Nozomu Mori *Editor*

Aging Mechanisms II

Longevity, Metabolism, and Brain Aging

Springer

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ISBN 978-981-16-7976-6 ISBN 978-981-16-7977-3 (eBook)
https://doi.org/10.1007/978-981-16-7977-3

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Preface

Japan is the world's longest-living country today. The average life expectancy is 81.64 years for men and 87.74 years for women, and the number of people over 100 years old (centenarians) is over 86,000 (as of 2021). However, of course, this was not the case in Japan 100 years ago. Health and long age the country enjoys today is based on the high economic growth after the Showa-era post-war reconstruction, improved dietary habits, and the expansion of the universal health care system. Although the country experienced the collapse of the bubble economy in the early Heisei period, the Japanese society has matured in a seemingly peaceful and prosperous manner up to the present day. However, the shadow of aging lurks over the whole society.

As per the famous Noh play "Atsumori," based on the Tale of Heike and sung and played by the renowned Sengoku Period Daimyo Oda Nobunaga, a man's life was said to be 50 years in the sixteenth century. Even in the late nineteenth century Meiji-era Japan, the average life expectancy was in the 40s. However, after World War II, Japan achieved the world's fastest increase in average life expectancy during the Period of Rapid Growth in the 1960s. This owes primarily to the establishment of the National Health Insurance, so-called *kaihoken*. In this relatively short period of time, Japan has quickly become a world leader in various health metrics, including longevity. Ikeda et al. analyze the key factors behind Japan's impressive historic achievements over the past half-century in *The Lancet* special issue (*The Lancet*, 378(9796), 1094–1105, Fig. 1).

On the flip side, Japan's entire society is now facing aging at the world's fastest rate. The Period of Rapid Growth has long passed, and the whole country is now "aging." On March 6, 2007, 14 years ago, something no one could have predicted happened; the collapse of a local city government. It was an unprecedented event. A town that once prospered from coal mining, Yubari city, Hokkaido, has gone bankrupt with a deficit of 350 billion yen. A coal-mining town once bustling with 120,000 people had been reduced to 10,000 people in 40 years. There used to be six elementary schools, but now there is barely a single one. There is one small clinic, but no general hospital. The number of city employees was reduced by two-thirds,

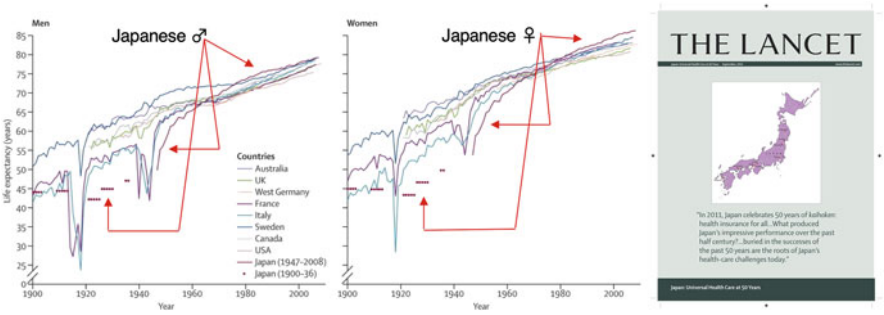

Fig. 1 Japan as No. 1 in the health-span growth and in longevity. (Adapted from *The Lancet*, 2011)

and the city was forced to pay off its debts. Exodos of residents started and became unstoppable, especially in the young people, and the situation of the town is getting worse. Over 50% of the population is above the age of 65. Yubari is the epitome of the negative aspects of Japan's aging society.

On November 18, 2010, The British business magazine *The Economist* introduced the situation in Yubari to the world, and sounded a warning about the future of Japan's super-aging population. The alarm was summed up in one word: "Japan's burden." Will Japan be able to cope with the various challenges of an aging society? The illustration of a child trying to support himself against the weight of the Japanese flag is painful to our eyes (Fig. 2).

This is not just a problem for Yubari, but will become a major issue soon in many parts of Japan. The baby boomer generation, born after World War II, has now become a large dark cloud of the elderly population of 75 years and older, shifting a massive burden to the future of a much smaller percentage of the young population. The origin of the problem lies in the imbalance between the extremely low birth rate and the growing aging population, with the birth rate of 1.4, lower than any other country. If this trend continues for the next 40 years, Japan's overall population will decrease by 40 million, and Japan's aging rate will reach 40% by the middle of this century (2050). That is to say, 4 out of 10 people will be 65 years or older. This is an average rate, however, and even if it can be lower in urban areas, the aging rate in the surrounding rural areas will easily exceed 50%. The situation Yubari is facing is expected to spread throughout the country soon.

While "aging" has become a major social problem, it is fundamentally a medical and biological issue. Every living thing ages and dies without exception. Aging is not an illness, but a natural process of life. Consequently, research should focus not only on age-related diseases or pathological aging but also on physiological processes of aging. We would like to understand the fundamental biological mechanisms of aging; how do we all grow old? Over the past several decades, our knowledge of the research findings on the biological mechanisms of aging has accelerated. However, we are still a long way from fully understanding all the mechanisms of aging; how we, as animals, age and how our lifespan is determined.

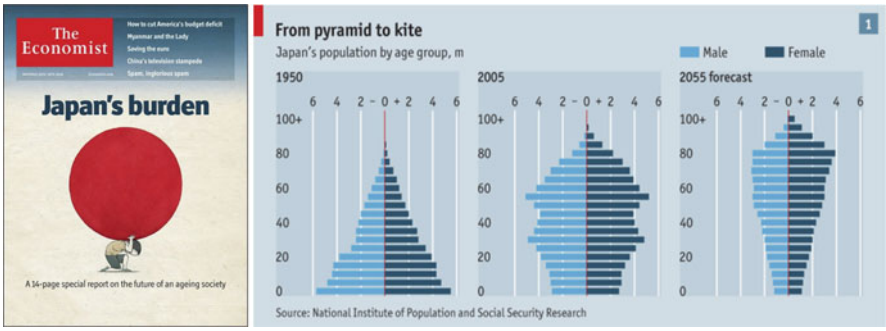

Fig. 2 Japan's burden and the population shift in the past and the future. (Source: *The Economist*, 2010)

Previously, we have released a book entitled *Aging Mechanisms: Longevity, Metabolism, and Brain Aging* (Springer, 2015), summarizing aging researches pursued in the leading laboratories in the two neighboring countries in East Asia, i.e., Japan and South Korea. It was compiled as a memorial of collaborative efforts of basic biomedical researchers on aging in the two countries, mainly through the binational discussion forum of AACL (Asian Aging Core for Longevity) initiated by the editor in 2006. Now, it is almost 15 years since the editor took a first step towards the discussion forum on aging research, and it is my sincere pleasure to find the discussion forum of AACL evolve into the Asian Society for Aging Research to promote the scientific discussion on aging in East Asia including Japan, Korea, and China. Herein, I deeply thank our former core members, Drs. Eun Seong Hwang (University of Seoul), Isao Shimokawa (Nagasaki University School of Medicine), Zhongjun Zhou (University of Hong Kong), Sang Chul Park (Seoul National University), Inhee Mook-Jung (Seoul National University), and Yong-Sun Kim (Hallym University).

I would note that this second volume *Aging Mechanisms II* (2021) is not a simple revision of the former Aging Mechanisms (2015), but it is intended to incorporate novel topics under the rapid progress of aging research in the leading laboratories in Japan. The only exception is Chap. 1, which is a revised version of the previous 2015 book chapter with a few modifications by Dr. Sataro Goto. The editor would like to express sincere thanks to everyone involved in the chapter contributions for their cooperation and enthusiasm, and hope that the book will be useful for many researchers and graduate students in biomedical aging research.

Fukuoka, Japan Nozomu Mori

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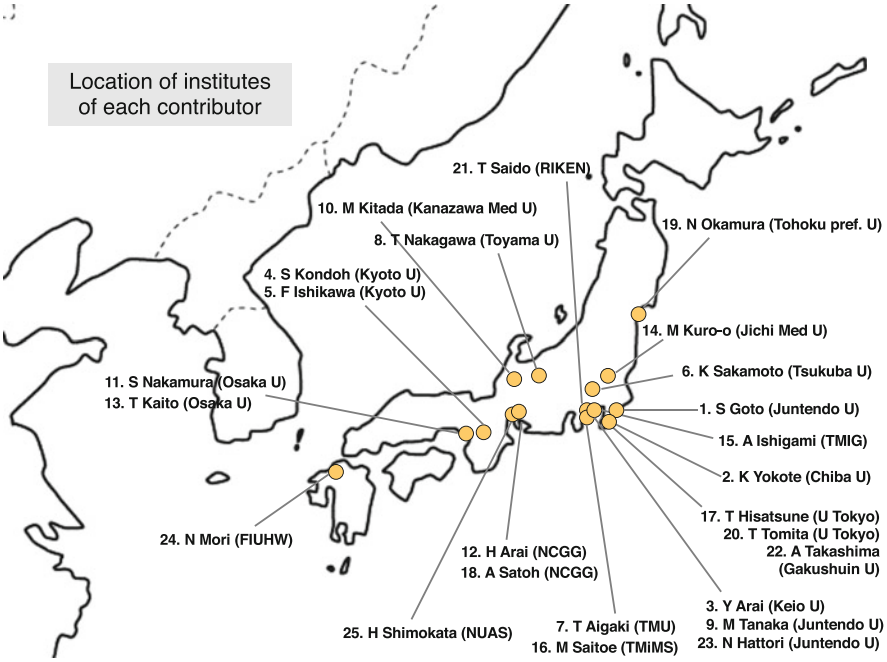
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Part I
From Hypothesis to Mechanisms

Chapter 1
An Unsolved Problem in Gerontology Yet: Molecular Mechanisms of Biological Aging—A Historical and Critical Overview

Sataro Goto

Abstract I discuss the historical background of the original proposals and modern versions of the selected theories of the molecular mechanisms of biological aging, i.e., the mutation or genome instability theory, the free radical or oxidative stress theory, the mitochondrial theory, the error catastrophe theory, the altered protein or protein homeostasis or proteostasis theory, the dysdifferentiation or epigenetic theory, and the hyperfunction theory, adding a brief comment on a recent popular theory of "epigenetic clock" in this revised version of my previous overview (Goto, Aging mechanisms. Longevity, metabolism and brain aging, Springer, Berlin, 2015). I have involved the development of some of the theories, which are therefore described in more detail than others. A discussion on the definition of aging and general comments on the aging theory are described. A most popular theory of aging, the free radical or oxidative theory, was proposed more than half a century ago but has recently faced severe criticisms to which I shall refer. So far, no single theory has been able to successfully explain the mechanism of biological aging. We are thus awaiting emergence of a new paradigm or an integration of the existing theories for better understanding of the mechanism.

Keywords Molecular mechanisms of aging · Mutation theory of aging/genome instability theory of aging · Free radical theory of aging/oxidative stress theory of aging · Mitochondria theory of aging · Error catastrophe theory of aging · Altered protein theory of aging/protein homeostasis or proteostasis theory of aging · Dysdifferentiation theory of aging/epigenetic theory of aging · Hyperfunction theory of aging

This article is a revised version of my previous contribution to the Springer book (Goto 2015).

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_1

1.1 Introduction

The average human life span in developed countries has increased by more than 20 years in the past several decades. Our major concern has shifted from an increase in the life span to an extension of the health span by retarding the progress of frailty due to lowered physical activities and inadequate nutrition in elderly people, thus reducing risks of potentially fatal diseases such as cancer, cardiovascular disease, stroke, kidney disorder, type 2 diabetes mellitus, etc. Currently, elderly people are more concerned of maintaining high quality of life by delaying frailty that results from the decline of physiological functions such as sarcopenia and osteoporosis, even when such conditions are not directly fatal by themselves. However, it is often stated that the major risk factor for developing the geriatric diseases mentioned above is old age, or rather, biological aging itself. This means that the biological mechanisms of aging are likely to underlie the etiologies and progress of age-related diseases, although aging itself is not technically a disease.

Since Peter Medawar stated in 1952 that aging is *an unsolved problem of biology* (Medawar 1952), the mechanisms of aging have been the subject of intensive research interest, and a large number of papers have been published on the mechanisms of aging. Half a century after Medawar's statement, leading scientists of biogerontology claimed that aging i*s* no longer an unsolved problem in biology (Holliday 2006; Hayflick 2007). Robin Holliday wrote that recently published major books on aging agree that the biological reasons for aging in mammals are now well understood and that the mechanism of biological aging is therefore no longer an unsolved problem. It is true that there appears to be similar, apparently common or conserved, senescent phenotypes in different species of animals in which longevity differs by several 100-fold (see Fig. 1.1 and Table 1.1); however, the very basic problems of the mechanism behind such species differences in longevity are not clear nor have been studied deeply enough.

In this chapter, I provide an overview of selected theories of the mechanisms of biological aging. The overview includes theories of historical interest that are not necessarily widely accepted currently and/or theories that have since been transformed into modern versions. The latter group is presented under the same sections as the original theories from which they are derived.

1.2 The Definition of Aging

There are two words with somewhat similar meanings that are commonly used in gerontology but are often confused, i.e., *aging* and *senescence*. Caeb Finch writes in his influential book that the term *aging* is mainly used to describe any changes that occur during the passage of physical time, during which there need be not common mechanisms, such as the aging of collagen, the aging of diploid cells in culture or of erythrocytes in circulation, the aging of populations or societies, or the aging of

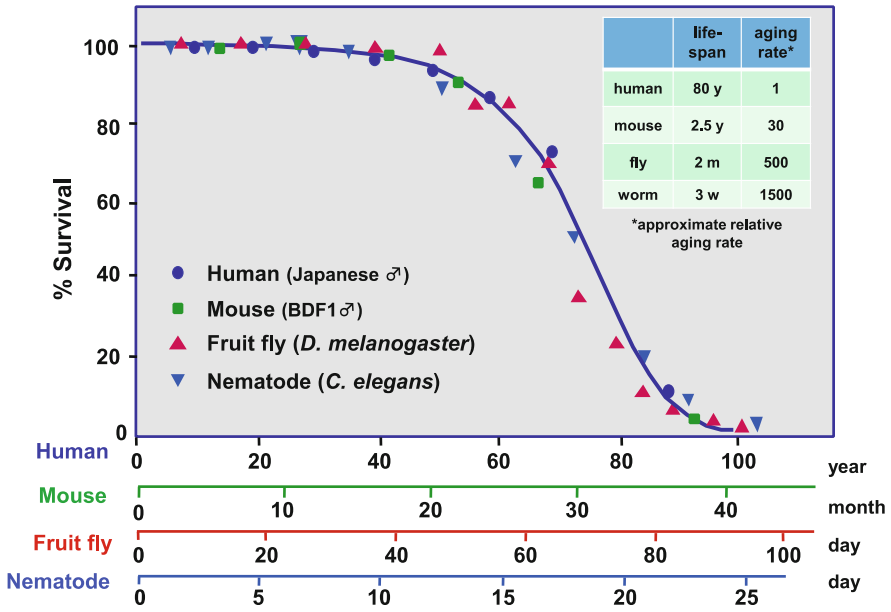

Fig. 1.1 Survival curves of human, mouse, fruit fly, and nematode. (Adapted and modified from Goto S (2002) Saibo kogaku 21: 704–708 (in Japanese))

genes and species during evolution. In contrast, the term *senescence* is used to describe age-related changes in an organism that adversely affect its vitality and functions and, most importantly, increase its mortality rate as a function of time (Finch 1990). Robert Arking states that "the terms *aging* and *senescence* seem to overlap considerably, and the difference between them may be one of emphasis rather than fundamentals" (Arking 1998). Because the term *aging* is often used to convey what he describes as *senescence* in most current gerontology writing, I use the term *aging* to discuss the mechanisms of aging (senescence) in this chapter.

To cite a few examples of the definition of *aging* (*senescence*) by leading scientists in biomedical gerontology books, Medawar wrote, as cited by Bernard Strehler in his book (Strehler 1977), "*Senescence* may be defined as that change of the bodily faculties and sensibilities and energies which accompanies aging, and which renders the individual progressively more likely to die from accidental causes of random incidence." Strehler himself defines it as "the changes which occur (1) generally in the postreproductive period and (2) which result in a decreased survival capacity of the part of the individual organism." He further notes that "different evolutionary lines might very well decline in their survival capacities for entirely different immediate reasons. It may also be, however, that there are one or more dominant mechanisms of aging, common to all higher forms of life." Alex Comfort defines *senescence* (*aging*) as a decrease in viability (leading to an increasing probability of death) with increasing chronological age and an increase in

Table 1.1 Common and uncommon aging phenotypes in human and model animals (Adapted from Vijg and Campisi (2008) and modified by Goto (2015))

Phenotype	Human	Mouse	Fly	Nematode
Decreased cardiac function	Yes	Yes	Yes	NA
Apoptosis, cellular senescence	Yes	Yes	Yes	?
Cancer, hyperplasia	Yes	Yes	No	No
Genome instability	Yes	Yes	Yes	Yes
Macromolecular aggregates	Yes	Yes	Yes	Yes
Reduced memory & learning	Yes	Yes	Yes	NA
Decline in GH, DHEA, testosterone, IGF	Yes	Yes	?	?
Increase in gonadotropins, insulin	Yes	Yes	?	?
Decreased thyroid function	Yes	Yes	NA	NA
Decrease in innate immunity	Yes	Yes	Yes	Yes
Increase in inflammation	Yes	Yes	No	No
Skin morphology changes	Yes	Yes	?	Yes
Decreased mitochondrial function	Yes	Yes	Yes	Yes
Sarcopenia	Yes	Yes	Yes	Yes
Osteoporosis	Yes	Yes	NA	NA
Abnormal sleep	Yes	Yes	Yes	?
Decrease in vision	Yes	Yes	?	NA
Demyelination	Yes	Yes	?	No
Decreased fitness	Yes	Yes	Yes	Yes
Arteriosclerosis	Yes	No	NA	NA
Changes in fat	Yes	Yes	?	?

Note: Highlights by yellow are common aging phenotypes in listed animals. *NA* not applicable

vulnerability (Comfort 1964). The term vulnerability may be rephrased as frailty, a term more commonly used in geriatric medicine in recent years.

Surveying the definition of aging in gerontology literatures, I note that aging can be defined as a progressive functional decline with advancing age that occurs in every individual, sooner or later, within a population of a species, beginning around the time of reproductive maturity and leading to an increased probability of death over time. Theories of the mechanisms of aging that can fit with this definition will be examined in this chapter.

1.3 Aging Theories

In 1990, Zhores Medvedev wrote that more than 300 theories about the biological mechanisms of aging could be found in the literature (Medvedev 1990). Among the theories cited in his review, some are still popular, and some have disappeared or have been transformed, while other new theories have emerged and are currently

being tested for validity. Theories of aging are mixed in that there are different levels of aging phenomena at the molecular, cellular, tissue, organ, or systemic levels.

George Martin has proposed a classification of the mechanisms of aging into two categories: public and private mechanisms (Martin et al. 1996a). The public mechanisms of aging are those that could potentially be applied to the aging of different animals and tissues or cells, while the private mechanisms of aging are those that appear to be only true in specific species, cells, tissues, or organs. For example, the immunological theory can only be true in animals such as mammals with appropriate immune system but may not be true in nematodes or insect models which lack in acquired immunity seen in mammals. When thinking about the aging that occurs in any somatic cells of different species of animals, it is more appropriate to focus on "public" mechanisms rather than "private" mechanisms for the purposes of our discussion. See discussion on "public" and "private" mechanisms of aging in a literature (Partridge and Gems 2002).

In this chapter, I therefore discuss the mechanisms of aging that can mainly, although not exclusively, be viewed as public. The private mechanisms of aging, however, are by no means unimportant. Indeed, they are useful by themselves to explain particular etiologies or the progress of individual age-related diseases. It should be noted that private mechanisms often involve public mechanisms. For instance, endocrinological decline with age, a private mechanism of aging, can be caused by public mechanisms, such as oxidative stress or protein alteration. It should be noted that each theory is naturally not mutually exclusive or incompatible each other, but may instead be regarded as a part of other theories.

Figure 1.1 illustrates age-related changes in the mortality rate of different animal species, with life span difference of more than 1000-fold (e.g., between human and nematode). The apparent similarity of the survival curves may suggest that the underlying mechanisms of aging are common among the shown animal species. In fact, many aging phenotypes are conserved in model animals and human, as shown in Table 1.1 (Vijg and Campisi 2008). It should be noted, however, that no overall correlation of age regulation was found in the gene expression database, at least between mice and humans, for example, and therefore, aging processes in mice and humans may be fundamentally different, despite certain commonalities in the observed transcriptional profiles in the genes, for example, of electron transport chain for aging mouse, human, fly, and nematode (Zahn et al. 2007). In the following sections, I examine selected public mechanisms of aging.

1.4 Mutation Theory of Aging/Genome Instability Theory of Aging

This theory predicts that mutations accumulating in the genome are responsible for aging, i.e., physiological decline with advancing age. One of the early proponents of the theory was Leo Szilard. As a nuclear physicist, he proposed that somatic cell

mutations induced by ionizing radiation generated in reactions such as the nuclear fission and fusion would accelerate aging (Szilard 1959). Ionizing radiation in fact shortened the life span of mice and rats, shifting the survival curves to the left, with similar shapes as unirradiated controls, apparently being reminiscent of an acceleration of normal aging (Lindop and Rotblat 1961). It was later shown, however, that the major cause of the observed life span shortening was an increased rate of carcinogenesis rather than an acceleration of physiological aging in general. Irradiated rodents have therefore not been used as models of accelerated aging. In the meantime, it has been reported that the DNA repair activity of skin fibroblasts in cultures irradiated with ultraviolet light depends on an animal's maximum life span (Hart and Setlow 1974). The activity of cells from long-lived animals, such as human, elephant, and cow, was nearly five times higher than that in short-lived animals such as rat and mouse. Although the repair capacity and life span were not proportional, it was thought that long-lived species may have a more active repair system that could therefore play a role in deceleration of aging rate. More recently, it was reported that base excision repair activity declines with age in mice in the brain, liver, spleen, and testes (Cabelof et al. 2002). To study the mutation frequency in vivo, selectable markers, such as hypoxanthine phosphoribosyltransferase (HPRT) of purine metabolism, have been used to detect 6-thioguanine-resistant cells that are defective in the HPRT gene. Using this method, it was reported that the mutation frequency increased with age (from 2 to 94 years of age) in cultured human kidney tubular epithelial cells (Martin et al. 1996b). To overcome the limitation that the cells to be assayed must proliferate in vitro in the assay, transgenic mice with reporter genes, such as the bacterial lacZ gene, have been developed. The DNA recovered from the transgenic mouse tissues, including the brain and heart, consisting of mainly postmitotic cells, was screened for mutations in the integrated shuttle vector in a bacterial host (Dollé et al. 2000). Significant age-related mutant frequency was found to increase from 10×10^{-5} (3 months old) to 25×10^{-5} (33 months old) in the small intestine and from 5 to 10×10^{-5} in the heart of mice. However, no change was observed in the brain (5×10^{-5}) between the young and old animals. It is noted that the increase was linear from young to old ages, with no larger changes at older ages.

Because functional decline with age is apparently more significant in the brain and heart than in the intestine and because the frequency of mutation is not high enough to account for the level of decline, it appears to be difficult to ascribe a cause of aging to the age-related accumulation of mutations. In fact, the serious proponents of this theory recognize one important question about this theory, stating that "it is not known whether the frequency of the random changes is sufficient to cause the phenotypic effects generally associated with aging" as cited from the abstract of a paper by Vijg and Suh (2013). The readers are advised to also refer to a recent general view on this theory (Moskalev et al. 2012).

1.5 Free Radical Theory of Aging/Oxidative Stress Theory of Aging

The free radical theory of aging is one of the most well-known and popular theories of aging proposed so far. The theory has currently been transformed into the "oxidative stress theory of aging" because oxidative stress most frequently involves reactive oxygen species (ROSs) and because the causative agents of the stress are not only free radicals but also include non-radical ROS such as hydrogen peroxide (Martin et al. 1996a). The principle of the theory was originally proposed by Denham Harman more than half a century ago (Harman 1956). The history of the theory and the inside story of how the idea came to him are found in an interview with him (Harman and Harman 2003). He was originally a chemist specializing in free radicals who later became interested in aging and established himself as a medical scientist. In the beginning, the theory apparently did not attract as much interest from scientists working on aging as other theories, such as the mutation theory and the protein cross-linking theory. This is likely because radicals were not familiar to biologically oriented scientists, and the theory appeared to be too simple and straight forward to explain the complex aging phenomena. However, after superoxide dismutase (SOD), which catalyzes dismutation of superoxide radical forming hydrogen peroxide, was reported to be widely distributed in mammalian tissues (McCord and Fridovich 1969), more researchers became interested in the capacity of free radicals to damage a variety of cellular constituents, potentially leading to aging. The major targets of free radical damage were believed to be membrane lipids, which contain many unsaturated fatty acids that are easily attacked by radicals to produce lipid peroxides. Lipid peroxides were thought to be components of the lipofuscin age pigment, a then well-known histological marker of aged cells that consume substantial amounts of oxygen, such as neurons and kidney cells. Lipid peroxidation has readily been measured as thiobarbituric acid reactive substances (TBARS), although the method to measure TBARS may be problematic in specificity and, recently, such substances as isoprostanes have been used to evaluate the oxidation. DNA was another molecule of interest for oxygen radical attack. It can form 8-hydroxy-2-deoxyguanosine (8-oxodG), which is relevant to cancers that increase with age (Fraga et al. 1990).

Oxidatively modified proteins have attracted the least interest mainly because of limitations in the methods to detect them despite the fact that the catalytic activities of enzymes have long been known to decrease with age (Stadtman 1988) and therefore can drive aging. Earl Stadtman and his collaborators established a convenient method to detect oxidatively modified proteins in which reactive carbonyl moieties are generated as oxidation products in amino acid residues such as lysine, arginine, and proline that can be measured by spectrophotometric or immunological methods after the reaction of proteins with 2,4-dinitrophenylhydrazine to derivatize the carbonyls to the hydrazones.

All cellular components (e.g., membrane phospholipids, nucleic acids, and proteins) have been reported to be oxidatively damaged with age, which could

potentially cause the physiological decline of the organisms (Cutler and Rodriguez 2003). The free radical theory of aging has prompted researchers to study radical scavengers and antioxidants to see if such chemicals can extend the life span of animals. Harman himself showed in his early studies that the synthetic antioxidants 2-mercaptoethylamine and butylated hydroxytoluene can extend the life span of mice (Harman 1968). Numerous studies have been conducted since then to try to extend the life span of experimental animals or to ameliorate age-related diseases in humans that are possibly caused by ROS, mostly using antioxidant vitamins, such as vitamins C and E, or natural products such as polyphenols and carotenes. The results, however, have been rather disappointing in human clinical trials attempting to reduce the risks of age-related diseases, although antioxidant supplements had been reported to be promising in experimental animals (Sadowska-Bartosz and Bartosz 2014). In human studies, it has been reported in a systematic review and meta-analysis of randomized trials with a total of 232,606 participants that antioxidant supplements (β-carotene, vitamins A and E) can even significantly increase all-cause mortality (Bjelakovic et al. 2007). In animal studies, for example, the popular "anti-aging" polyphenol resveratrol, which is not necessarily supposed to act as an antioxidant, has been shown to not extend the life span of genetically heterogeneous mouse strains that mimic human population in multiple laboratories (Strong et al. 2013).

The free radical theory of aging appeared to explain the rate of living theory of aging, which was first proposed many years ago (Pearl 1928), suggesting that there is an inverse relationship between the metabolic rate and longevity in different animal species. However, it turned out that this does not apply to mammals. The opposite was even true intraspecifically when energy expenditure and the life span of individual mice were studied, in that the higher the energy expenditure (indicating a larger consumption of oxygen), the longer the life span, contrary to what is expected from the free radical theory of aging (Speakman et al. 2004). Based on studies of genetically modified mice showing under- or overexpression of genes of antioxidant enzymes (e.g., cytoplasmic and mitochondrial superoxide dismutases, catalase, glutathione peroxidase), it was concluded that all of the antioxidant enzymes studied separately or in combination do not significantly influence the life span in mice (Pérez et al. 2009). On the other hand, it is true that oxidative damage in lipids, DNA, and proteins increases with age, as described above, suggesting an involvement of free radicals in aging. Additionally, a variety of mutant animals with longer life spans show increased resistance to oxidative damage (Brown-Borg 2006; Pickering et al. 2017). Thus, potential roles of ROS in driving aging should not be underestimated, although they may not play a crucial role in life span determination.

It has often been stated that the major source of ROS generation is mitochondria, as discussed later in the mitochondrial theory of aging. However, apart from ROS generated in the mitochondria as byproducts, oxidants can be generated as normal products in multiple enzyme reactions catalyzed by oxidases, such as NADPH oxidase, xanthine oxidase, and monoamine oxidase, contributing to overall cellular oxidative stress. Such oxidants can damage cellular molecules and also play important roles as signaling factor (Finkel 2011). Although the involvement of ROSs in

signal transduction have attracted more interest in recent years than their potential detrimental role in aging, I do not discuss details of this topic as it is beyond the scope of this overview.

I instead discuss the hormetic roles of ROSs that are relevant to aging. Hormesis is a dose-response relationship that exhibits stimulation at low doses and inhibition at higher doses, although whether a response is beneficial or harmful can be complex and is often not immediately obvious (Calabrese and Mattson 2011). Exposure to a variety of stressors, such as toxins, heat, ROS, and radiation, can induce an adaptive response if they are not too strong, making an organism more resistant to subsequent stronger challenges (Gems and Partridge 2008). Nematodes pretreated with hyperbaric oxygen became more resistant to semilethal oxygen exposure (Cypser and Johnson 2002). Interestingly, an oxidative stressor (juglone) could induce substantial resistance to a lethal challenge. The life span of the pretreated worms was increased compared to naive counterparts. We have shown that regular moderate exercise in old rats can reduce oxidative stress, as measured by protein and DNA oxidation, by upregulating anti-oxidation systems, including the glutathione, proteasome, and DNA repair enzymes (Goto and Radák 2009; Nakamoto et al. 2007; Radák et al. 2001). Other investigators have also demonstrated that exercise induces antioxidant enzymes (Gomez-Cabrera et al. 2008) and that antioxidant vitamins C and E ameliorate the beneficial effects of exercise (Ristow et al. 2009). Exercise hormesis is well recognized, as the ROS induced by moderate exercise constitutes a significant mechanism of beneficial effects of the regimen (Gomez-Cabrera et al. 2008; Radák et al. 2005). See also the discussion on mitohormesis in the mitochondrial theory of aging section.

