 2016). Consequently, endogenously generated platelet ROS are essential modulators of platelet activity.

Finally, inflammation is another relevant contributor to oxidative stress in platelets. Our work shows that ageing-associated platelet hyperreactivity in old mice is associated with TNF- α -driven inflammation and mitochondrial dysfunction. Recent evidence indicates that TNF- α directly induces ROS production in platelets, priming platelets for activation (Naime et al. 2019). Consequently, it is possible to hypothesize that TNF- α -driven inflammation in old mice promotes platelet hyperreactivity to a certain extent due to the direct induction of ROS by platelets.

Altogether, mitochondrial dysfunction is associated with platelet hyperreactivity in ageing. The current evidence points toward a multifactorial process that includes decreased antioxidant potential, direct stimulation of ROS production by inflammatory cytokines like TNF- α , or even dysregulation of the mechanisms for clearance of dysfunctional mitochondria like autophagy and mitophagy. While the employment of antioxidants for older individuals seems like an apparent intervention, historical evidence of serious bleeding complications in older adults taking over-the-counter antioxidants calls for caution (Schiavon et al. 1984; Steiner 1983; Freedman 2008). Several research groups devote our time to increasing our understanding of the mechanisms that underly platelet hyperreactivity to develop new safe, and cost-effective therapies to modulate this age-associated deleterious platelet phenotype.

Altered Intercellular Communication

Secretion occurs upon activation; thus, platelet-mediated secretion is a pivotal modulator of wound healing, inflammation, angiogenesis, and senescence. Platelet secretion is mediated by alpha granules, dense granules, lysosomes, and extracellular vesicles (EV) such as exosomes and microparticles. These granules contain over 250 different bioactive molecules that confer to platelets the potential to participate in modulating other functions beyond hemostasis and thrombosis. For example, vascular endothelial growth factor (VEGF) contained in alpha granules can promote angiogenesis (Etulain et al. 2013). Chemokines like RANTES, MIP-1 α , or CXCL4 facilitate the recruitment of lymphocytes and neutrophils at damaged sites (Fig. 13.3) (Gleissner et al. 2008). P-selectin present in alpha granules promotes cellular inflammation by assisting with the recruitment of monocytes through PSGL-1 present on the surface of monocytes and endothelial cells (da Costa Martins et al. 2007; Celi et al. 1994; Ivanov et al. 2019).

Diseases that affect platelet delta granules like Hermansky Pudlack Syndrome (HPS) or alpha granules such as Grey Platelet Syndrome (GPS) are characterized by either altered formation (decreased number) or alterations in the secretory machinery. Patients with these types of modifications in platelet granules typically exhibit a clinical bleeding phenotype due to reduced platelet numbers (impaired thrombopoiesis) or the inability to release granule contents upon activation to enhance primary hemostasis. This highlights the importance of platelet granule bioactive molecules that participate in hemostasis. In addition, alterations in the regulatory components of platelet granule formation or secretion often affect innate immunity or result in immune dysregulation. For example, some forms of HPS are accompanied by diminished degranulation of Natural-Killer cells (Boeckelmann et al. 2021). Similar alterations have been reported in patients with GPS where leukocytes exhibit altered granule secretion, which predisposes patients to immune dysregulation and autoimmunity.

Platelets can participate in endothelial remodeling through the release of chromogranin (a monocyte chemoattractant precursor), cyclophilin A, and vascular smooth muscle cell growth factor via EV, which colocalize in atherosclerotic lesions (Coppinger et al. 2004). Thus, platelets are active participants in arterial wall remodeling and plaque stability (Coppinger et al. 2004). Additionally, plaque rupture activates platelets, inducing arterial thrombosis, the cause of stroke and myocardial infarction.

The two leading causes of death in cancer are thrombotic events (Khorana 2010) and metastasis (Dillekås et al. 2019), of which platelets are essential modulators. Platelet–tumor cell interaction may induce platelet activation enhancing the release of growth factors from platelet granules and thus fueling tumor growth and epithelial–mesenchymal transition (Guo et al. 2019; Labelle et al. 2011). In this regard, activated platelets release TGF β , activating macrophages and neutrophils, which induce a pro-inflammatory microenvironment. Additionally, platelet–cancer cell aggregates protect cancer cells from shear forces and help them to colonize secondary niches (Lambert et al. 2017).

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by memory and cognitive impairment and is the most common worldwide cause of dementia (Breijyeh and Karaman 2020). The strongest risk factor for AD is ageing, and platelets contribute to the physiopathology of AD (Ferrer-Raventós and Beyer 2021). Platelets from patients with AD contain less dense granules (Kessler et al. 1995) which is directly proportional to the clinical severity of dementia (Tajeddinn et al. 2016). One of the main features of AD is the formation of senile neocortical plaques, which are mainly constituted by the amyloid- β peptide (A β) (Sun et al. 2015). Cleavage of amyloid precursor protein (APP) by γ/β -secretase generates A β . In AD, accumulation of A β in neurons increases the production of ROS that causes neurodegeneration (Sun et al. 2015). Platelets contain both APP and A β which can be released upon activation by collagen or arachidonic acid. Platelets are considered the primary source of the circulating APP. Platelets from AD patients exhibit approximately 17% more β -secretase activity than healthy elderly individuals (Johnston et al. 2008). Therefore, platelets are critical players in

the physiopathology of AD as they contain the elements required for the formation of A β . More recent evidence demonstrates that 22% of the total number of platelets from a mouse model of AD (APP mouse model) accumulate in the brain compared to controls, indicating a significant adhesion of AD platelets to the cerebral vasculature (Kniewallner et al. 2020). Platelets from mice with AD not only exhibit hyperreactivity but also, in the presence of A β these platelets form stronger clots resistant to thrombolysis (Cortes-Canteli et al. 2010). Moreover, platelets from older mice with AD (APP mouse model) transferred to control young mice led to the formation of A β accompanied by cognitive deterioration in the control young mice (Wu et al. 2021).

Cellular Senescence

Cellular senescence has been defined as a state of replicative cell death (Kumari and Jat 2021; Di Micco et al. 2021; Blagosklonny 2003). In general, nucleated senescent cells remain viable but can display morphological changes, metabolic alterations, gene expression changes, and senescence-associated secretory phenotype (SASP).

Given that platelets lack a nucleus, only a few senescence-associated features can be assessed. For instance, platelets are not subjected to the same checkpoints as nucleated cells due to the lack of nuclear DNA. Therefore, their senescence phenotype is substantially divergent from one of nucleated cells. Platelet senescence at an older age has not been addressed. Instead, the field has focused on studying platelet senescence throughout their lifespan in circulation. Studies focusing on understanding the mechanisms that regulate platelet senescence and apoptosis aim to identify interventions that could delay these processes. Such discoveries could significantly impact the way platelets are stored for transfusion.

The estimated lifespan of platelets is 5–10 days. In blood banks, platelets are stored for up to 5–7 days (Arbeithuber et al. 2020). Thus, considering that platelets are prone to activation ex vivo, a prolonged storage time can increase the likelihood of activation and even accelerate cell death. Some of the main senescence events found in platelets ex vivo are activation of an intrinsic platelet apoptotic pathway modulated by a decline of Bcl-X_L levels relative to Bak that would lead to the activation of Bak to trigger apoptosis (Dowling et al. 2010), exposure of phosphatidylserine (Dasgupta et al. 2010; Rand et al. 2004; Pereira et al. 2002) and the formation of platelet-to-platelet aggregates of apoptotic platelets. Shedding of the collagen receptor GpVI and the primary receptor for von Willebrand Factor (VWF) are also some of the manifestations of platelet senescence (Hartley 2007). Another critical characteristic of senescence is the senescence-associated secretory phenotype (SASP), which endows senescent cells with the ability to modify their microenvironment through the secretion of bioactive molecules (Venturini et al. 2020). This process is thought to alter the microenvironment to favor diverse physiological and pathological processes. In that regard, senescent platelets during storage release extracellular vesicles enriched for miRNAs distinct signatures

characteristic of cardiometabolic and vascular signature diseases (Pienimaeki-Roemer et al. 2017). Whether the release of miRNAs during platelet storage is due to SASP or secondary to platelet activation remains to be investigated.

Dysregulated Nutrient Sensing

Nutrient-sensing pathways are signaling pathways known to modulate ageing and age-related conditions. Some of the main pathways identified include insulin-like growth factor 1 (IGF-1) and mTOR. Briefly, overactivation of these pathways leads to ROS generation, therefore, inducing cellular ageing. Numerous studies have demonstrated that pharmacological and non-pharmacological interventions that modulate the activity of some of these nutrient-sensing pathways are linked to an extended lifespan. For example, caloric restriction and fasting, interventions that ameliorate signaling through the IGF-1 and mTOR pathways (decreasing the generation of ROS) have been associated with an increased life expectancy in short-lived species like rodents (Mitchell et al. 2019; Heilbronn and Ravussin 2003).

The role of nutrient-sensing pathways during ageing on platelet development in humans and its functional impact have not been performed. Despite this, a few studies provide an overview of possible ways nutrient-sensing pathways modulate platelet function during ageing. High mTOR activity and increased levels of ROS underly platelet hyperreactivity in aged mice as both mTOR inhibition by Rapamycin or the use of N-acetylcysteine (ROS scavenger) ameliorated their hyperreactive platelet phenotype (Yang et al. 2016).

Similarly, the role of IGF-1 on platelet development and function during ageing has not been investigated. Nutrient sensing through the IGF-1 pathway is another relevant signaling route associated with promoting longevity (Junnila et al. 2013). Platelets, in addition to expressing the receptor for IGF-1, also store and release IGF-1 upon activation (Hers. 2007). Released IGF-1 can enhance platelet activation in a paracrine manner (Kim et al. 2007). Therefore, it is possible to speculate that elevated levels of IGF1 or aberrant signaling through the IGF1 pathway might promote the development of platelet hyperreactivity and thrombosis at an older age.

Concluding Remarks

Platelet hyperreactivity is the most studied aspect of platelets at an older age. Chronic inflammation has been associated with the development of platelet hyperreactivity during ageing. However, given that ageing is a time-dependent loss of function of multifactorial origin it is necessary to expand our investigations to determine if and which of the many hallmarks of ageing contribute to ageing-associated platelet hyperreactivity. Understanding the mechanism that underly this

functional phenotype is of great importance to developing new therapies to decrease ageing-associated thrombosis.

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Chapter 14

Ageing Skeletal Muscle: The Ubiquitous Muscle Stem Cell



Claire E. Stewart

Abstract In 1999, in a review by Beardsley, the potential of adult stem cells, in repair and regeneration was heralded (Beardsley Sci Am 281:30–31, 1999). Since then, the field of regenerative medicine has grown exponentially, with the capability of restoring or regenerating the function of damaged, diseased or aged human tissues being an underpinning motivation. If successful, stem cell therapies offer the potential to treat, for example degenerative diseases. In the subsequent 20 years, extensive progress has been made in the arena of adult stem cells (for a recent review see (Zakrzewski et al. Stem Cell Res Ther 10:68, 2019)). Prior to the growth of the adult stem cell research arena, much focus had been placed on the potential of embryonic stem cells (ESCs). The first research revealing the potential of these cells was published in 1981, when scientists reported the ability of cultured stem cells from murine embryos, to not only self-renew, but to also become all cells of the three germ layers of the developing embryo (Evans and Kaufman Nature 292:154–156, 1981), (Martin Proc Natl Acad Sci U S A 78:7634–7638, 1981). It took almost 20 years, following these discoveries, for this technology to translate to human ESCs, using donated human embryos. In 1998, Thomson et al. reported the creation of the first human embryonic cell line (Thomson et al. Science 282:1145–1147, 1998). However, research utilising human ESCs was hampered by ethical and religious constraints and indeed in 2001 George W. Bush restricted US research funding to human ESCs, which had already been banked. The contentious nature of this arena perhaps facilitated the use of and the research potential for adult stem cells. It is beyond the scope of this review to focus on ESCs, although their potential for enhancing our understanding of human development is huge (for a recent review see (Cyranoski Nature 555:428–430, 2018)). Rather, although ESCs and their epigenetic regulation will be introduced for background understanding, the focus will be on stem cells more generally, the role of epigenetics in stem cell fate, skeletal muscle,

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skeletal muscle stem cells, the impact of ageing on muscle wasting and the mechanisms underpinning loss, with a focus on epigenetic adaptation.

Keywords Stem cell · Myoblast · Muscle · Ageing · Sarcopenia · Epigenetics

Stem Cells

Stem cells, regardless of whether they are embryonic or adult, have the defining features that they are capable of self-renewal and of specialisation. Following fertilisation of the human egg and the first three divisions of the zygote, 8 cells exist, which are referred to as **totipotent** cells and which have the capacity to form an intact human (Stewart 2004; Juliandi et al. 2011). As division continues, the number of stem cells increases, but their ability to form an intact embryo (individually) decreases. Within 5 days post-fertilisation of the human egg, the blastocyst forms, giving rise to an outer cell layer, which will generate the placenta and an inner cell mass of ~30–50 cells, from which the embryo develops. These inner cells are referred to as **pluripotent** embryonic stem cells (ESCs). Although they can individually develop into all cell types in the body (~ 216 different cell types), they are not individually capable of forming a human being (Stewart 2004; Juliandi et al. 2011). As embryonic development progresses, the cells become more specialised, committing to lineage-specific fates, with genes critical for earlier phases of development being switched off, with only those required for specific tissue function or phenotype remaining active. A small population of cells, within these tissues, however, remain only partially differentiated and are capable of limited, local specialisation, being generally activated during disease or following damage; these cells are referred to as **multipotent** stem cells (Stewart 2004; Juliandi et al. 2011).

Adult stem cells generally fall into this multipotent (or indeed unipotent) category. It is important to note, however, that adult somatic cell fate can be reprogrammed, via a process of de-differentiation back to an embryonic-like state. This reversal of potency was induced via the genomic integration of 4 key embryonic genes: Oct4/Sox2/Klf4/c-Myc, as demonstrated in the pioneering work of Takahashi and Yamanaka (2006). Using adult murine fibroblasts as a model, they were the first to demonstrate that differentiated adult murine skin cells could be induced to a pluripotent state, creating the first **induced pluripotent** stem cell (iPSC) model. One year later, the potential of this concept was independently proven using adult human fibroblasts by the groups of Yamanaka and Thomson (Takahashi et al. 2007; Yu et al. 2007). Although beyond the scope of this review, it is important to note that since these early discoveries, multiple adult cell types from a variety of different species have been used to develop iPSCs using retro-, lenti- and adeno-viral vectors for the transfer of the reprogramming factors (Ye et al. 2013). The model iPSCs have been used to study processes of development (Cui et al. 2022), reprogramming and disease (Meir and Li 2021).

Following fertilisation, as multicellular organisms develop, cellular functions diversify. These changes enable tissue development and function at the expense of

developmental potential. There is a fundamental need for committed cells, in multicellular organisms, to enable our performance and our survival. The control of stem cell differentiation and hence fate is in part under the control of epigenetic regulation (Meir and Li 2021).

Epigenetics

Lifestyle and environmental factors that result in modifications to DNA and histones, without changing the DNA sequence, occur as a result of epigenetic modifications or tags. These epigenetic ‘tags’, which open or condense chromatin, include (1) *histone acetylation* on lysine residues, performed by Histone Acetyl Transferases (HATs), which mark transcriptionally competent regions of DNA and culminate in chromatin opening, thereby facilitating target gene transcription. By contrast, *histone deacetylases* (HDACs) cause lysine deacetylation, resulting in hypoacetylated histones, which are often associated with transcriptionally inactive chromatin structures; (2) *histone methylation*, performed by Polycomb Repressive Complex 2, leads to chromatin condensation and results in repressed transcription. DNA methylation (hypermethylation) of cytosine–guanine (C–G) pairings, especially in CpG-rich regions and in important regulatory regions such as promoters, typically leads to chromatin condensation and repressed transcription (Jones et al. 1998; Bogdanović and Veenstra 2009). Reduced methylation (hypomethylation) provides a more relaxed chromatin structure, enabling gene expression to occur (Jones et al. 1998; Bogdanović and Veenstra 2009).

Epigenetics and Stem Cell Fate

As the embryo develops and the stem cells transition down a committed cell lineage route, extensive changes in gene expression occur. These changes are governed by alterations in DNA methylation and histone acetylation, both of which are critical for enabling the differing roles of stem cells, including self-renewal, specialisation and differentiation (Wu and Sun 2006). Driven by intrinsic and extrinsic cues, throughout embryonic and post-partum growth and development, reciprocal gene expression occurs, whereby genes associated with self-renewal are silenced, enabling transcription of cell type-specific genes during differentiation and vice versa. These fluid changes in gene expression and repression are underpinned by unique epigenetic mechanisms, which restrict developmental plasticity through changes in DNA methylation and chromatin modifications in an inheritable manner (for recent reviews see (Wu and Sun 2006; Meir and Li 2021)). Epigenetic regulation of adult stem cell behaviour is also critical. This is not surprising, even though they face lineage restrictions, since to be defined as stem cells, these cells need to have the capacity to self-renew and differentiate. The importance of epigenetic regulation of

adult stem cell behaviour will be revisited in the section ‘Ageing, Epigenetics and Skeletal Muscle’.

Adult Stem Cells and Their Potential

Technological advances in cell and molecular biology have contributed to our understanding of the physical cues and biological signals that control cellular phenotype and behaviour in health and disease, with injury and repair and across the ageing spectrum. These developments have enabled the manipulation of stem cells in culture, providing an understanding of how differentiation is controlled and how it can be manipulated. Historically, because of the perceived difficulties in working with adult stem cells (low numbers obtainable and problems of senescence), many argued that studying pluripotent ES cells would expedite the development of stem cell therapies. However, more recently and given the potential for autonomous stem cell therapies provided by adult stem cells, these cells have gained interest and traction. Indeed, to understand how adult tissues repair and regenerate (at a cellular and niche level), requires the study of adult stem cells—a field of research that is growing to include 3D and organoid investigations of adult stem cell behaviour *in vitro*. The growth, repair and regeneration of many adult tissues rely on the ability to resident adult stem cells to undertake these tasks, with evidence being generated using primary and immortalised adult stem cells, where ethical and religious constraints are less prevalent. For the purposes of this chapter, the focus will be on the adult skeletal muscle stem cell and the impact of ageing on its behaviour (for a recent review on the regulators of mesenchymal (bone, adipose, muscle and cartilage) stem cell differentiation see Rauch and Mandrup (2021))).

Skeletal Muscle

Muscle contributes the greatest tissue mass to the healthy human body, consisting of smooth and striated forms. The former is present in the walls of the gastrointestinal system, the genitourinary urinary tract and blood vessels and is under involuntary control. Cardiac and skeletal muscles are striated, with the former being under involuntary and the latter under voluntary control (Sweeney and Hammers 2018). Over 600 different skeletal muscles are present in the human body (Brooks 2003) and they comprise approximately 40% of the healthy young adult human mass. Skeletal muscle is essential for growth, posture, locomotion and storage/provision of energy (Csapo et al. 2020). Muscle mass increases during childhood, puberty and following resistance training interventions. By contrast, ageing, disease and disuse result in loss of muscle mass and strength. Skeletal muscle is essential for survival, with muscle wasting in cancer (cachexia) affecting approximately 50% of cancer

patients and accounting for 20–30% of all cancer deaths; indeed, death normally occurs when losses of mass exceed 30–40% (Saini et al. 2006).

Given the important metabolic, physiological and functional roles that skeletal muscle plays, its development and subsequent adaptability are key to health and quality of life.

Skeletal Muscle Development—Role of the Stem Cell

The development and adaptability of skeletal muscle is a highly coordinated process. Although debate exists regarding the precise source of myogenic cells in the trunk and limb, they are believed to be derived from the somites (temporary mesoderm-derived segments, located in pairs on either side of the neural tube in developing vertebrate embryos (Stockdale et al. 2000)). Progenitor cells originating from the hypaxial domain of the somites migrate to the limb buds, where they divide and begin to express muscle-specific determination factors, influenced by signals from neighbouring tissues (Hawke and Garry 2001; Buckingham et al. 2003). Furthermore, these cells derived from the hypaxial somites also reportedly give rise to the abdominal and diaphragm muscles, whereas most muscles of the head originate from cranial mesoderm (Ono et al. 2010).

Briefly, within murine models, primary muscle fibres form at approximately 11–14 embryonic days in the limbs. These are followed at embryonic days 14–16 by secondary fibre formation, which is followed by innervation (Ontell and Kozeka 1984a, 1984b; Cossu and Biressi 2005; Messina and Cossu 2009). A similar but slower pattern of primary and secondary fibre formation and innervation is reported in human embryonic development, where myogenic progenitor cells migrate to limb buds at approximately 6 weeks of gestation, where they differentiate into myoblasts. Following multiple rounds of proliferation between weeks 7 and 9, they fuse to form multinucleated primary myotubes attached via tendons to bones. Secondary myotubes, which initially share a common basement membrane, subsequently form, at which point, histologically recognisable muscle is well defined. From 11 weeks, the fibres grow in length and in cross sectional area and by 6 months of gestation in the human embryo, the number of fibres is reportedly set, with further growth occurring as a result of increased cross sectional area, not increased fibre numbers (Jones and Round 2007).

Skeletal Muscle Stem Cells

Given that skeletal muscle fibres are terminally differentiated and themselves incapable of replication, their ability to adapt to physiological and pathological cues is of particular scientific interest. Insight into the mechanisms enabling the adaptability of skeletal muscle was described over 60 years ago, by Mauro, who reported (using

electron microscopy) the presence of single cells beneath the basal lamina of the tibialis anticus of the frog (Mauro 1961). Due to their peripheral location, beneath the basal lamina, he designated these cells satellite cells. Mauro suggested that these cells were quiescent, non-fused myoblasts (muscle cells) that were present to provide a resident cell pool capable of facilitating growth and repair, following damage. Although frequently overlooked, Bernard Katz (Katz 1961) also reported the presence of satellite cells on the periphery of frog muscle spindles in the same month of the same year. He speculated that these cells were associated with muscle hypertrophy. The role of the satellite cell in enabling growth and repair of mature muscle fibres has gained significant interest, particularly in relation to protein turnover and cell turnover in muscle adaptation to ageing and disease (Pallafacchina et al. 2013).

Potential of Satellite Cell Models

Muscle cells retain a memory of the donor environment from which they were derived such as from cachectic, physically active, sarcopenic, or obese individuals (reviewed in Sharples et al. 2016, 2018). Therefore, the study of such satellite cells, obtained from biopsies samples, provides a relevant understanding of their *in vivo* behaviour. Since these cells provide the opportunity for detailed temporal studies to be performed, they enable research, ethically not possible *in vivo*, due to the number of biopsies that would need to be harvested. Satellite cells, therefore, provide relevant *in vitro* models and biopsies a snapshot in time, which if paired, provide an opportunity to monitor *in vitro* cellular and *in vivo* physiological/biochemical adaptation in response to exercise and nutrition, age, disease, disuse and injury. Damaging protocols, loading, electrical stimulation or serial passaging of the cells *in vitro*, also provide relevant models for the study of injury, hypertrophy and atrophy, which can all be manipulated with, e.g. growth factors, cytokines, nutraceuticals, or serum; providing knowledge, not only of mechanisms underpinning adaptation, but also the capacity to better inform *in vivo* questions.

One area of research, which is gaining particular interest in the muscle biology arena is the impact of age on muscle mass and strength. Research has focussed on the processes underlying muscle wasting with ageing (sarcopenia) as well as the potential benefits of exercise and nutrition in reducing the rates of muscle wasting and therefore improving functional ability and health, with age. Research has focussed on the roles of exercise and nutrition, with an emphasis on protein (Hesketh et al. 2020; Stansfield et al. 2021) and cellular turnover (Pallafacchina et al. 2013). With that in mind, it begs the question, what do we know about skeletal muscle ageing?

Skeletal Muscle Ageing

As we age, we see a progressive decline in muscle mass, strength, performance and metabolic potential (Cruz-Jentoft et al. 2018; Daily and Park 2022). Losses of

muscle mass associated with ageing are referred to as sarcopenia, whereas those more accelerated losses, associated with chronic catabolic diseases, are referred to as cachexia (Saini et al. 2006). For the purposes of this manuscript, the focus will be on muscle wasting with ageing, which can be further sub-categorised into lean or obese sarcopenia, dependent on the adipose burden of the individual (Molino et al. 2016).

Losses of muscle mass and strength with ageing are affected by processes that influence the balance between anabolism (protein synthesis) and catabolism (protein degradation) of muscle. The mechanisms of action of these factors are of fundamental biomedical interest, due to the risk of increased morbidity and early mortality associated with sarcopenia (Roubenoff 2003). It is estimated that we lose approximately 30–40% of our muscle mass and strength between the ages of 20 and 70 years and that most individuals aged 80 years or over suffer from sarcopenia; recent systematic reviews conducted to estimate the prevalence of sarcopenia in older populations, estimate prevalence to vary between 10 and 40%, depending on definitions used (Shafiee et al. 2017; Mayhew et al. 2018). The highest prevalence estimates were for measures using appendicular lean mass/weight as a defining factor (Mayhew et al. 2018). Given the current ageing demographic, the impact of sarcopenia not only on the quality of life of the older person but also on the workforce and the healthcare system warrants consideration. Indeed, a recent retrospective, prevalence based, economic burden study, consisting of 4011 adults aged ≥ 40 years with and without sarcopenia suggested that the total estimated US cost of hospitalisations in individuals with sarcopenia was \$40.4 billion (Goates et al. 2019), making sarcopenia and its associated health complications a major healthcare issue.

Cross sectional studies suggest the main source of muscle fibre loss with ageing is type II muscle fibres (Larsson et al. 2019). Importantly, with the loss of muscle fibres, there is also a decline in the abundance of satellite cells with age (Gibson and Schultz 1983; Chen and Goldhamer 2003). The role of the satellite cell in enabling growth and repair of skeletal muscle, across the lifecourse, must therefore be considered, when trying to determine the role of the satellite cell in delaying or reducing muscle mass with ageing. Gaining a better understanding of the ageing muscle stem cell (muscle stem cell and satellite cell are used interchangeably for the remainder of this chapter) is therefore critical to the development of future interventions targeted at reducing muscle loss with age.

Ageing and the Skeletal Muscle Stem Cell

Skeletal muscle growth and regeneration are facilitated by the resident pool of stem cells in mature muscle fibres. Following resistance training or injury and in response to local (niche), intrinsic (cell) or systemic factors, these muscle stem cells are activated, divide, migrate to the site of injury and fuse with the damaged fibre to repair the damaged tissue (McKinnell et al. 2005; Fu et al. 2015). In addition to activation and repair, the muscle stem cells have the required capacity to self-renew. This may occur via asymmetric division, resulting in a differentiation-committed and

a non-committed stem cell, the latter enabling replenishment of the resident muscle stem cell pool (Kuang et al. 2007). Symmetric division also occurs to increase the number of resident muscle stem cells that may be required to repair injury/rebuild a damaged fibre (Kuang et al. 2007). It has recently been reported that the resident satellite cells form a heterogenous pool, and whether they self-renew or they differentiate is dictated by intrinsic and extrinsic factors (Sousa-Victor et al. 2022). It is hypothesised that as we age, the number of muscle stem cells declines as does their potential to repair damage/elicit hypertrophy (although this is still under debate). Regardless, the mechanisms underpinning muscle wasting and the influence they have on muscle atrophy, hypertrophy and repair, warrant further investigation.

While it is not clear whether reduced muscle stem cell activity/numbers are the cause or the result of ageing muscle, what is clear is that the processes are more complex than altered cellular behaviour alone. As alluded to above, the stem cell niche (extracellular matrix proteins, growth factors, fibroblasts, immune cells, neurones and capillaries) as well as autocrine, paracrine and endocrine adaptations with age, all contribute to the altered behaviour of the muscle stem cell and to declining muscle mass (for recent and extensive reviews see Almada and Wagers (2016); Hong et al. (2022); Sahinyan et al. (2022)). One area gaining extensive interest in ageing and in ageing muscle stem cell behaviour, in particular, is the role of cell senescence in altered muscle stem cell behaviour and in sarcopenia (Almada and Wagers 2016; He et al. 2022). Recent data suggest that the ability of older satellite cells to engraft, following intramuscular transplantation into immunocompromised mice, is reduced compared to younger cells, in a cell-density-dependent manner (Cosgrove et al. 2014). The older donor cells displayed reduced replicative capacity, increased p38 α/β -MAPK activation and elevated levels of senescence regulator genes, thought to be involved in the reduced proliferative capacity of the aged cells (Cosgrove et al. 2014) and with the downstream elevation of the cyclin-dependent kinase inhibitor p16INK 4a (Muñoz-Espín and Serrano 2014).

While the above mechanisms begin to provide insight into what is happening to the ageing satellite cell, what they do not cover is the influence of lifecourse epigenetic adaptation, which also occurs with ageing and the impact this may have on stem cell behaviour and muscle adaptation with ageing.

Ageing, Epigenetics and Skeletal Muscle

DNA modifications, without changes to the DNA sequence itself, enable rapid cellular adaptation to environmental/behavioural situations. A fundamental hallmark of the ageing process is an altered epigenetic landscape reportedly responsible for altered metabolism, poorly regulated mitochondrial function, cellular senescence, stem cell reductions and altered intracellular signalling (López-Otín et al. 2013; Voisin et al. 2020). DNA methylation with ageing is reportedly a key feature of the ageing process in many tissues. The comparative study of younger ($n = 24$ healthy young males; age range 18–27) vs. older ($n = 24$ healthy aged males; age range

68–89 years) skeletal muscle samples using DNA methylation arrays (covering 450,000 CpG sites) revealed a hypermethylated pattern across the genome in older, compared to younger human muscle (Zykovich et al. 2014). It is speculated that the methylation changes in muscle, with ageing may underpin the sarcopenic adaptation, also witnessed, however, it is important to note that the majority of the hypermethylated sites were within the genes themselves, rather than within their promotor regions. That being said, the authors did successfully sub-classify the tissue correctly into young and old categories based on 500 of the CpG sites and were the first to report an epigenetic signature of ageing muscle (Zykovich et al. 2014). Subsequent studies have suggested that the methylation status of approximately 200 CpG sites can accurately predict the chronological age of skeletal muscle tissue (Voisin et al. 2020; Voisin et al. 2021).

Research from the group of Sharples, using technology allowing investigation of 850,000 CpG sites in skeletal muscle tissue and skeletal muscle-derived stem cells, also revealed an accumulation of hypermethylated sites in both muscle tissue and isolated muscle-derived stem cells from aged (mean 83 years) versus young (mean 27 years) adults (Turner et al. 2020). The methylation profile in aged tissue was associated with enriched hypermethylation in load/growth-associated gene pathways such as focal adhesion and mTORC signalling, as well as pathways-in-cancer (including focal adhesion, MAPK signalling, PI3K-Akt-mTOR signalling, p53 signalling, Jak-STAT signalling and TGF-beta and notch signalling), rap1-signalling, axon-guidance and hippo-signalling (Turner et al. 2020). The hypermethylation profile of aged muscle stem cells displayed enrichment in genes associated with axon-guidance, adherens junction and calcium signalling, particularly at later timepoints of myotube formation, where morphological differentiation was compromised in aged vs. young cells and reductions in MyoD/Myogenin gene expression were evident (Turner et al. 2020). Like the research of Zykovich et al. (2014), we also reported that genes involved in axon guidance, in both aged tissue and isolated muscle cells, displayed enriched hypermethylation compared with their younger adult counterparts (Turner et al. 2020).