Thus, ROSs have two sides, making this theory somewhat complex. On the one hand, ROSs are believed to have detrimental effects, as proposed in the original theory. On the other hand, they are also thought to have beneficial effects as signaling factors and factors that can protect an organism against stresses that they may encounter in life.

1.6 The Mitochondrial Theory of Aging

Mitochondria have long been known to be the power station of eukaryotic cells, generating the majority of ATP and therefore being vital to life. After the proposal of the free radical theory of aging, these organelles have attracted increased interest in the other side of life, as they use most of oxygen taken up by cells that could potentially be converted to damaging reactive oxygen species (ROSs) in the respiratory chain. Harman was the first to suggest that mitochondria can be a major source of free radicals and also a principal target of the damage that drives aging as an obvious extension of the free radical theory of aging (Harman 1972). In fact, mitochondrial DNA (mtDNA) and proteins are more vulnerable to oxidation than cytoplasmic or nuclear proteins and nucleic acids, likely due to their proximity to the electron transport chain, the lack of histones to protect the DNA, and their low repair

activities. Later, Jaime Miquel expanded the mitochondrial theory of aging (Miquel et al. 1980). A number of papers in support of the theory have been published. It has often been cited that ROSs (such as hydrogen peroxide) generated in the mitochondria account for 1–2% of the total oxygen uptake (Chance et al. 1979). Even higher values of 4–5% have been reported (Luft and Landau 1995). However, later studies have criticized these reports, and the current estimation for these values is as low as 0.15% (St-Pierre et al. 2002).

Point mutations that may occur due to oxygen radicals accumulate in mtDNA with aging, possibly also due to mtDNA polymerase errors, suggesting that this process may cause the age-related functional decline of cells and tissues (Michikawa et al. 1999). For this reason, mice with defective mtDNA polymerase have been constructed as a model of premature aging to prove or disprove this theory (Trifunovic et al. 2004). Studies of these mice demonstrated that the animals with a homozygous mutation (mtDNA mutator mouse) expressing proofreading-deficient mtDNA polymerase γ show reduced life span. They also show phenotypes of accelerated aging at 6–9 months of age, such as hair loss and graying, sarcopenia, osteoporosis, heart enlargement, and reduced subcutaneous fat, all of which are features that are typical of human aging (Trifunovic et al. 2004). Despite these premature aging phenotypes and the accumulation of mtDNA mutations, no increase in hydrogen peroxide production and oxidative stress markers (protein carbonyl, 8-OHdG, and F2-isoprostane) has been observed in isolated mitochondria and tissues of the mice. Thus, these findings did not support the idea that mtDNA mutations cause increased ROS production that might drive aging. One criticism of this research is that these mice may not represent natural human aging because the levels of mtDNA mutations in human tissues are an order of magnitude lower than in the mutator mice (Khrapko et al. 2006). It should, however, be noted that a recent report on the mtDNA mutator mice showed that the hydrogen peroxide levels in the aged animals were increased relative to the young mutator or wild type mice, suggesting that prolonged exposure to higher concentrations of ROSs could contribute to accelerated aging (Logan et al. 2014). Thus, the possible contribution of ROSs to aging in the mtDNA mutator mice remains controversial. Interestingly, however, 5 months of endurance exercise can rescue premature mortality in the mutator mice by inducing mitochondrial biogenesis, thereby mitigating the development of sarcopenia, brain atrophy, cardiac hypertrophy, and other age-related pathologies (Safdar et al. 2011). Endurance exercise rescued mtDNA depletion in multiple tissues and reduced the frequency of point mutations in the mutant mice. These data support the view that lifestyle can improve the systemic deterioration of mitochondrial function that could increase morbidity and mortality with aging.

Supporting evidence for the mitochondrial theory of aging has been obtained in transgenic mice overexpressing human catalase in the mitochondria, which exhibit increased life spans with reduced cardiac pathologies and cataract severity (Schriner et al. 2005). These mice exhibited higher aconitase activity, a marker of antioxidant capacity, in the heart and lower 8-OHdG in the DNA of the skeletal muscle, suggesting that oxidative stress can be ameliorated by the overexpression of catalase targeted to mitochondria.

In view of the controversy regarding the contribution of mitochondrial ROS in aging, it is worthy of referring to the concept of mitochondrial hormesis (or mitohormesis) (Schulz et al. 2007; Ristow 2014). It was found that nematodes treated with 2-deoxyglucose (2DG), an inhibitor of glycolysis, exhibited a prolongation of their life span with a compensatory increase in mitochondrial respiration, which is associated with increases in the level of ROS, followed by increased expression of catalase, which scavenges hydrogen peroxide (Schulz et al. 2007). When the worms were pretreated with VC, VE, or other antioxidants, the elevation of catalase was abolished, and the extension of life span of the worms treated with 2DG was blocked. It thus appears that mitochondrial oxidants induced an increased defense against oxidative stress as a hormetic response because excess oxidants are obviously detrimental.

The mitochondrial theory of aging has thus developed into a theory evaluating the roles of ROS generated from the organelle as signals for cellular homeostasis rather than simply as damaging chemicals, as originally suggested. Also, I should add that results incompatible with this theory are reported (Lapointe and Hekimi 2010).

1.7 The Error Catastrophe Theory of Aging

This theory was most prominently advanced by Leslie Orgel (1963) in accordance with the development of molecular biology of the gene expression in the 1960s, such as the research on the mechanisms of replication, transcription, and translation. This theory predicted that nucleic acids and proteins inevitably contain errors when they are synthesized because the information transfer in each step of gene expression and maintenance is not perfectly accurate and the synthesizing machineries consisting of error-containing molecules would make further errors, thus forming a vicious cycle of error propagation that could result in the gradual loss of cellular function, i.e., catastrophe, with age. Although this theory is usually regarded as being advocated by Orgel, it should be noted that Zhores Medvediev presented a similar idea independently (Medvediev 1962). This theory has attracted particular attention from scientists interested in the molecular mechanisms of aging because it suggests a hypothesis that is experimentally testable by means of emerging theoretical and technological developments of research in gene expression.

Possible detrimental consequences of the propagation of errors are likely more serious in nondividing cells than in dividing cells because error-containing dividing cells can be eliminated and replaced by new cells or can be diluted by cell division, while error-containing molecules may be repaired or replaced by metabolic turnover in nondividing and/or slowly dividing cells.

Of the types of errors in information transfer, translational errors had been most extensively studied. These errors can occur in two independent steps of translation:

(1) The charging of individual tRNAs by cognate amino acids and (2) the decoding of codon of mRNA. The former step is catalyzed by aminoacyl tRNA

synthetases that may mischarge amino acids to tRNAs by imperfect enzymes. The latter step occurs on ribosomes by matching codons with anticodons of charged tRNA. A number of studies on the rate of mistranslation (error frequency) in aging had been conducted mainly using young and senescent cells in culture. For example, the error frequency of actin synthesis was studied in human fibroblasts at different replicative ages (Harley et al. 1980): Histidinol, an analogue of histidine, was added to the culture medium and thereby blocked the charging of tRNA for histidine. The decrease in the histidine-charged tRNA concentration induces an incorporation of glutamine into actin in the place of histidine because the codons for glutamine (CAA or CAG) are similar to those for histidine (CAU or CAC) so that errors of translation can occur due to codon-anticodon mispairing at the third position. Late-passage cells from fetal, young, and old donors cultured in vitro showed similar or lower error frequencies than the corresponding early-passage cells, suggesting that error propagation does not occur and thus fails to support the error catastrophe theory of aging. In another study, age-related changes in the charging error were examined in vivo by the incorporation of ^{14}C-methionine and ^{3}H-ethionine, an analogue of methionine, into proteins of young and old mouse livers (Ogrodnik et al. 1975). It was expected that ethionine could be mischarged to tRNA in place of methionine by methionyl tRNA synthase if the fidelity of the enzyme would be decreased with age. The misincorporation of ethionine in the place of methionine was 10–50% higher in ribosomal proteins of old animals, indicating that the charging fidelity indeed declines in older animals, although it was not clear if the error rate propagates with age.

As for the recognition of natural amino acids in young and old animals, we have studied the age-related changes in the fidelity of aminoacylation by tyrosyl-tRNA synthetase isolated from the liver of rats (Takahashi and Goto 1988). The enzymes were purified from the livers of young (4–7-month-old) and old (27–29-month-old) rats, such that no detectable phenylalanyl-tRNA synthetase was contaminated to study the misrecognition of phenylalanine as tyrosine by the enzyme. The error frequency of the tyrosyl-tRNA synthetase (on the order of 10^{-8}) from the older animals was slightly lower than that from the younger animals, but this difference was not statistically significant. Thus, the fidelity of aminoacyl tRNA synthetase did not appear to decline significantly in old age, again suggesting that errors in translation would not increase with aging at the stage of tRNA charging with amino acid in translation.

The fidelity of decoding on ribosomes from young and old animals had been mostly studied by assessing the misincorporation of non-cognate amino acids using synthetic mRNA of homopolymers, such as poly(U) which codes for phenylalanine polymers. The misincorporation of leucine into the poly(U)-dependent synthesis of polyphenylalanine using ribosomes of tissues did not differ significantly between young and old mice (Mori et al. 1979). We have, instead, studied codon recognition fidelity using a unique group of natural mRNAs that code for limited species of amino acids. Protamines are highly basic nuclear proteins from fish sperm consisting of 33 amino acid residues.

They contain only seven different amino acid species, of which approximately two-thirds are arginine. It was therefore possible to study the incorporation of radioactive amino acids in vitro that are not coded in the mRNAs. The fidelity of the decoding of the mRNAs on ribosomes from the livers of mice between 2 and 29 months of age was found to not change significantly (Mori et al. 1983). Thus, these findings are not consistent with the error catastrophe theory of aging in terms of the predicted age-related changes in translational fidelity. This is probably because the proofreading mechanisms (Hopfield 1974; Fersht 1980) of translation are maintained throughout life, keeping the fidelity high enough, such that propagation of error would not occur.

More recently, the high fidelity of translation has been discussed from evolutionary perspectives as it can be important for survival by avoiding protein misfolding (Drummond and Wilke 2009) (see also Sect. 1.8). Another possibility that error-containing proteins do not increase with age is that such proteins may be preferentially degraded and replaced by intact molecules by metabolic turnover as discussed in the next session (Sect. 1.8).

Other steps of information transfer in which error catastrophe could occur are DNA replication and transcription. No age-dependent differences have been found between the fidelity of nuclear DNA polymerase-α and nuclear DNA polymerase-β that were partially purified from the regenerating livers of young (6-month-old) and old (28-month-old) mice when the enzymes were tested for copying bacteriophage φX174 DNA (Silber et al. 1985). The same group of investigators showed that the fidelity of highly error-prone DNA polymerase-β in the brain of young and old mice was not significantly different when copying the same bacteriophage DNA (Subba Rao et al. 1985). Thus, although available reports on the possibility of age-related changes in the fidelity of DNA polymerases are limited, it appears that the error catastrophe theory of aging is not supported by the information transfer in nuclear DNA replication. Although Orgel implied that transcription errors can lead to the catastrophe (Orgel 1963), I am not aware of a published paper on age-related changes in the fidelity of nuclear gene expression or of RNA polymerases in the nucleus (Imashimizu et al. 2013). The integrity of RNA coded in mitochondrial DNA has been studied in the brain of young (1-month-old) and older (18-month-old) mice (Wang et al. 2014). The transcriptional error of the mitochondrial RNA polymerase is site-specific and varied greatly among different genes. The error levels in two age groups, however, were not significantly different, suggesting that error propagation does not occur during aging. It is noted that transcriptional errors were independent of the DNA mutation frequency and were up to 200-fold more frequent than replication errors. The authors therefore conclude that the mitochondrial transcription fidelity limits the impact of mitochondrial DNA mutation.

Thus, the error catastrophe theory of aging, which was once a popular hypothesis, is not supported by the current experimental evidence. This theory thus seems to have been largely forgotten, but it should be noted that pathologist George Martin has argued that "it may have been given a premature death certificate" because drifts in gene expression may be responsible for the "quasi-stochastic" distribution of

lesions in geriatric pathologies, such as Alzheimer's disease and atherosclerosis and that errors in information transfer could feasibly contribute to this process (Martin 2012).

Although it is unlikely that error catastrophe occurs in genetic information transfer, it should be noted that errors in protein synthesis can occur as the misfolding of higher structures during translation. In fact, the rate of folding errors can be as high as 30% of newly synthesized proteins, even though misfolding may be mostly prevented by chaperons (Schubert et al. 2000) (see also: Sect. 1.8).

1.8 The Altered Protein Theory of Aging/Protein Homeostasis or Proteostasis Theory of Aging

The origin of this theory may be traced back to Friz Verzár, who reported an age-related increase in collagen cross-linking in rat tail tendons (see Nagy 1986). A large number of studies have confirmed that changes in collagen occur with age in various tissues and animals (Robert 2006). However, because collagen is an extracellular protein and its relevance to cellular metabolisms is limited, researchers interested in aging and inspired by the findings became more concerned about the age-related changes of enzymes and other proteins involved more directly in intracellular functions. In the meantime, studies on the error catastrophe theory of aging have failed to support the predicted propagation of errors in translation as described above and instead suggested the presence of altered forms of enzymes in aged cells and tissues. Thus, altered enzymes were interpreted to be formed not by translational errors but by posttranslational modifications.

Altered forms of enzymes in old cells and animal tissues have been detected by various means. They have been shown to have low specific activity (by between 30 and 70%) per unit weight of purified enzyme (Rothstein 1981). One problem with finding altered forms of an enzyme through purification is that altered enzymes with reduced activity are often lost during the purification process, as purification protocol usually depends on enzymatic activity. Altered enzymes have been detected in crude extracts without purification that depends on enzyme activity, since antibodies against an enzyme molecule can react with enzymes with no or reduced activity that remain immunologically cross-reactive as the native enzyme (Gershon and Gershon 1970). Another frequently used method was to examine the heat-stability of an enzyme in cell or tissue extracts. An enzyme likely becomes heat-labile if it is altered such that the mixture of native and altered enzymes has a biphasic or quasi-biphasic heat-inactivation kinetic curves for the activity so that the percentage of the altered form of an enzyme could be evaluated for the extent of alteration (Houben et al. 1984). Thus, many altered proteins, mainly enzymes, have been reported to increase in cells and tissues with aging, suggesting that they may be responsible for the age-related decline of physiological functions.

The causes of these alterations have been suggested to be posttranslational modifications, such as oxidation or nitrosylation by ROSs or RNSs (reactive nitrogen species) and glycation by glucose. In some cases, reactive aldehydes derived from lipid peroxides are responsible for the modifications. We and other investigators have shown that the heat-labile enzymes described above are generated by a reaction with ROSs in vitro (Takahashi and Goto 1990). The chemistry of modifications has been studied extensively, proving that the side chains of specific amino acid residues, such as lysine, arginine, and proline, are modified (Stadtman 1993). Notably, carbonyl moieties generated by oxidation have most frequently been used to evaluate oxidative stress on proteins by biochemical or immunochemical methods (Levine et al. 1990; Nakamura and Goto 1996), although this method is not without problems (Fedorova et al. 2014; Goto and Nakamura 1997). In addition to a correlative relationship between the oxidative modification of proteins and aging, a causal relationship between age-related increases in oxidative stress and functional decline has been suggested (Martin et al. 1996a; Martin and Grotewiel 2006). However, despite numerous reports on the possible involvement of protein oxidation in aging, it is hard to decide its major contribution, as multiple effects of oxidative stress on other molecules, such as DNA and membrane phospholipids, do occur in parallel.

The glycation caused by nonenzymatic chemical reactions of proteins with glucose is another well-recognized posttranslational modification that increases with age in long-lived proteins, such as collagens and elastin, as well as lens crystallins. The glycation of proteins ends up in generating a variety of products collectively called AGEs (advanced glycation end products). Because proteins exposed to a high concentration of glucose in the blood for a long period of time are susceptible to this modification, it accumulates frequently in extracellular matrix proteins and proteins with very low turnover rates. Glycation appears to be less involved in the age-related functional decline of cells as a general cause than other posttranslational modifications that occur more frequently inside cells. Nevertheless, there is no question that glycation is involved in age-related diseases of endothelial cells, such as in atherosclerosis, cardiovascular pathologies, and renal disorders, in which tissue microvessel dysfunction is involved.

More recently, apart from the posttranslational modifications described above, specific altered proteins with abnormal conformational structures in age-related neurodegenerative diseases, such as Alzheimer's disease (amyloidβ and tau tangles), Parkinson's disease (mutant α-synuclein), Huntington's disease (mutant huntingtin), and amyotrophic lateral sclerosis (misfolded SOD1), have been studied extensively (Stefani 2004; Labbadia and Morimoto 2015). More generally, amyloid diseases that impair the functions of different organs are also protein conformation diseases that increase with age. There are many other examples of protein misfolding and aggregation causing age-related diseases (Chiti and Dobson, 2017; Klaips et al. 2018). While numerous cases, especially in neurodegenerative diseases, have been reported in which protein alterations produce age-related pathologies, it is not clear whether such changes also contribute to the functional decline of cells and tissues in physiological aging. It is possible that minor alterations of individual proteins cause

undetected changes, yet result in significant physiological deterioration in a long period of aging.

The accumulation of altered proteins with age can be driven by either increases in the formation or the decline of degradation, or both processes. While the mechanisms involved in the formation of such proteins have been extensively studied, the decrease in degradation or elimination has attracted less interest. Rudolf Schoenheimer described for the first time the dynamic state of body constituents, such as lipids and proteins, as early as the late 1930s, when the stable isotope technique became available to label cellular and extracellular components for chasing the fate of the labeled materials, thereby highlighting the importance of metabolic turnover as a homeostatic life maintenance mechanism. Due to the difficulty of the access to the historical book *The Dynamic State of Body Constituents* (Harvard University Press, Cambridge, MA, 1949) written by him, I cite instead an excellent overview on this topic (Kennedy 2001). Schoenheimer's view, however, was challenged by Jacques Monod (*Nobel Prize* laureate for the operon theory) and collaborators, who studied the turnover of β-galactosidase in growing *E. coli* and concluded that most proteins in the cells are static rather than in a dynamic state (Hogness et al. 1955). They further suggested that the proteins in mammalian tissues would also be stable because the apparent dynamic state in these cells may be interpreted as some proteins being secreted or lost by cell death. However, it was shown that proteins in rabbit macrophages, nondividing cells, actually turnover, thus not supporting Monod's hypothesis (Harris and Watts 1958). Even so, protein degradation has not attracted the same intense research interest as other more positive biological processes such as protein and nucleic acid synthesis.

The degradation of intracellular proteins was originally thought to be mainly dependent on lysosomes, which were found to contain multiple proteolytic enzymes (cathepsins) with different specificities at acidic pH values (de Duve 1983). While lysosomal proteolysis is thought to be nonspecific with regard to the protein substrates degraded, the half-life of different proteins was reported to vary considerably. This fact facilitated studies on non-lysosomal protein degradation that were first performed in rabbit reticulocytes that do not have lysosomes. The extensive research on non-lysosomal protein degradation has established the mechanisms of the ubiquitin-proteasome system of proteolysis, showing that substrate proteins are marked with ubiquitin for degradation and digested by proteasomes (in the case of 26S proteasome, see below) (Ciechanover 2005). The proteasome is a multi-catalytic protease complex that exists in two forms, 26S and 20S, that differ in subunit composition but share a common catalytic specificity. The 26S proteasome degrades proteins tagged with ubiquitin chains and ATP dependently, while the 20S proteasome degrades non-ubiquitinated proteins without using ATP.

On the other hand, the lysosomal pathway of proteolysis has developed into the elucidation of autophagy-lysosome systems, in which protein aggregates and damaged organelles are specifically recognized and destroyed, contrary to what was originally believed to be nonspecific (Koga et al. 2011). Both systems of protein degradation have profound impacts on aging and age-related diseases, particularly in

neurodegenerative diseases (Rubinsztein et al. 2011; Saez and Vilchez 2014; Klaips et al. 2018).

The altered protein theory of aging prompted studies on protein turnover in aging (Van Remmen et al. 1995; Goto et al. 2001). For example, it was demonstrated that the half-lives of enolase in nematodes and aldolase in mice are extended in old animals compared with their younger counterparts, as determined by pulse-chase experiments. We found that the half-life of the various proteins introduced into mouse hepatocytes in primary culture were extended by 40–60% in the cells from old animals (Ishigami and Goto 1990; Goto et al. 2001). It was also shown in vivo that prematurely terminated puromycinyl peptides, as a model of altered proteins, are much more slowly degraded in the livers of old mice than in those of younger animals (Lavie et al. 1982). Thus, the degradation of normal and abnormal proteins was shown to be impaired in old animals, and these findings were comparable with the age-related accumulation of altered proteins in different tissues. In the meantime, it was firmly established that the ubiquitin-proteasome system and the autophagy-lysosome system are responsible for intracellular protein degradation as described above. Many studies have demonstrated that proteasome activity declines with age (Saez and Vilchez 2014; Shibatani et al. 1996). We have shown that the activities of both the 20S and 26S forms of the liver proteasome decline similarly with aging in three age groups of rats of from 8–10 to 25–28 months of age (Hayashi and Goto 1998). Despite the decline in the enzyme activities, the amount of catalytic subunits measured by immunoblot did not change with age, suggesting that posttranslational modifications or subunit replacement are responsible for the decreased activities. In fact, other investigators have reported that the subunit composition of the proteasome is altered in aged tissues. Furthermore, a subunit of the proteasome is sensitive to oxidative modification (Ishii et al. 2005), suggesting that oxidative stress can accelerate the accumulation of oxidized proteins in aging by reducing the efficiency of damaged proteins. It is interesting to note that the 20S proteasome degrades oxidatively modified proteins selectively (Davies 2001) and that the 26S proteasome can be reversibly dissociated to produce the 20S proteasome by removing 19S regulators upon oxidative challenge, thereby facilitating adaptation to stress (Grune et al. 2011). It should be mentioned that the Lon protease plays an important role in the degradation of oxidized mitochondrial proteins, the activity of which declines with age and contributes to the accumulation of damaged proteins in the organelle (Ngo et al. 2013).

When the damage to proteins is extensive, forming insoluble cross-linked aggregates that are not degraded by proteasomes, the autophagy-lysosome system degrades them in addition to removing the damaged organelles (Wong and Cuervo 2010). The autophagy-lysosome system is considered to act via microautophagy, macroautophagy, and chaperon-mediated autophagy, and the latter two systems are the predominant mechanisms of autophagy in animals. Macroautophagy refers to the digestion of contents of cytoplasmic regions engulfed in membrane vesicles, which then fuse with lysosomes for degradation. Chaperon-mediated autophagy is the digestion of substrates bound to the chaperon heat-shock cognate protein (hsc70), which is recognized by lysosomes via an interaction with the receptor protein on the

surface. Substrates translocated across the lysosomal membrane are then digested. The activities of these autophagic processes decline with aging (Rubinsztein et al. 2011). The age-associated decline in the chaperon-mediated autophagy can be caused by decreased content of the substrate receptor (lysosome-associated membrane protein type 2a) (Cuervo and Dice 2000) and the age-associated impairment of lysosomal function (Kurz et al. 2008).

A number of studies have established the extensive involvement of altered protein conformation in age-associated neurodegenerative diseases. These are mainly due to the impaired functions of ubiquitin-proteasomes and/or autophagy-lysosome systems and the chaperon dysfunctions described in many excellent reviews (Takalo et al. 2013; Hipp et al. 2019). However, I do not go into the details of these studies as this subject is of little relevance to the scope of this overview, although it is conceivable that these mechanisms are also involved in the general age-related functional decline of housekeeping proteins.

Thus, the original idea that accumulation of altered proteins causes a variety of aging phenotypes has expanded to include different aspects of life processes. The altered protein theory of aging/proteostasis theory of aging has now become one of the most widely accepted theories to explain the basic mechanisms of aging.

1.9 Dysdifferentiation Theory of Aging/Epigenetic Theory of Aging

Richard Cutler suggested that differentiated cells can undergo changes in transcription during aging, such that the strict pattern of gene expression is gradually relaxed, leading to the deterioration of the functions of cells and tissues (Cutler 1991). This idea, called the dysdifferentiation theory of aging, was based on the finding that the expression of globin or its related mRNA and murine leukemia virus RNA is increased in the brains and livers of aged mice compared to their younger counterparts (Ono and Cutler 1978). More recently, it has been shown that gene expression becomes gradually heterogeneous in the tissues of individuals with advancing age, including the cerebral cortex and hippocampus (Somel et al. 2006). These findings are compatible with the dysdifferentiation theory of aging.

This theory had never been popular, but has been recently revived as the epigenetic theory of aging. Epigenetics is a phenomenon in which a fixed pattern of gene expression in a cell, or an organism is inherited from one generation to the next without changes in the genomic nucleotide sequence. This definition has been broadened to include the long-term stable control of gene expression in differentiated cells in a body without changes in the nucleotide sequence, as manifested in various physiological and pathological situations, including aging and age-related diseases. The epigenetic regulation of long-term cell-specific gene expression is determined by a variety of mechanisms, including DNA methylation, histone modifications, and microRNA expression (Brunet and Berger 2014; Raj and Horvath

2020). These epigenetic mechanisms of gene modulation are influenced throughout life by both internal and external stimuli, such as energy metabolism, nutrition, and exercise, and can therefore impact on the physiological aging and the incidence of age-related diseases (Lopez-Otin et al. 2013; Goto et al. 2015).

It has been shown in twin studies that there are far more differences in the patterns of DNA methylation and histone acetylation in the circulating lymphocytes of older (50 years of age) monozygotic twins compared with younger (3 years of age) twins (Fraga et al. 2005). Interestingly and consistently with the findings, the differences in the gene expression between the older pairs were much greater than those in the younger pairs. These findings suggest that an identical genome in early life could undergo different epigenetic modifications throughout life, potentially resulting in differences in the aging rates and/or in their vulnerability to diseases. This type of variable epigenetic modifications may partly explain the relatively low contribution (approximately 30%) that genes have on longevity compared with environmental factors (Ljungquist et al. 1998; Dato et al. 2017).

Frailty is a common manifestation of physiological aging. It has been reported that a worsening frailty status, as measured by the loss of body weight, the development of sarcopenia and muscle weakness, and the reduction in physical activity, is associated with decreased global DNA methylation in the peripheral blood cells of individuals aged 65–105 years old over a 7-year follow-up period (Bellizzi et al. 2012). Aging is often associated with reduced levels of global DNA methylation (hypomethylation), mostly in cytosine base of CpG sequences, but its physiological implications remain mostly unclear. However, it should be mentioned that the age-related hypermethylation can occur in some cases of cancer such as promoter regions of tumor suppressor genes increasing the risk of carcinogenesis with age (Kulis and Esteller 2010).

In recent years, epigenetic modifications have attracted a particular interest following Steve Horvath published an influential paper on DNA methylation (DNAm) and aging covering a variety of tissues of different organisms in normal and pathological situations, coining a term "epigenetic clock" that appears to predict biological age rather than chronological age (Horvath 2013; Levine et al. 2018; Ryan 2021). I do not discuss this emerging topic in detail as many review articles have been published (see, e.g., an article by Jylhava et al. (2017) for a comparison among potential age predictors such as telomere attrition including DNAm age). It should, however, be noted that it is not clear whether DNAm is simply a marker of aging or has a causal or mechanistic relationship with changes of gene expression that should be relevant to physiological decline of cells and tissues with age, i.e., biological aging. In fact, Horvath admits that "I do not find that age effects on DNAm levels affect gene" and "the relationship between DNAm levels and expression levels is complex" (Horvath 2013). In a recent systematic survey of the epigenetic clock, Oblak et al. (2021) state that a majority of parameters potentially related to the epigenetic clock is age-related diseases such as cancer, cardiovascular disease, lung disease including air pollution caused disorders, diabetes mellitus and mental disorders, etc. but so far apparently not clearly relevant to physiological decline in normal aging. Notably, the authors describe that frailty, a hallmark of human biological

aging, does not have any significant effects. Therefore, I would think that DNAm age could not predict biological or physiological age but possibly can predict the remaining time of life or health span as DNA methylation being predictive of susceptibility to some kinds of age-related diseases.

Changes in the posttranslational modification of histones also occur with age, which can lead to reduced gene expression, as decreased acetylation allows the chromatin to more tightly condense by increasing the interactions with DNA. As an example, we have shown that acetylation of lysine 9 in histone H3 is reduced in aged rat livers compared to younger counterparts, suggesting a possible mechanism of decrease in the expression of certain genes with age (Kawakami et al. 2009). Memory impairment is a common feature of old animals and a serious problem for elderly people. It has been reported that the acetylation of specific lysine residues in histone H3 and H4 are transiently increased in the hippocampus of young (3-month-old) mice subjected to contextual fear conditioning but not in their older (16-month-old) counterparts (Peleg et al. 2010). These findings suggest that memory impairment in old animals is correlated with defects in learning-induced histone acetylation. Intriguingly, the administration of histone deacetylase inhibitors, such as sodium butylate, to old mice prior to the memory conditioning increased the acetylation significantly in the coding regions of learning-regulated genes. These findings suggest that the dysregulation of histone acetylation is causally related to age-associated memory impairment, raising a possible mechanism for the treatment of this disorder.

MicroRNAs (miRNA) are another epigenetic modifier of aging that have been widely studied in recent years (Bushati and Cohen 2007; Grasso et al. 2014). The RNAs are short, noncoding RNAs coded in the nuclear genome affecting transcription or mRNA stability and thus can influence gene expression in aging and diseases. Different kinds of miRNA have been reported to change with age in invertebrate models such as nematode and fruit fly as well as normal tissues (brain, skeletal muscle, heart, etc.) of mice and rats (Kinser and Pincus 2020). miRNAs secreted from cells and tissues exist in the circulation and thus have been studied for a possible biomarker of aging. It should be mentioned that functional roles of miRNA and regulation of its gene expression have remained to be defined, and therefore appeared to have limited significance at present to explain the mechanisms of aging.

1.10 The Hyperfunction Theory of Aging

This recently proposed new theory of aging that is apparently against the traditional view of aging deserves mentioning, as it particularly opposes the influential free radical theory of aging and may open up a new door to explain the mechanisms of aging. In most of the aging theories described so far, aging is believed to be due to an accumulation of detrimental molecular changes in protein and nucleic acid that is induced by ROSs and other chemicals or by errors in critical life maintenance

processes. Mikhail Blagosklonny proposed that aging is instead caused by the hyperfunction of growth, such as hypertrophy and hyperplasia, rather than an increase in the damage that occurs later in life, leading to age-related pathologies (Blagosklonny 2008). His claim is based on reports that contradict the ideas that aging is caused by an accumulation of molecular damage. According to such ideas, the molecular damage is mainly due to ROS. The reduced translation activity due to the deletion of ribosomal S6 protein kinase 1, a component of the target of rapamycin (TOR) pathway, is believed to lead to an increased life span and resistance to age-related pathologies (Selman et al. 2009). TOR is an evolutionarily conserved protein kinase that regulates growth and metabolism and is involved in the modulation of aging (Kapahi et al. 2010). Blagosklonny admits that damage accumulation can cause the deterioration of cellular functions over time but also predicts that an organism could not live long enough to accumulate a lethal level of damage (Blagosklonny 2008). It is possible, however, that damage accumulation would increase the probability of death when exposed to internal and external stress, thus constituting a mechanism of aging. He stresses the role of the TOR pathway by placing it in the center of the hyperfunction theory of aging because most factors that appear to reduce the activity of TOR retard aging and extend the life span of model organisms (Blagosklonny 2012). Gems and Partridge support the idea of hyperfunction as a mechanism of aging but state that it remains unclear how the pathway controls the rate of aging and life span (Gems and Partridge 2013). This theory predicts a form of antagonistic pleiotropy (Austad and Hoffman 2018) in which hyperfunction increases fitness early in life but can be harmful in old age. The identity of the intrinsic or extrinsic factors that maintain hyperfunction in the face of declining metabolic activity with age remains unknown. It should be noted that a recent report describes that rapamycin extends the life span of mice but ameliorates few aging phenotypes, such that its effects are not due to a modulation of aging but are instead related to aging-independent drug effects (Neff et al. 2013).

1.11 Summary and Perspectives

Despite extensive efforts to solve *an unsolved problem of biology* for nearly three quarter of a century since Medawar wrote a book with this title, no single theory has yet fully explained the mechanism of aging. As all animals are considered to be the products of evolution, it is assumed that there are conserved aging mechanisms even between species with remarkably different life spans, such as humans, mice, fruit flies, and nematodes (see Fig. 1.1 and Table 1.1). Although there appear to be conserved pathways that potentially drive aging (Kenyon 2010), it is not known how these very basic molecular mechanisms result in such great life span variation. The mechanism has remained as *an unsolved problem in gerontology*. The leading theories that have so far been proposed are apparently acceptable at least in part, but not without objections, and different theories interrelate with each other by one theory being a part of the others, suggesting that each one can contribute partly to be

integrated into the whole process of aging. In addition, it has been proposed that chance or stochasticity in addition to genes and environments can play a role in aging regardless of the mechanisms in both humans and model organisms (Kirkwood and Finch 2002; Vaupel et al. 1998). Nevertheless, no one would think that a lucky mouse can live for 100 years and an unlucky normal human would die of aging in 3 or 4 years, showing that the gene undoubtedly play a definitive role for the rate of aging and life span determination. But nobody knows which gene or genes are responsible, and a little effort has appeared to be made so far to identify one.

A major target of future studies of aging will be how to integrate the different theories to understand the mechanisms of varied aging rates in different animal species and individual differences of the aging rate within a species.

We are perhaps in the stage of awaiting a new paradigm or an integration of the existing theories to provide us with an improved understanding of the mechanism of aging.

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Part II
Human Longevity: Accelerated Aging and Centenarians

Chapter 2
Clinical and Basic Biology of Werner Syndrome, the Model Disease of Human Aging

Yoshiro Maezawa, Masaya Koshizaka, Hisaya Kato, and Koutaro Yokote

Abstract Werner syndrome is an autosomal recessive genetic disorder first described in 1904 by Otto Werner, a German ophthalmologist. It is considered the representative progeroid syndrome because various signs of aging, such as gray hair, cataracts, diabetes, and skin ulcers, appear after puberty. The onset of the disease begins in the 20s or 30s, leading to diabetes and atherosclerosis, and death in mid-50s due to myocardial infarction or malignant tumors. The number of patients in Japan is estimated to be between 700 and 2000, and 60% of the world's reports are from Japan, suggesting that this accelerated aging disease is more common in Japan. The cause of Werner syndrome was identified in 1996 as a mutation in the WRN gene, a RECQ helicase located in chromosome 8. Since then, various studies have shown that the syndrome is associated with decreased DNA damage repair and genomic instability, shortened telomeres, chronic inflammation due to cellular senescence- and senescence-associated secretory phenotype (SASP), decreased mitochondrial function and accumulation of oxidative stress, stem cell senescence, and epigenetic changes. While most premature aging syndromes occur in childhood and involve a growth and developmental disorder, only Werner syndrome occurs after normal growth and puberty, suggesting that this syndrome is a model of human aging. The elucidation of the pathogenesis and molecular mechanisms of this disease and the development of a treatment strategy are expected to lead to the elucidation of the pathogenesis of general human aging and of aging-related diseases such as diabetes and malignant tumors.

Keywords Werner syndrome · Adult progeria · Segmental aging · Skin ulcer · Genome instability · Cellular senescence

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_2

2.1 Clinical Features and Pathogenesis of Werner Syndrome

2.1.1 Introduction

Werner syndrome (WS) is a rare autosomal recessive disorder, resulting from genetic instability (Gray et al. 1997), and is sometimes referred to as "accelerated aging syndrome" because beginning in puberty, it presents with symptoms such as cataracts and loss and graying of hair, which makes individuals look relatively old for their age. It is estimated that there are approximately 700–2000 patients in Japan (Matsumoto et al. 1997a; Satoh et al. 1999; Yokote et al. 2017; Yamaga et al. 2017). Of the world's reported cases to date, 60–80% are from Japan. The life expectancy of people with WS is shorter than that of healthy individuals because of its frequent comorbidities, such as diabetes, arteriosclerosis, and malignant tumors (Onishi et al. 2012). The characteristics of patients with WS include a bird-like face and a high-pitched voice. Moreover, patients with WS have a high incidence of intractable ulcers in the legs and feet, resulting in pain, infections, and even amputation of the lower limbs, which can have a major negative impact on the person's quality of life and vital prognosis.

In Japan, a guide for the diagnosis of WS was created in 1984. In 1996, a mutation in the DNA helicase Werner syndrome protein (WRN) encoded by the RecQ genes on chromosome 8 was identified as the cause of the disease. Although no fundamental treatment has been developed for the disease, many studies are beginning to suggest that patient life expectancy could potentially be extended with appropriate treatment interventions. The diagnostic criteria for WS were revised, and the first guideline for treatment was developed in 2012. WS was designated as an intractable disease in Japan in 2015. A nationwide survey in 2017 identified 116 patients diagnosed with WS (Koshizaka et al. 2020). The Werner Syndrome Registry (case registration system) was established in 2017 (Koshizaka et al. 2020). Treatments for WS were standardized, and the first guideline for its diagnosis and treatment in English were developed in 2020 (Takemoto and Yokote 2021).

2.1.2 Diagnostic Criteria

Based on a nationwide epidemiological study carried out from 2009 to 2011, the diagnostic criteria for WS were revised (Takemoto et al. 2013). From the results of the aforementioned study, progeroid faces, bilateral cataracts, skin atrophy, clavus and callus, flat feet, bird-like face, and abnormal voice were found in >85% of the genetically confirmed cases of WS. Calcification in the Achilles tendon is a frequent symptom of WS, and 80% of people with WS presented with this symptom. Furthermore, the segmental and flame-like patterns of calcification were highly specific to WS (Fig. 2.1) (Takemoto et al. 2013).

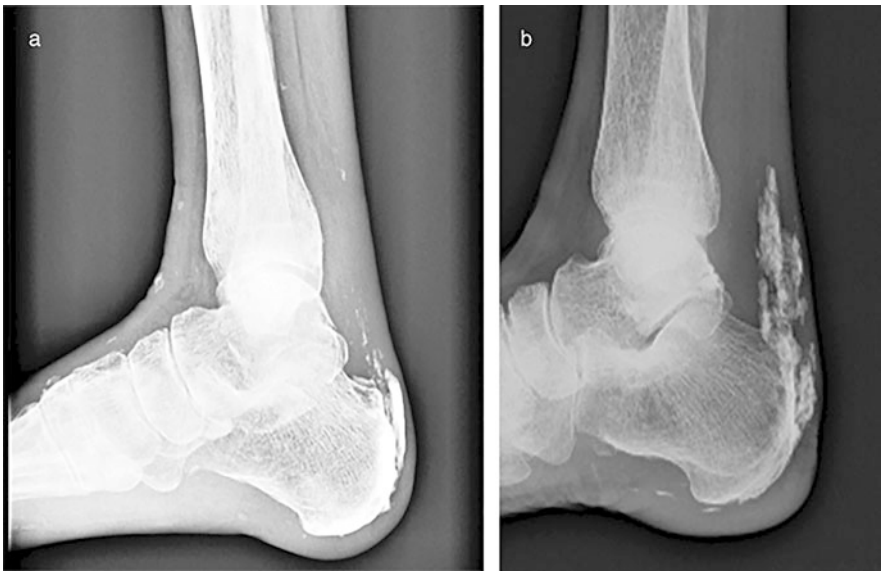

Fig. 2.1 Calcification in the Achilles tendon seen in Werner syndrome patients. (**a**) Segmental and (**b**) flame-like calcifications in the Achilles tendon

Table 2.1 Revised diagnostic criteria for Werner syndrome

I. Cardinal signs and symptoms (onset over 10 until 40 years of age)	
1. Progeroid changes of hair	Gray hair, baldness, etc.
2. Cataract	Bilateral
3. Changes of skin and intractable skin ulcers	Atrophic skin, tight skin, clavus, callus
4. Soft tissue calcification	Achilles tendon, etc.
5. Bird-like face	
6. Abnormal voice	High-pitched, squeaky, hoarse voice
II. Other signs and symptoms	
1. Abnormal glucose and/or lipid metabolism	
2. Deformation and abnormality of the bone	Osteoporosis, etc.
3. Malignant tumors	Non-epithelial tumors, thyroid cancer, etc.
4. Parental consanguinity	
5. Premature atherosclerosis	Angina pectoris, myocardial infarction
6. Hypogonadism	
7. Short stature and low bodyweight	
III. Genetic testing	

On the basis of these results and the extensive clinical experience with Japanese cases of WS accumulated over many years and with unanimous agreement from the Japanese Werner Syndrome Working Committee, new diagnostic criteria (Table 2.1) (Takemoto et al. 2013) were formulated.

Addendum: Mental retardation is seldomly found in WS, and cognitive function is often appropriate for the patient's age. Confirmed: All cardinal signs are present or a gene mutation in addition to at least three cardinal signs. Suspected: One or two of the cardinal signs (hair changes and cataract) plus at least two more from all the signs.

Because the incidence of bird-like face is high (>93%), it is used as a cardinal sign. The characteristic WS face (Fig. 2.2) (Takemoto et al. 2013) includes a pinched nasal bridge and diminished subcutaneous tissue. The patient's voice might be high-pitched, squeaky, and/or hoarse; an example of the voice in a WS case is available on the committee website, with the patient's consent (http://www.m.chiba-u.jp/class/clin-cellbiol/werner/index.html).

Regarding hypogonadism, it has been reported that WS is associated with secondary sexual underdevelopment, decreased fertility, and testicular or ovarian atrophy. It is significant that fewer than 35% of patients in the present study had children [Takemoto. et al. 2013].

On the basis of extensive clinical experience and published medical literature, the cognitive function of WS patients is usually appropriate for their age.

Genetic analysis is now included in the diagnostic criteria. *WRN* is the only gene associated with classic WS, and mutations can be identified by DNA sequence analysis (Yu et al. 1996; Oshima et al. 1996; Friedrich et al. 2010) or Western blotting (Goto et al. 1999; Shimizu et al. 2002; Takada-Watanabe et al. 2012). However, these molecular genetic tests are not feasible at all institutions, and genetic testing is not essential to confirm the diagnosis of WS.

For differential diagnosis, atypical WS (Chen et al. 2003a); mandibuloacral dysplasia (Cavallazzi et al. 1960; Novelli et al. 2002); Hutchinson-Gilford progeria syndrome (Eriksson et al. 2003), caused by *LMNA* mutation; Rothmund-Thomson syndrome, caused by *RECQ4* mutation (Kitao et al. 1999); and Bloom syndrome, caused by *RECQ2* mutation (Ellis et al. 1995), are listed. These syndromes typically present with progeroid symptoms earlier than WS and are extremely rare in Japan.

2.1.3 Werner Syndrome Registry

The Werner Syndrome Registry in Japan was established in 2017. Forty-three patients were enrolled and registered in the registry. Table 2.2 shows the major signs of WS observed in registered patients. Almost all patients exhibited some of the major signs, such as graying hair, hair loss, cataracts, skin atrophy changes, and soft tissue calcification. Approximately 90% of patients had a characteristic bird-like face and high-pitched voice. Over half of the patients had diabetes, impaired glucose tolerance (67.5%), dyslipidemia (65.0%), and fatty liver (52.5%). The major signs and clinical symptom at the time registered to the registry are shown in Table 2.2. A small percentage of patients in the registry had a history of atherosclerosis (0%), angina pectoris or myocardial infarction (2.5%), or arteriosclerosis obliterans (15.0%). Angina pectoris or myocardial infarction significantly decreased

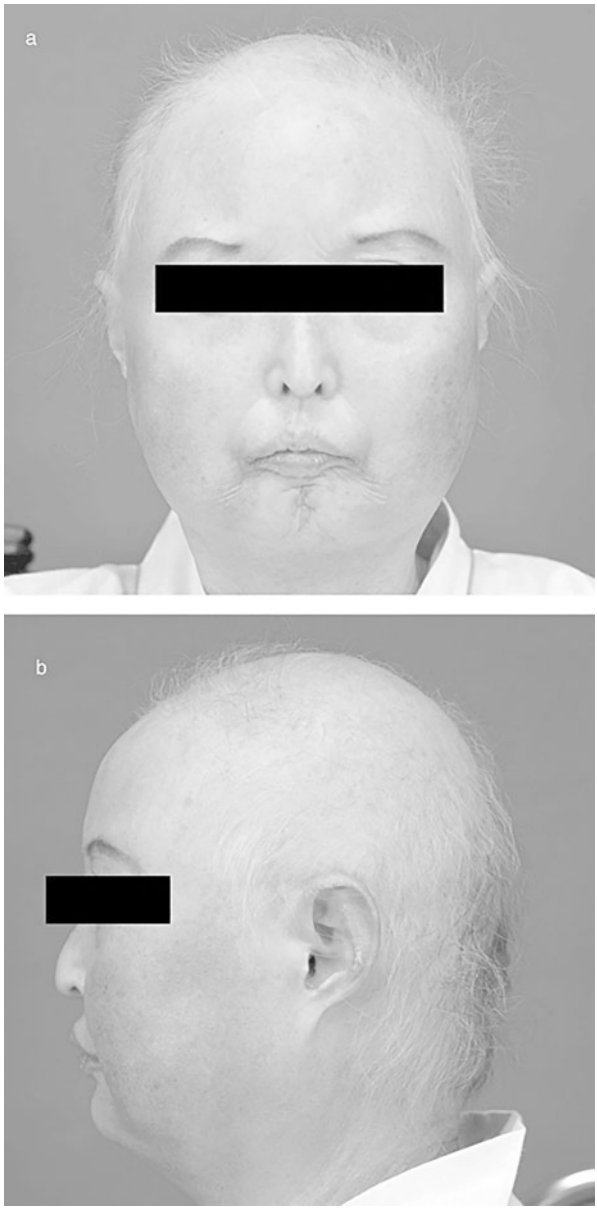

Fig. 2.2 A bird-like face is typically seen in Werner syndrome patients. The nasal bridge of a 57-year-old woman appears pinched, and subcutaneous tissue is diminished. (**a**) front of face, (**b**) side of face

(2.5% vs. 14.8%, $P = 0.049$) compared with the previous survey conducted in 2009 (Takemoto et al. 2013). This result may have arisen because of improved comprehensive control of diabetes, dyslipidemia, and hypertension with new treatments such as HMG-CoA reductase inhibitors (statins) or peroxisome proliferator-activated receptor gamma agonists (Yokote and Saito 2008; Yokote et al. 2004a;

Table 2.2 The frequency of major signs and clinical symptoms of and medications administered to patients with Werner syndrome at the time registered to the registry

	%	*n*	*N*
Major signs			
Graying of hair, hair loss	97.5	39	40
Cataracts	100	40	40
Skin changes	97.5	39	40
Intractable skin ulcers	67.5	27	40
Soft tissue calcification	87.5	35	40
Bird-like face	90	36	40
High-pitched voice	87.5	35	40
Clinical symptoms			
Diabetes, IGT	67.5	27	40
Dyslipidemia	65	26	40
Hypertension	42.5	17	40
Fatty liver	52.5	21	40
Cerebral bleeding	0	0	40
Cerebral infarction	0	0	40
AP or MI	2.5	1	40
ASO	15	6	40
Amputation	15	6	40
Malignant tumor	20	8	40
Medications			
Diabetes, IGT			
DPP-4 inhibitor	37.0	10	27
Biguanide	33.3	9	27
Thiazolidine	48.1	13	27
Alpha GI	7.4	2	27
Sulfonylurea	11.1	3	27
SGLT2 inhibitor	3.7	1	27
Glinide	0	0	27
GLP-1 analog	3.7	1	27
Insulin	14.8	4	27
Dyslipidemia			
Statin	65.4	17	26
Fibrate	3.8	1	26
Ezetimibe	0	0	26
EPA	11.5	3	26
Resin	0.0	0	26
Nicotinic acid	19.2	5	26
Probucol	0	0	26
Hypertension, among others			
Ca blocker	47.1	8	17
ARB	35.3	6	17
ACE inhibitor	0.0	0	17

(continued)

Table 2.2 (continued)

	%	*n*	*N*
Alpha1 blocker	0.0	0	17
Beta blocker	11.8	2	17
Diuretics	0.0	0	17
Antiplatelet	5.0	2	40
Anticoagulant	12.5	5	40

The percentages of medications administered to treat abnormal glucose metabolism, dyslipidemia, and hypertension are shown for the total number of patients with each disorder. *N* number of patients, *n* number of patients with symptoms or treatment drugs, *IGT* impaired glucose tolerance, *AP* angina pectoris, *MI* myocardial infarction, *ASO* arteriosclerosis obliterans, *DPP-4* dipeptidyl peptidase-4, *alpha GI* alpha glucosidase inhibitor, *SGLT2* sodium-glucose cotransporter-2, *GLP-1* glucagon-like peptide-1, *EPA* eicosapentaenoic acid, *Ca* calcium, *ARB* angiotensin II receptor blocker, *ACE* angiotensin-converting enzyme inhibitor

Yasuda et al. 2010). These treatments appeared to have ameliorated the arteriosclerotic outcomes in the patients with WS.

Two thirds of patients with WS had an intractable ulcer. The nationwide survey in 2017 revealed that a higher percentage of patients with WS were reported by plastic surgeons (7.7%) and dermatology specialties (7.9%) than by other departments. It is speculated that patients with WS visited the hospital for the treatment of ulcers or to receive more specialized treatments in dermatology and plastic surgery departments. Limb amputation was observed in 15.0% of patients.

It is noteworthy that approximately 30% of the patients' parents had a consanguineous marriage.

2.1.3.1 General Information

Age at Onset and Diagnosis

The patients' average age at registration was 50.1 ± 7.5 years. The average age at WS onset was 26.1 ± 9.5 years; however, the age at diagnosis was 42.5 ± 8.6 years (Table 2.3). There was a delay between the age at onset and the age at diagnosis. This tendency was also reported in 2006 in the international WS Registry (Huang et al. 2006). In the Japanese registry, the age at onset of cataracts was 31 years, and the age at diagnosis or referral was 43 years. These results suggest that it is necessary to consider measures for early diagnosis and early intervention. WS onset is usually marked by bilateral cataracts or gray hair and hair loss, which are the first symptoms (Takemoto et al. 2013). Patients undergo an ophthalmologic operation for cataracts around their third decade of life. However, around their fourth decade, patients tend to have intractable ulcers and visit dermatologists or plastic surgeons. The national survey showed that many patients were reported by dermatologists or plastic surgeons, rather than ophthalmologists. Improvements in earlier diagnostic methods are needed. As one possible solution, calcification in the Achilles tendon is easily

Table 2.3 Patient background, physical findings, body composition, and physical function at the time registered to the registry

	Total				Men				Women			
	Mean		SD	*n*	Mean		SD	*n*	Mean		SD	*n*
Patients' backgrounds												
Age (years)	50.1	±	7.5	40	49.4	±	7.6	22	50.9	±	7.5	18
Onset age (years)	26.1	±	9.5	30	28.2	±	8.5	16	23.7	±	10.2	14
Diagnosed age (years)	42.5	±	8.6	39	42.0	±	6.4	21	43.2	±	10.8	18
Physical findings												
Height (cm)	154.0	±	10.7	40	159.7	±	8.6	22	147.2	±	9.0	18
Body weight (kg)	44.1	±	9.5	40	49.0	±	9.3	22	38.1	±	5.4	18
BMI (kg/m^2)	18.5	±	3.1	40	19.2	±	3.5	22	17.6	±	2.5	18
Waist circumference (cm)	77.3	±	12.0	24	80.4	±	12.2	14	73.0	±	10.8	10
Visceral fat area (cm^2)	102.3	±	61.4	10	112.4	±	81.5	4	95.6	±	51.7	6
SMI (kg/m^2)	4.3	±	0.8	9	4.5	±	0.9	5	4.1	±	0.6	4
Physical function												
Mean grip strength (right) (kg)	17.1	±	8.7	23	20.8	±	8.6	13	12.3	±	6.3	10
Mean grip strength (left) (kg)	16.0	±	7.6	23	19.5	±	7.3	13	11.4	±	5.3	10
Mean walking speed (m/s)	0.8	±	0.6	13	0.9	±	0.6	6	0.8	±	0.6	7

BMI body mass index, *SMI* skeletal muscle mass index, *SD* standard deviation

examined on routine X-ray and is highly indicative of WS, and proactive X-ray images are recommended when WS is suspected. However, Achilles tendon calcification might not be present in young patients, and minute calcifications might require diagnosis by a specialist, such as an orthopedic surgeon (Takemoto et al. 2013).

Physique

In the registry, the patients' average height, body weight, and body mass index (BMI) (159.7 cm, 49.0 kg, and BMI 19.2 kg/m^2 in men and 147.2 cm, 38.1 kg, and BMI 17.6 kg/m^2 in women, respectively) (Table 2.3) were lower than those of the average Japanese individual in the fifth decade of life (169.2 cm, 68.1 kg, and BMI 23.5 kg/m^2 in men and 156.6 cm, 55.0 kg, and BMI 22.2 kg/m^2 in women, respectively). Patients with WS present with central obesity; the average abdominal circumference was 80.4 ± 12.2 cm in men and 73.0 ± 10.8 cm in women. The average abdominal circumference was large, although the respective BMIs were low (Table 2.3). In other words, patients with WS have lipodystrophy.

Life Expectancy

Previously, it was reported that patients with WS die around the fifth decade of life (Goto and Matsuura 2008). However, the life expectancy for Japanese patients with WS has steadily increased compared with that two decades (Goto 2000) or even one decade ago (Koshizaka et al. 2020; Takemoto et al. 2013).

Laboratory Test

Regarding laboratory tests, on average according to the registry, patients had more than twice the levels of gamma-glutamyl transpeptidase than the upper normal limit [Koshizaka et al. 2020]. The levels of glycated hemoglobin, plasma glucose, and low-density lipoprotein cholesterol (LDL-C) levels were well controlled.

2.1.3.2 Symptoms

Patients with WS display various signs of aging that appear from the second decade of life. Gray hair and hair loss appear at 20 years of age, and bilateral cataracts appear at 30 years of age. They exhibit lipodystrophy and sarcopenic obesity and often have the following symptoms: sarcopenia, diabetes, dyslipidemia, fatty liver, osteoporosis, foot skin ulcers, ulcer infection, and calcification in tendons. They also often have myocardial infarctions, and malignant tumors appear at 40 years of age [Goto et al. 2000; Takemoto et al. 2013]. However, these symptoms in patients with WS are different from those of simple aging in that the incidence of some common aspects of aging, such as dementia, are rare.

Sarcopenia

Sarcopenia is defined as a condition that combines decreased skeletal muscle mass with weakness or decreased physical function. Patients with WS are characterized by visceral fat accumulation and thin limbs. A decrease in skeletal muscle mass frequently occurs in patients with WS before 40 years of age.

According to the registry, the average of the total limb skeletal mass index, identified using dual-energy X-ray absorptiometry, was 4.5 ± 0.9 kg/m^2 for men and 4.1 ± 0.6 kg/m^2 for women. Grip strengths were (right) 20.8 ± 8.6 kg and (left) 19.5 ± 7.3 kg for men and (right) 12.3 ± 6.3 kg and (left) 11.4 ± 5.3 kg for women. Walking speed was 0.8 ± 0.6 m/s on average (Table 2.3). The average grip strength, walking speed, and skeletal muscle mass index met the diagnostic criteria for sarcopenia. Therefore, most patients aged over 40 years had sarcopenia.

Although the mechanism is still unclear, various potential factors including aged skeletal muscle, metabolic abnormality, and inflammation, or a decreased amount of activity due to low physical function, have been considered. Resistance exercise may

prevent the appearance of sarcopenia, and early intervention is required in patients with WS (Kuzuya et al. 2021). Sarcopenia appears early in most patients with WS; therefore, sarcopenia may be prevented by early intervention with strength training and treatments including amino acids such as leucine, whey protein, calcium, and vitamin D (Cruz-Jentoft et al. 2020; Martinez-Arnau et al. 2019).

Diabetes

More than 60% of patients with WS have diabetes (Koshizaka et al. 2020). Diabetes associated with WS is classified as "accompanied with other diseases and conditions and the one occurring mainly in association with other genetic syndromes." The patients used to require high amount of insulin and were still on poor glucose control. Diabetes due to WS is marked by accumulated visceral fat and high insulin resistance, despite low BMI (Takemoto et al. 2021). Therefore, thiazolidine derivatives and metformin (Yasuda et al. 2010) are effective for glycemic control. There have been many reports on the effectiveness of a thiazolidine derivative, an agonist, of peroxisome proliferator-activated receptor γ, an insulin sensitizer (Yokote et al. 2004a, b; Honjo et al. 2008; Takino et al. 1994; Izumino et al. 1997; Imano et al. 1997; Hattori et al. 2004; Yamamoto et al. 2007). Dipeptidyl peptidase-4 (DPP-4) inhibitors (Watanabe et al. 2013; Kitamoto et al. 2012) and glucagon-like peptide-1 receptor agonists (Ide et al. 2016) are also beneficial for patients with WS. More than 30% of patients with diabetes were treated with a DPP-4 inhibitor, biguanide, or thiazolidine in the registry (Koshizaka et al. 2020).

Dyslipidemia

Arteriosclerosis is one of the two leading causes of death in patients with WS, along with malignancy (Goto 1997). Among the various forms of arteriosclerosis that patients with WS develop, coronary artery diseases and peripheral arterial disease have a high incidence, and the latter plays a role in causing skin ulcers in patients with WS to become refractory (Takemoto and Yokote 2012). Disorders of carbohydrate metabolism and lipid metabolism associated with WS act as promoting factors for atherosclerosis (Tsukamoto et al. 2021). Previous guidelines showed that hypercholesterolemia occurred in 53% of patients with WS (Takemoto and Yokote 2012). The incidence of dyslipidemia in patients with WS is high, at 85%. The most common type of dyslipidemia is hypertriglyceridemia, occurring in 76% of patients, followed by hyper-LDL cholesterolemia/non-high-density lipoprotein (HDL) cholesterolemia in 68% of patients and hypo-HDL cholesterolemia in 32% or patients. Patients with WS and dyslipidemia develop diabetes at a high rate ($\geq$90%). The mean BMI of patients with WS and hypertriglyceridemia was 18.2, showing a lack of association with obesity. The rates of achieving the lipid control target values among patients with WS are high, at 91% for LDL cholesterol, 91% for HDL cholesterol, and 82% for triglycerol. Strong statin dosage is mainly used as an

antidyslipidemic drug and contributes to the achievement of the control target values (Tsukamoto et al. 2021). Two-thirds of patients in the registry with dyslipidemia were treated with statins (Koshizaka et al. 2020).

Fatty Liver

WS patients with fatty liver had a mean BMI of 18.8 and a maximum BMI of 22.6, and 83% of these patients were underweight. The L/S ratio showed a positive correlation with HDL cholesterol levels and a negative correlation with triglyceride levels. It did not correlate with liver enzyme levels (Tsukamoto et al. 2021). Insulin resistance associated with a fatty liver (nonalcoholic fatty liver disease) and accumulation of visceral fat has been considered to play a major role in metabolic abnormalities (Kitade et al. 2017; Kahn and Flier 2000; Hardy et al. 2012). There is evidence regarding treatments with pioglitazone (Belfort et al. 2006; Aithal et al. 2008), vitamin E (Sanyal et al. 2010), and ursodeoxycholic acid (Leuschner et al. 2010) in the general population, and Takemoto et al. reported that astaxanthin, a carotenoid, improved fatty liver in patients with WS (Takemoto et al. 2015).

Atherosclerosis

Early-onset atherosclerosis occurs in WS patients, and the incidence of ischemic heart disease and arteriosclerosis obliterans is particularly high. Because of the high possibility of silent myocardial ischemia, proactive and regular tests for arteriosclerosis are recommended for confirmed cases of WS. In contrast, the prevalence of morbidity because of stroke in WS patients is similar to that found in the general population of the same age in Japan (Okabe et al. 2012).

Malignancy

Malignant tumors were observed in 20.0% of patients in the registry. The morbidity of malignant tumors is still high in patients with WS. Reportedly, age at cancer diagnosis in patients with WS advanced by 20 years when compared with that of the general Japanese population (Lauper et al. 2013). Because neoplastic lesions start developing from a young age, regular screening for malignancies is necessary for confirmed cases of WS. Epithelial and non-epithelial tumors are equally common in WS cases, in contrast to the 10:1 (epithelial-to-non-epithelial) ratio observed in the general population. Cancer was significantly more prevalent in WS patients with diabetes. Therefore, routine cancer screening is especially important in this particular subgroup. Epithelial tumors with high frequency include thyroid cancer, lung cancer, gastric cancer, hepatic cancer, and pancreatic cancer. Non-epithelial tumors with high frequency were malignant fibrous histiocytoma, melanoma, meningioma, and myelodysplastic syndrome (Onishi et al. 2012).

Osteoporosis

Osteoporosis has been observed in approximately 41% of WS patients (Murata and Nakashima 1982). Although osteoporosis was relatively rare in younger patients, almost all patients who were at least 40 years of developed osteoporosis. It is likely to be more severe in the femur than in the lumbar spine (Mori et al. 2017, 2021). Osteoporosis is considered to occur because bone formation is inhibited, while bone resorption is normal in WS (Rubin et al. 1992). Research showing the relation between the *WRN* gene polymorphism and osteoporosis suggests that genetic factors might also be involved in osteoporosis associated with Werner syndrome (Ogata et al. 2001; Zhou et al. 2015). No clear evidence to date regarding treatment for osteoporosis associated with WS has been found. Therefore, the treatment of osteoporosis according to the guidelines for this purpose is considered appropriate (HO osteoporosis prevention and treatment guideline 2015).