Epigenetic adaptation regulates gene expression, by making the DNA conducive (euchromatin) or non-conducive (heterochromatin) to transcription. Increased DNA methylation, as evident with ageing, generally results in a reduction in transcription, suggesting that gene transcription in muscle and muscle-derived stem cells declines with ageing. These changes may contribute to impaired muscle function and fusion with ageing. Further studies in other models do suggest an important role for epigenetics in ageing. Studies investigating transposase-accessible chromatin using sequencing (ATAC-seq) of old and young murine muscle stem cells also revealed extensive differences in chromatin accessibility in aged vs young cells (Shcherbina et al. 2020). Further, a general loss of heterochromatin with ageing has also been described in numerous model organisms and many adult stem cells, which is thought to culminate in inappropriate gene expression profiles and cell behaviour (for details see Sahinyan et al. (2022)). While the focus here has been on altered epigenetic patterns in muscle and muscle stem cells, it is important to remember that DNA damage, absence/presence of senescence, inappropriate signalling and metabolism,

mitochondrial dysfunction, impaired autophagy and the increased abundance of damaged proteins, all contribute to the changes cells and tissues experience with ageing (for reviews see Voisin et al. (2021); Hong et al. (2022); Sahinyan et al. (2022)). A recent review examining the role of myofibre repair vs. stem cell-driven regeneration begins to interrogate why repair and regeneration are impaired in sarcopenia and raises interesting questions for future research in the study of the mechanisms underpinning muscle wasting with ageing (Roman and Muñoz-Cánores 2022).

Summary

Skeletal muscle wasting with ageing is known to impact not only function, metabolism, quality of life and falls risk, but also impacts compromised cardiovascular health, increased general rates of morbidity and early mortality. While exercise and nutrition are known to reduce the rate of muscle loss, even master's athletes do lose muscle mass, indicating an element of anabolic resistance with ageing. Extensive research exists, but more is needed in this arena. However, if we do not fully understand the cause of muscle loss (in the face of healthy nutrition and continued activity), then the likelihood of success for interventions will be compromised. The questions now being addressed relating to lifecourse influences on epigenetic profiles in muscle with age, the fluidity of such profiles and the potential distinctions between fibre-driven self-repair vs. satellite cell-mediated regeneration with age pose interesting research questions. Ultimately understanding if ageing drives compromised muscle function, or if compromised function culminates in ageing is an interesting concept remaining to be defined.

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Chapter 15

Age-Related Changes in Central Nervous System 5-Hydroxytryptamine Signalling and Its Potential Effects on the Regulation of Lifespan



Sara Fidalgo and Mark S. Yeoman

Abstract Serotonin or 5-hydroxytryptamine (5-HT) is an important neurotransmitter in the central nervous system and the periphery. Most 5-HT (~99%) is found in the periphery where it regulates the function of the gastrointestinal (GI) tract and is an important regulator of platelet aggregation. However, the remaining 1% that is found in the central nervous system (CNS) can regulate a range of physiological processes such as learning and memory formation, mood, food intake, sleep, temperature and pain perception. More recent work on the CNS of invertebrate model systems has shown that 5-HT can directly regulate lifespan.

This chapter will focus on detailing how CNS 5-HT signalling is altered with increasing age and the potential consequences this has on its ability to regulate lifespan.

Keywords Serotonin · central nervous system · ageing · serotonin transporter · 5-HT_{2A} receptor · lifespan

Overview of 5-HT in the Central Nervous System

Discovery of 5-HT

5-HT was originally isolated from the GI tract by Vittorio Ersperger as a substance that was able to contract a range of smooth muscles (Ersperger and Viali, 1937). He named the substance enteramine. A little later a substance called serotonin was

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isolated and named by Rapport, Green and Page of the Cleveland Clinic in 1948 and enteramine and serotonin were shown to be the same molecule (Rapport et al. 1948a, b) and later identified as 5-hydroxytryptamine (5-HT) (Rapport, 1949). In the early 1950s, Twarog published a paper that was the first to show that 5-HT was a neurotransmitter in the mammalian brain (Twarog and Page, 1953). This finding has led to the development of a range of drugs to treat a range of mental health conditions.

5-HT Distribution in the CNS

5-HT is present in relatively few neurons in the CNS that have their cell bodies located in a region of the CNS called the pons and the medulla in several nuclei called the raphe nuclei (Fig. 15.1). Despite the relatively low numbers of CNS serotonergic neurons, their processes are widespread and input into many regions of the brain (Fig. 15.1). This allows 5-HT to regulate a wide range of physiological processes detailed above. Serotonergic neuronal cell bodies originating from the midbrain region (B1, B2 and B3) have descending projections in the spinal cord allowing them to regulate pain perception, motor function and the autonomic nervous system (Fig. 15.1B). B6 and B7 make up the principal dorsal raphe group which projects to the cortex where they regulate sleep, the dorsal striatum where they influence motor control and the amygdala where they can affect mood. B5, B8 and B9 which form the median raphe nuclei project to cortex and hippocampus where they regulate learning and memory formation. Serotonergic projections are also found in the hypothalamus, where they have been shown to affect circadian rhythm, food intake and thermoregulation, and the thalamus where they have a role in sleep and the regulation of epileptic activity.

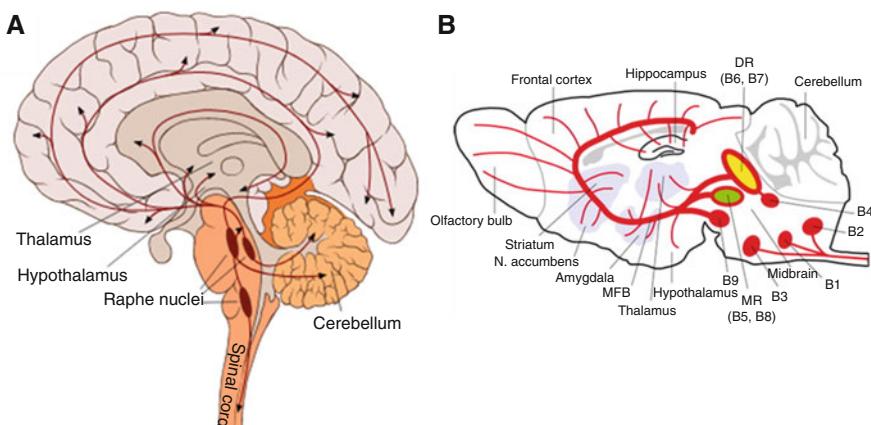


Fig. 15.1 Diagrammatic representation of the central serotonergic pathways in (A) Human brain (Wikipedia, 2021) and (B) the rat brain (Lesch and Waider, 2012)

5-HT Synthesis

5-HT is synthesised from the precursor amino acid tryptophan. Tryptophan is an essential amino acid derived from proteins in the diet. It is taken up into neurons by a range of different transporters (Vumma et al. 2011). Here it is converted to 5-hydroxytryptophan by the rate-limiting enzyme tryptophan hydroxylase (TpH). There are two isoforms of TpH in mammals. Of these TpH-2 is located in neurons and is responsible for the hydroxylation of tryptophan in the CNS. Subsequent decarboxylation by a non-specific amino acid decarboxylase forms 5-hydroxytryptamine (Fig. 15.2, lower panel). 5-HT is then packaged into vesicles by the vesicular monoamine transporter (VMAT) prior to release. Increases in intracellular Ca^{2+} , typically caused by the arrival of an action potential at the presynaptic terminal, act as a stimulus to allow the vesicles to fuse with the plasma membrane allowing them to release some or all of their contents into the synapse. To limit 5-HT signalling, 5-HT is taken back up into neurons via the actions of the serotonin transporter (SERT) where it can be re-packaged into vesicles or metabolised by monoamine oxidase. There are two monoamine oxidase (MAO) enzymes in the nervous system, termed A and B. MAO_A preferentially metabolises 5-HT, however, monoamine oxidase B (MAO_B) is the major monoamine oxidase in 5-HT-containing neurons but its affinity for 5-HT is low (O'Carroll et al. 1983), and therefore whilst it may deaminate some 5-HT most will be re-packaged into vesicles

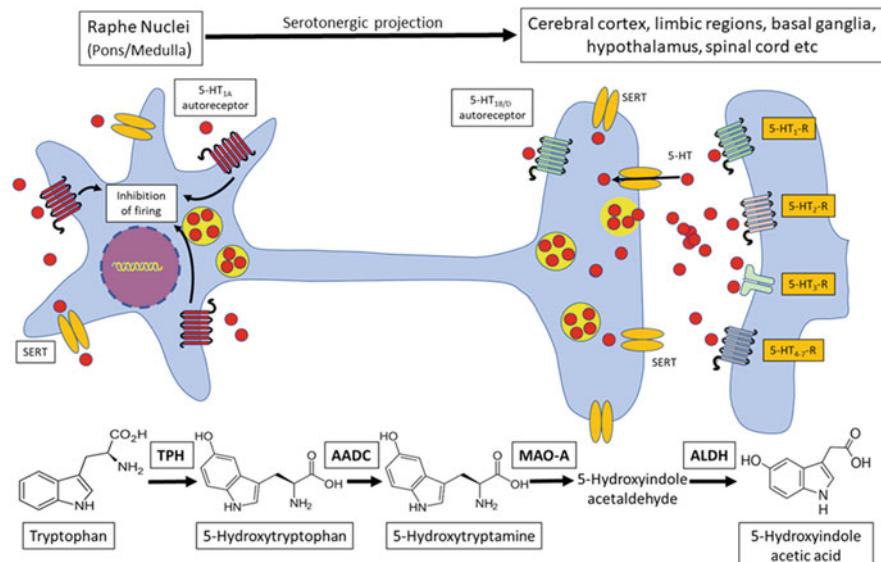


Fig. 15.2 Diagrammatic representation of a serotonergic neuron (top) and the pathway for the synthesis and metabolism of 5-hydroxytryptamine (below). Adapted from (Millan et al. 2008)

to be used again for synaptic signalling. MAO_A has also been shown to be expressed in glial cells (Westlund et al. 1988; Fitzgerald et al. 1990) and has been shown to be functionally active (Fitzgerald et al. 1990). In addition, human astrocytes express SERT mRNA (Kubota et al. 2001) and 5-HT uptake into astrocytes has been demonstrated (Bel et al. 1997; Hirst et al. 1998; Malynn et al. 2013). Therefore, glial uptake and metabolism could also contribute to the removal of 5-HT from the synapse. In addition to SERT other transporters such as the organic cation transporter 3 can transport 5-HT into neurons and glial cells (Takeda et al. 2002; Vialou et al. 2008; Gasser et al. 2009), whilst the plasma membrane monoamine transporter (Dahlin et al. 2007), allows re-uptake of 5-HT in both serotonergic and non-serotonergic neurons. Following deamination to 5-hydroxyindoleacetaldehyde an aldehyde dehydrogenase converts this product to 5-hydroxyindole acetic acid, which being water soluble, can then be excreted in the urine (Fig. 15.2).

5-HT Receptors

5-HT has its actions via seven different receptor types classed as 5-HT₁–5-HT₇ receptors. 5-HT₁, 5-HT₂, 5-HT₄, 5-HT₅, 5-HT₆ and 5-HT₇ are all G-protein-coupled receptors. 5-HT₁ receptors reduce intracellular cAMP whilst 5-HT_{4,5,6} and 7 receptors all increase intracellular cAMP (Alexander et al. 2011). 5-HT₂ receptors act via Gq to increase intracellular calcium and activate protein kinase C. 5-HT₃ receptors are ionotropic receptors with an integral ion channel that transduces the binding of extracellular 5-HT into a depolarisation of the target cell. Within each class of receptors, there are a range of sub-types of 5-HT receptors making a total of 14 different 5-HT receptors through which 5-HT can have its actions on other neurons, microglia, astrocytes and oligodendrocytes within the CNS (Alexander et al. 2011). Inhibitory 5-HT_{1A/B} autoreceptors can inhibit the firing rate of serotonergic neurons and therefore reduce 5-HT release (Fig. 15.2).

Effects of Age on Serotonergic Signalling

Given the ability of 5-HT to regulate a range of behaviours that have been shown to be affected by the natural ageing process (e.g. learning and memory, sleep and mood) research has focused on whether age-related changes in serotonergic signalling can help explain these deficits. In the next section, we will describe how the natural ageing process affects the different elements involved in regulating serotonergic signalling in the CNS.

Effects of Age on Serotonergic Neuronal Number

A landmark study in 1997 by Morrison and Hof quantified the numbers of neurons in the ageing neocortex and hippocampus and showed limited neuronal death that was unlikely to lead to the losses in function observed in older people (Morrison and Hof, 1997). A more focused study of the ageing raphe nuclei in humans has shown that the numbers of serotonergic neurons are not significantly altered by the ageing process (Klöppel et al. 2001). Interestingly, similar studies in non-human primates showed a decrease in cell packing density and neuronal number in aged rhesus monkeys (Kemper et al. 1997). However, in this study, the phenotype of the neurons was not determined, although the authors believed that at least one of the populations that were decreased in number was serotonergic, based on their size (Kemper et al. 1997). A similar age-related decrease in the density of 5-HT-positive neuronal cell bodies was observed in the raphe of the rat (Lolova and Davidoff, 1992). In the absence of a loss in neuronal cell bodies, an imaging study in humans has demonstrated a loss of fibres in the raphe—hippocampal tract, inferring some peripheral neurodegeneration (Sekul and Ikuta, 2021). A similar reduction in the innervation of the neocortex from the raphe has also been observed in aged rats (Descarries et al. 1975).

These data suggest that the natural ageing process either reduces the numbers of connections that the serotonergic neurons are making with their target or decreases the numbers of serotonergic neurons. Which of these effects is observed is probably reflective of the biological age of the subjects, with younger subjects showing milder neuronal process degeneration, whilst older subjects may experience some neuronal loss. Whether these changes are sufficient to impair the functioning of the CNS is unclear, although in at least one study correlations were observed between loss of neurons and memory function (Kemper et al. 1997).

Tryptophan Hydroxylase

Tryptophan Hydroxylase (TpH) activity and CNS expression has been explored in a range of organisms from snails and mice to humans. In general, the activity of TpH2 was found to decrease with increasing age in snails (Morgan et al. 2021) and rats (Hussain and Mitra, 2000; Hussain and Mitra, 2004) in a range of CNS regions. Immunohistochemical analysis of TpH2 expression in the rat neural and neurointermediate lobes of the pituitary showed reduced labelling with increased age, suggesting a decrease in TpH2 protein expression (Saland et al. 1993). However, other studies using immunohistochemistry failed to show any change in the expression of TpH2 in SAMP8 mice (Pérez-Cáceres et al. 2013), rats (Timiras et al. 1984) or in humans (Klöppel et al. 2001).

Work by Hussain and Mitra strongly suggested that the decrease in TpH2 activity was due to age-related decreases in the expression of key antioxidant enzymes. This

would increase oxidative stress and reduce TpH2 activity, an effect that was blocked by catalase and/or glutathione peroxidase. They also suggested that the phosphorylation status of TpH 2 was also a possible site of the age-related decline in TpH2 activity but did not show any evidence to support this hypothesis (Hussain and Mitra, 2000; Hussain and Mitra, 2004). Our work on the snail has shown that 14-3-3, a protein known to facilitate TpH-2 phosphorylation, is downregulated with increasing age and this downregulation could explain the reduced activity of TpH in the CNS (Morgan et al. 2021). Interestingly, whilst a regional analysis confirmed that TpH activity was decreased in old snails, analysis at the level of a single identifiable serotonergic neuron showed that 5-HT expression increased with increasing age. These data strongly suggest that analysis at the level of the single neuron is required to provide a complete picture of how 5-HT synthesis is affected by the ageing process.