Skin Ulcers

Approximately 40% of patients with WS have skin ulcers (Kubota et al. 2021). WS is characterized by symptoms such as atrophy of subcutaneous tissues, decreased blood flow (Okabe et al. 2012), and lower activity of fibroblast cells (Hatamochi et al. 1994) due to metabolic disorders in connective tissues (Muftuoglu et al. 2008), which may easily cause refractory skin ulcers (Yeong and Yang 2004). Skin ulcers in patients with WS often arise from hyperkeratotic lesions and trauma to pressure points such as the plantar region and are more difficult to treat than wound healing in healthy individuals. The ulcers are often located at the distal one-third of the lower legs (Kubota et al. 2021). Skin ulcers lead to reduced quality of life of patients. Callosities in the foot also often form. A callosity in WS is an important therapeutic target for the prevention of ulcers.

Macroscopic evaluations of ulcers are important. Plain radiography and computed tomography are helpful for examining the shape of the entire foot and the conditions of the individual bones of the foot. Vascular evaluation is necessary. Magnetic resonance imaging examination is useful for suspected osteomyelitis.

Treatment includes topical application of a keratolytic agent for keratosis around the ulcer. The treatment of skin ulcers is the same as for normal ulcers, and if the ulcer is associated with infection and necrotic tissue, surgical debridement with a scalpel or scissors should be performed as much as possible after washing with saline or mildly warm water or with an antibacterial agent. Topical medications that promote softening and debridement of the necrotic tissue can be used with careful control of moisture in the wound. Topical agents that promote granulation should be used in wounds where necrotic tissue has been removed without infection. Dressings to maintain a moist environment in the wound may also be useful. If the wound does not improve with conservative treatment, surgical treatment should be considered (Motegi et al. 2021). The combination of surgical treatment and wound bed preparation is important in the treatment of skin ulcers (Kubota et al. 2021).

Infection

Generally, foot skin ulcers may often become severe, leading to the failure of conservative treatment and necessitating surgical excision of the infected site. The goal in the treatment of an infection caused by refractory skin ulcers in patients with WS is to minimize the exacerbation of the ulcerated skin lesion by detecting signs of infection early and treating it. It is important to identify the bacterial etiology causing an infection in the skin ulcer and treat with an effective antimicrobial. For poorly controlled infection, debridement and surgical excision are needed at an appropriate time (Taniguchi et al. 2021a).

Calcification in Tendons

It can be presumed that a considerable number of Japanese individuals with WS are never correctly diagnosed. Achilles tendon calcification was observed in 76.1% of patients with WS, whereas it was observed in only 0.88% of patients without WS, accompanied by 1–4 calcified masses with a maximum diameter ranging from 9.7 mm to 63.2 mm. The frequency of Achilles tendon calcification in patients with WS is far higher than that in patients without WS. Achilles tendon calcification could contribute to the diagnosis of WS (Taniguchi et al. 2021b).

2.2 Basic Research and Molecular Mechanisms of Werner Syndrome

2.2.1 Werner Gene and Protein

The causative gene of WS, WRN, was cloned in 1996 (WRN gene: OMIM 604611) (Yu et al. 1996; Oshima et al. 1996; Matsumoto et al. 1997b). It is a RecQ-type DNA helicase and consists of 1432 amino acids (Gray et al. 1997). To date, 83 different mutations have been reported, and additional novel mutations have been identified (Yokote et al. 2017). Among them, the type 4 mutation (the mutation of the base immediately before exon 26 from G to C results in the formation of a truncation mutant protein: c.3139-1G > C) is found in approximately 70% of Japanese WS patients and is considered to be the founder mutation (Oshima et al. 2017).

The accelerated aging mechanism of WS involves the loss of function of the RecQ-type DNA helicase, which plays an important role in various nuclear functions such as DNA repair, replication, recombination, and transcription (Oshima et al. 2017). In the presence of ATP, WRN protein converts DNA double strands into a single strand in the $3' \rightarrow 5'$ direction. In addition, the N-terminus of WRN protein contains an exonuclease domain that removes bases one by one in the $3' \rightarrow 5'$ direction. As a result of structural analysis using the large synchrotron radiation

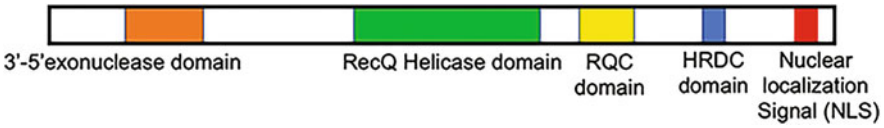

Fig. 2.3 A scheme of WRN protein. WRN has exonuclease domain, RecQ helicase domain, and nuclear localization signal at C-terminus

facility Spring 8, it has recently been reported that the winged-helix motif of the WRN protein acts as a "molecular knife" to unravel the double-stranded DNA at G quadruples, holiday junctions, or other unusual complex structures of DNA (Kitano et al. 2010; Gilson and Geli 2007; Huang et al. 2000). The C-terminus of WRN protein contains a nuclear translocation signal. The mutant WRN protein reported so far is a truncated protein that does not form a C-terminus and lacks the nuclear translocation signal, which inhibits nuclear translocation. This may be the reason why there is no obvious correlation between mutations and symptoms in WS. Indeed, mutant WRN protein that lacks C-terminal 30 amino acid residues cannot localize in the nucleolus where WRN usually resides (Suzuki et al. 2001). WRN contains two other domains, namely, RecQ helicase-conserved (RQC) region and the helicase, RNase D, C-terminal-conserved (HRDC) region (Fig. 2.3).

2.2.2 WRN and DNA Damage Repair

The role of WRN gene in DNA damage repair, especially after double-strand break (DSB), has been extensively reported. WRN protein is usually located in the nucleolus and moves to DNA damage sites upon CBP/P300-mediated acetylation (Blander et al. 2002). Sirt1 deacetylates WRN and regulates its reentry to the nucleolus from the nucleoplasm after DNA damage response (Li et al. 2008). When exonuclease domain-deficient WRN protein, helicase domain-deficient WRN, and WRN protein lacking both domains were expressed in fibroblasts from WS patients, DNA damage repair was most improved when the WRN protein lacking both domains was expressed. Interestingly, DNA damage repair requires balanced activities of helicase and exonuclease domains (Chen et al. 2003b).

There are two types of repair systems after DSB: homologous recombination (HR) that produces the new strands using genomic homologous sequences as templates and nonhomologous end joining (NHEJ), which connects both damaged ends. In addition, NHEJ includes two pathways, classical NHEJ and alternative NHEJ, which utilize different mechanisms. HR is preferentially active in S and G2 as sister chromatid is available after DNA replication, and NHEJ is active in all the cell cycle phases. HR has a high fidelity, and NHEJ is more prone to produce errors because it does not refer to intact homology sequence. Ku70/80 heterodimer protein, along with DNA-dependent protein kinase catalytic subunit (DNA-PKcs), is the essential regulator of DNA damage response that initiates the cascades of classical

NHEJ (Walker et al. 2001). WRN has two putative Ku-binding motifs: one at the N-terminus next to the exonuclease domain and one at the C-terminus next to an XLF-like motif. The N-terminal Ku binding motif enhances exonuclease activity of WRN (Grundy et al. 2016). DNA-PK also interacts with WRN, thereby phosphorylating its serine 440 and 447 sites and regulating relocalization of WRN to the nucleoli (Kusumoto-Matsuo et al. 2014). WRN also interacts with XRCC4-DNA ligase IV complex (X4L4), and this binding promotes the exonuclease activity of WRN to generate DNA ends suitable for XRCC4-LIG4-mediated ligation (Kusumoto et al. 2008). In addition, it has recently been reported that WRN promotes classical NHEJ via helicase and exonuclease enzymatic activities and inhibits alternative NHEJ using nonenzymatic functions (Shamanna et al. 2016). Taken together, the enzymatic activity of WRN, especially the exonuclease activity to process damaged DNA ends, promotes classical NHEJ.

On the other hand, WRN is also involved in HR. The critical regulators of HR are MRE11/RAD50/NBS1 (MRN) complex in conjunction with CtIP (Garcia et al. 2011). The endonuclease and 3′–5′ exonuclease activities of MRE11 are required for the initiation of HR. Following this, exonuclease 1 and/or the nuclease DNA2 with the RECQ helicase Bloom syndrome protein mediates extensive DNA resection (Nimonkar et al. 2011). WRN interacts with MRE11 and NBS1 (Cheng et al. 2004), and this results in the enhancement of WRN helicase activity. WRN also binds to DNA2 and facilitates extensive DNA resection (Sturzenegger et al. 2014). Moreover, cyclin-dependent kinase 1 (CDK1) phosphorylates at Ser1133 and promotes the DNA end resection by DNA2 at replication-related DSBs (Palermo et al. 2016). The Ser1133 phosphorylation of WRN is required for the interaction with the MRE11 complex. WRN also interacts with other HR proteins including RAD51, RAD54, RAD52, and BRCA1/BARD1 complex, and these interactions stimulate WRN helicase activity. These studies indicate the critical role of WRN also in HR (Lachapelle et al. 2011; Otterlei et al. 2006; Lu and Davis 2021) (Fig. 2.4).

2.2.3 WRN and Telomeres

WRN proteins are also involved in telomere maintenance. Telomeres are chromosome-terminating complexes composed of the characteristic repeating DNA sequence TTAGGG and a protein called the shelterin complex, which protects the DNA ends of chromosomes. Telomeres shorten with aging, and when telomeres exceed a certain length, cells irreversibly stop proliferating, leading to cellular senescence. The shelterin complex mainly consists of six proteins, TRF1, TRF2, RAP1, TIN2, TPP1, and POT1. WRN interacts with POT1 and TRF2 (Opresko et al. 2004) (Machwe et al. 2004), and this interaction facilitates WRN to resolve G4 quadruplex, holiday junctions formed at D- and T-loops of telomeres (Opresko 2008; Nora et al. 2010). WS patients aged 40–60 years show prominent telomere reductions compared to WS patients younger than 30 years or age-matched non-WS patients, indicating that the loss of WRN function leads to accelerated telomere

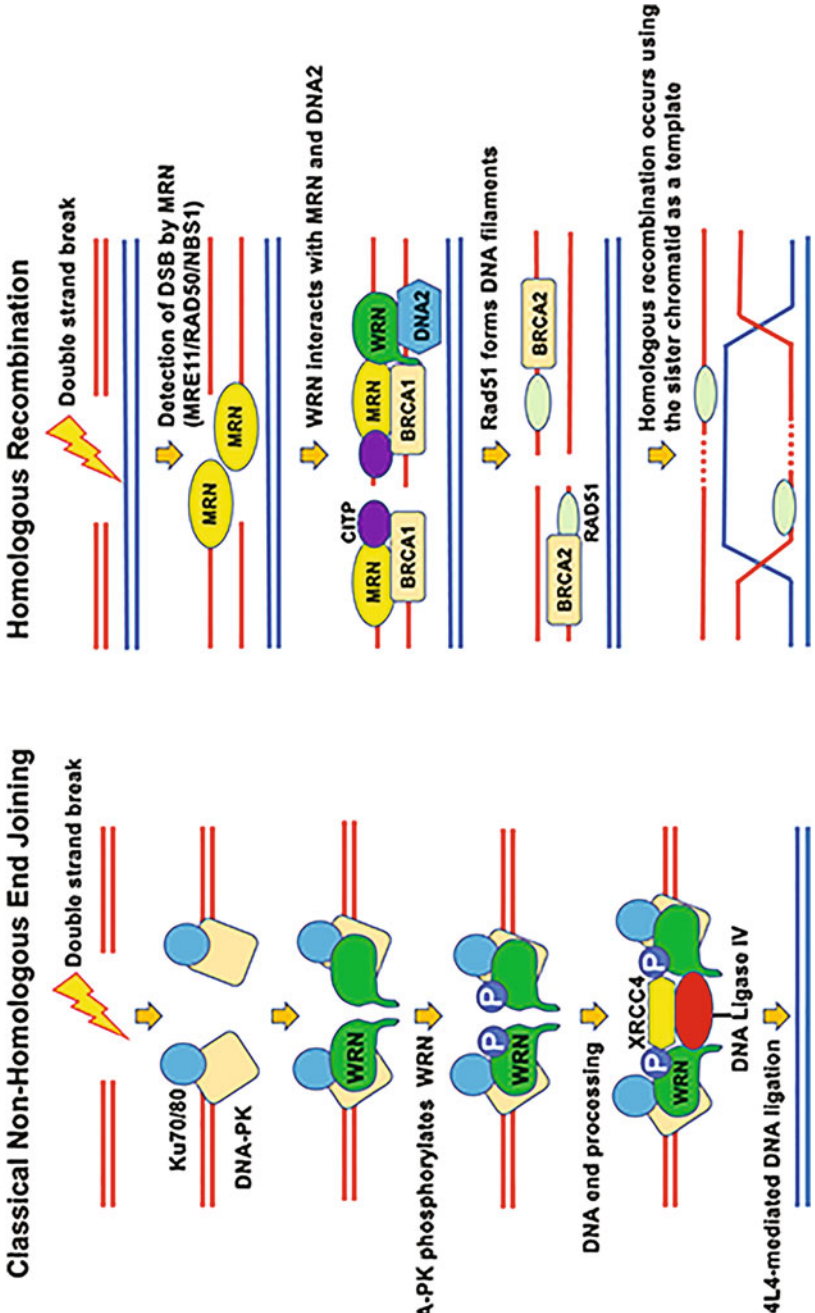

Fig. 2.4 A scheme of the roles of WRN in classical NHEJ and HR after DNA double-strand break

attrition (Ishikawa et al. 2011). In fibroblasts derived from WS patients and genetically created WRN-deficient cells, telomere loss leads to fusion, segregation defects, and instability of chromosomes (Laud et al. 2005; Crabbe et al. 2007). When WRN is mutated, the guanine tetramer produced during telomere replication cannot be unraveled, and the lagging strand of DNA synthesis is stopped, preventing replication and accelerating telomere shortening (Shimamoto et al. 2015). In fact, it has been reported that reintroduction of telomerase activity can inhibit telomere loss, new chromosomal aberrations, and cellular senescence in cells derived from WS patients. Therefore, at least part of the WS phenotype is thought to be due to telomere defects (Fig. 2.3) (Crabbe et al. 2007; Wyllie et al. 2000). At telomeres, SIRT6, one of the longevity genes, sirtuin, removes the acetylation of histone H3K9 in a nicotinamide adenine dinucleotide (NAD+)-dependent manner. SIRT6-mediated histone acetylation is required for WRN to bind to telomere chromatin (Michishita et al. 2008). On the other hand, shortening of telomeres was not observed in hepatocytes derived from WS patients, and keratinocytes in the skin retained telomerase activity and did not show replicative senescence (Ibrahim et al. 2016), suggesting that telomere damage caused by WRN deficiency may vary depending on the tissue (Tokita et al. 2016).

Concerning G4 quadruplex and WRN, in fibroblasts from WS patients, significant association was observed between G-quadruplex loci and the loci whose gene expressions were upregulated (Johnson et al. 2010). Significant enrichment of G4 motifs has also been observed at the transcription start site and 5′ end of first introns of genes downregulated in WS fibroblasts. WS fibroblasts display senescence-associated gene expression programs, disease-associated miRNAs, and dysregulation of canonical pathways that regulate cell signaling, genome stability, and tumorigenesis. Together, WRN regulates transcription by binding to G4-DNA motifs (Fig. 2.5).

In addition, we recently reported the difference of telomeres between regions of the body. The differences of gene expression profiles were compared in fibroblasts between the limbs and trunk in WS patients, in whom the trunk is relatively plump, but the limbs are extremely atrophic, leading to intractable skin ulcers. We found increased cellular senescence and shortened telomeres in the periphery compared to the abdomen. Interestingly, fibroblasts in the periphery restored osteo-differentiation capacity and markedly reduced adipogenicity, reflecting the pathogenesis of WS (Kato et al. 2021a).

2.2.4 WRN and Mitochondria, mTOR, and Autophagy

Cells from WS patients and genetically modified WRN-deficient cells show phenotypes with mitochondrial dysfunction. WRN depletion leads to a global alteration in the gene expressions that regulates energy production and redox condition. This change attenuates antioxidative defenses and increases mitochondrial oxygen consumption leading to increased reactive oxygen species (ROS) and oxidative DNA

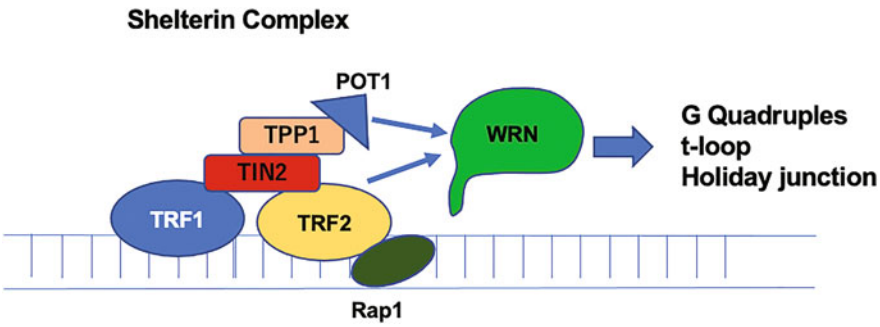

Fig. 2.5 The interaction between the shelterin complex and WRN: WRN plays a role in unraveling the G quadruples that occurs during telomere replication and the DNA loops called t-loop structures at telomeres. POT1 and TRF2, which are part of the telomeric shelterin complex, facilitate this function of WRN

damage. In cancer cells, this metabolic change counteracts the Warburg effect and results in the suppression of cell proliferation and senescence-like phenotype. This effect may be preferable in cancer settings, and a reason why WRN-deficient cells show the synthetic lethality that will be discussed later (Li et al. 2014). Cells that lack WRN accumulate oxidative bases including 8-oxoguanine, FapyG, and FapyA, and the double-strand break marker gamma H2AX, indicating the function of WRN in oxidative damage repair in the genome. NEIL1 is a mammalian DNA glycosylase required for repairing oxidatively damaged DNA bases. Upon exposure to oxidative stress, WRN binds to NEIL1 via the RQC region, colocalized in the nuclei, and functions to excise oxidative regions from bubble DNA substrates (Das et al. 2007). In 2019, Fang et al. reported impaired mitophagy, accumulation of ROS and depletion of NAD+, the co-enzyme of sirtuins, and many metabolic enzymes, in WS patient cells and WRN-deficient invertebrates. NAD+ repletion using nicotinamide riboside (NR) improves the transcriptome profile of the WRN-deficient worms and their mitochondrial quality through DCT and ULK1-dependent mitophagy. Moreover, NR extends the life span of *Caenorhabditis elegans* and *Drosophila melanogaster* models of WS. Thus, mitochondrial dysfunction is a critical mechanism for the accelerated aging phenotype of WS and simultaneously may be a potential therapeutic target (Fang et al. 2019).

The mammalian target of rapamycin (mTOR) is an essential signaling molecule that forms the hub of multiple metabolic pathways, and WRN may be involved in this pathway and autophagy. In WRN knockdown primary fibroblasts, increased autophagy was observed (Saha et al. 2014; Talaei et al. 2013). Short-term rapamycin treatment, which inhibits mTOR, increased autophagy activity, while long-term rapamycin treatment led to improved cell growth, reduced accumulation of DNA damage marker 53 BPI, improved nuclear morphology, and reduced autophagy markers LC3II and P62 (Saha et al. 2014). It is speculated that reduced protein aggregates by early enhancement of autophagy resulted in better cellular function.

2.2.5 *Phenotype of WRN KO Mice*

Although WS has a pronounced phenotype in humans, another feature of WRN is that there is almost no phenotype in deficient mice. The first WRN mutant mice were generated in 1998 by deleting the catalytic site of the helicase domain, but they did not show any signs of aging such as gray hair or short life span until 13 months of age. On the other hand, when heterozygotes were crossed, the birth rate of wild type: heterozygote:homozygote was 1:1.9:0.6, and the birth rate of KO mice was reduced. In addition, Embryonic stem cells derived from KO mice showed increased sensitivity to topoisomerase inhibitors such as camptothecin (Lebel and Leder 1998). Long-term observation of these mice showed mild shortening of life span after 20 months, the accumulation of ROS in the liver, and fatty liver (Massip et al. 2010). In contrast, Guarente's group created mice lacking exon 18 and later part of the WRN gene (most of the C-terminal side of the helicase domain), which resulted in no signs of aging. But, double KO mice of WRN KO with P53 KO, a molecule that protects the genome from DNA damage, showed shortened life span (Lombard et al. 2000). Interestingly, double KO of WRN KO mice with telomere RNA component-deficient mice, a noncoding RNA of the telomere maintenance complex, resulted in the development of aging phenotypes such as gray hair, cataracts, hunchback, and short life span after five to six generations (Chang et al. 2004). The mechanism of this minor phenotype of WRN KO mice is not clear, but it may be due to the redundancy of five subtypes of RecQ helicases that are complementary to the WRN deficiency, and the possibility that the DNA damage accumulation is insufficient because of the short life span. Intriguingly, telomerase activity is relatively high in mice. Because the phenotype of WS is telomere dependent, the relatively high telomerase activity in mice may mask the phenotype.

2.2.6 *WRN and Stem Cell Senescence and Epigenome Regulation*

WS is also involved in stem cell senescence and epigenetic regulation. As cells undergo senescence, the cytoplasm enlarges and the nucleus swells, and the traditional transcriptionally inactive heterochromatin structure loosens and becomes euchromatin dominant, a structure that facilitates transcription (Zhang et al. 2020). In 2015, Belmonte and colleagues at the Salk Institute generated WRN-deficient ES cells and found no abnormalities in their ability to differentiate into endoderm, mesoderm, and ectoderm, or in ES cell proliferation. Interestingly, when mesenchymal stem cells (MSCs) were induced from these WRN-deficient ES cells, they showed decreased proliferation; senescence-associated beta-Gal staining; expressions of p16 and p21 and other cell cycle-inhibitory senescence markers, IL6 and IL8, and other senescence-associated secretory phenotypes (SASPs). In addition, WRN protein interacts with histone methyltransferase complex proteins such as

SUV39H1, LAP2beta, and HP1alpha to regulate histone methylation H3K9me3 and H3K27me3. In WRN-deficient cells, this mechanism is impaired, and H3K9me3 and H3K27me3 are reduced, resulting in an aging phenotype (Zhang et al. 2015). WRN-deficient MSCs and MSCs derived from ES cells carrying the mutation of other early-onset progeroid syndrome, Hutchinson-Gilford progeria syndrome (HGPS), have been compared (Wu et al. 2018). HGPS is caused by a mutation of lamin A, shows symptoms of early-onset premature aging from infancy, and develops severe atherosclerosis leading to myocardial or cerebral infarction. Contrary to expectations, WRN-deficient MSCs show more early-onset cellular senescence than HGPS MSCs; however, the phenotype was much milder. The HGPS MSCs show late-onset acute premature aging phenotype, providing a useful tool that recapitulates the pathology of WS and HGPS. Using this system, a flavonoid, quercetin, and the antioxidative vitamin, vitamin C, were identified as compounds that retard cellular senescence of WS (Li et al. 2016; Geng et al. 2019).

In addition, CRISPR Cas9-based screen was performed using mesenchymal precursor cells that have pathogenic mutations for WS and Hutchinson-Gilford syndrome. The result identified KAT7, a histone acetyltransferase, as a factor whose deficiency alleviated cellular senescence. The lentivirus treatment encoding Cas9 and Kat7 targeting guide RNA improved hepatocyte senescence and liver aging and extended the life span in aged mice and progeroid model Zempste24 null mice, indicating that epigenetic modification may also be a target of antiaging interventions (Wang et al. 2021).

Epigenomic findings in WS patients have also been reported. Horvath et al. proposed the epigenetic clock, which estimates the aging of cells and tissues by analyzing DNA methylation in 391 CpG regions of the genome. Using this method to analyze DNA methylation in the whole blood of WS patients, the average age of methylation in WS is 6.4 years more than that in the healthy group (Maierhofer et al. 2017). However, further analysis showed that DNA methylation in WS did not show differences in retrotransposons such as LINE1 and ALU, which are reportedly altered during aging. This suggests that WS aging is partly different from the normal aging epigenome (Maierhofer et al. 2019).

2.2.7 WS Patient-Derived iPS Cells

As mentioned above, ES cells lacking WRN do not show an obvious phenotype, but then what about iPS cells created from WS patient cells that have accumulated DNA damage over a lifetime? In 2014, Shimamoto et al. established iPS cells from skin fibroblasts of WS patients, which had infinite proliferative potential for more than 2 years, maintained an undifferentiated state, and showed no increase in chromosomal aberrations during the culture period. On the other hand, when the cells were induced to differentiate into somatic cells, signs of premature senescence were observed. Furthermore, it was speculated that part of this phenotype was due to the extension of telomeres by reprogramming (Shimamoto et al. 2014). At the same

time, WS-specific iPS cells were also established in the National Institute of Health in the USA, and early senescence was observed when induced into MSCs, but not when differentiated into neural progenitor cells in the ectoderm. Recently, genomic gene correction was performed on iPS cells generated from a WS patient's blood using CRISPR/Cas9 technique and enabled the isogenic comparison between patient-derived iPS cells and normal iPS cells (Kato et al. 2021b). MSCs derived from WS-specific iPS cells showed decreased expression of various growth factors and reduced ability to differentiate into cartilage, bone, and fat (Tu et al. 2020). These abnormalities in MSCs and the relative preservation of other germ-derived tissues are consistent with the symptoms of WS, in which abnormalities are concentrated in mesenchymal tissues, resulting in subcutaneous tissue atrophy, visceral fat accumulation, myocardial infarction, and mesenchymal sarcoma, but not cognitive decline. In recent years, MSCs have shown promise as a cell therapy for a variety of conditions, including skin ulcers and diabetes, and may be a potential therapeutic strategy for WS.

2.2.8 Malignancy and WRN

Patients with WS have a high incidence of malignancy, as described in the previous section. The mechanism is considered to be genomic instability caused by the loss of WRN, leading to the accumulation of DNA damage and tumorigenesis. On the other hand, WRN is attracting attention as a synthetic lethal gene in cancer therapy, although it appears to be quite the opposite. Synthetic lethality refers to a phenomenon in which the loss of a single gene is not lethal to cells, but the simultaneous loss or disruption of two or more pathways causes cell death. In 2019, a CRISPR- or RNAi-based screen showed that tumors exhibiting microsatellite instability (MSI) caused by defective DNA mismatch repair showed a significant decrease in cell survival when WRN was deleted or suppressed. In these cells, the loss of WRN leads to DNA breaks and apoptosis. On the other hand, the survival of cancer cells without MSI remains unchanged even when WRN is reduced (van Wietmarschen et al. 2020; Chan et al. 2019). Furthermore, in 2021, a study using 60 preclinical cancer models with MSI reported that the phenomenon of cancer cell death after loss of WRN was widely observed. This means that WRN-targeted therapy may be effective in a wide range of settings, such as initially or after the acquisition of anticancer drug resistance in malignancies with MSI (Picco et al. 2021). These studies may suggest that WRN deficiency relatively suppress epithelial cancers. Indeed, increased chemotherapeutic activity of camptothecin in cancer cells by siRNA-induced silencing of WRN helicase has been reported in 2007 (Futami et al. 2007). Although these findings are about the function of the WRN gene in tumors, not in patients with WS, the fact that there is a biological advantage to suppressing the WRN gene may indicate the significance of the existence of a substantial population with heterozygous WRN mutations.

2.3 Conclusion

Although more than 20 years has passed since the identification of the WRN gene, it has been difficult to elucidate the pathogenesis of WS because of the lack of phenotype in WRN-deficient mice and the difficulty in culturing cells due to premature aging. In recent years, however, advances in genetic modification technology, material delivery systems, and stem cell biology have gradually revealed the molecular mechanisms of WS. On the other hand, the average life expectancy of patients with WS has extended due to the improved management of metabolic complications such as diabetes and dyslipidemia and advances in the treatment of intractable skin ulcers. The combined knowledge of clinical and basic medicine will lead to further improvement in the prognosis of WS, as well as to a better understanding of the mechanisms of aging and aging-related diseases.

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Chapter 3
Biomarkers of Healthy Longevity: Lessons from Supercentenarians in Japan

Yasumichi Arai and Nobuyoshi Hirose

Abstract As the global population ages, achieving a long and healthy life is becoming an increasingly important social issue. Since 1992, we have conducted the Tokyo Centenarians Study and, subsequently, the Japan semi-supercentenarians study to explore the biological, genetic, social, and environmental factors associated with healthy longevity. We have found a subset of centenarians who are independent in their activities of daily living and have a high probability of becoming supercentenarians (110 years or older). Identifying specific biomarkers conducive to healthy longevity in supercentenarians may provide insights into protective and/or delaying mechanisms against aging-related diseases. By using a unique dataset of 1427 elderly individuals, including 36 supercentenarians, 572 semi-supercentenarians (105–109 years), 288 centenarians (100–104 years), and 531 very old people (85–99 years), we found that N-terminal pro-B-type natriuretic peptide (NT-proBNP), interleukin-6, cystatin C, cholinesterase, and albumin were associated with all-cause mortality. Of these, low NT-proBNP levels were strongly associated with survival beyond 105 years, while albumin levels were associated with high mortality across all age groups. Results of single-cell RNA analyses showed that supercentenarians had an excess of cytotoxic CD4 T cells, which was unique to this exceptional population. In the near future, elucidation of the supercentenarian aging process by multi-omic approaches will provide valuable perspectives for developing translational clinical strategies for the prevention of age-related diseases and disabilities.

Keywords Centenarian · Supercentenarian · Biomarker · NT-proBNP · Albumin

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_3

3.1 Introduction

As the global population ages, achieving a long and healthy life is becoming an increasingly important social issue. In 1992, we started the Tokyo Centenarian Study (TCS) to explore the biological, genetic, and psychosocial factors associated with healthy longevity in centenarians (Gondo et al. 2006; Takayama et al. 2007). Many centenarians are independent in their activities of daily living (ADL) until their 90s. However, according to the TCS results, only about 20% of centenarians were independent at the age of 100 years or older, and the majority of centenarians needed some kind of assistance in their daily lives (Gondo et al. 2006). Subsequent follow-up studies have shown that centenarians who were physically independent had a high probability of becoming semi-supercentenarians (105 years or older) or even supercentenarians (110 years or older). In other words, ADL and survival are correlated in individuals well beyond the age of 100, and supercentenarians have an extremely long and healthy life expectancy. We started the Japan Semi-supercentenarian Study (JSS), a nationwide visiting survey, to recruit mainly those aged 105 years or older (Arai et al. 2014, 2015) to determine the genetic and biological factors of healthy longevity. Biomarkers generally refer to biological indicators that reflect the progress of a disease or the response to treatment and include biological substances such as proteins and genes contained in body fluids and tissues, such as blood and urine. The identification of specific biomarkers that faithfully reflect health indicators in supercentenarians will expand our knowledge on the biology of human longevity and improve the quality of medical care for age-related diseases and disabilities. In this chapter, we have summarized the previous and current findings on biomarkers for healthy longevity observed in the JSS.