Vesicular 5-HT Content

5-HT is synthesised in the cytoplasm and transported into vesicles by the vesicular monoamine transporter 2 (VMAT2). An imaging study in rats showed no change in the expression of VMAT2 with increasing age but showed a change in the subcellular localisation of the protein, which correlated with age-related decreases in function (Cruz-Muros et al. 2009). Consistent with this finding, an imaging study in humans demonstrated a small decrease in VMAT2 function of 0.77% per annum (Frey et al. 1996), whilst another showed no significant change in VMAT2 function (Lin et al. 2013). Together these studies strongly suggest that vesicular neurotransmitter content is unlikely to change significantly with increasing age.

5-Hydroxytryptamine

Invertebrates lend themselves to the study of ageing due to their relatively short lifespans. In the nematode worm *C. elegans*, immunohistochemical methods demonstrated that CNS 5-HT decreased with increasing age (Yin et al. 2014). Similar decreases were observed in *Drosophila* (Liao et al. 2017). In the pond snail, *Lymnaea*, there was no significant change in regional 5-HT with increasing age (Yeoman et al. 2008; Morgan et al. 2021), however, HPLC analysis at single cell level showed that somatic 5-HT was increased in a key identified neuron, the cerebral giant cell (CGC) responsible for learning and memory formation (Morgan et al. 2021). Using immunohistochemistry with a secondary fluorescent antibody, we have confirmed the HPLC findings as 5-HT fluorescence was also more intense in the old CGC somata (Fig. 15.3, lower panels). However, 5-HT fluorescence in the terminals (buccal ganglia) is reduced with increasing age, indicating that the

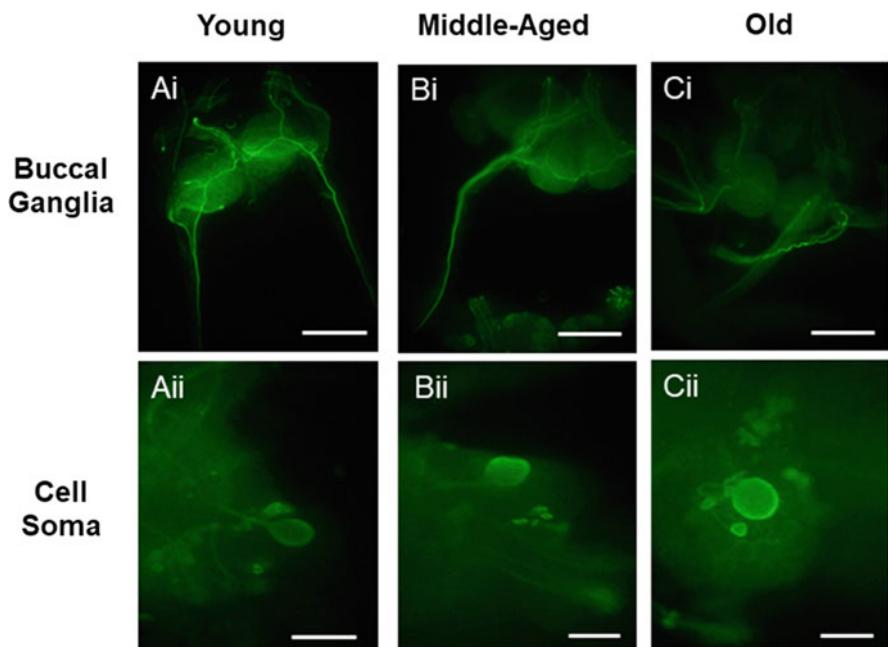


Fig. 15.3 Photomicrographs showing 5-HT labelling (bright green fluorescence) in the processes of the cerebral giant cell (CGC) in the buccal ganglia and the CGC soma in the cerebral ganglia of young, middle-aged and old CNSs

subcellular distribution of 5-HT changes with increasing age (Yeoman unpublished data; Fig. 15.3).

In mammals, despite the potential for age to decrease the activity of TpH2, many studies exploring the impact of the natural ageing process on CNS 5-HT production did not show a decrease in CNS 5-HT in humans (Robinson, 1975; Rosa-Neto et al. 2007) and rodents (Bhaskaran and Radha, 1983; Moretti et al. 1987; Sparks et al. 1985; Venero et al. 1991a, b; Venero et al. 1993a) across a range of CNS regions. A number of these studies by Venero and colleagues explored age-related changes in 5-HT in a range of different CNS regions. They showed either no change in 5-HT expression in the striatum and substantia nigra (Venero et al. 1991a, b), increases in the hippocampus (Venero et al. 1993a) or decreases in the pre-frontal cortex (Venero et al. 1993b) in 26-month-old rats compared to young controls, illustrating that the changes were brain region specific. Consistent with the decrease seen in the pre-frontal cortex, similar decreases were observed in 22 months (Petkov et al. 1988) and 24 months (Míguez et al. 1999) old rat cortex when compared to mature adult rats. However, similar changes were not seen in other rat studies or in humans and in one study an increase in rat cortical 5-HT was observed at ages greater than 30 months (Timiras et al. 1984). Whilst many studies infer that CNS 5-HT does not change with increasing age, publications that demonstrate decreases or increases in regional 5-HT infer that the direction of effect could be related to subject age and in

the case of rats, the strain studied and the housing conditions all of which could affect the rate of ageing (Jackson et al. 2017).

Firing of Serotonergic Neurones

Many aged neurons show reduced spontaneous and evoked firing frequencies when compared to young controls (Yeoman et al. 2012). In a range of species, these changes reflect increases in intracellular calcium and the activation of one or more calcium-dependent potassium currents and an increase in the amplitude and duration of the after-depolarisation that follows the action potential (for review see Yeoman et al. 2012). Studies in the mouse raphe nuclei demonstrated that raphe neurons fire tonically with a very low coefficient of variation and those recorded from old mice fire at a lower frequency and are sometimes silent. An increased after-spike current that corresponds to the after-hyperpolarisation was observed in aged raphe neurons consistent with findings in other central neurons (Rood et al. 2014). Work using the pond snail has also shown that the identified serotonergic CGC also fires tonically and its spontaneous and evoked firing frequency is reduced with increasing age due to the appearance of a large after-hyperpolarisation caused in part by increased current flow through a slow Ca^{2+} -dependant potassium current (Scutt et al. 2015). Therefore, even in the absence of any regional change in CNS 5-HT the reduced firing rate of serotonergic neurons would tend to infer that less 5-HT is being released into the synapse, potentially impacting the efficacy of transmission.

Release of 5-HT

The effects of age on neuronal 5-HT release have been little studied. Our work has explored this in the pond snail, *Lymnaea* (Patel et al. 2010). Using carbon fibre electrodes, we utilised constant potential amperometry to measure the dynamics of release events that represent the binding of individual neurotransmitter vesicles with the membrane and the subsequent release of the neurotransmitter 5-HT. The technique relies on holding the tip of the carbon fibre at a constant potential (+750 mV) that allows for the complete oxidation of the released molecules of 5-HT. The oxidation of these molecules is monitored as the flow of electrons to the electrode and the subsequent current can be monitored. Release events are fast to rise and have a slower fall time. Their amplitude and duration are variable, and this reflects a combination of the size of the vesicle that fuses with the presynaptic membrane and the proportion of transmitter released from that vesicle. The size of the current is directly proportional to the number of molecules of 5-HT released according to the Faraday Law. Analysis of 5-HT release from the serotonergic CGCs showed that ageing increased the number of molecules of 5-HT released per release event (Fig. 15.4A) by increasing the time constant for decay of the events (Fig. 15.4B).

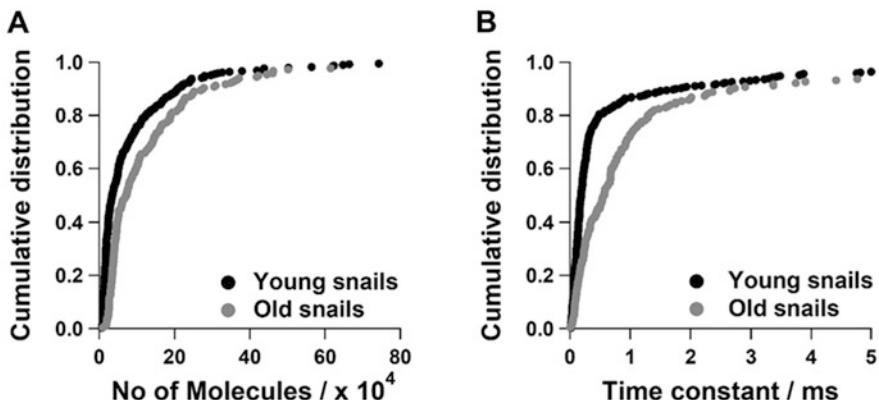


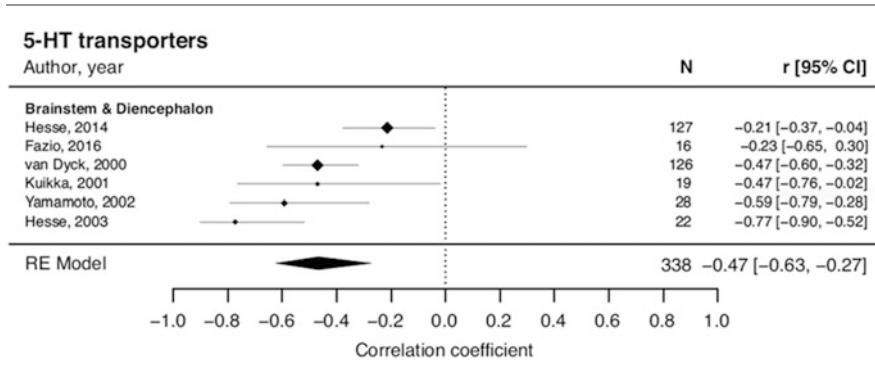
Fig. 15.4 Effects of age on the properties of vesicular release events from an identified serotonergic neuron in the pond snail, *Lymnaea*. Effects of age on (A) the number of molecules released per event and (B) the time constant for decay of each event (Patel et al. 2010)

These age-related changes are unlikely to reflect an increase in CGC vesicular 5-HT concentration as previous studies have shown either no change or a decrease in VMAT2 expression with ageing (Scutt et al. 2015). The precise causes of these changes are currently unclear, but they could be due to age-related changes in intracellular Ca²⁺ that have previously been described in these neurons (Scutt et al. 2015) and/or altered membrane lipid composition which has the potential to change the dynamics of vesicular release (Gu et al. 2020). In support of this hypothesis, changes in membrane lipid composition have previously been observed in the ageing CNS (Egawa et al. 2016). Fewer release events were observed in old neurons raising the possibility that the increase in release per event was a compensatory mechanism by the neuron to maintain levels of 5-HT in the synapse given the reduced frequency of action potential firing. Previous work exploring glutamatergic synapses has shown that the dynamics of release can have a marked impact on signalling, with multiple low-level stimuli, as might be observed in a young neuron, having markedly different postsynaptic effects to fewer larger stimuli that would represent signalling in old neurons. Thus, changes in the release kinetics at serotonergic synapses could impact the efficacy of synaptic transmission.

Age-Related Changes in 5-HT Clearance from the Synapse

5-HT can be cleared from the synapse by diffusion or re-uptake into neurons/glial cells via the action of SERT. A wide range of studies have explored the effects of age on SERT expression in humans and several rodent species. In living humans and non-human primates, imaging studies have been performed using single photon emission computed tomography (SPECT) and positron emission tomography

Table 15.1 Forest plot of age effects in 5-HT transporters. Depicts reported effect size (diamond) and 95% confidence interval (line) for each included study. Weight of each study (size of diamond) depends on sample size and between-study variance. Summary effects sizes (polygon) and 95% confidence interval (width of polygon) for separate brain regions and in total. N represents the number of individual subjects included in each study and in the computation of the summary effect sizes. From Karrer et al. (2019)



(PET). These studies used a range of radiolabelled ligands for SERT to measure uptake via SERT and consistently showed that CNS SERT was downregulated with increasing age (van Dyck et al. 2000; Kakiuchi et al. 2001; Kuikka et al. 2001; Hesse et al. 2003; Koch et al. 2014; Yamamoto et al. 2002; Fazio et al. 2016). In non-human primates, similar decreases in SERT activity were observed with increasing age (Kakiuchi et al. 2001). More recently, Karrer and colleagues (Karrer et al. 2019) performed a meta-analysis of human PET and SPECT studies and showed a moderate decrease in human CNS SERT with increasing age (Table 15.1). This decrease would again act to increase 5-HT in the synapse and therefore help to compensate for the decreased firing rate observed in serotonergic neurons with increasing age.

Contrary to the imaging studies, when SERT expression was explored in post-mortem human brain tissue the effects of age were less conclusive. Studies showed either a decrease (Marcusson et al. 1987), no change (Andersson et al. 1992; Arranz et al. 1993; Ryding et al. 2004), or an increase in SERT expression in several brain regions (Severson et al. 1985). Unlike the *in vivo* imaging studies that measured SERT activity, these post-mortem studies explored the expression of SERT and the different techniques may explain these discrepancies.

In rodent studies, the effects of age on SERT expression are more complicated. In the majority of the papers cited below, studies explored post-mortem brain tissues and therefore explored the expression of SERT and not its activity. Many studies showed increases in SERT expression with increasing age in a range of CNS regions including the cortex (Brunello et al. 1988; Druse et al. 1997; Severson, 1986), raphe nucleus (Meister et al. 1995), nucleus accumbens (Druse et al. 1997), amygdala (Druse et al. 1997), hippocampus (Druse et al. 1997, Severson, 1986) and the

hypothalamus (Severson, 1986). In some studies, increased SERT expression was observed between young and adult rodents and this was followed by a decreased expression in old age. In the hippocampus, SERT expression (measured by paroxetine binding) was increased in 15-month-old Fisher 344 rats and decreased in 24-month-old animals (Arora et al. 1993). Similarly, in hamsters, SERT binding was increased in middle-aged animals (Duncan et al. 2000) and decreased in old age (Duncan and Hensler, 2002).

These discrepancies between the human and animal studies most likely reflect the different methods for analysing the effects of age on SERT, but it could infer that the effects of age on SERT are different in rodents and humans. To test this latter hypothesis several studies have attempted to measure SERT function in aged rodents. In one such study, SERT function was assessed by using micro-PET to mimic the human *in vivo* studies (Hoekzema et al. 2011). The authors compared 4 months with 19-months-old male OFA rats and demonstrated that ageing was associated with an increase in SERT activity in the frontal cortex and the thalamus/midbrain of the rat (Fig. 15.5). This strongly suggested that the effects of age on SERT function were different in rodents and humans. However, another study that explored cortical 5-HT uptake in 24-month-old male Sprague Dawley rats, showed a decrease in aged rats, consistent with the human studies (Brunello et al. 1988). Additionally, in the same study the authors showed that expression, measured by imipramine and paroxetine binding was increased with age, strongly suggesting that whilst the expression of SERT may increase with increasing age its function is decreased. The differences in the findings of these two studies may be reflective of the strain or age of the rats used and highlight the necessity for further studies using imaging techniques in other strains of rats over a range of age groups to more fully explore the timeline of changes in SERT expression and activity in aged rodents.

Our work using the pond snail as a model system for exploring the effects of age on serotonergic signalling explored the ability of the selective 5-HT re-uptake inhibitor to affect the strength of the monosynaptic connection between the serotonergic CGC neuron and the B1 motor neuron. In this study, single action potentials were evoked in the CGC and the corresponding excitatory post-synaptic potential (EPSP) was recorded intracellularly in a B1 motor neuron. In young (3–4 months) and middle-aged (6–7 months old) snails, the amplitude and the duration of the EPSP was enhanced dramatically in the presence of fluoxetine, consistent with it blocking the re-uptake of 5-HT and enhancing the concentration of 5-HT in the synapse. In old (11–12 months) snails, the control EPSP was not significantly enhanced by fluoxetine consistent with a decrease in either the expression or activity of SERT with increasing age (Yeoman et al. 2008).

Effects of Age on Monoamine Oxidase A/B

There are two isoforms of monoamine oxidase, namely MAO_A and MAO_B encoded by different genes (Weyler et al. 1990). Their role is to deaminate and thereby

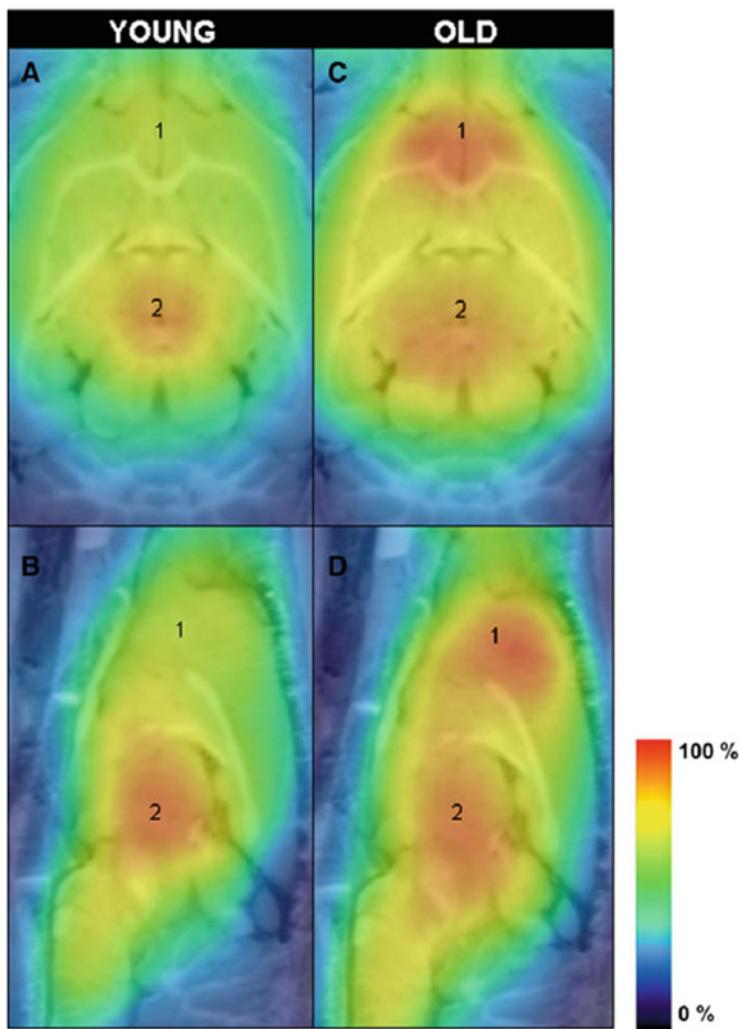


Fig. 15.5 Radioligand uptake in an old and young rat. Horizontal and sagittal views depicting averaged time frame image for [¹¹C]-DASB binding in a young (A, B) and an old (C, D) animal, overlaid on an anatomical rat brain template. 1: Frontal cortex; 2: Thalamus/Midbrain (Hoekzema et al. 2011)

inactivate monoamines such as norepinephrine, dopamine and 5-HT. MAO_A is the main isoform that degrades 5-HT and is predominantly located in astrocytes within the CNS (Fowler and Tipton, 1984). MAO_B is expressed in serotonergic neurons (Jahng et al. 1997; Kitahama et al. 1991; Luque et al. 1995), but its affinity for 5-HT is very low and therefore whether it significantly affects intracellular 5-HT or whether its presence is to deactivate other monoamines that may be brought into the cell by SERT is not known. A wide range of studies have explored how the

ageing process affects the expression/activity of MAO_A. Whilst a few studies have shown that ageing is associated with an increase in MAO_A expression or activity (Shih, 1979; Petkov et al. 1988; Pradhan 1980), most studies showed no significant change in MAO_A activity or expression with increasing age in humans (Carlsson et al. 1981; Gottfries et al. 1975; Fowler et al. 1980a, b; Kornhuber et al. 1989; Sparks et al. 1991; Volchegorskii et al. 2001), the squirrel monkey (Irwin et al. 1997) or the rat (Timiras et al. 1984; Sparks et al. 1985). Despite the lack of change in MAO_A a number of studies have shown changes in a measure of 5-HT turnover, namely the 5-HIAA:5-HT ratio (Lee et al. 2001; Sparks et al. 1985; Venero et al. 1993b). These changes may be reflective of the observed increase in MAO_B expression and activity seen with increased age in humans (Carlsson et al. 1981; Gottfries et al. 1975; Fowler et al. 1980a, b; Kornhuber et al. 1989; Sparks et al. 1991; Volchegorskii et al. 2001; Fowler et al. 1997) and rodents (Benedetti and Keane, 1980; Arai and Kinemuchi, 1988; Venero et al. 1993a; Irwin et al. 1992; Pardon et al. 2000; Saura et al. 1994). Additionally, they could represent increased metabolism by astrocytes/glial cells facilitated by impaired neuronal re-uptake of 5-HT in the synapse (see previous section). Further studies are required to clarify the causes of these changes in the absence of any significant change in MAO_A.

Effects of Age on 5-HT Receptors

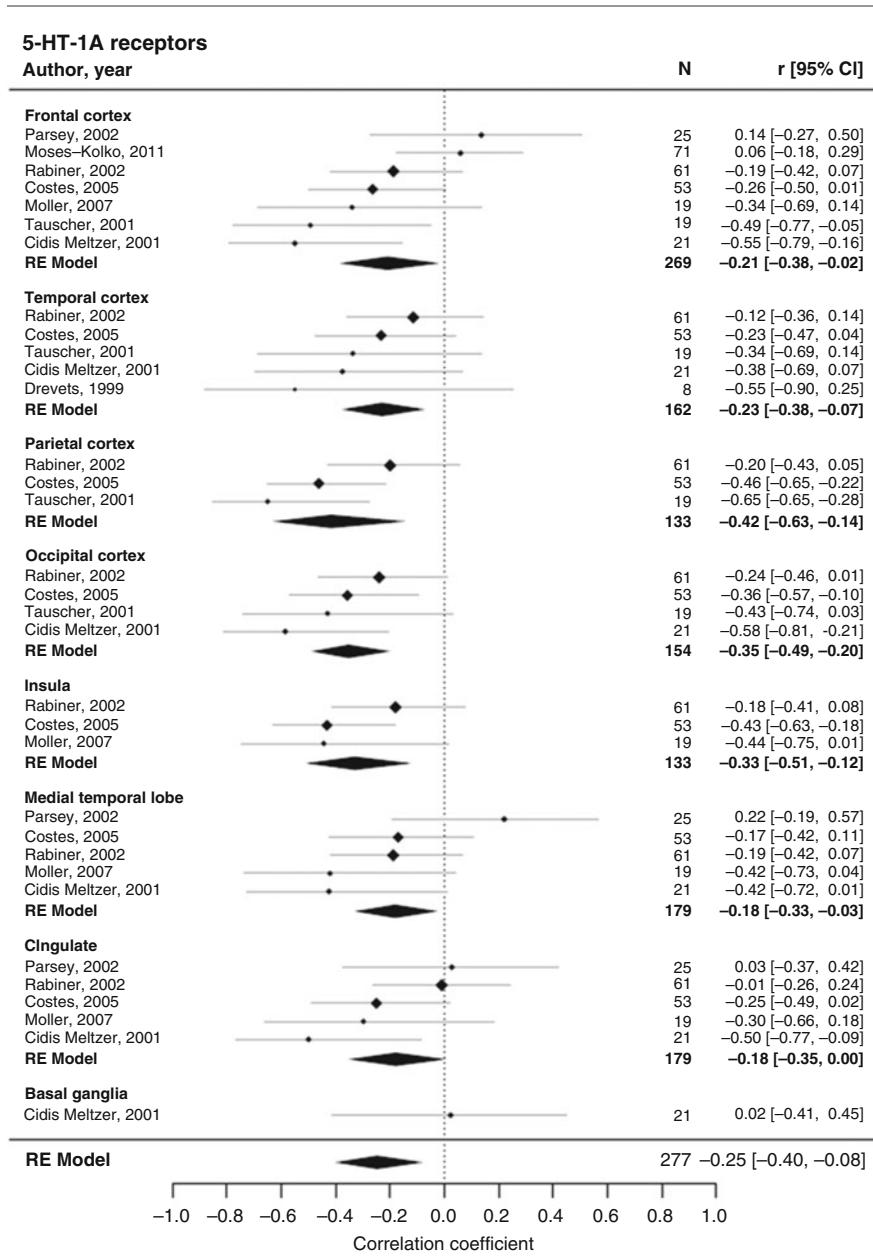
5-HT receptors can be subdivided into seven main classes many of which have additional sub-types yielding a total of 14 different receptor types. Changes in the expression of any of these receptors could have profound effects on 5-HT signalling, both in neurons and in glial cells within the CNS.

5-HT_{1A} Receptors

5-HT_{1A} receptors act as autoreceptors inhibiting the release of 5-HT from serotonergic neurons. In addition, they can also act as heteroreceptors regulating the release of transmitters from non-serotonergic neurons. Inhibitory 5-HT_{1A} receptors have been linked with regulation of long-term memory and the sleep/wake cycle, both of which can be disrupted in older humans (Gaspar et al. 2003; Hannon and Hoyer, 2008). Imaging studies in humans have shown changes in 5-HT_{1A} receptor binding in a range of brain regions (Parsey et al. 2002; Moses-Kolko et al. 2011; Rabiner et al. 2002; Costes et al. 2005; Møller et al. 2007; Tauscher et al. 2001; Cidis Meltzer et al. 2001; Drevets et al. 1999). A meta-analysis of these studies has been recently published by Karrer and colleagues and demonstrated that increasing age decreased the expression of 5-HT_{1A} receptors by between 1.5% and 7.0% per decade depending on the brain region examined (Karrer et al. 2019) (Table 15.2).

Similar age-related decreases in 5-HT_{1A} receptors have been observed using PET scanning in the frontal and temporal cortices of aged non-human primates (Tsukada

Table 15.2 Forest plot of age effects on 5-HT_{1A} receptors. Depicts reported effect size (diamond) and 95% confidence interval (line) for each included study. Weight of each study (size of diamond) depends on sample size and between-study variance. Summary effects sizes (polygon) and 95% confidence interval (width of polygon) for separate brain regions and in total. N represents the number of individual subjects included in each study and in the computation of the summary effect sizes. Taken from Karrer et al. (2019)



et al. 2001). Studies have also shown a decrease in 5-HT_{1A} binding sites in the brains of old rats (Nyakas et al. 1997) and hamsters (Duncan and Hensler, 2002). Other investigators did not find any difference in the *Htr1a* gene mRNA level in brains of young and old rats (Yau et al. 1999, Mitchell et al. 2009). However, the relationship between this lack of change in *Htr1a* gene mRNA to 5-HT_{1A} receptor protein expression is unclear as this was not explored in these studies.

5-HT_{1B} Receptors

Similar to the 5-HT_{1A} receptor, 5-HT_{1B} receptors can be located presynaptically where they act as autoreceptors to regulate 5-HT release (Hoyer and Middlemiss, 1989), but they are also present on non-serotonergic neurons where they act as heteroreceptors regulating neurotransmitter release (Middlemiss and Hutson; 1990, Maura and Raiteri, 1986). Relatively few studies have explored how the expression of the 5-HT_{1B} receptor is altered with increasing age. In humans, 5-HT_{1B} receptors were shown to decrease with increasing age at a rate of approximately 8% per decade (Matsumoto et al. 2011). In rats, ageing caused a decrease in the *Htr1b* mRNA level in the brains of old rats compared to young animals (Mitchell et al. 2009). These changes do not appear to be due to neurodegeneration of serotonergic neurons as there is no change in the expression of 5-HT_{1A} or 5-HT₆ mRNA in same study.

5-HT_{2A} Receptors

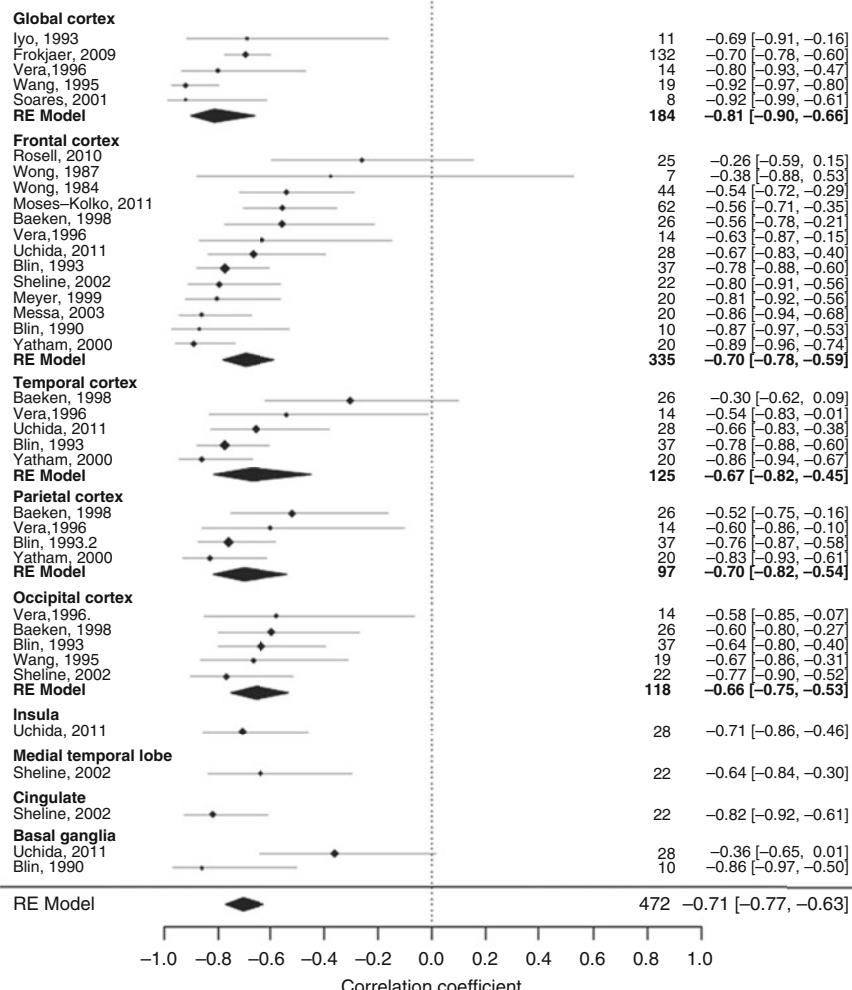
5-HT_{2A} receptors are located on the post-synaptic neuron and have high concentrations in the cerebral cortex, piriform cortex and entorhinal cortex and are also present in the basal forebrain, the septal nuclei and in the CA1–3 regions of the hippocampus and the dentate gyrus. These latter locations relate to the involvement of this receptor in learning and memory formation. These receptors are also involved in the aetiology of a range of psychiatric conditions including schizophrenia, anxiety and depression with drugs that alleviate these conditions acting either to reduce 5-HT_{2A} receptor expression or directly reduce its activation by endogenous 5-HT (Raote et al. 2007).

Imaging studies in humans have shown consistent age-related decreases in 5-HT_{2A} receptor expression (Iyo and Yamasaki, 1993; Frokjaer et al. 2009; Véra et al. 1996; Soares et al. 2001; Rosell et al. 2010; Wong et al. 1987; Wong et al. 1984; Moses-Kolko et al. 2011; Baeken et al. 1998; Uchida et al. 2011; Blin et al. 1993; Blin et al. 1990; Sheline et al. 2002; Messa et al. 2003; Yatham et al. 2000). A meta-analysis of human PET and SPECT studies demonstrated a marked decrease in 5-HT_{2A} receptor expression with increasing age (Karrer et al. 2019) (Table 15.3). These data are unequivocal and are supported by studies in non-human primates in a range of brain regions (Kakiuchi et al. 2000). Studies also report an age-dependent reduction of 5-HT_{2A} receptor binding sites in brains of old rats (Gozlan et al. 1990; Druse et al. 1997). However, other studies did not find any difference in the *Htr2a*

Table 15.3 Forest plot of age effects in 5-HT-2A receptors. Depicts reported effect size (diamond) and 95% confidence interval (line) for each included study. Weight of each study (size of diamond) depends on sample size and between-study variance. Summary effects sizes (polygon) and 95% confidence interval (width of polygon) for separate brain regions and in total. N represents the number of individual subjects included in each study and in the computation of the summary effect sizes (Karrer et al. 2019)

5-HT-2A receptors

Author, year



gene mRNA in the brains of young and old rats (Yau et al. 1999), or in 5-HT_{2A} receptor expression (Robson et al. 1993; Yamaguchi and Yamagata, 1991).