3.2 Demography and Functional Status of Supercentenarians

Based on the increase in life expectancies over the recent decades, the world's population of centenarians is growing rapidly. However, the total number of supercentenarians is extremely limited, and only few countries have reported on their exact status. According to the 2015 census, the total population in Japan is approximately 127 billion including 61,763 centenarians. Of the centenarians, 3916 are over 105 years old, and 146 are over 110 years old. The ratio of females to males is 6.36 among all centenarians, 8.99 among the supercentenarians, and 15.22 among the supercentenarians, indicating that the ratio of females increases with age. The ratio of centenarians to the total population is 1 per 2000 individuals, while the ratio is 1 per 32,000 individuals for the semi-supercentenarians and 1 per 870,000 individuals for supercentenarians. Even in Japan, a country with the highest number

of the world's oldest elderly, the presence of supercentenarians is extremely rare. Compared to the results of the 2010 census, the number of people aged 100 years and above increased by 129% in the 5 years leading up to 2015, the number of people aged 105 years and above increased by 153%, and the number of people aged 110 years and above increased by 187%. Further analysis of demographic trends is needed to conclude whether the supercentenarians represent the limit of human longevity or whether environmental factors such as the introduction of long-term care insurance are at play. Maintaining physical independence and cognitive function is a major component of healthy longevity. In the TCS and the JSS, we classified 642 centenarians, aged 100 years or above, into 3 groups according to the age at death: (1) 100–104 years old (centenarians), (2) 105–109 years old (semi-supercentenarians), and (3) 110 years old and above (supercentenarians) (Arai et al. 2014). The results showed that the supercentenarians had a higher ADL (Barthel Index) and cognitive function (mini-mental state examination, MMSE) than the semi-supercentenarians or centenarians when assessed at the age of 100–104 years (Arai et al. 2014). When the supercentenarians' ADLs at age 105–109 were compared between the semi-supercentenarians and the centenarians, the Barthel Index was significantly higher in the supercentenarians. Those with a higher degree of independence in daily life had a longer life expectancy after the age of 100 years. The supercentenarians who reached the age of 110 or more had an extremely high degree of independence at the age of 100 years. Supercentenarians also had higher cognitive function (MMSE) at 100–104 years of age than the other 2 groups, and maintenance of cognitive function was significantly associated with independence in ADLs after 100 years of age (Arai et al. 2014). The New England Centenarian Study in the United States tracked the cognitive function of more than 1400 centenarians and showed that the onset of dementia and age-related cognitive decline was slower in centenarians, supercentenarians, and supercentenarians, in that order (Andersen 2012). These studies indicate that supercentenarians are characterized by an extraordinarily long life span with relatively high physical and cognitive functions.

3.3 Cardiovascular Biomarkers and Exceptional Survival

Aging is a dominant risk factor for most fatal diseases, such as cardiovascular disease, type 2 diabetes mellitus, Alzheimer's disease, and cancers (Kennedy et al. 2014). Of these, cardiovascular disease is a major cause of death and disability in older adults. We hypothesized that supercentenarians are able to approach the current human longevity limit by preventing or surviving cardiovascular diseases. To test our hypothesis, we examined the cardiometabolic risk factors, electrocardiogram (ECG) results, and a series of circulating biomarkers that reflected distinct cardioprotective and pathogenic pathways in a combined cohort of 1427 oldest individuals including 36 supercentenarians, 572 semi-supercentenarians

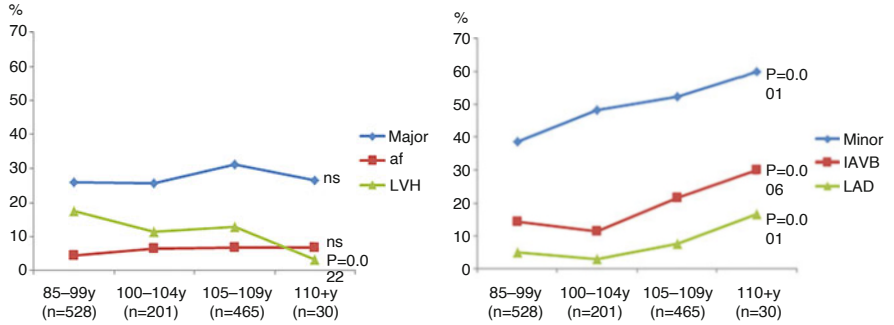

Fig. 3.1 ECG characteristics of the oldest individuals. Major abnormalities include past myocardial infarction, pacemaker rhythm, atrial fibrillation or flutter, left ventricular hypertrophy, advanced atrioventricular block, left bundle branch block, and Wolff-Parkinson-White syndrome. Minor abnormalities include nonspecific ST-T change, first-degree atrioventricular block, left anterior hemiblock, right bundle branch block, left axis deviation, sinus bradycardia, sinus tachycardia, low voltage in limb lead, poor progression, nonsignificant Q wave, premature atrial contractions, and premature ventricular contractions. (Constructed based on the results from Hirata et al. 2020)

(105–109 years old), 288 centenarians (100–104 years old), and 531 very old people (85–99 years old) (Hirata et al. 2020). In terms of traditional cardiovascular risk factors, the most striking feature of the centenarians is the low prevalence of diabetes mellitus (Table 3.1). The prevalence of diabetes among centenarians, semi-supercentenarians, and supercentenarians was 7.3%, 5.6%, and 5.6%, respectively, which was less than half of that among the very elderly population aged 85–99 years (18.6%) (Hirata et al. 2020). The prevalence of hypertension (being treated medically or diagnosed) in centenarians was also lower than that in the very old (62.9%) at 38.3%, 44.7%, and 38.9%, respectively. In contrast, centenarians show a relatively high prevalence of moderate-to-severe chronic kidney disease (i.e., stage 3b-5), suggesting that age-related decline in renal function becomes apparent beyond the age of 100 years. Regarding the electrocardiographic characteristics, the major abnormalities such as myocardial infarction, atrial fibrillation, and left ventricular hypertrophy were not common among the centenarian groups (Fig. 3.1 and Table 3.1). The most common ECG findings in supercentenarians were minor abnormalities, such as first-degree atrioventricular block (31.0%) and nonspecific ST-T change (27.6%), followed by left anterior hemiblock (20.7%). Overall, both centenarians and supercentenarians were characterized by low cardiovascular risks including low cholesterol levels and low prevalence of diabetes and left ventricular hypertrophy on ECG.

In our study, we tested nine circulating biomarkers that reflected distinct cardioprotective and pathogenic pathways in relation to mortality as follows: (1) four endogenous cardioprotective molecules [N-terminal pro-B-type natriuretic peptide (NT-proBNP), erythropoietin, adiponectin, and extracellular superoxide

Table 3.1 Demographic and clinical characteristics of centenarians, semi-supercentenarians, and supercentenarians compared to the very old

Characteristics	*N*	Very old (85–99 years)		*N*	Centenarians (100–104 years)		*N*	Semi-supercentenarians (105–109 years)		*N*	Supercentenarians (110+ years)		*p* for trend
Age at enrollment, years (IQR)	531	87.4	(86.3–88.8)	288	100.8	(100.2–102.3)	572	106.6	(105.8–107.4)	36	110.7	(110.4–111.3)	<0.001
Female, *n* (%)	531	298	(56.1%)	288	225	(78.1%)	572	502	(87.8%)	36	34	(94.4%)	<0.001
Current smoker, *n* (%)	511	36	(7.1%)	282	3	(1.1%)	564	7	(1.2%)	35	1	(2.9%)	<0.001
High education, *n* (%)	513	193	(37.6%)	275	61	(22.2%)	540	63	(11.7%)	34	3	(8.8%)	<0.001
Body mass index, kg/m^2	528	21.5	±3.2	187	19.5	±3.2	353	19.4	±3.3	21	18.4	±2.9	<0.001
Barthel index	529	95	±12	280	48	±35	564	28	±28	34	22	±25	<0.001
Mini-mental state examination	524	26.2	±4.1	243	13.9	±8.2	365	7.8	±7.5	26	5.2	±6.7	<0.001
Medical history													
Coronary heart disease, *n* (%)	531	53	(10.0%)	283	41	(14.5%)	566	78	(13.8%)	36	3	(8.3%)	0.124
Stroke, *n* (%)	531	92	(17.3%)	283	46	(16.3%)	566	123	(21.7%)	36	2	(5.6%)	0.268
Hypertension, *n* (%)	531	334	(62.9%)	287	110	(38.3%)	568	254	(44.7%)	36	14	(38.9%)	<0.001
Hyperlipidemia, *n* (%)	530	251	(47.4%)	288	40	(13.9%)	572	83	(14.5%)	36	8	(22.2%)	<0.001
Diabetes mellitus, *n* (%)	531	99	(18.6%)	288	21	(7.3%)	572	32	(5.6%)	36	2	(5.6%)	<0.001
Chronic kidney disease (stage 3b-5), *n* (%)	530	77	(14.5%)	288	101	(35.1%)	572	214	(37.4%)	36	11	(30.6%)	<0.001
Anemia, *n* (%)	531	231	(43.5%)	288	205	(71.2%)	572	387	(67.7%)	36	20	(55.6%)	<0.001
Medication													
Nitrate, *n* (%)	527	53	(10.1%)	279	39	(14.0%)	561	79	(14.1%)	32	3	(9.4%)	0.084
Oral anticoagulant, *n* (%)	527	20	(3.8%)	279	1	(0.4%)	561	6	(1.1%)	32	0	(0.0%)	<0.001
Antiarrhythmic drug, *n* (%)	527	21	(4.0%)	279	3	(1.1%)	561	9	(1.6%)	32	0	(0.0%)	0.007
Digoxin, *n* (%)	527	16	(3.0%)	279	11	(3.9%)	561	32	(5.7%)	32	1	(3.1%)	0.050

(continued)

Table 3.1 (continued)

Characteristics	*N*	Very old (85–99 years)		*N*	Centenarians (100–104 years)		*N*	Semi-supercentenarians (105–109 years)		*N*	Supercentenarians (110+ years)		*p* for trend
Diuretics, *n* (%)	527	61	(11.6%)	279	62	(22.2%)	561	166	(29.6%)	32	9	(28.1%)	<0.001
Calcium-channel blocker, *n* (%)	527	213	(40.4%)	279	47	(16.9%)	561	101	(18.0%)	32	3	(9.4%)	<0.001
ACE inhibitor or ARB, *n* (%)	527	157	(29.8%)	279	26	(9.3%)	561	70	(12.5%)	32	6	(18.8%)	<0.001
Beta-blocker, *n* (%)	527	47	(8.9%)	279	4	(1.4%)	561	7	(1.3%)	32	0	(0.0%)	<0.001
Antiplatelet, *n* (%)	527	141	(26.8%)	279	25	(9.0%)	561	60	(10.7%)	32	1	(3.1%)	<0.001
Statin, *n* (%)	527	81	(15.4%)	279	5	(1.8%)	561	10	(1.8%)	32	1	(3.1%)	<0.001
No circulatory drugs, *n* (%)	527	167	(31.7%)	279	136	(48.8%)	561	262	(46.7%)	32	18	(56.3%)	<0.001
Electrocardiogram													
Normal, *n* (%)	521	151	(29.0%)	193	41	(21.2%)	453	57	(12.6%)	29	4	(13.8%)	<0.001
Old myocardial infarction, *n* (%)	521	21	(4.0%)	193	8	(4.2%)	453	52	(11.5%)	29	4	(13.8%)	<0.001
Pacemaker, *n* (%)	521	6	(1.2%)	193	3	(1.6%)	453	5	(1.1%)	29	2	(6.9%)	0.409
Atrial fibrillation, *n* (%)	521	23	(4.4%)	193	13	(6.7%)	453	29	(6.4%)	29	1	(3.5%)	0.257
Atrial flutter, *n* (%)	521	2	(0.4%)	193	1	(0.5%)	453	2	(0.4%)	29	0	(0.0%)	0.984
Left ventricular hypertrophy, *n* (%)	521	90	(17.3%)	193	21	(10.9%)	453	56	(12.4%)	29	1	(3.5%)	0.008
Advanced atrioventricular block, *n* (%)	521	0	(0.0%)	193	2	(1.0%)	453	5	(1.1%)	29	0	(0.0%)	0.045
Major abnormality, *n* (%)	521	134	(25.7%)	193	50	(25.9%)	453	139	(30.7%)	29	7	(24.1%)	0.140
Non-specific ST-T change, *n* (%)	521	87	(16.7%)	193	53	(27.5%)	453	126	(27.8%)	29	8	(27.6%)	<0.001
First-degree atrioventricular block, *n* (%)	521	74	(14.2%)	193	20	(10.4%)	453	99	(21.9%)	29	9	(31.0%)	<0.001
Left anterior hemiblock, *n* (%)	521	30	(5.8%)	193	7	(3.6%)	453	44	(9.7%)	29	6	(20.7%)	0.002
Minor abnormality, *n* (%)	521	236	(45.3%)	193	102	(52.9%)	453	257	(56.7%)	29	18	(62.1%)	<0.001

IQR interquartile range, *ACE* angiotensin-converting enzyme, *ARB* angiotensin II receptor blocker

Plus-minus values are means ± SD. Trends in each characteristic of participants across four age groups were analyzed using the trend test for continuous variables, and the Cochran-Armitage test for trend for categorical variables (Modified citation to Hirata et al. 2020)

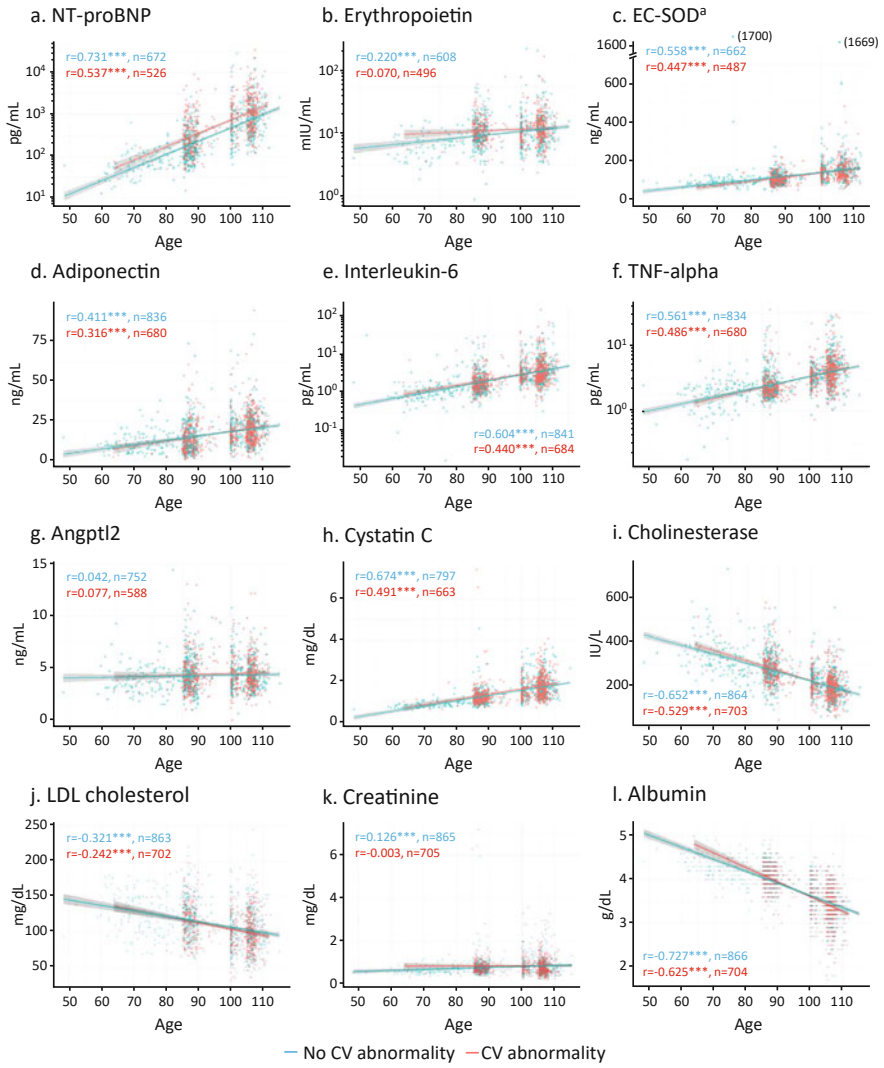

Fig. 3.2 Cross-sectional associations between circulating biomarkers and age by cardiovascular status. Scatter plots showing cross-sectional associations between biomarkers of cardioprotective pathways (**a**–**d**), inflammation (**e**–**g**), organ reserve (**h**, **i**), and select traditional risk factors (**j**–**l**), and age at enrollment, according to the presence (red) or absence (blue) of a cardiovascular abnormality. All the biomarkers were assessed at the time of enrollment. Spearman's correlation coefficients between biomarkers and age at enrollment and sample numbers are shown for those with (red) or without (blue) cardiovascular abnormality. The solid lines represent the correlation lines, and the shaded area represents the 95% confidence interval of the correlation line. Unrelated family members of the centenarians (spouses of the first-degree offspring of the centenarians) aged between 48 and 94 years (mean age, 73.1 years) were included as a younger control group ($n = 167$ at the maximum). Characteristics of this population are described in Hirata et al. 2020. Population sizes for the 12 biomarkers differ due to variations in the bio-banking of the samples. Participants were considered to have a cardiovascular abnormality when one or more of the following criteria were fulfilled: (1) a history of coronary heart disease or stroke, (2) cardiovascular medication use (i.e., nitrate, oral anticoagulant, antiarrhythmic drug, or digoxin), and (3) a major

dismutase (EC-SOD)]; (2) three inflammatory mediators [interleukin-6, tumor necrosis factor-alpha (TNF-alpha), and angiopoietin-like protein 2 (Angptl2)]; and (3) indicators of the functional reserves of the kidneys and liver [cystatin C and cholinesterase]. As shown in Fig. 3.2, plasma levels of endogenous cardioprotective molecules and inflammatory mediators, except Angptl2, were correlated with ages up to 115 years. Of these, only NT-proBNP showed age-related distributions, with or without a cardiovascular abnormality, supporting this biomarker's sensitivity for cardiovascular disease up to extreme old age.

Using multivariate Cox hazard models, we identified that high levels of NT-proBNP, interleukin-6, and cystatin C and low levels of cholinesterase and albumin were associated with an increased risk of all-cause mortality in the oldest people. In particular, NT-proBNP was strongly associated with survival beyond 105 years of age. In contrast, plasma albumin, a biomarker of nutrition, was consistently associated with mortality across all age groups.

In a retrospective analysis, where we classified our centenarians into three groups according to age at death (decedent centenarians who died between 100 and 104 years, decedent semi-supercentenarians who died between 105 and 109 years, and decedent supercentenarians who died at 110 years or above), we examined the correlations between the levels of prognostic biomarkers and age at enrollment across the three groups (Fig. 3.3). Only NT-proBNP showed age-specific distributions capable of distinguishing decedent supercentenarians from the younger cohorts (Fig 3.3a). These findings indicate that NT-proBNP levels were significantly lower in decedent supercentenarians than in other decedent centenarians at any given age at assessment. Based on these results, we proposed a working hypothesis that intrinsic aging of the cardiovascular system and possibly the renal system may ultimately deteriorate hemodynamic homeostasis and subsequently limit current human longevity (Fig. 3.4). Supercentenarians, by virtue of a postponed age-related increase in circulating NT-proBNP, may be equipped with efficient mechanisms for delaying the processes of cardiovascular aging. Future studies incorporating detailed assessments of the cardiac structure and functions using ultrasound cardiography are warranted to further validate this hypothesis.

Fig. 3.2 (continued) electrocardiographic abnormality (Table 3.1). The classification of cardiovascular abnormality in the unrelated family of centenarians was based on medical history and medication lists because of a lack of ECG assessment in this population. *NT-proBNP* N-terminal pro-brain natriuretic peptide, *EC-SOD* extracellular superoxide dismutase, *TNF-alpha* tumor necrosis factor-alpha, *Angptl2* angiopoietin-like protein 2. [a]Only individuals with 213RR genotype (noncarrier) in SOD3 (rs1799895) were included in the analysis. *$p < 0.001$. (Adopted from Hirata et al. 2020)

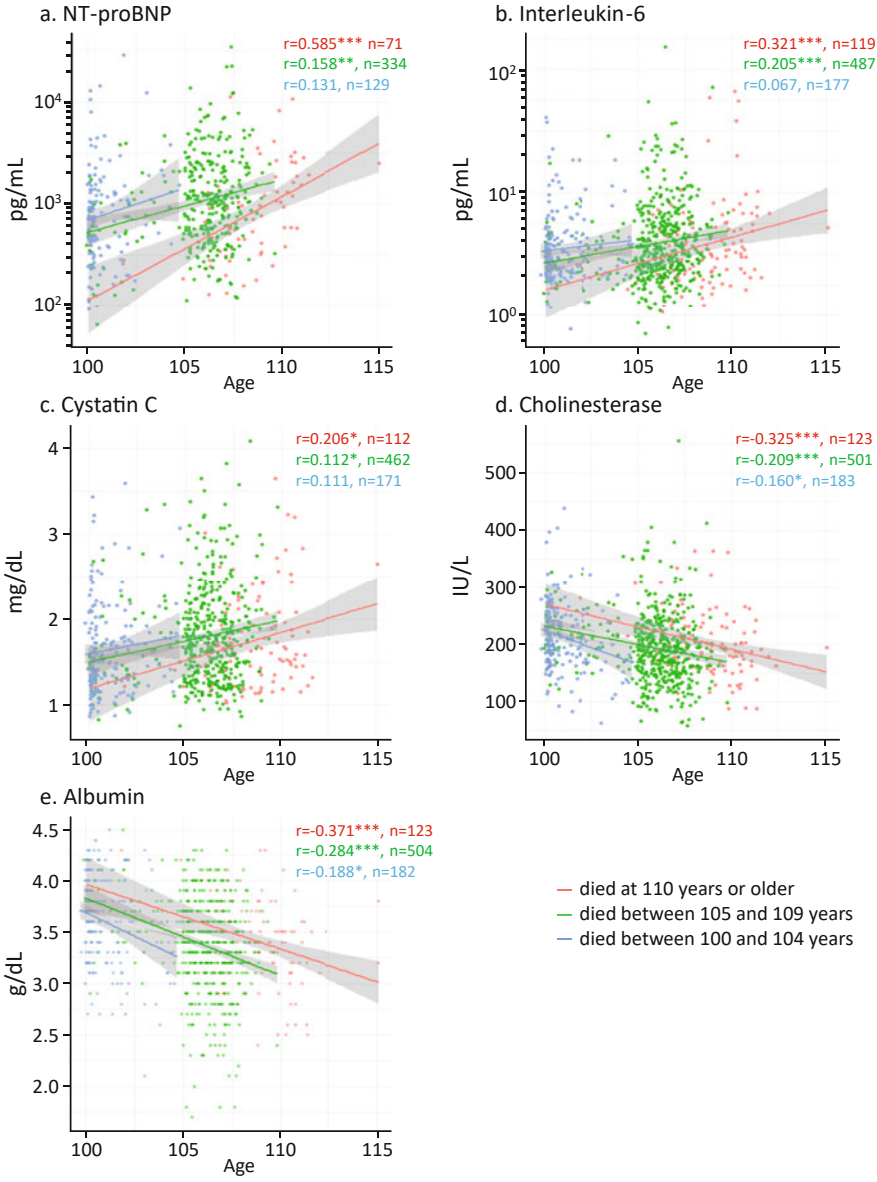

Fig. 3.3 Prognostic biomarkers in centenarians stratified by the age at death. Spearman's correlation coefficients of the relationships between prognostic biomarkers (NT-proBNP, interleukin 6, cyctatin C, and cholinesterase (**a-d**) identified in Fig. 3.4 and albumin (**e**) and age at enrollment were calculated for the three decedent centenarian groups: decedent centenarians (died between 100 and 104 years, blue line), decedent semi-supercentenarians (died between 105 and 109 years, green line), and decedent supercentenarians (died at ≥110 years, red line)). The shaded area represents the 95% confidence interval of the correlation line. All the biomarkers were assessed at the time of enrollment. Population sizes for the five biomarkers differ due to variations in the bio-banking of the samples. NT-proBNP indicates N-terminal pro-brain natriuretic peptide. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. (Adopted from Hirata et al. 2020)

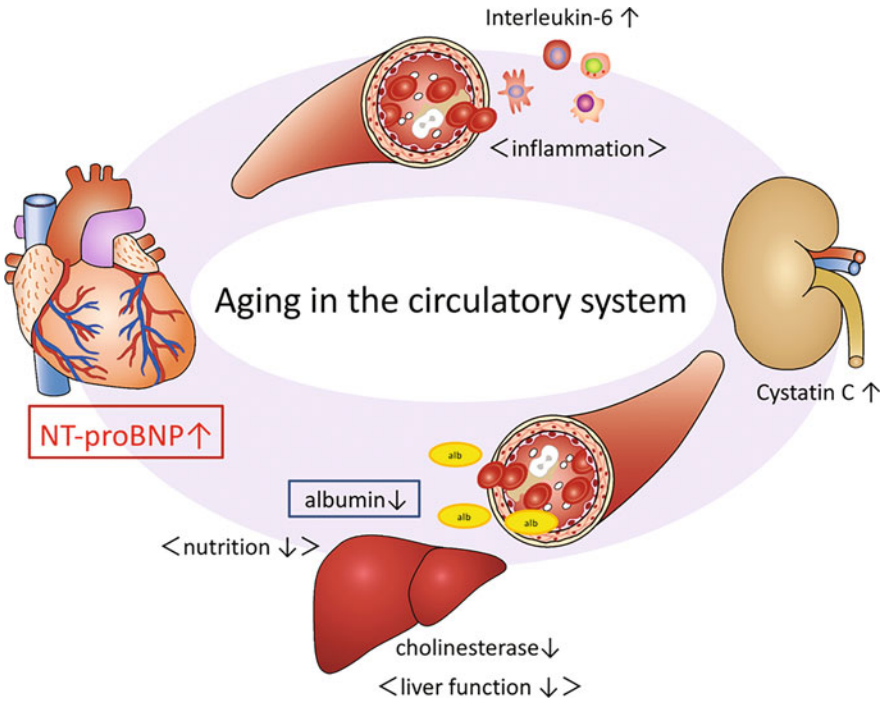

Fig. 3.4 Slow cardiovascular aging as a key biological pathway to healthy longevity (working hypothesis). Based on the observed association between cardiovascular biomarkers and exceptional survival, we hypothesized that the disruption of circulatory homeostasis due to age-related decline in cardiac and renal function may underlie the high levels of NT-proBNP in supercentenarians and that intrinsic aging of the cardiovascular system and possibly the renal system may limit current human longevity. In order to test our working hypothesis, ultrasound cardiography and histopathological analysis of cardiovascular system in the oldest old is necessary. Elucidating regulatory mechanism of cardiovascular aging in supercentenarians may lead to the development of prevention of age-related heart diseases such as heart failure with preserved ejection fraction (HFPEF)

3.4 Adiponectin

Advancing age is frequently associated with impaired glucose tolerance, insulin resistance, and the development of diabetes, predominantly type 2 diabetes. Nevertheless, several studies have demonstrated that the low prevalence of diabetes and preserved insulin sensitivity are the metabolic peculiarities of centenarians (Paolisso et al. 1996; Wijsman et al. 2011), suggesting that these may have a possible protective factor to maintain insulin sensitivity and glucose homeostasis. To date, vigorous basic research has been conducted on the mechanism underlying the association between insulin sensitivity and longevity. Adiponectin, one of the adipokines secreted from the adipose tissue, has emerged as a possible mechanistic link (Matsuzawa 2006; Rasouli et al. 2008). Adiponectin is an immensely beneficial adipokine (Matsuzawa et al. 2004). It plays an antidiabetic role within the liver and

skeletal muscles by facilitating glucose uptake at these sites, thereby enhancing insulin sensitivity. Adiponectin also has anti-inflammatory and anti-atherogenic properties. Several observational studies including our study have shown high plasma adiponectin levels in centenarians (Bik et al. 2006; Arai et al. 2006; Atzmon et al. 2008). These findings support the beneficial metabolic effects of this adipokine.

Interestingly, accumulating observational studies have demonstrated an unexpected association between high adiponectin levels and increased mortality in patients with cardiovascular diseases, particularly those with heart failure (Kistorp et al. 2005). This finding is counterintuitive to its salutary metabolic effects and is referred to as the "adiponectin paradox." A meta-analysis of 24 prospective studies suggested that the paradoxical association between high adiponectin levels and increased all-cause mortality risk is more significant in those with coronary heart disease (CHD) at the baseline than in those without CHD (Sook Lee et al. 2013). If that is the case, how should we interpret the high adiponectin levels in centenarians? To answer this question, we first examined the correlation between plasma adiponectin levels and cardiometabolic biomarkers in the centenarians. High adiponectin levels were correlated with high levels of high-density lipoprotein (HDL) cholesterol and low levels of HbA1c. This was compatible with this adipokine's beneficial metabolic effects. High adiponectin levels were also correlated with high levels of EC-SOD, an antioxidant enzyme in extracellular fluids (Sasaki et al. 2021), suggesting potential coordination of the anti-inflammatory and antioxidative pathways. To examine the prognostic significance of adiponectin, we investigated the association between adiponectin and mortality in a combined cohort with 1427 oldest individuals (Hirata et al. 2020). Intriguingly, high adiponectin levels were associated with high mortality in the very old, aged 85–99 years. This finding reflects the "adiponectin paradox." In contrast, high adiponectin levels were associated with low mortality in centenarians aged 100–104 years but not associated with mortality in semi-supercentenarians aged 105 years or above (Hirata et al. 2020). These results suggest that the association between adiponectin and mortality is complicated in the oldest individuals.

Some aspects of the complicated relationship between adiponectin and health outcomes remain unresolved. Based on the findings so far, we hypothesized that high adiponectin levels in centenarians may reflect the compensatory response to maintain metabolic and redox homeostasis and to counteract oxidative stress and inflammation, which are relevant in catabolic states, such as sarcopenia and chronic heart failure (Arai et al. 2019). Further longitudinal studies with sequential measurements of adiponectin and other biomarkers are warranted to gain a better understanding of the role of adiponectin in promoting healthy aging and longevity.

3.5 Immunological Biomarkers of Healthy Longevity

Over the past three decades, observational findings have shown that centenarians are relatively immune to illnesses, such as infections and cancers, during their entire lifetime. These findings have led to immunological investigations, one of the most

exciting topics in centenarian study. Recently, leukocyte telomere length (LTL), an indicator of senescence in circulating immunological cells, has been recognized as a promising biomarker of aging and age-related diseases (Demanelis et al. 2020). In collaboration with Prof. von Zglinicki of Newcastle University, we measured LTL in 684 centenarians and semi-supercentenarians, 167 pairs of centenarian offspring and their spouses, and 536 community-dwelling very old individuals, aged 85–99 years (Arai et al. 2015). Among the unrelated individuals, LTL shortened with age up to 100 years at rates of 21 $\pm$ 8 (males) and 29 $\pm$ 4 (females) bp/year. However, after 100 years of age, LTL increased in length by 59 $\pm$ 25 (males) and 48 $\pm$ 11 (females) bp/year. Interestingly, LTL in centenarian offspring was maintained for more than 20 years at a length corresponding to 60 years of age in the general population. LTL from centenarians and their offspring, and, especially, from semi-supercentenarians, was significantly longer than that expected for their age. LTL was not associated with ADL, cognitive functions, or life expectancy in the centenarians. However, we found that it was correlated with the CD28-positive cell rate, which indicated the extent of aging of the lymphocytes. The higher the CD28-positive cell rate, the longer the life expectancy above 105 years. These results suggest that slow immune senescence may be related to the maintenance of telomere length and longevity in semi-supercentenarians, demonstrating the usefulness of biomarker research in elucidating the mechanisms of healthy longevity.

Single-cell RNA sequencing is a powerful new technology that enables unbiased, high-throughput, and high-resolution transcriptomic analysis of individual cells. Given the importance of cell-to-cell variations, investigation at the single-cell level rather than a group of cells and consideration of the average can provide great insights into the biological process of extreme longevity. Recently, Hashimoto et al. used single-cell RNA analysis to study circulating immune cells from a group of supercentenarians and younger controls. They acquired a total of 41,208 cells from 7 supercentenarians (an average of 5887 per person) and 19,994 cells (an average of 3999 per person) from 5 controls in their 50s to 80s (Hashimoto et al. 2019). They found that while the number of B cells was lower in the supercentenarians, the number of T cells was approximately the same. Moreover, the number of cytotoxic CD4-positive T cells was considerably increased in the supercentenarians. Normally, CD8-positive T cells exert cytotoxic activity, and CD4 T cells do not. As such, the finding suggests that the CD4-positive cells of supercentenarians had acquired cytotoxic status. Hashimoto et al. examined the blood cells of two supercentenarians in detail and found that they had arisen from a process of clonal expansion. Although the pathophysiological roles of CD4 cytotoxic cells in supercentenarians and the clinical implications remain unclear, the study showed how single-cell transcription analysis can contribute to our understanding of immunological pathways associated with healthy longevity.

3.6 Future Prospects

So far, phenotyping and biomarker studies have shown that slow aging in the cardiovascular, renal, nervous (cognition), and musculoskeletal (ADL) systems is the main characteristic of supercentenarians. There remains a gap between our results and innovations in health promotion and preventive medicine for the general elderly population. In recent years, basic aging research using genetically modified model organisms, such as yeast, nematodes, and mice, has identified evolutionarily conserved signal transduction systems and molecular mechanisms that regulate aging and life span (López-Otín et al. 2013). Elucidation of ultimate aging process of supercentenarians will provide valuable perspectives for developing translational strategies for clinical application of the findings from longevity models. Moreover, multi-omic analyses, such as whole-genome sequencing, transcriptome, and metabolome analyses, are becoming more common. These may replace conventional approach by focusing on the candidate biomarkers that we described in this chapter. The "epigenetic clock" that predicts age by the methylation status of the hundreds of CpG region is a promising biomarker of biological aging (Horvath et al. 2015). Applying multi-omic technology and unraveling the molecular and genetic basis of slow aging in supercentenarians may contribute to the identification of therapeutic targets for the prevention or delay of age-related diseases, particularly cardiovascular diseases, in aging.

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Part III
Cellular Aging and Lower Animal Models

Chapter 4
Cellular Aging and Metabolites in Aging

Hiroshi Kondoh, Takumi Mikawa, and Masahiro Kameda

Abstract Diversity is observed in the wave of global aging, as aging is a complex biological process exhibiting individual variability. To assess aging physiologically, markers for biological aging is required in addition to the calendar age. The discovery of replicative senescence by Hayflick developed into telomere research, while stress-induced senescence (SIS) is known as a telomere-independent event. SIS serves as physiological barrier to oncogenesis in vivo, while it activates senescence-associated secretary phenotype (SASP), inducing chronic inflammation. Thus, beside telomere length, p16, p53, and inflammatory cytokines have been utilized as biomarkers for cellular senescence. From a metabolic perspective, the aging hypothesis includes the mitochondrial and the calorie restriction (CR) hypothesis. However, little is known whether CR is applicable to human longevity, as human aging is greatly affected by a variety of factors. Comprehensive analysis of the human blood metabolome captures complex changes with individual difference, detecting metabolites for aging or aging-relevant conditions. These information are valuable for future clinical applications in diseases relevant to aging.

Keywords Telomere · Stress-induced senescence · SASP · Metabolites · Frailty · Fasting · Antioxidant

4.1 Introduction

Currently, Japan is the only country in the world with a societal aging rate (% of population over 65 years old) exceeding 25% (global average = 12.38%) (ONU 2015). However, a wave of global aging is steadily approaching, and the global average is expected to reach 23.4% by around 2050. Indeed, many European and Asian countries will reach aging rates of more than 25%, while Japan is expected to exceed 42.5% by that time (ONU 2015).

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_4

In such super-aging society, we observe greater "diversity of aging." As the number of healthy elderly people increases, the number of bedridden patients and people who need long-term nursing care also increases. Because "diversity of aging" reflects individual variability, clinical symptoms among the elderly are also diverse. Thus, "aging" is multifaceted and complex, and variability is characteristic of aging.

Calendar age has commonly been used as an indicator of human aging. Calendar age defines the elderly as over 60 or 65 years old, proposed by the United Nations in 1956. In addition to calendar age, the importance of "biological age" has also been proposed, mainly based on findings in basic aging research, as we are now facing with diversification of the elderly in globally aging society.

As redefinition of "elderly" itself is becoming necessary (the proposal of the Japanese Geriatrics Society in 2017), here we overview the progress both in the cellular aging and the metabolomic aspects of aging.

4.2 Historical Theory and Replicative Senescence

In 1882, August Weismann proposed the "wear and tear" theory as the earliest aging model (Kirkwood and Cremer 1982). In this theory, the major cause of organismal aging is speculated to be accumulated damage due to stresses, followed by functional exhaustion of organs and cells; during juvenile stages, the ability to recover from damage is preserved, which gradually declines and vanishes in adulthood.

Weismann also presented the first experimental evidence of germ cell immortality (Weismann 1892). First, he cut off the tails of young mice before they achieved sexual maturity. When later bred, the tails of their offspring were perfectly normal. He repeated this process for 22 generations and observed that the acquired tail abnormality was never inherited. He drew the conclusion that the modulated properties in somatic cells in vivo were never inherited due to their definite life spans, while the genetic properties in reproductive germ cells are transmitted from generation to generation due to their potential immortality. Terminally differentiated somatic cells in vivo could be vulnerable to various stresses and eventually enter senescence state, during aging. In 1891, Weismann proposed that once somatic cells are isolated from the body, they do not senesce.

When cell culturing in vitro became feasible, Carrel and Ebeling tested Weismann's idea. They reported that fibroblasts from fetal chicken hearts could grow on laboratory glassware for at least 34 years under certain conditions (Carrel and Ebeling 1921), supporting Weismann's hypothesis. However, their experiments proved to be irreproducible because their primary cell cultures became contaminated by unnoticed cells in the culture medium, which was changed once a week. This created the appearance of continuous cell growth for several decades (Witkowski 1980).

Later, Hayflick also addressed the question of whether cells senesce in vitro. He observed that during serial culturing in vitro, human primary fibroblasts divided a

certain number of times, but eventually stopped dividing. This phenomenon became known as the Hayflick limit or "replicative senescence" (Hayflick 1965).

4.3 Telomere-Dependent and Telomere-Independent Senescence

Around the same time, James D. Watson, who discovered the DNA double helix, predicted that genomic DNA is shortened during chromosome replication (Wellinger 2014). He proposed that the ends of genomic DNA are not replicated during cell division, since the machinery for DNA replication progresses only in the 5′ to 3′ direction on the "antiparallel" DNA strand. This notion regarding the behavior of chromosome end structure led to the concept of "telomeres."

In 1986, Cooke et al. reported that the ends of sex chromosomes are shortened in senescent human somatic cells, consistent with Watson's idea (Cooke and Smith 1986). Strikingly, repetitive "TTAGGG" nucleotide sequences were found in the ends of chromosomes (Blackburn and Gall 1978). Telomeric sequences become shorter as cells undergo senescence, implying that the Hayflick limit would be linked to the shortening of telomeres. Later, telomerase was identified, which functions for the maintenance of telomere length by adding a specific DNA sequence to telomeres (Greider and Blackburn 1985). Finally, Shay and Wright reported that the ectopic expression of telomerase immortalizes human primary cells (Bodnar et al. 1998), providing definite evidence that telomere length is the causal factor for cellular senescence. Thus, telomeres have come to be called "biological clocks" or "tickets for aging."

The identification of telomere raises another question. Telomere length varies greatly, depending on the species; 25–50 kb for mice and 10–15 kb for humans (Whittemore et al. 2019). However, the in vitro life span of rodent primary cells is much less than that of human cells, indicating that telomere shortening is not the only factor for limiting life span. Indeed, even in primary cells with sufficient telomere length, cellular senescence is induced by stress due to harmful substances and by environmental factors, such as DNA damage, acid, oxidative stress, histone deacetylase inhibitors, culture stress, etc. These telomere-independent factors collectively cause stress-induced senescence (SIS) (Sherr and DePinho 2000).

Ras-val12 is a point mutation often observed in cancer cells. Manuel Serrano et al. noticed that in sharp contrast to normal cells, the arrested primary cells by the introduction of Ras-val12 displayed a "fried egg" morphology with a huge nucleus and enlarged cytoplasm, resembling senescent cells. Consistently, Ras-val12 provoked premature senescence, called oncogene-induced senescence (OIS), a subtype of SIS (Serrano et al. 1997).

4.4 Double-Edged Sword of SIS

Cancer cells exhibit multiple oncogenic mutations. In the experimental settings, the combination of activated Ras-val12 and ablated tumor suppressor p53 renders the primary cells escape from senescence, like cancerous cells. The reasonable explanation for this senescence-bypassing event is that OIS, a subtype of SIS, is a biological protective mechanism that suppresses carcinogenesis by preventing cells with oncogenic mutations from progressing into cancer cells. However, its physiological impact in vivo was doubted when Ras-val12 knock-in mice were reported as cancer prone (Johnson et al. 2001). Indeed, both types of tumor, benign and malignant, coexist in Ras-val12 knock-in mice. Benign tumors consist of senescent cells, while senescent cells disappeared in malignant lesions (Collado et al. 2005). These findings in vivo are consistent with the in vitro inhibitory effect against cancerous immortalization by SIS or OIS.

Recently, a close association between SIS and chronic inflammation has been discovered. Senescent cells produce more inflammatory cytokines (e.g., IL-1 and IL-6) than non-senescent cells, a state defined as "senescence associated secretary phenotype" (SASP) (Coppe et al. 2008). SASP promotes senescence of neighboring cells. In such contexts, "chronic inflammation" provoked by cellular senescence has a significant influence on organismal aging (Freund et al. 2010). Thus, SIS serves as "double-edged sword": the suppression of cancer in vivo vs. the promotion of deleterious chronic inflammation. Interestingly, Nuclear factor-κB (NF-κB) was identified as a highly activated transcription factor during cellular senescence (Fig. 4.2b). NF-κB activation induces anti-apoptosis genes, Bcl-2 and Xiap, followed by resistance of senescent cells against cell death. However, NF-κB also activates inflammatory responses in senescent cells (Adler et al. 2007). Thus, "chronic inflammation due to cellular senescence (SASP)" exerts causal effect on the progression of age-related diseases.

4.5 Senescence Markers

The irreversible cell cycle arrest with a metabolically active state is one of the prominent features of cellular senescence (Campisi 2013). In senescent cells, the enzymatic activity of cyclin-dependent kinase (CDK), a driver for cell cycle, is greatly diminished (Campisi and d'Adda di Fagagna 2007). Comparative analysis of protein profiles between senescent and non-senescent cells identified CDK inhibitors, p21 and p16Ink4a protein, as senescence markers (Fig. 4.1). Thus, in senescent cells, accumulated p21 and p16 Ink4a inhibits cell cycle progression and causes cell cycle arrest.

The other several markers for cellular aging were also identified, in addition to telomere length and "fried-egg"-like morphology. Senescence-associated beta-galactosidase (SA-β-gal) staining efficiently detects senescent cells (Dimri et al.

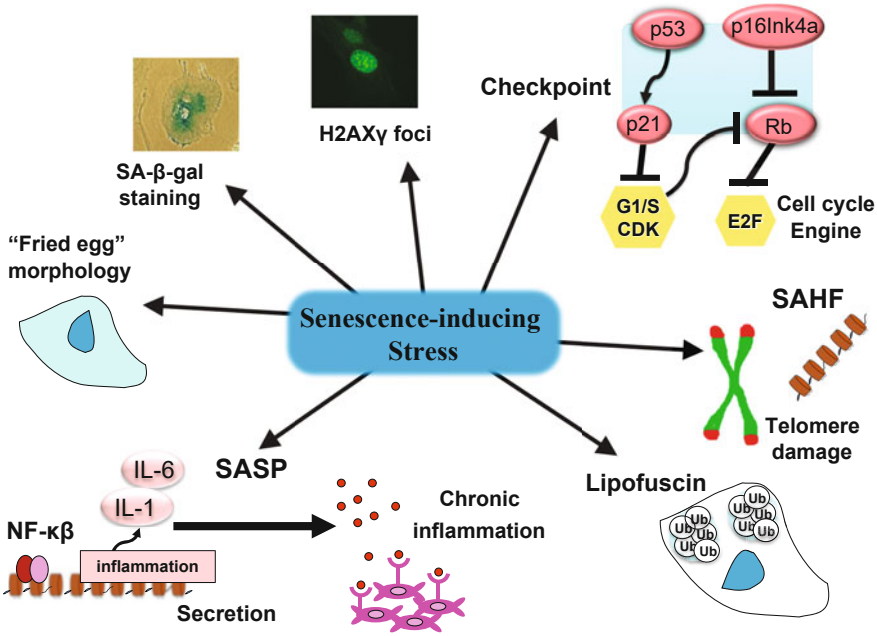

Fig. 4.1 Hallmarks of stress-induced senescence (SIS). Senescence-inducing stress provokes several features of cellular senescence: "fried egg" morphology, positive staining of SA-β-gal, H2AXγ foci formation, senescence-associated heterochromatin foci (SAHF), and others. Cyclin-dependent kinases (CDKs) drive the progression of the cell cycle, while CDK inhibitors, checkpoint genes, exert an opposite effect. As senescent cells suffer permanent cell cycle arrest, p21 and p16Ink4a, senescence markers, accumulate. Moreover, the activation of transcription factor NF-κB in senescent cells induces inflammation-related genes. Such inflammatory cytokines are secreted from senescent cells (senescence-associated secretary phenotype; SASP), which aggravates chronic inflammation in surrounding tissues

1995). Senescent nuclei are frequently accompanied with phosphorylated histone 2AX variant (H2AXγ) foci as results of DNA damage and senescence-associated heterochromatin foci (SAHF) (Narita et al. 2003). Accumulated lipofuscins are sometimes observed in senescent cytoplasm, representing aggregates of ubiquitinated proteins (Moreno-Garcia et al. 2018). These cellular senescence markers are useful device to evaluate proliferative limits of senescence as an index. In addition, inflammatory cytokines are also recognized as markers of both senescence and chronic inflammation.

4.6 The Aging Hypothesis Relevant to Metabolic Profiles

Oxidative stress has been proposed as a cause of aging and more recently as a factor involved in chronic inflammation. "Free radical theory" is one of the most popular aging hypotheses (Harman 1956). Damage to macromolecules, including telomeric

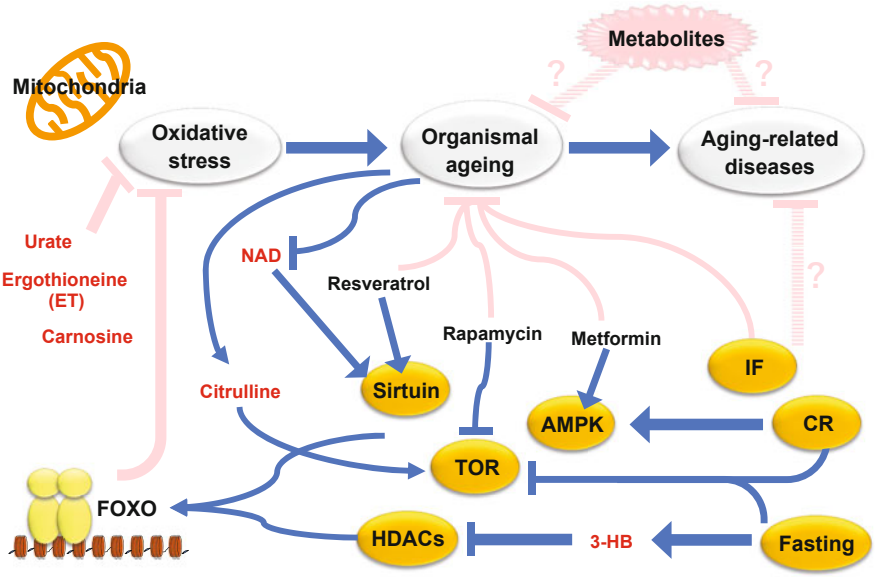

Fig. 4.2 The aging hypothesis relative to metabolism. Earlier, "the oxidative damage hypothesis" or "mitochondrial aging hypothesis" was proposed by Harman, as oxidative stress reduces organismal life span. Calorie restriction (CR) or intermittent fasting (IF) effectively extends life spans of experimental models. During CR, several signal modules, including sirtuin, Tor kinase, and AMPK, are activated or inactivated. Consistently, chemicals or compounds mimicking CR conditions increase longevity (resveratrol for sirtuin, rapamycin for Tor kinase, and metformin for AMPK). One of the targets of these signals is FOXO transcriptional factor, which activates radical scavengers. However, little is known about the effect of CR or fasting on human longevity. Some of the metabolites described in this review (red color) are known to interact with the signal molecules or to serve as antioxidants. *HDACs* histone deacetylase

DNAs, caused by free radicals is known as "oxidative stress" (von Zglinicki 2002). In addition, as more than 90% of free radicals has been attributed to mitochondria (Balaban et al. 2005), the "mitochondrial aging hypothesis" was proposed (Fig. 4.2) (Harman 1972). Consistently, the genetically manipulated mice with the extended life span, super Arf/p53 mice, displayed the increased levels of antioxidants, including glutathione (Matheu et al. 2007).

Calorie restriction (CR) by 20–30% extended life span by 20% or more in many model organisms, such as mice, flies, fish, spiders, etc. (McCay and Crowell 1934). CR activates or modulates several signal modules, such as Tor kinase, AMPK kinase, sirtuin, and FOXO transcription factor (Fig. 4.2) (Haigis and Guarente 2006; Onken and Driscoll 2010; Stanfel et al. 2009). FOXO upregulates several radical scavengers. Consistently, oxidative stress is reduced during CR, indicating its antioxidative roles (Sohal et al. 1994).

Strikingly, these CR-relevant signaling pathways could be modulated by several metabolites, which are effective both for extension of life span in experimental

models and for the treatment of human aging-related diseases (Fig. 4.2). Rapamycin inhibits the activity of Tor kinase, utilized as an immunosuppressor or anticancer drug, while metformin stimulates AMPK kinase for diabetic therapy (Mouchiroud et al. 2010). In addition to resveratrol, NAD+ also activates sirtuins. The life span is extended by the overexpression of adipose-tissue-specific Nampt, which catalyzes the biosynthesis of NAD+ (Yoshida et al. 2019). However, it is still controversial whether the modulation of FOXO would be efficacious in clinical applications, as FOXO overexpression compromises metabolic regulation in mice (Nakae et al. 2002).

Moreover, the mitochondrial aging hypothesis has been challenged, since partial impairment of mitochondrial functions provoked longevity in nematodes and fly (Houtkooper et al. 2013). Consistently, certain limited but defined overload of mitochondrial oxidative stress prolonged life span in nematodes, known as "mitohormesis" (Yang and Hekimi 2010). These opposing outcomes on oxidative stress and longevity cannot be ignored. In human clinical trials, supplementation with the antioxidants, beta-carotene, vitamin A, or vitamin E exacerbated mortality (Bjelakovic et al. 2014). In sharp contrast to the success of the "calorie restriction hypothesis" in experimental models, some epidemiological studies suggest that slightly overweight people live longer (Corrada et al. 2006). Compared to that in model organisms, aging research in human beings may require alternative approach, in part due to its variability and complexity.

Besides CR, the supplementations of other metabolites are under examination as intervention approach against human aging. A clinical trial of NMN (nicotinamide mononucleotide), a precursor of NAD+, has been started (Irie et al. 2020). The combination of exercise and supplementation for branched-chain amino acids improved muscle strength in aged frailty (Ikeda et al. 2016). Thus, metabolites themselves could be potentially one of the promising strategies.

4.7 Metabolomic Approach for Human Whole Blood

Metabolites are defined as small organic compounds, produced by the metabolic activity in living organisms, from bacteria to humans. The metabolome detects and quantifies numerous metabolites with, e.g., liquid chromatography-mass spectrometer (LC-MS). The analysis of the metabolome (metabolomics) is a growing branch of chemistry, not only to search for biomarkers but also to provide valuable metabolic information on physiology and pathophysiology.

Among other things, blood reflects physiological states in vivo affected by epigenetics, genetics, lifestyle, and health (Blackburn et al. 2015). Thus, metabolomics of human blood presents extensive evidence on metabolic aspects not only of physiological responses, health, aging, and diseases but also of biological effects of chemicals, nutrients, stressors, and environmental factors (van der Greef et al. 2013).

Blood contains cellular and noncellular components: red blood cells (RBCs), white blood cells (WBCs), platelets, and plasma. Various investigations have been mainly conducted on plasma or serum, the noncellular component (Srivastava 2019). This is probably due to the difficulty in handling cell-derived metabolites, part of which are rather labile (Gil et al. 2015).

We have established novel method for analyzing whole blood, plasma, and RBCs. The whole blood metabolome includes about 130 metabolites, comprising over 14 subgroups (nucleotides, nucleosides, nucleobases, vitamins and coenzymes, nucleotide-sugar derivatives, sugar phosphates, sugar derivatives, choline and ethanolamine derivatives, organic acids, antioxidants, amino acids, methylated compounds, acetylated compounds, carnitines, etc.) (Chaleckis et al. 2014). These compounds reflect diverse cellular metabolisms: energy production, DNA and RNA synthesis, lipid metabolism, mitochondrial respiration, redox homeostasis, protein synthesis, and so on. Consequently, the amounts of these compounds are influenced or regulated by various activities of tissues, activation or inactivation, secretion or absorption, regeneration or degradation, contraction or relaxation, digestion or condensation, accumulation, excretion, etc.

Among the ~130 compounds assessed by whole blood metabolomics, 50–60 are enriched in RBCs (Chaleckis et al. 2016). Based on the coefficient of variation (CV: SD divided by the mean), individual differences of metabolites are classified into two subgroups: those that are rather invariable (CV $<$ 0.4) and others with large variability (CV $>$ 0.4 or higher). The former covers vitally essential metabolites (ATP, glutathione, phospho-sugars, etc.), while the latter include dietary compounds such as caffeine, carnosine, ergothioneine, 4-aminobenzoate, and others (Chaleckis et al. 2016). Thus, whole blood metabolomics comprehensively captures complex changes with individual differences and is useful for human aging research. Indeed, whole blood metabolomics have revealed metabolites that serve as biomarkers relevant to aging, fasting, and frailty (Chaleckis et al. 2016; Teruya et al. 2019; Kameda et al. 2020).

4.8 Blood Metabolites for Aging Markers

Although targeted metabolomic analysis has identified aging-relevant compounds (Srivastava 2019), a comparative, non-targeted analysis of the whole blood metabolome was carried out to compare healthy young and elderly people (Chaleckis et al. 2016). Among the 126 metabolites, there were statistically significant differences in 14 metabolites (11%) between young (29 $\pm$ 4 years old) and elderly people (81 $\pm$ 7 years old), which can be regarded as aging markers. Six of them are RBC-enriched, implying that whole blood metabolomics is an efficient device for human aging research.

Nine of the 14 metabolites decreased in the elderly, while 5 of them increased (Table 4.1). The former include 1,5-anhydroglucitol (1,5-AG), acetyl-carnosine, carnosine, ophthalmic acid (OA), leucine, isoleucine, nicotinamide adenine

Table 4.1 Fourteen metabolites for aging overlap with those for fasting and frailty

Metabolites	Levels in elderly	Levels in frailty	Levels in fasting	Role in blood
1,5-Anhydroglucitol (1,5-AG)	↓	↓		Antioxidant
Acetyl-carnosine	↓	↓		Antioxidant
Carnosine	↓		↑	Antioxidant
Ophthalmic acid (OA)	↓	↓	↑	Antioxidant
Leucine	↓	↓	↑	Muscle maintenance
Isoleucine	↓	↓	↑	Muscle maintenance
NAD^+	↓			Redox homeostasis
$NADP^+$	↓			Redox homeostasis
UDP-acetyl-glucosamine	↓			Sugar nucleotide
2-ketobutyrate		↓	↑	Butyrate
Urate		↓	↑	Antioxidant
Ergothioneine (ET)		↓	↑	Antioxidant
Trimethyl-histidine		↓		Antioxidant
S-methyl-ET		↓		Antioxidant
Tryptophan		↓		Amino acid
Methionine		↓		Amino acid
Proline		↓		Amino acid
Citrulline	↑			Urea cycle
Pantothenate	↑		↑	Precursor of CoA
Dimethyl-guanosine	↑			Urine compound
N-acetyl-arginine	↑			Urea cycle
N6-acetyl-lysine	↑			Acetylated amino acid
UDP-glucuronate		↑		Sugar derivative
Creatine		↑		Energy storage

Whole blood metabolomics reported 14 metabolites (red) as aging markers. The upper panel (blue box) shows metabolites that decrease in the elderly or frailty, while the lower panel (gray boxes) list metabolites that increase in those. Please notice that several compounds for aging or frailty overlap with markers which increase in fasting. Properties of metabolites are added

dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$), and UDP-acetyl-glucosamine, while the latter comprise citrulline, pantothenate, dimethyl-guanosine, *N*-acetyl-arginine, and *N*6-acetyl-lysine.

1,5-AG, the 1-deoxy form of glucose, is derived from many foods. Circulating 1,5-AG is excreted from renal glomeruli, but reabsorbed through renal proximal tubules. As glucose is a competitive inhibitor against reabsorption of 1,5-AG, diabetic patients with high blood glucose levels show lower 1,5-AG levels. Thus, 1,5-AG is known as diabetes marker. However, levels of HbA1c and serum glucose, other diabetic parameters, are normal in both healthy young and elderly in this study; hence, it is possible that reabsorption of 1,5-AG in the kidneys is reduced with aging even in healthy people (Chaleckis et al. 2016). Carnosine and acetyl-carnosine are

dipeptides with antioxidant properties, which are abundant in muscles and neurons (Park et al. 2005).

OA is a tripeptide analog of glutathione, and L-γ-glutamyl-L-α-aminobutyrylglycine is synthesized by the same enzyme (glutathione synthase) utilized for glutathione production. Because glutathione is one of the most abundant antioxidative compounds in cells, OA is known as an oxidative stress marker (Soga et al. 2006). NAD^+ and $NADP^+$ are coenzymes involved in various redox reactions. Leucine and isoleucine are essential amino acids involved in the maintenance of skeletal muscle (Buse and Reid 1975). Recent blood metabolomics study consistently identified the reduction of essential amino acids in elderlies (Chen et al. 2020). UDP-acetyl-glucosamine is a substrate for glycosyl-based modifications and is involved in the synthesis of proteoglycans and glycolipids (Hirschberg and Snider 1987).

Among metabolites that increase in the elderly, citrulline is synthesized in the urea cycle and is involved in excretion of nitrogen. Dimethyl-guanosine and *N*-acetyl-arginine are also related to nitrogen metabolism (Niwa et al. 1998; Mizutani et al. 1987). These results suggest that urinary discharge of nitrogen-related metabolites is possibly reduced in the elderly. Pantothenate is a precursor of coenzyme CoA, involved in the TCA cycle and β-oxidation.

Correlation analysis among these 14 aging compounds suggests strong correlations among metabolites with declining values in elderly and also among those that increase in the elderly. Interestingly, there was no negative (reverse) correlation between these two subgroups, indicating at least two distinct subgroups for aging metabolites.

4.9 Blood Metabolites for Fasting Markers

The evidence on CR (calorie restriction) and longevity has been well documented in experimental models. Recent reports suggest that intermittent fasting, a cycle of fasting and feeding, enables *C. elegans* to live about 50% longer than conspecifics on a normal diet (Honjoh et al. 2009). Thus, CR and intermittent fasting have overlapping roles in the prolongation of life span (Fig. 4.2).

Although little is known about the link between fasting and aging in humans, it is well known that humans can withstand 30–40 days of fasting if dehydration is avoided, in sharp contrast to the vulnerability of mice to hunger (average survival of only several days of starvation). For example, Gandhi experienced hunger strikes of up to 21 days, 14 times or more in his lifetime as a form of political protest (Peel 1997).

Historically, research on fasting physiology has focused particularly on energy substitution (Owen et al. 1969). Glucose normally constitutes the major fuel source under non-fasting conditions, but during fasting, glycogen stores are rapidly exhausted in an effort to maintain minimum glucose levels in the blood. After the

depletion of glycogen storage, in addition to gluconeogenesis, fasting forces the human body to utilize various noncarbohydrate metabolites, such as lipids and branched-chain amino acids (BCAAs) as energy sources (Cahill 2006). Hormonal changes stimulate lipolysis in the white adipose tissue (WAT) and liver. First, 3-hydroxybutyrate (3-HB) increases 30- to 60-fold and is converted into acetyl-CoA in the brain or other tissues as an alternative energy source (Owen et al. 1969). Second, elevated acylcarnitines during fasting are essential for lipid transport into mitochondria (Hoppel and Genuth 1980). Third, increased concentrations of branched-chain amino acids (BCAAs), mainly released from muscle, are also utilized in the mitochondrial TCA cycle or in liver lipogenesis (Pozefsky et al. 1976). Thus, the elevation of butyrates, BCAAs, and acylcarnitines in circulating blood (quantified by targeted metabolomics or other techniques) serve as indicators of fasting.