The decrease in 5-HT_{2A} receptor expression demonstrated by the imaging studies in humans is convincing and these age-related changes could contribute to the well-described age-related impairments in learning and memory formation.

Gene sequencing studies in a range of invertebrate species have identified a number of different 5-HT receptors including 5-HT_{1, 2, 4, 6} and 7 receptors (Tierney, 2020). However, to our knowledge there are no studies that have directly explored how ageing affects the expression of these receptors in *C. elegans*, *Drosophila* or in molluscs *Lymnaea* or *Aplysia*. Our work on *Lymnaea* has shown that signalling through a cinanserin sensitive (5-HT₂-like receptor) is inhibited with increasing age, consistent with the mammalian data (Yeoman et al. 2008).

5-HT_{4, 6} and 7 Receptors

5-HT₄ and 6 receptors have been linked with facilitating learning and memory formation (Meneses, 2015; Branchek and Blackburn, 2000). A limited number of studies have explored the effects of age on the expression of 5-HT₄ (Madsen et al. 2011; Lorke et al. 2006) and 5-HT₆ (Lorke et al. 2006; Radhakrishnan et al. 2018) receptors in humans using imaging techniques. In both cases, the expression of the receptors decreased with increasing age.

No studies have been performed to explore the effects of age on human 5-HT₇ receptor expression but studies in hamsters (Duncan and Franklin, 2007) and rats (Yau et al. 1999) showed no change with increasing age.

Overview of Effects of Age on Serotonergic Signalling

Under conditions of healthy ageing, several factors appear to be acting to reduce serotonergic signalling in the CNS with increasing age. These are a reduction in the numbers of connections that serotonergic neurons make with different regions of the CNS, a decrease in the firing frequency of serotonergic neurons and a reduction in post-synaptic 5-HT_{1B, 2A, 4} and 6 receptors. Several factors appear to act to compensate for these deficits. First, studies in *Lymnaea* show that ageing increases the amount of 5-HT released per release event. This would increase synaptic 5-HT and provide a stronger stimulation of the post-synaptic cell in the presence of reduced numbers of connections and reduced receptor expression. Second, in humans decreases in presynaptic autoreceptors, would reduce the brake on transmitter release and increase the amount of 5-HT in the synapse. Finally, age-related decreases in SERT function would also act to increase and prolong synaptic 5-HT signalling, by reducing clearance from the synapse.

Table 15.4 Table showing a comparison across species of the effects of age on 5-HT signalling. NE—not examined

Variable Examined	Humans	Non-human primates	Rats	Hamsters	Mice	Drosophila	Molluscs	C.elegans
Serotonergic neuronal number	no change, but loss of fibres	decrease	decrease and loss of fibres	NE	NE	NE	no change	NE
Tryptophan hydroxylase	no change	NE	decrease	NE	decrease, NE	decrease	decrease	NE
Vesicular content	no change	NE	NE	NE	decrease	NE	NE	NE
Serotonin expression	no change	NE	increase decrease no change	NE	no change decrease	decrease	no overall change but results cell dependent	decrease
Neuronal firing rate	NE	NE	NE	NE	decrease	NE	decrease	NE
Release of serotonin	NE	NE	NE	NE	NE	NE	increase	NE
SERT expression/activity	decrease no change increase	decrease	decrease Increase no change	increase no change	NE	NE	decrease	NE
MAO _A	no change	no change	no change; increase	NE	NE	NE	NE	NE
5-HT receptors								
5-HT _{1A}	decrease	decrease	decrease	decrease	NE	NE	NE	NE
5-HT _{1B}	decrease	NE	decrease	NE	NE	NE	NE	NE
5-HT _{2A}	decrease	decrease	decrease no change	NE	NE	NE	NE	NE
5-HT _{4/6/7}	5-HT _{4,6} decrease 5-HT ₇ NE	NE	5-HT _{4,6} NE 5-HT ₇ no change	NE	NE	NE	NE	NE

Taken together it is difficult to predict how all these changes might affect serotonergic signalling. Indeed, variation in the species used, the brain region examined and animal age adds complexity to the analysis. What is clear is that with ageing, synaptic 5-HT concentration will be higher and these high levels will be prolonged compared to the young synapse, due to impaired clearance via SERT. If this is the case then there is the potential for spill over of the transmitter from the synapse and activation of extrasynaptic autoreceptors or receptors on other neurons/glia cells. Prolonged exposure to high concentrations of synaptic 5-HT will also lead to post-synaptic receptor desensitisation, reducing post-synaptic signalling. In the short-term, the desensitisation would most likely represent a phosphorylation of the receptor by G-protein receptor kinases and the subsequent recruitment of β -arrestin preventing an interaction between the G-protein and the receptor. In the longer term, desensitisation would lead to internalisation of the receptor potentially contributing to the age-related decrease in 5-HT receptor expression. In the absence of desensitisation, signalling through the post-synaptic 5-HT receptors is going to be altered with signalling through 5-HT_{1A, B}, 5-HT_{2A}, 5-HT₄ and ₆ receptors being weakened, whilst other receptors would be strengthened (5-HT₇), with similar potentiation observed at the 5-HT_{2C, D} and 5-HT_{3, 5} receptors, although no data could be found on how the expression of these latter receptors change with increasing age. The effects of age on 5-HT signalling are summarised in Table 15.4.

Ability of 5-HT to Regulate Lifespan

Studies in the late 1980s and early 1990s were the first to show that organismal ageing was a genetically malleable trait. These studies demonstrated that mutations in *C. elegans* insulin-like growth factor receptor, *daf-2*, were able to double lifespan (Friedman and Johnson, 1988; Kenyon et al. 1993; Kimura et al. 1997). *Daf-2* is part of a complex insulin signalling pathway that acts to connect nutrient levels in the environment to metabolism, growth, development and longevity (Murphy and Hu, 2013). These studies were vitally important as they showed that it was possible to intervene in the ageing process. The lifespan-extending effects of the *daf-2* mutant require *daf-16* a forkhead box transcription factor (FOXO) that is involved in regulating the animal's response to stress (Gottlieb and Ruvkun, 1994), see Fig. 15.6. Wild type *Daf-2* inhibits the translocation of *daf-16* to the nucleus strongly inferring that translocation to the nucleus is important for longevity.

This pathway is conserved across a range of organisms and shortly after the work on *C. elegans* studies demonstrated that mutations in the single *Drosophila* insulin receptor *dInR*, could extend lifespan (Clancy et al. 2001) as could mutations in the insulin receptor substrate, *chico* (Tatar et al. 2001). These early studies are reviewed in more detail by Kannan and Fridell (Kannan and Fridell, 2013). Subsequent studies in mice demonstrated that reduced insulin and insulin-like growth factor signalling was also capable of extending lifespan (Brown-Borg et al. 1996) demonstrating that the ability to prolong lifespan was conserved through evolution (Partridge and Gems, 2002).

The proportion of the population over the age of 65 years is set to double between now and 2050 (United Nations, 2015, Office for National Statistics, 2021). This increase in older people should be celebrated, but given that ageing is the number one risk factor for a range of diseases, this brings with it the potential for huge economic costs, in healthcare needs. Interestingly, many studies have shown that extending lifespan also extends health span in animal models leading to a wealth of research to further understand the regulation of lifespan.

A number of studies in invertebrates have demonstrated that 5-HT signalling is capable of regulating lifespan. Studies to determine the differences in longevity between two strains of *Drosophila* identified a quantitative trait locus for DOPA decarboxylase. DOPA decarboxylase is the enzyme that catalyses the last step in the formation of both 5-HT and dopamine suggesting the involvement of these amines in longevity (De Luca et al. 2003). Analysis of the lifespan of a range of *C. elegans* mutants showed that a mutation in tryptophan hydroxylase gene (*tph-1* mutant) failed to extend lifespan. However, a deletion mutation in the *Ser-1* 5-HT receptor (equivalent to mammalian 5-HT_{2A} receptor) significantly extended lifespan whilst mutations in *Ser-4* (equivalent to the mammalian 5-HT_{1A} receptor) shortened lifespan although these effects failed to reach significance ($p < 0.07$) (Murakami and Murakami, 2007). Interestingly, the lifespan-extending effects of the deletion mutation in *Ser-1* were blocked using *daf-16* (RNAi) suggesting that the effects of 5-HT were via insulin/insulin-like growth factor signalling (IIS) pathway. Further

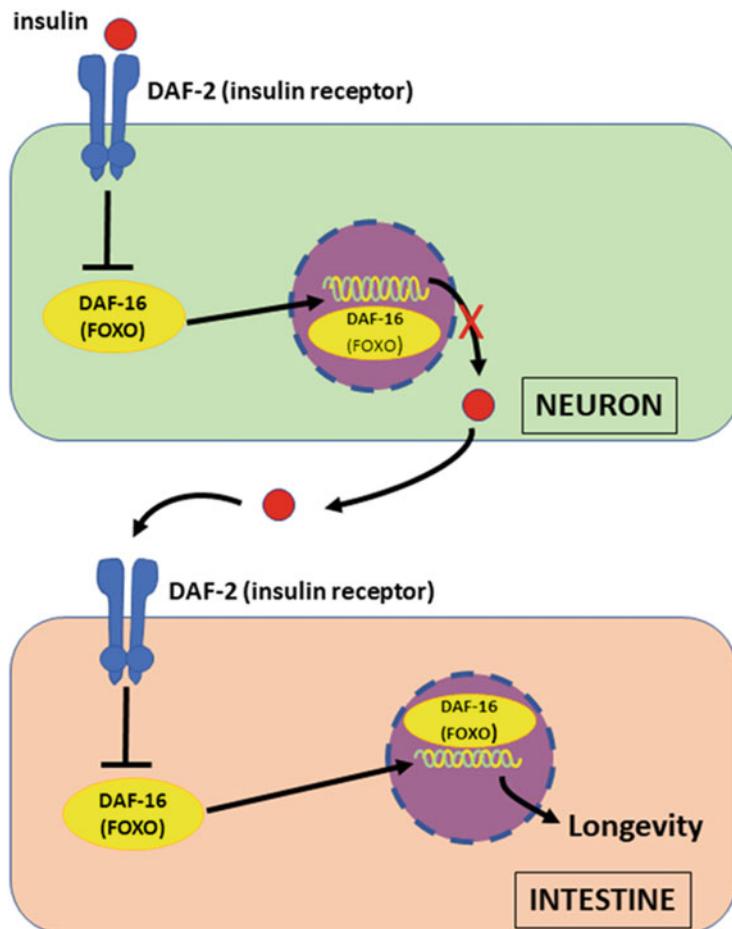


Fig. 15.6 Summary diagram showing how changes in insulin signalling can extend lifespan in *C. elegans*. Adapted from Miller et al. (2020)

evidence supporting the involvement of IIS in the regulation of lifespan comes from observations that 5-HT can directly target the daf-16/FOXO signalling pathway in *C. elegans* (Liang et al. 2006). Two additional studies have shown that antidepressants that target 5-HT signalling, namely mianserin and methiothepin can both regulate lifespan (Zarse and Ristow, 2008; Petrascheck et al. 2007). Both drugs were shown to have their effects via the IIS signalling cascade as mutations in daf-2 and daf-16 significantly attenuated the lifespan-extending effects of the drugs (Petrascheck et al. 2007). However, the two studies showed opposite results on lifespan with one showing lifespan-extending effects (Petrascheck et al. 2007), whilst the other showed lifespan-shortening effects (Zarse and Ristow, 2008). The pharmacology of the drugs used is complicated with both drugs having effects on 5-HT₁ and 5-HT₂ receptors as well as having off-target effects on other neurotransmitter systems.

Given the opposing effects of 5-HT_{1A} (*Ser-4*) and 5-HT_{2A} (*Ser-1*) *C. elegans* receptors on lifespan (Murakami and Murakami, 2007), it is possible that the different culture conditions highlighted by Zarase and Ristow are responsible for the differences in results as these could have affected the density or distribution of these two 5-HT receptors in the two different studies leading to the discrepancy in the data.

5-HT signalling was also shown to affect lifespan in *Drosophila* (Talaei and Atyabi, 2013). The authors fed *Drosophila* the selective 5-HT_{2A} receptor antagonist ketanserin by mixing it in their food. Ketanserin was shown to increase lifespan by 35%, although while the mechanisms of action were not explored directly in *Drosophila*, the authors did show that ketanserin had anti-ageing effects on human skin fibroblasts by decreasing the activity of mTOR, stimulating autophagy and wound closure and decreasing oxidative stress.

Taken together these data infer that active 5-HT signalling can shorten lifespan through the activation of the 5-HT₂-type of receptor whilst activation of the 5-HT₁-type receptor can increase lifespan in *C. elegans*.

More recent work exploring this area of research has examined how an animal's perception of its food can regulate lifespan. Interestingly, 5-HT signalling can regulate our body's response to the perception of food, affect the physical intake of food, what food is ingested and how much food is ingested. In addition, it is able to prepare the body for the intake of nutrients by deciding where the nutrients go and how they are metabolised. Together these effects are likely to contribute to the ability of 5-HT to regulate lifespan.

Work using *C. elegans* was the first to demonstrate the sensory modulation of lifespan (Apfeld and Kenyon, 1999). They showed that disruption to the structure or sensory pathways in the amphid organs that contact the external environment of the worm could extend lifespan. These disruptions were shown not to affect feeding rate, reproduction or development suggesting that the effects on lifespan were solely due to altered sensory perception. Interestingly, whilst disruption of some sensory neurons extended lifespan (ASI, ASG, AWA and AWC), loss of others shortened lifespan (ASK) (Alcedo and Kenyon, 2004).

In *Drosophila*, the perception of live yeast, a major food source of *Drosophila*, was able to partially reverse the lifespan extension observed following dietary restriction (Libert et al. 2007). In mammals, the work linking sensory perception to lifespan is less advanced. Olfactory dysfunction is a strong predictor of 5-year mortality although this most likely represents some underlying problem with the nervous system rather than being the primary driver of mortality (Pinto et al. 2014). Interestingly, whilst the effects of olfactory and gustatory neurons on lifespan have been little studied, work exploring the potential role of TRPV1 sensory nerves in regulating lifespan has shown that they are capable of antagonising insulin signalling (Melnyk and Himms-Hagen, 1995), a pathway that is involved in lifespan regulation in both invertebrates and vertebrates. Additionally, inhibition of the main transmitter of TRPV1 sensory neurons, calcitonin gene-related peptide (CGRP), can restore metabolic health in old mice as does a genetic deletion of TRPV1 (Riera et al. 2014).

The precise mechanisms underlying the effects of CGRP on lifespan are currently unknown.

Molecular Mechanisms Involved in Sensory Regulation of Lifespan by 5-HT

Food in the environment is capable of activating IIS. Indeed, worms, flies and mice that have disrupted IIS have extended lifespans (Partridge and Gems, 2002). Therefore, it is easy to see the link between food perception and lifespan in a wide range of organisms. Many lines of evidence show crosstalk between IIS signalling pathways and 5-HT in *C. elegans* (Sze et al. 2000; Liang et al. 2006) and *Drosophila* (Luo et al. 2012). However, the ability of sensory perception to impact lifespan is not solely down to altered IIS. Indeed, recent work has explored in more detail the molecular mechanisms underlying how sensory perception of food can influence lifespan and has found strong links between 5-HT signalling pathways and lifespan.