We conducted an exhaustive analysis of nearly 130 metabolites from the blood of 4 healthy young people for 58 h of starvation. In this study, over one-third of metabolites increased, indicating much greater metabolic activation during fasting than expected. Among the 44 increased metabolites, well-known fasting markers figure prominently (ketone bodies, carnitines, and BCAAs), consistent with previous findings (Fig. 4.3) (Hoppel and Genuth 1980).

In addition, several novel aspects of fasting were discovered involving (1) TCA cycle metabolites, (2) antioxidants, and (3) signaling molecules (Fig. 4.3). First, an increase in TCA cycle metabolites reflects the activation of mitochondrial function throughout the body during starvation, since red blood cells are not equipped with mitochondria. Increases of well-established fasting metabolites, ketone bodies, carnitines, and BCAAs also support mitochondrial activity during fasting. Second, an increase in antioxidant compounds was also observed, in addition to urate, xanthine, carnosine, OA, and ergothioneine (ET). Urate is one of the most abundant antioxidants in the blood (El Ridi and Tallima 2017), the precursor of which is xanthine. ET is abundant in mushrooms and fungi. In yeast, ET also increases in a low-glucose environment (Pluskal et al. 2014). Additionally, four metabolites (6-phosphogluconate, glucose-6-phosphate, pentose phosphate, and sedoheptulose-7-phosphate) generated via the pentose phosphate pathway (PPP) increased in plasma, but not in RBCs during fasting. The activation of the PPP produces NADPH, which is essential for redox control (Patra and Hay 2014). As sugar phosphate compounds in blood are enriched in RBCs, PPP metabolite increases only in plasma, suggesting that responses in tissues are largely responsible for these altered profiles during fasting. Collectively, one of the most significant metabolic changes during starvation is antioxidant enhancement.

Third, whole blood metabolomics identified purines and pyrimidines (GTP, CTP, ADP, IMP, cytidine, and adenine) and some signal-modulating metabolites (3-hydroxybutyrate and 2-oxoglutarate) as fasting markers (Teruya et al. 2019). It is conceivable that fasting provokes global remodeling of transcriptional networks to adapt to metabolic changes. Increased purines and pyrimidines may support anabolic

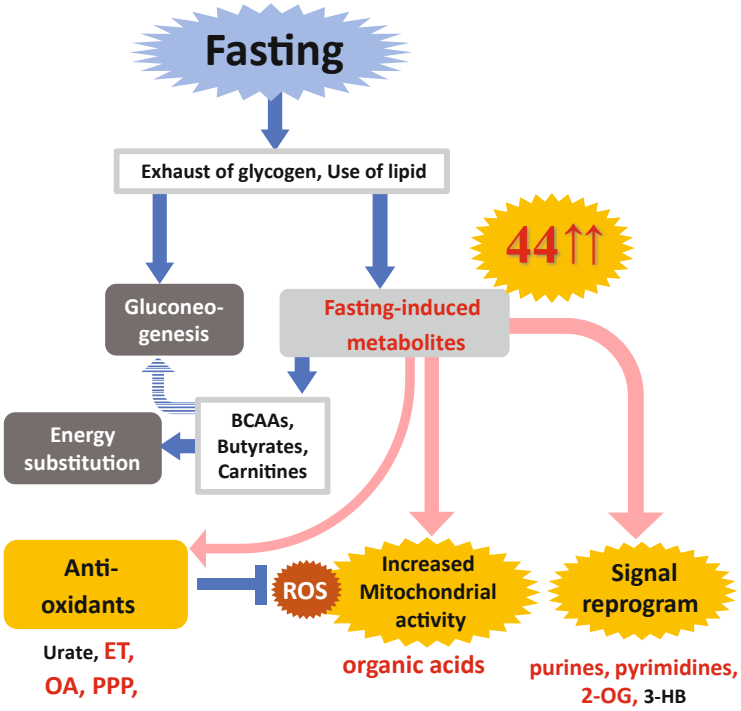

Fig. 4.3 Forty-four metabolites that increase during fasting include antioxidants, organic acids, and signaling-related compounds. Non-targeted comprehensive metabolomics of whole blood detected increases of one-third (44) of metabolites identified during 58 h of fasting. In addition to metabolites for energy production, antioxidative metabolites were identified as fasting markers, which may combat oxidative stress resulting from enhanced mitochondrial activity. Moreover, signaling metabolites would contribute for remodeling of metabolic homeostasis during fasting. See the text for details. *ET* ergothioneine, *OA* ophthalmic acid, *PPP* pentose phosphate pathway, *3-HB* 3-hydroxybutyrate, and *2-OG* 2-oxoglutarate

metabolism for RNA and protein synthesis. 3-Hydroxybutyrate (3-HB), a major energy substitute during fasting, is also known as a histone deacetylase inhibitor (Shimazu et al. 2013), while 2-oxoglutarate activates 2-oxoglutarate oxygenase, functioning in the demethylation of histones and nucleic acids and destabilization of transcriptional factors (Loenarz and Schofield 2008). Fasting may genetically or epigenetically modify transcriptional networks via such metabolites (Loenarz and Schofield 2008).

Interestingly, four metabolites that increase during fasting (carnosine, OA, leucine, and isoleucine) overlap with the decreased metabolites in elderly (Table 4.1 and Fig. 4.4). It is possible that fasting may exert antiaging or rejuvenile effect through the upregulation of these aging metabolites.

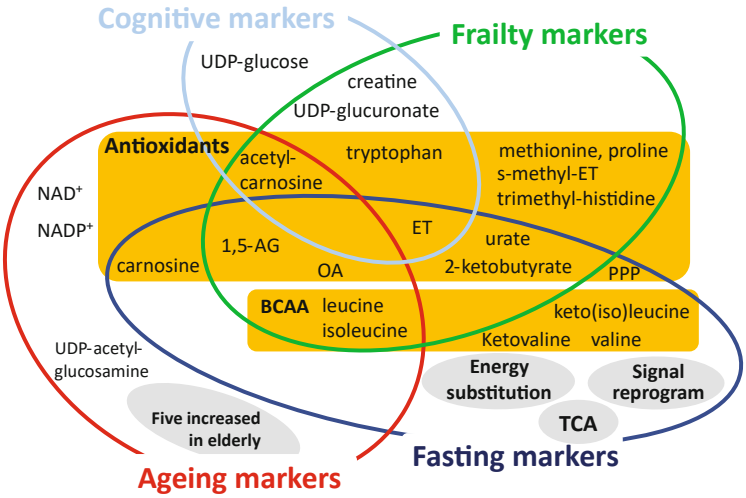

Fig. 4.4 Summary of metabolites for aging, fasting, frailty, and cognitive impairment. Overlapping but distinct markers for aging, fasting, and frailty are identified by non-targeted, comprehensive metabolomic analysis of human whole blood. Various antioxidative metabolites are included in the list of aging and fasting markers. Please notice that four metabolites decreased in elderly (OA, carnosine, leucine, and isoleucine) were increased during fasting, indicating the possible antiaging effect of fasting. Among 15 frailty markers, 11 antioxidative metabolites are decreased, indicating the involvement of antioxidative defense in the pathogenesis of frailty. Among 14 aging markers, 5 metabolites (acetyl-carnosine, 1,5-AG, OA, leucine, and isoleucine) overlapped with frailty markers. Among six cognitive markers, five compounds (acetyl-carnosine, 1,5-AG, OA, UDP-glucuronate, and creatine) overlapped with frailty markers, reflecting the cognitive aspects of frailty. Thus, cognitive markers are much involved in frailty markers, while one compound (acetyl-carnosine) is listed as overlapping both in aging and cognitive markers. *BCAA* branched-chain amino acid, *ET* ergothioneine, *1,5-AG* 1,5-anhydroglucitol (1,5-AG), *OA* ophthalmic acid, *PPP* pentose phosphate pathway

4.10 Frailty Markers for Antioxidation, Cognition, and Mobility

Many aging-related diseases are increasing in our globally aging society, including lifestyle diseases (hypertension, diabetes, obesity, osteoporosis, atherosclerosis, etc.), dementia, cancer, and others. Among other factors, the severity of frailty is most closely correlated with health risks of advanced age (Li et al. 2020). Frailty is a vulnerability to stressors, due to the declining physiological capacity of organs as a result of aging (Fried et al. 2001). Because it results from age-related deterioration of multiple organ systems, frailty displays complex features, including cognitive dysfunction, hypomobility, and impaired daily activity.

Frailty is currently defined by three major diagnostic tools (Dent et al. 2017b). The Fried Cardiovascular Health Study (CHS) index is useful to detect physical frailty (Fried et al. 2001), while the Rockwood Frailty Index covers its

multimorbidity (Rockwood et al. 1999). In contrast, the Edmonton Frailty Scale (EFS) or Tilburg Frailty Indicator efficiently evaluates both physical and psychosocial aspects of frailty (Rolfson et al. 2006).

Four recent studies reported the metabolomic analysis of the blood from frail elderly subjects (Pujos-Guillot et al. 2018; Marron et al. 2019; Rattray et al. 2019; Livshits et al. 2018). However, these reports drew divergent, nonoverlapping conclusions, stemming from different experimental designs. For example, the former two reports applied the Fried CHS index as a diagnostic tool, which lacks cognitive assessment, while the latter two utilized the Rockwood Frailty Index. Since these studies were based on serum samples, we performed whole blood metabolomics for frailty. Our study was designed to cover multiple domains of frailty, by applying the EFS, the Japanese version of the Montreal Cognitive Assessment (MoCA-J) (Fujiwara et al. 2010) and the Timed Up and Go (TUG) test (Podsiadlo and Richardson 1991) as diagnostic tools (Rolfson et al. 2006), because the EFS is recognized as a valid and reliable measurement tool for the identification of frailty (Dent et al. 2017a) and is widely recommended in clinical guidelines (Dent et al. 2017b).

Using the EFS as a guide, our study identified 15 blood metabolites involved in antioxidation, cognition, and mobility as frailty markers (Table 4.1 and Fig. 4.4) (Kameda et al. 2020), while studies based on the Fried CHS index reported blood metabolites related mainly to physical or sarcopenic frailty (Marron et al. 2019).

First, among 15 frailty markers, 7 compounds that decreased in frailty are associated with antioxidative defense: acetyl-carnosine, ergothioneine (ET), *S*-methyl-ET, trimethyl-histidine, OA, 2-ketobutyrate, and urate (Fig. 4.4). Trimethyl-histidine and *S*-methyl-ET are involved in ET synthesis (Asmus et al. 1996). 2-Ketobutyrate is a precursor of OA. Thus, the ergothioneine and OA pathways are greatly affected in frailty. Second, we observed that five amino acids (methionine, proline, tryptophan, isoleucine, and leucine) decreased significantly in frail subjects. Among these five amino acids, methionine, proline, and tryptophan have been reported as radical scavengers in vitro (Marcuse 1960) (Meucci and Mele 1997). Thus, our whole blood metabolome for frailty revealed antioxidant enrichment in cellular components. Consistently, recent findings of longitudinal studies support our notion that diminished antioxidative defenses are heavily involved in the pathogenesis of frailty (Marron et al. 2020). It is noteworthy that most cognitive markers and some hypomobility markers overlap with frailty markers, supporting the notion that frailty is an integrated spectrum of age-related disorders (Fig. 4.4). On the other hand, only acetyl-carnosine overlapped in aging and cognitive markers, indicating the gap between physiological and pathological aging in cognition.

Interestingly, metabolites affected in frailty largely overlap with metabolites that decrease during aging (acetyl-carnosine, OA, 1,5-AG, isoleucine, and leucine) and compounds that increase during fasting (2-ketobutyrate, OA, isoleucine, leucine, urate and ET), indicating an intriguing metabolic link between frailty and human aging (Table 4.1 and Fig. 4.4). Various antioxidative metabolites are conspicuously included among frailty and aging markers, suggesting that one of the key stressors to which frail elderly are vulnerable is oxidative stress. In this context, increased

oxidative defense during fasting may effectively moderate aging or aging-relevant disorders.

4.11 Summary

The diversity of aging has a significant impact not only on clinical and basic research but also on society and economies and facilitates structural changes in entire societies. The answer to the fundamental question, "What is aging?", is not yet fully known. Accumulating evidence suggests that defining "aging" only on the basis of calendar age does not explain the entire situation.

Recent advance in aging research identified mechanisms and biomarkers for biological aging. The research on cellular senescence suggests the significance of telomere length, p16, p53, inflammatory cytokines, and others. On the other hand, the aging research on metabolic aspects, including metabolomics, disclosed the metabolites closely involved in aging and its relevant diseases.

Interestingly, some of the metabolites described in this review are known to interact with the signal molecules (Fig. 4.2). NAD^+, decreased in elderly, activates sirtuin, while citrulline, accumulated in elderly, stimulates TOR kinase. 3-HB inhibits histone deacetylases, followed by FOXO activation. Thus, metabolites might affect aging or aging-related diseases by modulating signal modules. As one hint of human aging research, results of whole blood metabolomics help to better understand biological age, especially given the variable nature of aging.

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Chapter 5
To G0 or Not to G0: Cell Cycle Paradox in Senescence and Brain Aging

Shoma Ishikawa and Fuyuki Ishikawa

Abstract Organisms can cope with ever-changing environments by inducing various adaptive responses that increase fitness. Cellular senescence is a stress-activated program characterized by the irreversible arrest of the cell cycle in damaged, proliferating cells. Senescence is a potent tumor-suppressor cell state; by the same token, however, it is also a primary driver of the aging process and age-related diseases. Outside of this conventional view, several recent studies have suggested that a senescence-like response can occur in terminally differentiated post-mitotic cells in non-regenerative tissues, such as neurons in the brain. We have provided evidence for multifaceted attributes of senescence in post-mitotic neurons in the context of homeostatic control mechanisms in the aged brain. Here we highlight an emerging paradigm shift regarding senescence and discuss the potential physiological relevance of post-mitotic neuronal senescence in aging.

Keywords Adaptation · Alzheimer's disease · Cellular senescence · Post-mitotic cells · Proteostasis failure · Neurons

5.1 Alzheimer's Disease

Species evolve by maximizing survival or reproductive success (also known as the Darwinian fitness) in changing environments, the cornerstone of evolution. Nonetheless, traits that enhance fitness early in life can exert unselected adverse effects late in life as selective pressure declines with age. These trade-offs between early- and late-life fitness—termed antagonistic pleiotropy, originally proposed by George C. Williams in 1957 (Williams 1957)—undergird the prevailing evolutionary

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_5

explanation of aging. Hence, biological aging is viewed as a gradual functional deterioration of biological systems, ineluctably increasing the risk of mortality.

The human brain regulates virtually all aspects of physiological processes in the body (perception, feeding, circulation, respiration, digestion, motion, reproduction, etc.), yet it is, without doubt, highly susceptible to age-dependent functional decline. Cognitive functions including memory, learning, and processing speed are most affected by both normal and pathological aging, including mild cognitive impairment (MCI) and Alzheimer's disease (AD). AD is a major cause of dementia in the elderly population, and it is diagnostically defined by three pathological criteria: (1) the deposition of senile plaques, insoluble protein aggregates comprised of β-amyloid (Aβ) peptide; (2) the accumulation of neurofibrillary tangles, hyperphosphorylated forms of the microtubule-associated protein tau; and (3) the progressive loss of terminally differentiated mature neurons in the medial temporal lobes, including the hippocampus and entorhinal cortex, which form essential circuits for memory formation, retrieval, and processing (Palop and Mucke 2010; Price et al. 2001; Yankner et al. 2008). While both amyloid and tau proteins are appreciated to play important physiological roles in neurogenesis, neuronal activity, and neuroprotection (Kent et al. 2020; Müller et al. 2017; Wang and Mandelkow 2016), age-related dysregulation of protein homeostasis (proteostasis) leads to an accumulation of misfolded proteins and toxic protein aggregates (Hipp et al. 2019; Kaushik and Cuervo 2015). Amyloid and tau pathologies can also occur in cognitively healthy older people (Bennett et al. 2006; Davis et al. 1999) who have a greater risk of AD onset (Knopman et al. 2012). Because of these clinically silent cases, AD is now considered to begin with an asymptomatic "preclinical" AD stage several decades before clinical symptoms appear (Langbaum et al. 2013; Petersen 2018; Sperling et al. 2014). In contrast to the morphological phenotypes indicative of proteotoxic burden, a loss of hippocampal neurons is not observed in cognitively normal older individuals (i.e., normal aging or preclinical AD). Instead, the reduced number of neurons is strongly associated with cognitive impairment in the symptomatic stages (i.e., MCI and AD) (Burke and Barnes 2006; West et al. 1994), reflecting the difficulty in treating late-stage AD patients with medications (Cummings et al. 2020; Mangialasche et al. 2010; Mullard 2021). With this insight, there is now a rush to develop therapeutic interventions in early stages of AD (Langbaum et al. 2013). To this end, it is urgently necessary to understand the molecular pathologies occurring in preclinical AD and the normal aging process. It has been proposed that neuronal loss is provoked by the improper localization of the transcriptional repressor REST (repressor element-1 silencing transcription factor) to the cytosol in hippocampal neurons of AD patients. In healthy aged individuals, REST normally accumulates in the nucleus of hippocampal neurons and plays a key role in neuroprotection by repressing genes involved in stress responses, apoptosis, and AD pathologies (Lu et al. 2014). However, it is not clear whether other mechanisms are involved in the characteristic neuronal loss in AD.

5.2 Cellular Senescence

Dating back over half a century, the finite replicative potential of normal human fibroblasts undergoing serial passages in vitro was first described by Hayflick and colleagues, a phenomenon called replicative senescence (also known as the Hayflick limit) (Hayflick and Moorhead 1961). Senescent cells are metabolically active and viable, yet irreversibly cease proliferation, even under conditions of adequate mitogens and nutrients. Later, it was demonstrated that the gradual attrition of telomeres—the nucleoprotein complexes at chromosomal termini that protect the DNA at the physical ends of linear chromosomes—during successive rounds of cell division eventually causes deprotection of telomeres and activation of the DNA damage response (DDR), inducing cellular senescence (d'Adda di Fagagna et al. 2003; Herbig et al. 2004; Karlseder et al. 2002). It takes a long time for this process to occur, because it requires many cell divisions to reduce the telomere length to a critical point, called replicative senescence. Later, it was found that features indistinguishable from replicative senescence are induced as stress responses in a relatively short period. A variety of endogenous and exogenous stresses, including oncogene activation, DNA replication stress, mitochondrial dysfunction, exposure to genotoxic reagents, and oxidative stress, lead to cellular senescence, collectively called premature senescence or stress-induced senescence (Kuilman et al. 2010; Salama et al. 2014). The molecular basis behind the initiation of senescence depends on the p53 and/or $p16^{INK4A}$-retinoblastoma (Rb) tumor suppressor pathways (Gorgoulis et al. 2019). This dependence establishes cellular senescence as a tumor suppressive mechanism, which prevents the malignant progression of benign tumors, as in the case of melanocytic nevi. With the passage of time, senescent cells accumulate in various tissues with age and are associated with organismal aging and age-related pathologies (Childs et al. 2015; Muñoz-Espín and Serrano 2014; van Deursen 2014). Indeed, it has been demonstrated, using INK-ATTAC (apoptosis through targeted activation of caspase) transgenic mice in which $p16^{INK4A}$-positive cells can initiate apoptosis, that selective elimination of senescent cells from progeroid mice and naturally aged mice can delay tumorigenesis and mitigate age-related functional decline of multiple organs, including the muscle, kidney, and fat, thereby extending both median and healthy life span (Baker et al. 2011, 2016).

Cells undergoing senescence display transcriptome reprograming (Casella et al. 2019; Hernandez-Segura et al. 2017) and epigenetic remodeling of chromatin (Cheng et al. 2017; Hernandez-Segura et al. 2018; Rai et al. 2014; Sen et al. 2019), which potentiate the induction of phenotypic features of senescent cells, including senescence-associated apoptosis resistance (SAAR) (Ryu et al. 2007; Sanders et al. 2013; Wang 1995). Senescent cells are also characterized by the secretion of a myriad of proinflammatory cytokines, growth factors, and matrix metalloproteases that is termed the senescence-associated secretory phenotype (SASP) (Coppé et al. 2008) or senescence-messaging secretome (SMS) (Kuilman and Peeper 2009). The SASP mediates beneficial and detrimental outcomes

associated with senescence in different pathophysiological contexts. For instance, the SASP can be tumor-protective when reinforcing senescence cell cycle arrest in autocrine and paracrine manners (Acosta et al. 2008; Kuilman et al. 2008) and when stimulating the immune surveillance machinery that eliminates senescent, preneoplastic, and/or transformed cells (Krizhanovsky et al. 2008; Prata et al. 2018; Sagiv et al. 2013; Xue et al. 2007). In contrast, it can have tumor-promoting effects by stimulating tumor growth and metastasis as well as angiogenesis in a cell nonautonomous fashion (Faget et al. 2019). In the short term, the SASP also exerts tissue homeostatic functions during embryonic development and wound healing. Conversely, if sustained for the long term, it can lead to chronic inflammation and tissue destruction, thereby driving pathophysiological processes with aging (Childs et al. 2015). Thus, in this scenario, the acute senescence program under intact immune surveillance has likely evolved in mitotic cells as an adaptive response to preserve tissue homeostasis and integrity in early life, but it becomes maladaptive later in life with declining immune function.

5.3 Cellular Senescence in Post-mitotic Cells

Adult stem cell senescence is at the nexus of replicative exhaustion and the functional decline of renewable tissues (e.g., gut, hematopoietic system, pancreas, and muscle) (Choudhury et al. 2007; Cosgrove et al. 2014; Janzen et al. 2006; Krishnamurthy et al. 2006; Signer et al. 2008; Sousa-Victor et al. 2014). In stark contrast, replicative exhaustion of adult stem cells has been considered to only negligibly pertain to the impaired integrity and the consequent functional attrition of non-regenerative tissues, like the brain and heart. Rather, terminally differentiated post-mitotic cells in these tissues (neurons in the brain and cardiomyocytes in the heart, respectively) physically exit the cell cycle, persist indefinitely in G0 phase, and ostensibly last for the entire life of the organism, with only minimal replacement; these long-lived, post-mitotic cells are thus essential for the respective tissue function and maintenance. Thus, the progression of such slow-onset diseases as AD, Parkinson's disease (PD), and heart failure can be ascribed to the relentless cell death of post-mitotic cells. The cellular mechanisms governing homeostasis and life-and-death decisions in these non-regenerative, privileged cells remain a long-standing enigma.

Alongside the discovery of the Hayflick limit (Hayflick and Moorhead 1961) seminal studies in neurons and cardiomyocytes showed age-dependent accumulation of lipofuscin (autofluorescent lysosomal degradation residues that contain highly oxidized proteins and lipids) (Reichel 1968; Strehler et al. 1959), one of the cardinal features of senescence in vitro and in vivo (Evangelou et al. 2017). Although it appeared inapt to categorize the neurons and cardiomyocytes in aged individuals as "senescent" due to their stable, functional, post-mitotic nature, there is accumulating evidence demonstrating classical senescence signatures in these cells (summarized in Table 5.1) (reviewed in Sapieha and Mallette 2018; von Zglinicki et al. 2021).

Table 5.1 Senescence-associated phenotypes in post-mitotic neurons during in vitro and in vivo aging

Molecular markers	Related phenotypes	Primary neurons	Normal aging	AD
SA-β-Gal	Lysosomal dysfunction	○	●	○
Lipofuscin	Lysosomal dysfunction	○	◎	◎
p16	Stable cell cycle arrest	○	◎	◎
p21	Stable cell cycle arrest	○	○	○
Proinflammatory factors	SASP	○	◎	◎
GATA4	SASP regulator	○	◎	–
p53	DDR/transcription	–	[a]	[b]
Phospho-p38	Stress sensing pathway	○	○	◎
macroH2A	SAHF	–	○	–
H3K9me3	SAHF	[c]	○	◎
Bcl-2	SAAP	○	○	◎
DNA damage (DSBs, oxidative DNA lesions)	Senescence-inducing stimuli	○	◎	◎
Proteotoxicity/ER stress	Senescence-inducing stimuli	○	◎	◎

● human, ○ rodent, ◎ both, – not characterized
SAHF senescence-associated heterochromatin foci (Narita et al. 2003)
[a] Age-dependent loss of global p53 levels has been reported in the mouse brain (Lee et al. 2019)
[b] Deposition of p53 aggregates has been implicated in impaired DDR and accumulation of DNA damage in the brain of AD patients and AD model mice (Farmer et al. 2020)
[c] Global loss of H3K9me3 has been implicated in dynamic chromatin reorganization in LTC-neurons (Ishikawa and Ishikawa 2020)

These observations include the elevation of senescence-associated beta-galactosidase (SA-β-gal) activity, the most commonly used surrogate marker for senescence (Dimri et al. 1995), in aged rat hippocampus and long-term primary cell cultures (LTC) of rat hippocampal, cortical, and cerebellar granule neurons (~30 days in vitro (DIV)) (Bhanu et al. 2010; Dong et al. 2011; Geng et al. 2010). In addition, Jurk and colleagues monitored age-dependent changes in multiple senescence markers in the murine central nervous system (Purkinje neurons and cortical neurons) as well as peripheral (myenteric) neurons (Jurk et al. 2012). Their work demonstrated that neurons from old (32 months), but not young (4 months), mice exhibited SA-β-gal activity, upregulation of a p53 downstream effector p21, an age-related elevation of the prominent SASP component IL-6 (Coppé et al. 2008), active (phosphorylated) p38 (Iwasa et al. 2003), microH2A (Zhang et al. 2005), and γH2AX (phosphorylated H2AX at S139) (d'Adda di Fagagna et al. 2003; Herbig et al. 2004; Rodier et al. 2011), a well-established DNA double-stranded break (DSB) marker, all collectively indicative of senescence. Of note, in TERC (telomerase RNA component) knockout (KO) mice bearing critically short telomeres over

several generations, as well as in p21 single- and TERC and p21 double-KO mice, there is evidence of a sustained DDR at telomeres that triggers a senescence-like response in neurons in a p21-dependent manner (Jurk et al. 2012). Recently, telomere DNA damage-associated senescence has also been observed in other post-mitotic cell types, such as cardiomyocytes and osteocytes, in both aged humans and mice (Anderson et al. 2019; Farr et al. 2016). Senescent cell clearance from aged mouse tissues, using the INK-ATTAC model or by treatment with Navitoclax (ABT-263), which inhibits anti-apoptotic Bcl-2 family proteins, attenuates cardiac fibrosis and hypertrophy that contributes to cardiac dysfunction (Anderson et al. 2019) as well as age-related bone loss (Farr et al. 2017). Thus, irreparable DNA damage provoking persistent DDR activation is a common source of senescence-inducing stimuli, both in proliferation-competent cells and nondividing cells (von Zglinicki et al. 2021). Collectively, this insight has upended the long-held paradigm of senescence since the discovery of the Hayflick limit, and therefore the mechanism and physiological importance of post-mitotic cell senescence require further investigation.

5.4 Proteostasis Failure as a Driver of Neuronal Senescence

Despite the general credence of senescence-inducing stimuli, persistent DDR activation is not the only event initiating the senescence program. Recently, we have demonstrated that the LTC protocol can induce a series of unique features seen in conventional senescence (SA-β-gal, p16, lamin B1 loss, and SASP) in primary post-mitotic neurons from the hippocampus and cortex of embryonic rat brains (Ishikawa and Ishikawa 2020). Intriguingly, neither accumulation of DSBs, as determined by levels of γH2AX foci throughout the LTC nor the induction of p21 expression was associated with the senescent hippocampal neurons. These findings contrast with reports that mouse and rat LTC-cortical neurons accumulate DSBs (Moreno-Blas et al. 2019; Piechota et al. 2016), as was also reported for normal aging rodent brains (Jurk et al. 2012; Moreno-Blas et al. 2019). It should be noted, however, that DNA damage is unlikely to be causative of the LTC-induced neuronal senescence, since LTC neurons showed a delayed appearance of DSBs, after the induction of SA-β-gal activity (Piechota et al. 2016). Moreover, DNA damage-inducing drugs doxorubicin and bleomycin did not accelerate the elevation of SA-β-gal activity in rat primary neurons irrespective of their origins (Piechota et al. 2016; Ishikawa and Ishikawa, unpublished). Instead, our LTC model has demonstrated that those senescent cells show proteostasis failure, such as Aβ burden and protein aggregate accumulation, which is likely to be attributed to reduced autophagic flux (Ishikawa and Ishikawa 2020; Moreno-Blas et al. 2019), akin to brain aging-associated changes and/or early AD pathologies. AD-related proteotoxicity has been shown to induce conventional senescence in various types of mitotic cells, including human tracheal epithelial cells (Chong et al. 2018), human umbilical endothelial cells (Donnini et al. 2010), mouse astrocytes (Bussian et al. 2018), and mouse neural stem/progenitor cells (He et al.

2013; Zhang et al. 2019). Likewise, manipulating Aβ toxicity through genetic and biochemical approaches—ectopic expression of the amyloid precursor protein (APP) gene that carries mutations found in familial AD (FAD), exposure to recombinant aggregate-prone $A\beta_{42}$, or treatment with a compound that disassembles Aβ aggregates—has implicated AD-relevant proteostasis failure in neuronal senescence (Ishikawa and Ishikawa 2020). In line with this evidence, aggressive Aβ deposition is capable of promoting an age-dependent increase in p16-positive neurons in the hippocampus of 5xFAD mice, an early-onset FAD mouse model carrying five mutations in APP and PSEN1 (Wei et al. 2016), suggestive of Aβ-driven neuronal senescence in vivo. Interestingly, in sporadic AD, Aβ pathology can also be attributed to the physiological processing of APP that regulates neuronal activity and/or neuronal cell survival (Müller et al. 2017). In fact, age-dependent increments in L-type voltage-sensitive Ca^{2+} channel (L-VSCC) currents, membrane density, and expression levels have been reported in both the LTC model (Porter et al. 1997) and aged animals (Campbell et al. 1996; Herman et al. 1998; Thibault and Landfield 1996), which may contribute, at least in part, to the Aβ burden during in vitro aging (Bertrand et al. 2011; Ishikawa and Ishikawa 2020) and vice versa (Kim and Rhim 2011). Thus, it is of particular interest to evaluate functional APP products, such as neuroprotective APPsα and AICD (APP intracellular domain) (Müller et al. 2017), as pro-senescence effectors.