In *C. elegans* longevity is not only linked to nutrient resources in the environment but also to temperature and oxygen levels (Miller et al. 2020). In both cases, the presence of hypoxia or heat stress leads to the production of 5-HT which impacts the stress response by inhibiting the translocation of *daf-16* to the nucleus and as a consequence reducing lifespan (Fig. 15.7). In the case of hypoxia, the effects of 5-HT are inhibited in *Ser-1* mutants suggesting the involvement of the 5-HT_{2A} receptor. In the case of heat stress the downstream 5-HT receptor is likely *Ser-7* a homologue of the 5-HT₇ receptor found in mammals.

Research using *Drosophila* has generated some profound and interesting results concerning the impact that sensory perception has on lifespan and the involvement of 5-HT in this process (Miller et al. 2020).

Effects of Dead Conspecifics on Lifespan and the Role of 5-HT

Work by Chakraborty and colleagues has shown that *Drosophila* lifespan can be regulated through a combination of the visual and olfactory cues of dead conspecifics (Chakraborty et al. 2019). The lifespan-shortening effects required that: the flies were not killed by freezing; were recently dead (<11 days); and dead flies were a closely related species to the flies under test. The observation that both visual and olfactory cues were important suggests that the test flies can both recognise a conspecific and presumably determine the time and type of death through olfactory cues. Interestingly, the ability of dead conspecifics to attenuate lifespan was not observed in flies administered a 5-HT_{2A} receptor blocker or in mutant flies that lack the 5-HT_{2A} receptor (Fig. 15.8). Similarly, the effects of exposure to dead

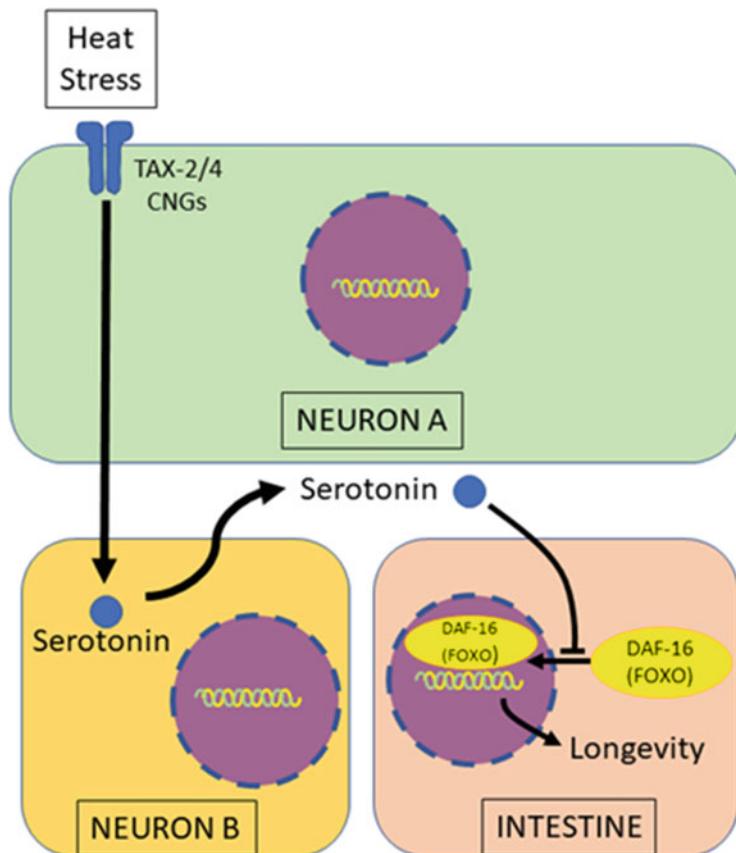


Fig. 15.7 Heat stress can reduce lifespan in *C. elegans* in a 5-HT-dependent manner. Adapted from Miller et al. (2020)

conspecifics could be mimicked in naïve flies by making neurons that normally express the 5-HT_{2A} receptor constitutively active when the temperature was raised above 25 °C.

As mentioned previously dietary restriction is capable of extending lifespan in a range of animals from *C. elegans*, to *Drosophila*, mice, non-human primates and even humans. In *Drosophila*, exposure of dietary-restricted flies to attractive food odours attenuates the effects of dietary restriction on lifespan, although the mechanism is not completely understood (Libert et al. 2007). The main component of food that drives the effect of dietary restriction on lifespan is its protein content. Flies kept on a low-protein diet will swap to ingesting protein from a primary sugar diet when protein becomes available. This switch requires the presence of the 5-HT_{2A} receptor as the switch was blocked in flies lacking functional 5-HT_{2A} receptors. More interesting was the observation that when flies were able to choose between a fixed diet (containing 10% w/v sugar and 10% w/v protein) or a choice diet

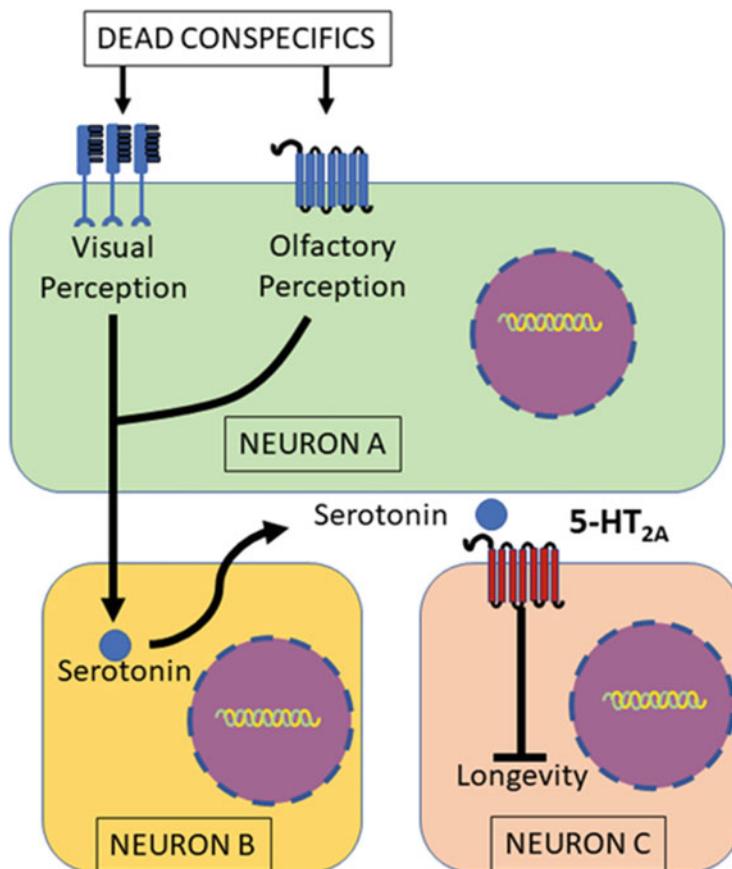


Fig. 15.8 Visual and olfactory cues on lifespan in *Drosophila* are regulated by 5-HT acting through a 5-HT_{2A} receptor. Adapted from Miller et al. (2020)

consisting of protein (10% w/v) and sugar (10% w/v) presented separately, lifespan was decreased. This decrease, which was particularly strong in males, did not depend on the amount of sugar and protein ingested as this was essentially the same between groups, but was dependent on the level of protein in the choice diet (Lyu et al. 2021b). Previous studies had shown that the 5-HT_{2A} receptor was important for determining the choice of a protein diet (Ro et al. 2016). Using a fly strain with a P-element insertion in the promotor region of the 5-HT_{2A} receptor gene produced a fly with a loss of function of the 5HT_{2A} receptor. Using this strain blocked the effect of food choice on lifespan, strongly suggesting that 5-HT signalling through the 5-HT_{2A} receptor is important for regulating lifespan changes due to food choice (Lyu et al. 2021b). The authors further showed that knockdown of glutamate dehydrogenase a key component of the tricarboxylic acid (TCA) cycle was also capable of abrogating the effects of food choice on lifespan suggesting a link between 5-HT_{2A}

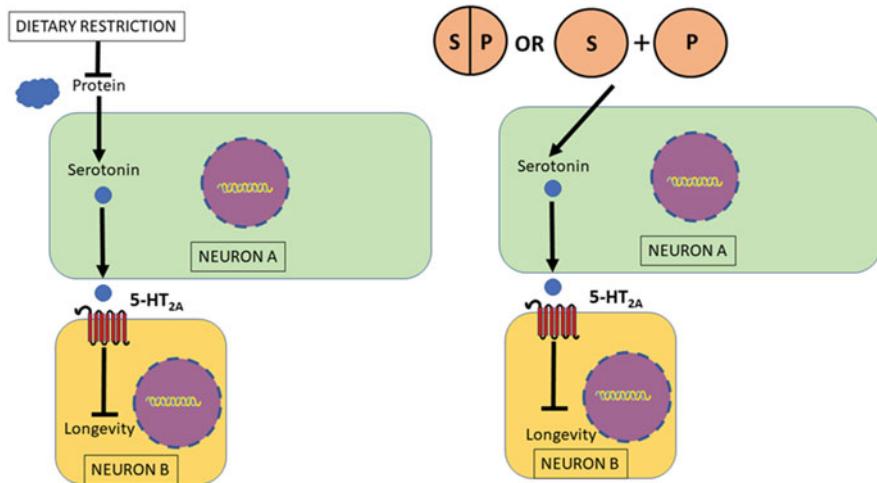


Fig. 15.9 The effects of dietary restriction and food choice on longevity require 5-HT acting via a 5-HT_{2A} receptor. Adapted from Miller et al. (2020)

receptor signalling and the TCA cycle. In a recent paper, Lyu and colleagues have further investigated the mechanism by which nutrient choice decreases lifespan (Lyu et al. 2021a). In this paper, they demonstrate that nutrient choice changes the state of metabolic networks from one that is highly connected and robust to one that is fragmented and fragile. Importantly, these changes were also dependent on the 5HT_{2A} receptor. The authors proposed that these changes are important as they may allow the animals to adapt their physiology to match the availability of key nutrients in their environment and that in turn, this may increase fitness (Fig. 15.9).

Finally, Miller and colleagues have further explored how the perception of food odours can attenuate the lifespan-extending effects of dietary restriction (Miller et al. 2022). In this paper, the authors confirm the work of Libert and colleagues (Libert et al. 2007) by showing that exposure to attractive food odours in the absence of food ingestion can abrogate the effects of dietary restriction on lifespan. In addition, they show that mianserin, a 5-HT receptor antagonist, is capable of blocking the effects of attractive food odours and increases longevity. Given that mianserin can block both *Ser-1* (5-HT_{2A}) and *Ser-4* (5-HT_{1A}) receptors and that both receptors have previously been shown to be important in regulating lifespan (Petrascheck et al. 2007), it was important to determine which receptor was involved in allowing attractive odours to block the lifespan-extending effects of caloric restriction. Using a series of knockdown experiments, the authors showed that *Ser-4* appeared to be the main receptor involved in the perception of attractant odours. Conditional knockouts strongly suggested that this receptor was located in the nervous system.

So How Does the Natural Ageing Process and 5-HT Signalling Interact?

The research highlighted in the previous section has demonstrated convincingly that 5-HT can affect lifespan in a range of invertebrate species. More specifically, 5-HT is capable of decreasing lifespan, mainly through its effects on the 5-HT_{2A} receptor, but also with involvement of the 5-HT_{1A} receptor. It is interesting to note that during the natural ageing process both these receptors are downregulated in higher organisms, suggesting that as animals age signalling through the 5-HT_{2A} and to a lesser extent the 5-HT_{1A} receptor is impaired. Given that signalling through these two receptors can decrease lifespan it is interesting to speculate that a compensatory decrease in receptor expression in old age may act to try to maintain organismal function albeit by reducing signalling through pathways that act to shorten lifespan. Indeed, whilst reduced signalling through the 5-HT_{2A} and 5-HT_{1A} receptors may bring beneficial effects to lifespan, the trade-off detrimental effects should not be underestimated. Reduced signalling through 5-HT_{2A} receptors would decrease the flexibility/plasticity of the metabolic pathways to adapt to the environmental changes in nutrient availability. In terms of fitness, this is not an issue as most of the changes observed in receptor expression occur at a time of life where fecundity is very low or non-existent. However, this may seriously impact the ability of the animal to choose the appropriate nutrients to maintain homeostasis (i.e. the lack of ability to select appropriate protein diet if required). Additionally, 5-HT is an important regulator of olfaction in rodents (Gaudry, 2018) and 5-HT_{2A} and 5-HT_{1A, B} receptors are crucial to the function of this sense. Therefore, a further detriment to the observed downregulation of these receptors with increasing age is that sensory perception of food will be impaired, which in humans is critical to taste and the palatability of food. Together, the potential for ageing to decrease the flexibility of metabolic systems and to reduce the palatability of food could contribute significantly to a condition known as anorexia of ageing, defined as a failure to ingest an adequate amount of food to meet their essential energy and nutrient requirements (Landi et al. 2016).

Of course, there are several caveats to this interpretation of the data that elude to a requirement for experiments to be carried out in the future. First, whilst 5-HT levels in the CNS of both *C. elegans* and *Drosophila* have been shown to decline with increasing age, no data is available detailing how the expression of the 5-HT₁ and 5-HT₂ receptors changes over the life course of these animals. Second, the data presented at the beginning of this chapter has focused strongly on receptor expression in the CNS and not in the periphery and whilst some of the effects described in the invertebrates above rely on central serotonergic signalling, namely those described for *Drosophila*, the *C. elegans* data requires the involvement of peripheral 5-HT receptors located in the intestine and how these receptors change with increasing age is currently not known. Finally, whilst we have tried to illustrate that similar serotonergic signalling mechanisms may be present in higher organisms including humans this has yet to be determined.

In summary, the recent work by Miller and colleagues (Miller et al. 2020, Miller et al. 2022) has opened a new chapter in the role of 5-HT in the nervous system and the potential to develop therapeutics to target the serotonergic system to improve health span in our ageing population.

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Chapter 16

Systems Biology of Ageing



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Abstract The ageing process is highly complex involving multiple processes operating at different biological levels. Systems Biology presents an approach using integrative computational and laboratory study that allows us to address such complexity. The approach relies on the computational analysis of knowledge and data to generate predictive models that may be validated with further laboratory experimentation. Our understanding of ageing is such that translational opportunities are within reach and systems biology offers a means to ensure that optimal decisions are made. We present an overview of the methods employed from bioinformatics and computational modelling and describe some of the insights into ageing that have been gained.

Keywords Systems · In silico · Bioinformatics · Computational modelling and simulation

Introduction

Ageing is an inherently complex process driven by dysregulation in intracellular molecular reactions to entire organ systems. At the cellular level, ageing phenotypes are associated with changes to complex molecular networks arising from genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication, as highlighted by López-Otín et al. (2013) as the hallmarks of ageing. These cellular phenotypes may differ from cell to cell and between cell types and their host tissues, so that the ageing phenotype is one

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of the highest complexity. A full understanding necessitates a hybrid reductionist-integrative strategy addressing the multiple levels of complexity in a systematic manner. Individual contributions may be addressed initially as isolated systems and then combined with other contributions to build up an integrative view. This is the basis of systems biology. It adopts principles and methodologies from computer science, physics, engineering, and mathematics together with experimental biology to gain a mechanistic understanding of biological systems and networks (Kirkwood 2011). The methodologies employed in computational systems biology are primarily data mining with bioinformatic analysis and/or computational or mathematical simulation-based analysis. The former, known as the top-down approach, is used to gather knowledge from large biological datasets, resulting in the formulation of a hypothesis. Whereas a bottom-up approach of simulation-based analysis allows the testing of a hypothesis using *in silico* models to make predictions, which can aid the design of *in vivo* and *in vitro* experiments (Kitano 2002). Applying a systems biology approach brings together existing data and knowledge from disparate and individual experiments to provide the details of the underlying complexity without which we could never truly understand the mechanisms of ageing.

Bioinformatics

Bioinformatic methods are used in top-down systems biology. The overall purpose is to probe omic data to identify molecules and subsystems involved in ageing. The first stage in bioinformatic analysis involves linking experimental observations from many sources to create an interconnected framework of key interactions, generating modular, cohesive and interconnected networks which can describe the system as a whole (Song et al. 2018). A variety of computational tools, databases, reporting and formatting standards have been developed for use by ageing researchers (Wieser et al. 2011); however, the integration, interpretation, formatting, storage and sharing of data, clinical and phenotypic information remain major challenges (Dato et al. 2021).

Developments in biotechnology, technology, sequencing, bioinformatics/computing, statistics, as well as reductions in costs and increases in speed have enabled biologists to generate data sets covering entire genomes, transcriptomes, epigenomes, metabolomes, lipidomes, proteomes, protein conformation, and protein binding from a single experiment. Moreover, we can observe changes in these large datasets throughout the life-course of individual organisms and cells (Dato et al. 2021; Song et al. 2018). Transcriptomics no longer requires RNAs to be captured using poly-A tails, enabling measurement of non-protein-coding RNA and isoforms, identifying functional roles for non-coding ‘junk DNA’ (Bainbridge et al. 2006; Palazzo and Gregory 2014; Pettersson et al. 2009). Extension of RNA-seq to single cells has enabled the study of cellular variation within tissues to investigate the role of stochasticity or the location of cells relative to key structures such as veins and arteries. Spatial transcriptomics using technologies such as laser-based precision

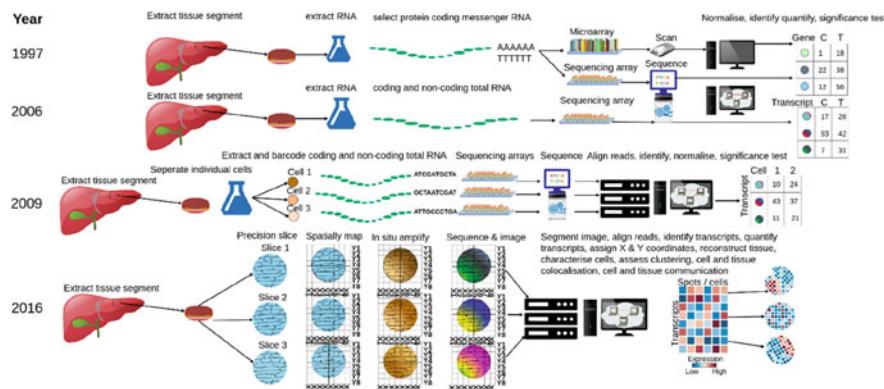


Fig. 16.1 Timeline of the evolution of transcriptomics showing the basis of experimental methods, bioinformatic analysis steps, data types produced and recent advancements that have enabled spatial transcriptomics

slicing, in situ DNA sequence cell barcoding and spatial array image-based sequencing is opening opportunities to study the specific impacts of cellular location, cell subtypes and cell–cell interactions on health and disease states. The size and complexity of resulting data, technical noise and the scale of genomic, epigenetic and transcriptomic interactions represent major future challenges in bioinformatics and systems biology alike (Aldridge and Teichmann 2020; Rao et al. 2021). An overview of the evolution of transcriptomics technologies is shown in Fig. 16.1.

Figure 16.1 presents the rapid expansion of omic analysis which can be illustrated in the study of senescent cells. Initial studies used microarray data to assess the expression of specific RNAs between proliferating cells and cells that had divided until uncapped telomeres prevented further division (Zhang et al. 2003). However, our understanding of senescent cells evolved alongside omic technologies, and later studies used RNA-seq to identify the differential expression of existing as well as novel transcripts at specific timepoints during senescence induction (Young et al. 2009). This utilised multiple types of senescence that unlike replicative senescence could be induced at a point of choosing. More recent studies included large numbers of interventions to identify the mechanisms behind cellular senescence (Georgilis et al. 2018). The role of the senescence-associated secretory phenotype (SASP) in paracrine senescence induction (Acosta et al. 2013), and the role of Notch signalling in juxtacrine senescence induction (Hoare et al. 2016) were both identified in part from transcriptomic data, which also allowed an unbiased comparison between the profiles of these new types of senescence and the initial inducer-senescent cells. These studies helped to enrich our understanding of cell senescence, and studies such as Hernandez-Segura et al. (2017) used meta-analyses of existing transcriptomic data to identify universal markers of senescent cells. Later studies did the same for proteomics (Basisty et al. 2020; Neri et al. 2021), while single-cell transcriptomics revealed subpopulations within populations of senescent cells from the same mode of induced arrest (Chen et al. 2020).

Single-cell transcriptomics has also been used to create an atlas of ageing in mice (*Tabula Muris Senis*), identifying double the proportion of cells expressing the senescence marker CDKN2A in older mice (Almanzar et al. 2020). This finding was corroborated by Uyar et al. (2020) analysing data from multi-tissue mouse single-cell transcriptomic data sets. They identified that the highest proportion of cells expressing ten (pre-specified) senescence-associated genes were found in the liver, with Interleukin 1 β (IL1 β), a cytokine involved in inflammageing, showing the highest expression. Thus, bioinformatic analysis helps us identify the important molecules in ageing using unbiased methodologies. Computational modelling can then be used to put these molecules in context to establish their importance within networks of interacting molecules.

Computational Modelling

Models can be dynamic or static, discrete or continuous and stochastic or deterministic, to reflect the system being modelled and the hypothesis being tested. Dynamic models explore how variables evolve temporally, while static models explore a process at a given timepoint. Dynamic models are a more useful tool as processes inevitably undergo temporal change with age, for example Dalle Pezze et al. (2014) used dynamic modelling techniques to investigate mitochondrial function in cellular senescence. Living systems are by definition dynamic but often achieve prolonged periods of homeostasis which sometimes lend themselves to static mathematical modelling. Discrete variables have defined integer values, while continuous variables have an infinite number of states or values in between discrete integers. Stochastic models account for the randomness of cellular processes—cellular and genetic damage is random and is therefore an example of an age-related stochastic behaviour. Examples of stochastic modelling include the Sozou and Kirkwood (2001) model which investigated the heterogeneity in entry of cells into cellular senescence, and the Proctor et al. (2005) study which used stochastic modelling to investigate the role of molecular chaperones in ageing. On the other hand, deterministic models have set initial parameter values and initial conditions which are not influenced by random dynamics. Models can also have a spatial element that incorporates not just the levels of the various molecules but their positions relative to each other on a two- or even three-dimensional plane. This can involve the use of partial differential equations such as in reaction-diffusion equations (Turing 1952), grid-like systems such as cellular potts models (Graner and Glazier 1992), or agent-based systems where elements such as cells or macromolecules are modelled as agents with defined position and motion (Byrne and Drasdo 2008). Common ways to model systems with these frameworks are shown in Fig. 16.2, adapted from McAuley et al. (2017). When variables do not have predetermined values from experimental data, modellers can utilise parameter estimations to guide variable behaviour, determining how different values affect model dynamics. Parameter estimation is a particularly challenging task in developing computational models: success is

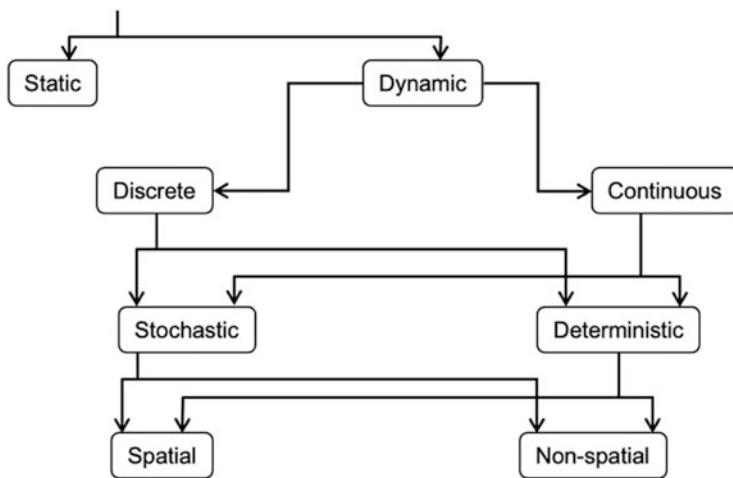


Fig. 16.2 Modelling framework flowchart

often dependent on data quality and quantity, requiring careful selection of assumptions and often extensive high-performance computing resources. Multiple software packages are available but additional programming is often required (Hoops et al. 2006; Welsh et al. 2018).

One example of successfully using a model to investigate a gap in knowledge was by Sozou and Kirkwood (2001) who wanted to understand why there was heterogeneity in cell division potential and entry into replicative senescence, as the shortening of telomeres on its own could not account for the heterogeneity observed. The model suggested that oxidative damage from mitochondrial ROS alongside nuclear somatic mutations could contribute to the observed heterogeneity in the number of cell divisions before cells entered replicative senescence. The model findings were later reinforced by Passos et al. (2007) through experiments which showed that the production of mitochondrial superoxide increases with replicative cell age. They further showed that mild mitochondrial uncoupling led to reduced superoxide generation, slowed telomere shortening and delayed replicative senescence entry, demonstrating the importance of mitochondrial ROS in contributing towards the heterogeneity of cell entry into replicative senescence.

Computational modelling has been widely used to investigate the oscillatory nature of the p53 response (Geva-Zatorsky et al. 2006; Proctor and Gray 2008), the role of molecular chaperones in ageing (Proctor et al. 2005; Proctor and Lorimer 2011), mitochondrial function in ageing and senescence (Dalle Pezze et al. 2014; Passos et al. 2010), and the dysregulation of various signalling pathways with age (Hui et al. 2016; Smith and Shanley 2010). As well as modelling specific processes which occur in ageing, computational models can investigate the molecular level of age-related diseases—for example the role of cytokine interactions in rheumatoid arthritis has been modelled (Baker et al. 2013), as has the role of amyloid- β dimer aggregation in Alzheimer's disease (Proctor et al. 2012).

Many modelling studies are conducted in collaboration with laboratory-based research allowing the model to inform the experiments and vice versa. For example, Dalle Pezze et al. (2014) combined in silico and in vitro experimental approaches to explore the role of different signalling molecules in cellular senescence and mitochondrial function. They experimentally identified that inhibition of ROS or mTOR partially rescued the dysfunctional mitochondrial phenotype observed in senescent cells, developing a model which replicated these outcomes and predicted the effects of dual inhibition which they then confirmed experimentally. Although the interventions were not able to completely prevent mitochondrial dysfunction in senescence, the model identified that mitochondrial fission may drive the increased mitochondrial mass found in senescence.

Another example of successful collaboration between computational and experimental biologists is the work by Passos et al. (2010) who used modelling alongside in silico interactome analysis and live cell microscopy to identify a dynamic feedback loop between p21 and ROS in cellular senescence by which DNA damage activated p21 through p53 which then increased ROS production to increase DNA damage and thus ensure permanent cell cycle arrest.

A good understanding of the underlying intracellular molecular events is crucial to study the ageing process, informing when higher level phenotypes such as functional decline and disease should manifest both for individual organisms and even at the population level through the use of multiscale modelling.

Multiscale Models of Ageing

Ageing is a systemic phenomenon resulting from complex interactions between multiple functional and organisational levels, including interactions between cells and between cells and their surrounding microenvironment. These include the biomechanical forces affecting cells and the dynamics of tissue-level organisation. These processes operate across different spatiotemporal domains, making ageing a multiscale problem consisting of age-related defects at the level of molecules, cells, tissues and organs all contributing to the whole-body frailty and disease we define as ageing (Kirkwood 2005b).

One approach to addressing a multiscale system is through the construction of cell-based models representing a system such as a tissue as an aggregate of individual agents (cells), each with its own set of distinct characteristics and mechanisms. This type of framework allows us to take an integrative approach by analysing the spatiotemporal dynamics of individual elements and how they collectively result in a particular phenotype. Furthermore, multi-cell models have the capacity to combine different model types of varying resolution, for example an ordinary differential equation (ODE) model, to dictate individual cell behaviour, coupled with an agent-based model to ascertain tissue-level behaviour. In keeping with this, Martinez Guimera et al. (2017) developed a multi-cell senescence model coupled with an intracellular ODE model of irradiation-induced senescence (Dalle Pezze et al. 2014).

The model showed that a tissue level of just 10% senescent cells could be sufficient to drive a whole tissue into senescence, highlighting the importance of the bystander effect (Acosta et al. 2013; Nelson et al. 2012). Additionally, further model simulations showed that senescent cell clearance by immune cells largely determined progression of the model towards a steady state, suggesting that targeted immunotherapy against senescent cells would be a more effective therapeutic approach than targeting the SASP.

Other approaches to multiscale modelling include whole-body modelling, which integrates molecular and physiological data to study whole-body metabolism. For instance, McAuley et al. (2012) developed a set of ODEs representing a whole-body model of cholesterol metabolism to explore how changes in Low-Density Lipoprotein Cholesterol (LDL-C) clearance by the liver and absorption by the intestine contributed to the age-related rise in plasma LDL-C. The multiscale nature of cholesterol metabolism was addressed in the model through compartmentalisation of the various processes involved including cholesterol intake, synthesis of intestinal cholesterol, excretion and clearance from the plasma, hepatic and peripheral tissue. Their results suggested clearance by the liver was the more pertinent factor.

Although multiscale modelling in ageing is still in its infancy, some notable work has been done to explore the evolution of ageing. Nelson and Masel (2017) developed a series of equations for changes in cell vigour and cooperativity which suggested that ageing might be an unavoidable result of mutations that reduced the cooperativity or increased the vigour of cells relative to wild-type cells. This ran in stark contrast to earlier theories that suggested ageing resulted from reversible mutations such as mutation accumulation theory (Medawar 1952) as well as the likely incorrect assumption that tissue-level selection would be inconsequential to fitness (Szathmáry and Smith 1995).

Goldsby et al. (2014) developed a computational model consisting of ‘digital’ multicellular organisms to study the mutagenic effects of metabolism that could have led to the evolution of division of labour between the somatic and germline cells. The division of labour theory posits that ageing might be linked to the asymmetrical separation of resources between reproductive functions and somatic functions such as maintenance and repair (Kirkwood 2005a). By analysing the lineages of simulated organisms, Goldsby et al. (2014) demonstrated that organisms can evolve to harbour the potential for cell differentiation resulting in reproductive or somatic maintenance functions, and that somatic phenotypes could be associated with ageing, while the reproductive cells remain protected. Similarly, Radzvilavicius et al. (2016) developed an agent-based evolutionary model showing that mitochondrial fitness drives the evolution of the germline, and that gamete sequestration from the soma reduces mutational load since copying errors in gametes are reduced. They also showed that greater organismal complexity and higher numbers of mitochondria do not contribute to the evolution of germline sequestration. More recently, Pen and Flatt (2021) developed a simple cell-based model to investigate the role of parent–child asymmetry in the evolution of division of labour and cellular senescence. From their model, they concluded that parent–offspring asymmetry and the division of labour was not required for the evolution of ageing. This claim was supported by model

simulations that showed that symmetrically reproducing cells with equally partitioned damage, and their cell lineages, were still able to develop senescence.

Conclusion

Systems biology has made a significant contribution to our understanding of ageing, and its potential will only increase with ongoing development of associated technologies. However, as the systems addressed become more extended the technical expertise required to understand them also rises, and communicating the results becomes increasingly difficult. Progress is highly dependent on the understanding developed using the reductionist approach, without which we could not hope to build models or comprehend large omic datasets, but it is also true that we cannot hope to understand the significance of reductionist approaches to complex systems without the approaches and techniques described in this chapter.

Biological systems do not always behave in expected ways, reflecting feedback loops, tipping points, levers, hubs, stable states, self-organisation, distributed control, emergence and non-linearity among other phenomena that are not easily understood without big picture, unbiased datasets and computational models. Successful interventions to slow or prevent the ageing process will necessitate in large part an understanding of the underlying complexities developed through these methods.

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