Integral to proteostasis control is the mechanistic/mammalian target of rapamycin (mTOR) pathway, which coordinates protein synthesis and degradation depending on available nutrients and cellular conditions (Laplante and Sabatini 2012). It has been recognized as a nexus in aging at both cellular and organismal levels. Blocking the mTOR pathway has been shown to restore proteostasis and extend life span in various organisms (Johnson et al. 2013). Administration of rapamycin, an mTOR complex 1 (mTORC1) inhibitor, or its related reagents ameliorates Aβ and tau pathology and cognitive impairment in several AD model mice (Talboom et al. 2015). Moreover, the mTOR pathway is critical for a transition to senescence (Iglesias-Bartolome et al. 2012; Leontieva and Blagosklonny 2010; Young et al. 2009) as well as the establishment of the SASP in proliferative cells (Herranz et al. 2015; Laberge et al. 2015). Consistently, our recent findings have indicated that chronic treatment with rapamycin fosters proteostasis, thereby circumventing induction of senescent phenotypes in LTC neurons (Ishikawa and Ishikawa 2020). Importantly, in both human and murine fibroblasts, constitutive activation of mTORC1 has been shown to induce senescence via either the p16 or p53-p21 pathways, which are dependent on the major mTORC1 downstream substrates S6K and 4E-BP1/2, respectively, even without overt DDR activation (Alimonti et al. 2010; Astle et al. 2012; Barilari et al. 2017; Petroulakis et al. 2009). Given that Aβ can affect the mTOR pathway (Baik et al. 2019; Caccamo et al. 2010; Talboom et al. 2015), proteostasis failure may tilt the mTOR/S6K axis to the p16 pathway during LTC, ultimately triggering neuronal senescence. Overall, these recent findings lead to a unifying paradigm of cellular senescence, where both mitotic and post-mitotic cells, with some differences due to their nature, manifest stress responses that share common features based on a molecular mechanism that

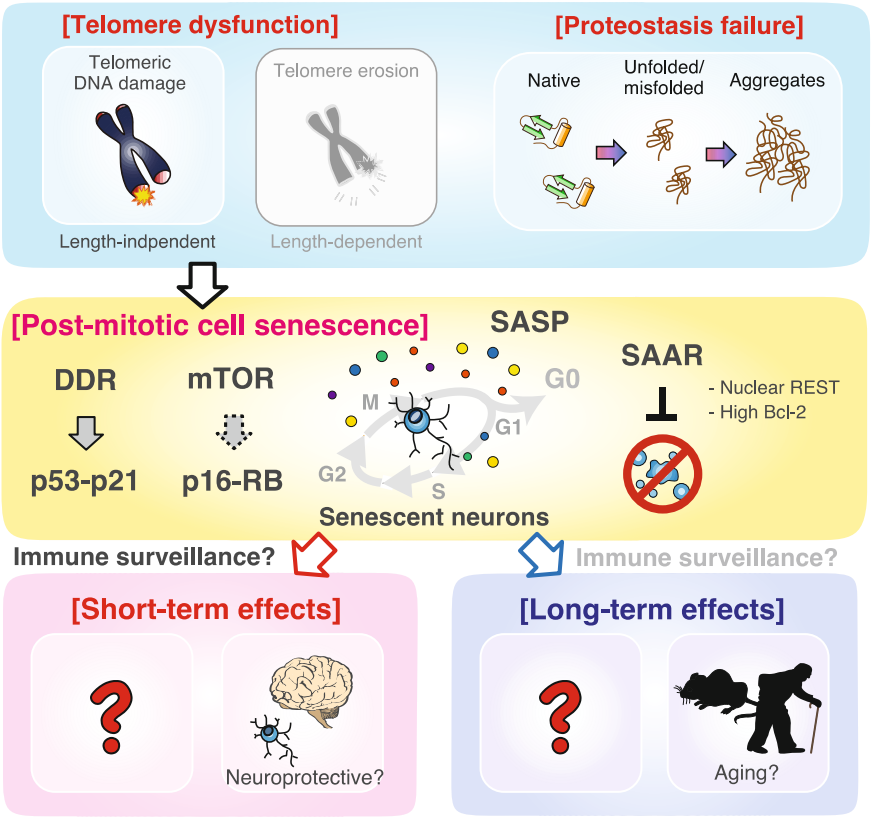

Fig. 5.1 Post-mitotic neurons undergo cellular senescence in vitro and in vivo. Owing to its narrow definition, most of our knowledge of cellular senescence, stress-induced permanent cell cycle exit, is restricted to stem or mitotically active cells. However, several recent studies have described a senescence-like phenomenon in terminally differentiated neurons provoked by persistent DDR activation and proteostasis disruption—major senescence-inducing stimuli for proliferation-competent cells. Given the fact that differentiated nondividing cells are believed to be long-lived cells, neurons could conceivably engage pro-survival responses, such as senescence

involves mTOR-mediated control of proteostasis (Fig. 5.1). Albeit mostly based on experiments using the cell culture model for studying brain aging, we believe that neuronal senescence may also occur in vivo aging through the mTOR-dependent mechanism, as proteostatic collapse would appear to be the culprit of brain aging.

5.5 Neuronal Senescence: Pleiotropic Response

Recent evidence from animal model studies has suggested that senescent glial cells and oligodendrocyte progenitor cells burdened by tau and Aβ proteotoxicity, respectively, contribute to AD pathologies and clinical symptoms (Bussian et al. 2018; Zhang et al. 2019). Besides AD, senescent cells have also been shown to be involved in the etiologies of atherosclerosis (Childs et al. 2016), type I diabetes (Thompson et al. 2019), and osteoporosis (Farr et al. 2017). Thus, senescence is now thought to be a primary source driving both physiological and pathological aging. However, the physiological consequence of post-mitotic cellular senescence remains to be addressed.

AD is an indolent neurodegenerative disease in which the pathological changes including tau and Aβ deposition begin decades prior to neurodegeneration, a bottleneck in the AD continuum. In other words, pathological proteotoxicity does not always impose neuronal cell death, so that it may provoke neuroprotective responses in the brain of AD patients at the preclinical stage. Strikingly, our LTC model has revealed that Aβ and proteostatic collapse trigger neuronal senescence, with simultaneous acquisition of adaptations to multiple age-cumulative stresses: genotoxic damage, oxidative stress, and proteotoxicity (Ishikawa and Ishikawa 2020). Accordingly, we propose that beyond serving as an intrinsic tumor-suppressor in mitotic cells, senescence may act as a cell-autonomous safeguard mechanism against the transition from an asymptomatic stage to symptomatic ones (MCI and AD). In support of this, the SAAR in senescent neurons is correlated with the nuclear localization of REST (Ishikawa and Ishikawa 2020; Piechota et al. 2016). Moreover, increased levels of pro-survival protein Bcl-2 in the senescent neurons would caution against the use of Bcl-2 inhibitors as agents that eliminate senescent cells, the so-called senolytics (Chang et al. 2016; Ogrodnik et al. 2019; Thompson et al. 2019; Wang et al. 2017; Yosef et al. 2016; Zhang et al. 2019; Zhu et al. 2016).

Though contrary to the prevailing idea of cell cycle exit coupled with terminal differentiation, partial reactivation of unscheduled cell cycle entry in post-mitotic neurons has been widely recognized to be a cellular event that precedes neuronal death of damaged neurons under various stress and neurodegenerative disease conditions, such as AD (Greene et al. 2004; Herrup and Yang 2007; Kruman et al. 2004). Of note, a recent study has demonstrated that senescent melanocytes escape stable cell cycle arrest, resume proliferating, and ultimately become cancerous by eliminating senescence-associated heterochromatin foci (SAHF) (Narita et al. 2003) through the upregulation of histone demethylases, such as LSD1 (lysine-specific demethylase 1) and JMJD2C (jumonji C domain-containing oxygenase D2C) (Yu et al. 2018). In addition, the reversal of therapy-induced senescence in lymphoma cells has also been implicated in aggressive tumor relapse due to acquisition of cancer stemness (Milanovic et al. 2018). Although further experimental validations are required, our view might provide an unprecedented perspective for the full spectrum of brain aging: continuous changes in cell cycle state from reversible (quiescence) to a stable state (senescence) and partial reactivation (neuronal death).

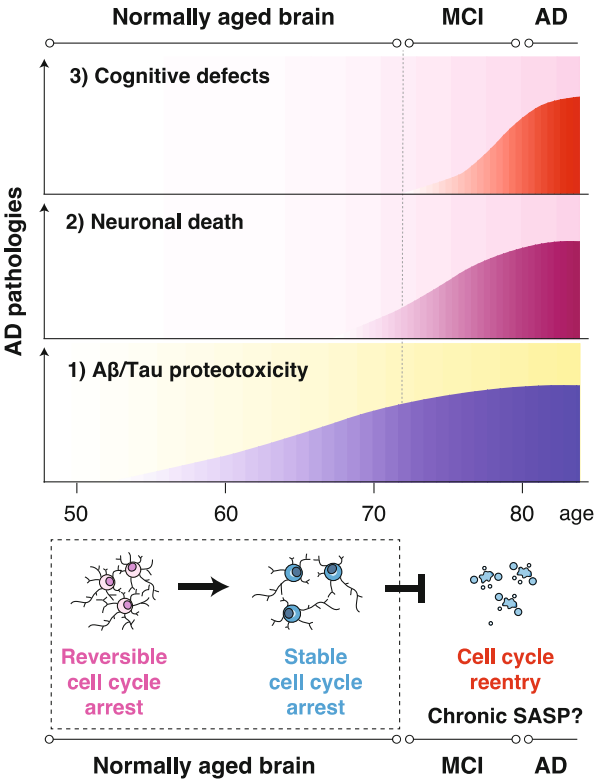

Fig. 5.2 The hypothetical model of neuronal senescence in the aging brain. During aging, proteostasis failure is coupled with an abnormal accumulation of amyloid and tau proteins in the brain, which synergistically drives neurodegeneration and cognitive impairment, ultimately leading to overt AD. Post-mitotic neurons could activate the senescence program (stable cell cycle arrest) upon loss of proteostasis, which may act to safeguard against asymptomatic proteotoxic burden

Chronic neuroinflammation has been suggested to further exacerbate the AD pathologies. Such destructive inflammation is thought to largely depend on highly active astrocytes and microglial cells. Nonetheless, Cxcl1 expression has been shown to be elevated in hippocampal neurons of AD patients and can accelerate tau pathology (Xia and Hyman 2002). Consistently, evidence from our recent study has revealed that LTC-induced senescent neurons show SASP (*Cxcl1*, *Igfbps*, and *Pai-1*) (Ishikawa and Ishikawa 2020). Furthermore, as it is specifically upregulated in the LTC neurons, the elevated Cxcl1 levels can be a reliable marker for senescent neurons. Cxcl1 has been shown to mediate non-cell autonomous/paracrine regulation of senescence in mitotic cells via its receptor CXCR2 (CXC chemokine receptor 2) (Acosta et al. 2008; Yang et al. 2006). Despite the importance in future investigations of non-cell autonomous senescence in neurons, neuronal SASP may illustrate antagonistically pleiotropic effects on neuronal cell fate during brain aging, depending on its spatiotemporal regulation: the short-term protective role (senescence induction) and the long-term destructive role (neurodegeneration) (Fig. 5.2).

5.6 Conclusion

There is growing support for the view that sustained cellular senescence is associated with age-dependent functional decline and pathology in various tissues and diseases. However, it has also been substantiated that senescent cells benefit from a number of physiological processes in vivo during embryonic development, tissue repair, and tumor suppression, endowing organisms with enhanced reproductive fitness in early life. More studies providing proof-of-concept of post-mitotic cell senescence may broaden our long-standing perspective of senescence and compel us to consider its essence apart from irreversible cell cycle arrest (Gorgoulis et al. 2019). We propose a model in which post-mitotic cell senescence can provide a counterintuitive homeostatic function in preserving tissue integrity at the very end of the life cycle, even though there would be a cost-effectiveness threshold counter-balanced by SASP. Further elucidation of the adaptive mechanisms of neuronal senescence may provide valuable insights into promising therapeutic approaches for early intervention to prevent neurodegenerative diseases, such as AD. Certainly, additional work is warranted to fully elaborate the new paradigm of senescence by addressing some major questions. Is post-mitotic cell senescence induced early in life? If so, is it restricted to specific cell types? What is the physiological consequence of post-mitotic cell senescence? What is the cellular fate of senescent post-mitotic cells (e.g., immune surveillance or escape from stable cell cycle exit)? What molecular markers delineate the different cellular states (terminal differentiation, quiescence, and senescence)? With the intensifying interest in aging and age-related diseases, the answers to these questions will no doubt be forthcoming.

Acknowledgments We are grateful to Dr. J. Hejna for the critical reading of the manuscript. This study was supported by JSPS KAKENHI Grant Number 19H05655 and the Core-to-Core Program (JPJSCCA20200009) from the Japan Society for the Promotion of Science (JSPS) (to F.I.). S.I. is supported by a scholarship of the Alexander von Humboldt Foundation.

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Chapter 6
C. elegans Longevity Genes

Takaya Sugawara and Kazuichi Sakamoto

Abstract In recent years, due to animal ethics, there has been increasing public criticism to avoid using mammals in experiments unnecessarily. For such reason, the search for alternative animal models becomes necessary. *Caenorhabditis elegans* is a transparent nematode about 1 mm in length and often used as an experimental model animal. This is because *C. elegans* has a short life span and can be grown in simple laboratory facilities, and many of its genes are homologous to those of mammals. Nematodes have some genes related to aging called longevity genes. Deletion of these genes alters various physiological functions, including metabolism, antioxidant activity, and life span. It is now known that many of them are contained in some signaling pathways and that their activity is altered by various stimuli. Stimulation from signaling pathways promotes the nuclei localization of several transcription factors and regulates the transcription of various genes and consequently the life span of *C. elegans* will change. In this chapter, we will present the basic characteristics of *C. elegans*, classical and up-to-date methods of measuring life span, aging phenotype using fluorescence and well-known longevity genes. This article will convince you that *C. elegans* can be an alternative model organism for mammals.

Keywords *Caenorhabditis elegans* · Longevity genes · Aging · Model organism · Signaling pathway

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_6

6.1 *Caenorhabditis elegans*

Caenorhabditis elegans, which is often used as a model organism for aging experiments, is a nonparasitic, molting animal belonging to the phylum Nematoda and is a linear animal with a thick cuticular layer covering its epidermis. Under unfavorable conditions, such as inadequate food or high temperatures, the first stage (L1) larvae can survive for several months by transitioning to dauer at a late stage of L1 larvae, thus slowing down their metabolism until the environment changes to a more favorable one (Fig. 6.1) (Golden and Riddle 1984).

Under natural conditions, *C. elegans* live in the soil and feed on bacteria by moving their pharynx back and forth. Analyses of the bacteria in the gut of *C. elegans* suggest that in the natural environment, it feeds on a variety of bacterial species (Dirksen et al. 2016; Zhang et al. 2017). In contrast, in a laboratory environment at 20 °C, nematodes generally feed on *Escherichia coli* and can survive for approximately 20 days on the agar media (Stiernagle 2006). They are easy to culture and can be observed with a 10× optical microscope and do not require sophisticated laboratory environments (Nigon and Félix 2017). There are two sexes in nematodes: hermaphrodites and males. Most nematodes are hermaphroditic, which means they can self-fertilize and lay eggs. Due to self-fertilization, the nematodes used in experiments have the same genetic background with little individual variation (Stiernagle 2006; Nigon and Félix 2017). Males have a hooked tail tip, and mating with hermaphrodites is thought to produce individuals that can tolerate environmental changes (Sulston et al. 1980). The percentage of males is

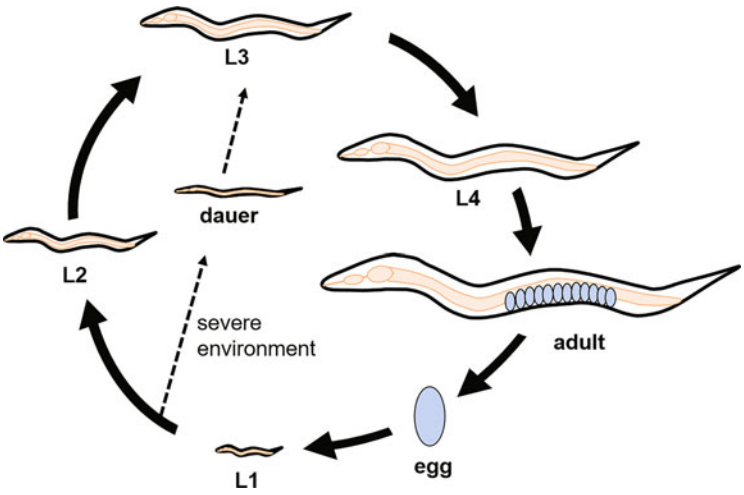

Fig. 6.1 The life cycle of *C. elegans*. *C. elegans* hatch from eggs and molt four times before becoming adults. *C. elegans* in severe environments become dauer larvae and wait for the environment to improve

very low, less than 1%, but it can be artificially increased by exposing them to a high temperature of 30 °C (Wb 1988). The hermaphrodite can lay the next generation of eggs within 4 or 5 days after hatching, so the generation change is rapid. Nematodes are widely used as model organisms for aging experiments because of their easy rearing and short life span. The life span of mice, which is often used as a model organism for experiments, is several years. Thus, nematodes can facilitate the completion of experiments in a shorter period of time (Son et al. 2019). The study of nematodes began actively about half a century ago. At the very beginning, Sidney Brenner and his colleagues identified all the cell lineages of *C. elegans* (Brenner 1974). In 1998, the entire genome of a multicellular organism was sequenced for the first time using *C. elegans*, and approximately 19,000 genes on 6 chromosomes were identified (C. elegans Sequencing Consortium 1998). It has been shown that hermaphroditic nematodes have 959 somatic cells, while male nematodes have slightly more than 1000 somatic cells (Ellis and Horvitz 1986). Whole genome analysis has shown that many genes in *C. elegans* are homologous to those in higher animals (Kim et al. 2018). *C. elegans* was the first organism in which RNA interference (RNAi) was established by Fire et al. and has since been studied using various genetic manipulation techniques, including genetic recombination using CRISPR (Fire et al. 1998; Friedland et al. 2013). Owing to the advantages of these technologies for easy genetic modification, there are abundant gene-deficient mutants and recombinants that have been created by numerous research groups. Many of them are available from libraries such as Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, USA) and National BioResource Project (NBRP, Tokyo Women's Medical University, Tokyo, Japan), and these mutants are also used as models for the analysis of specific nerves and proteins. Therefore, *C. elegans* is often used as a model for the analysis of pathologies of Alzheimer's disease and Parkinson's disease (Alexander et al. 2014; Cooper and Van Raamsdonk 2018). In addition to their nervous and reproductive systems, they also have digestive organs, such as the intestine. Thus, substances ingested as food are digested and absorbed. Therefore, they are often used as experimental model organisms for the functional evaluation of orally administered food extracts and functional substances (Sugawara and Sakamoto 2018). In recent years, alternative model organisms for mammalian experimental animals have become necessary due to increasing public criticism of animal ethics. *C. elegans* is considered to be the most promising alternative model organism for mammals for the reasons mentioned above.

6.2 Methodology

Life span measurements of nematodes are usually initiated by rearing newly emerged larvae in isolation or by crushing adult nematodes and extracting their eggs for a synchronized treatment to align the developmental stages (Corsi et al. 2015). *C. elegans* usually feed on bacteria, and when reared in a laboratory

environment, OP50, an *E. coli* strain, is generally used as food. When RNAi is performed, HT115, an *E. coli* strain, is used as food (Timmons et al. 2001). When analyzing the physiological effects of bacteria, such as *Lactobacillus*, which are known as probiotics, it is possible to analyze the life span of nematodes by feeding only those bacteria instead of *E. coli* (Nakagawa et al. 2016). Nematodes are commonly cultured on an agar medium coated with *E. coli*. The common method used to measure their life span is by contact with a platinum picker. Although this method is reliable, it has some limitations. A major problem is that it causes a heavy burden on the experimenter. Although it does not take much time to measure the life span of each individual, measuring the life span of hundreds of nematodes requires an enormous amount of time and effort. One way to solve this problem is to measure the autofluorescence (Coburn et al. 2013). It has been confirmed that *C. elegans* emits blue fluorescence at the time of death under stressful environments, such as heat and oxidative stress, and it is expected to be applied to the aging life span analysis (Coburn et al. 2013). This autofluorescence, called death fluorescence, is caused by anthranilic acid in the nematode. Anthranilic acid is present in the gut granule in the intestine of the nematode, and several hours before and after the death of the nematode, the gut granule collapses due to Ca^{2+} stimulation causing anthranilic acid to leak out, which is observed as a blue fluorescence. The collapse of the gut granule is accompanied by the migration of Ca^{2+} through the channel from the anterior to posterior part of the nematode, and the blue fluorescence is observed in a wave-like pattern from the anterior to posterior direction (Coburn et al. 2013). This autofluorescence is also observed in other organisms; however, the transparent body of the nematode facilitates the observation of the fluorescence inside the individual. In recent years, as bioinformatics has become more sophisticated, many methods have emerged to reduce human labor. One of these methods is to take photographs or videos of nematodes and then process the data to automatically determine whether they are alive or dead (Felker et al. 2020). This is expected to greatly reduce the burden of life span measurements. When measuring the life span of *C. elegans*, it is very difficult to measure if larvae are mixed in the media. Therefore, in many cases, fluorouridine is added when measuring the life span of *C. elegans* (Mitchell et al. 1979). Under normal conditions, fluorouridine has almost no effect on the physiology of *C. elegans*, but it is known to have some effects on certain genetic mutants (Aitlhadj and Stürzenbaum 2010). To eliminate this effect, few previous studies have used continuous water flow to eliminate the larvae (Xian et al. 2013). Although it requires a special device, it has succeeded in further reducing labor by combining it with automatic determination using the computer described above. In this way, although artificial measurements on solid media are still the mainstream method for life span measurement, semi-automated and fully automated methods are emerging with the development of bioinformatics technology.

6.3 Aging Phenotype

Anthranilic acid, which emits autofluorescence, is not the only phenotype that becomes apparent with aging. Similar to other organisms, aging in nematodes is accompanied by a decrease in motility and a delay in metabolism. In *C. elegans*, the inside of the body can be observed since they are transparent, whereas in other organisms, tissue sections must be used for analysis. For example, lipofuscin, known as the age pigment, is a lysosomal remnant that accumulates with aging (Terman and Brunk 1998). The level of lipofuscin accumulation can be observed and quantified in the whole nematode body without using tissue sections (Clokey and Jacobson 1986). Phenotypes associated with aging can be observed using GFP fluorescence. Since genetic recombination technology is well developed in *C. elegans*, it is easy to create recombinants that have GFP. Therefore, by using recombinants that fuse GFP into proteins specific to muscles and mitochondria, muscle shrinkage and mitochondrial aggregation associated with aging can be observed in the body (Palikaras et al. 2015). Reproductive capacity is closely related to aging, and there is usually a trade-off between life span and reproductive capacity (Cox et al. 2010). This is no exception for nematodes. Nematodes without reproductive organs, either artificially removed or genetically absent, have their life span extended by more than 50% (Antebi 2013). In the past, nematodes with such changes in life span have also been discovered by attempting to analyze the emerging phenotype by deleting specific genes. In these attempts, many genes related to life span and aging have been identified.

6.4 Longevity Genes

Many genes are known to be responsible for the life span of animals, and these are called longevity genes. Among these, sirtuin is well known. Sirtuin was initially discovered in yeast as the Sir2 gene and is an NAD-dependent histone deacetylase that plays an important role in DNA silencing and other functions that control life span (Imai et al. 2000). In addition to yeast, genes homologous to sirtuin have been found in nematodes and mammals (Wood et al. 2004; Michan and Sinclair 2007). The *age-1*, a gene that regulates life span in *C. elegans*, was discovered in 1988 and acts as a phosphoinositide 3-kinase (PI3K) (Friedman and Johnson 1988). Deletion of this gene increases the life span of nematodes by approximately 40–60% (Friedman and Johnson 1988). A number of other longevity genes have been discovered in *C. elegans*, and they are often a component of a continuous signaling pathway. For example, the insulin/IGF-1 signaling (IIS) pathway, p38 mitogen-activated protein kinase (MAPK) pathway, and c-Jun N-terminal kinase (JNK) pathway are well-known signaling pathways in *C. elegans*. The signals from these pathways activate transcription factors and regulate the transcription of various genes that regulate the life span (Table 6.1) (Lin et al. 2001; Oh et al. 2005; Troemel et al. 2006; Lapierre

Table 6.1 The components and target transcription factors of signaling pathways

Signaling pathway	Components	Target transcription factor
IIS (Kim et al. 2018)	DAF-2, AGE-1, PDK-1, PPTR-1, SGK-1, AKT-1/2, DAF-18	DAF-16
p38 MAPK (Inoue et al. 2005)	TIR-1, NSY-1, SEK-1, PMK-1	SKN-1
JNK (Oh et al. 2005; Neumann-Haefelin et al. 2002)	MEK-1, JKK-1, JNK-1	DAF-16
TOR (Blackwell et al. 2019)	LET-363, DAF-15, RICT-1, MLST-8, SINH-1, RAGA-1, RHEB-1, NPRL-2/3	PHA-4

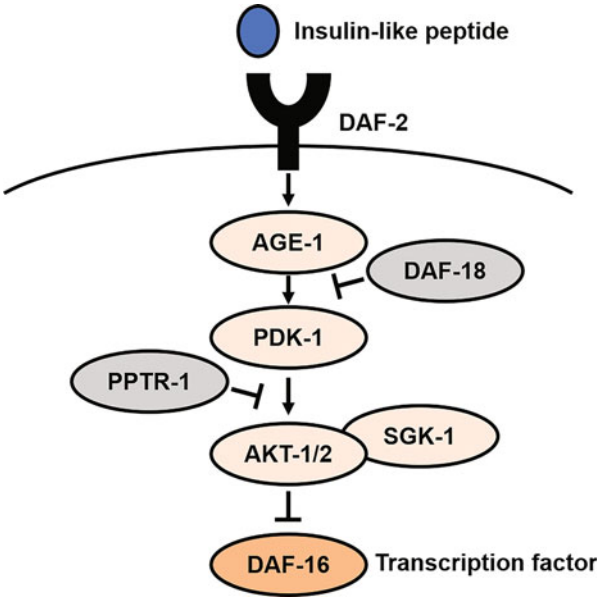

Fig. 6.2 Component proteins in the IIS pathway. The insulin/IGF-1 signaling (IIS) pathway is a well-known cascade. DAF-16 is dephosphorylated and translocated into the nucleus by stimulation from the IIS pathway, where it regulates the transcription of genes involved in stress tolerance, resulting in the extension of life span

and Hansen 2012; Blackwell et al. 2019).These signaling pathways are intricately related to each other, and the IIS pathway has been one of the most studied signaling pathways since the beginning. The IIS pathway, including *age-1*, was discovered by searching for abnormal dauer formation (daf) (Fig. 6.2) (Guarente and Kenyon 2000). The effect of the IIS pathway on life span is so dramatic that a mutant lacking the *daf-2* gene, which accepts insulin-like peptides, has been shown to increase life span by more than twofold (Kenyon et al. 1993). The DAF-16, a transcription factor, is dephosphorylated and translocated into the nucleus by stimulation from the IIS pathway, where it regulates the transcription of genes involved in stress tolerance, resulting in the extension of life span (Lin et al. 2001). Signaling pathways other than the IIS pathway are also known to be activated by stress and caloric restriction. Their stimulation leads to the nuclei translocation of various transcription factors, which in turn regulate the transcription of genes involved in energy metabolism and stress

tolerance (Inoue et al. 2005; Hansen et al. 2007; Pastuhov et al. 2016). This prolongs the life span of *C. elegans*. These cascades are also known to be homologous in many other organisms, including humans (Ogg et al. 1997; Kim et al. 2002; Oh et al. 2005; McCormick et al. 2011). The signal cascades in *C. elegans* is now being elucidated in much detail, and the elucidation of these cascades suggests the possibility of elucidating the mechanisms of human life span and aging (Lee et al. 2015). These pathways are intricately related, and the analysis of pathways in mammals is extremely difficult. Hence, *C. elegans*, as the simplest multicellular model organism, is expected to bring a breakthrough in the development of aging research.

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Chapter 7
Understanding the Functions of Longevity Genes in *Drosophila*

Toshiro Aigaki and Manabu Tsuda

Abstract The fruit fly, *Drosophila melanogaster*, has been an excellent model organism to study aging and longevity. A number of genes affecting longevity have been identified by forward and reverse genetic approaches. Historically, antioxidant genes were the first target to study their roles in aging and longevity, as predicted by the "free radical theory of aging." Superoxide dismutase (SOD), catalase (Cat), and thioredoxin (TRX) have been examined with transgenic flies. SOD and TRX have multiple copies, each of which has a unique expression pattern and functional property. The next target was the insulin/insulin-like growth factor-1 (IGF-1) signaling pathway, which controls growth, body size, oxidative stress resistance, and longevity. The Jun N-terminal kinase (JNK) signaling pathway plays a critical role in regulating organismal physiology upon oxidative stress and longevity. More recently, an emerging target is epigenetic mechanisms, which appear to control longevity with novel pathways.

Keywords Antioxidant · Stress resistance · Insulin/IGF-1 signaling · JNK signaling · Epigenetic mechanism

7.1 *Drosophila melanogaster* as a Model System to Study Aging

The fruit fly, *Drosophila melanogaster*, has been used as a model organism to study aging and longevity. Its genome is relatively small (180 Mbp), containing approximately 14,000 genes (FlyBase, http://flybase.org/). More than 50% of them have homologs in humans and share 75% of known human disease-related genes. It takes

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_7

10–14 days from eggs to adult flies, which produce the next generation within a few days. A mated female can lay 50–100 eggs per day, and because of its small body size, it is easy to collect a large number of flies and perform experiments in a limited space. The longevity of adult flies is from 1 to 2 months, depending on the genetic and environmental conditions. Finally, advanced genetic techniques and resources are available.

Identification of mutants is the first step of the genetic approach to the mechanism of aging and longevity determination. Genes affecting longevity have been identified by forward and reverse genetic approaches. The gene functions have been assessed using gain-of-function (overexpression/misexpression), loss-of-function mutants, or RNA-mediated knockdown. In this chapter, we will review some of the representative longevity genes, which are related to (1) antioxidant, (2) insulin/IGF-1 signaling, (3) JNK signaling, and (4) epigenetic mechanism.

7.1.1 Antioxidant

Oxidative stress is the primary cause of aging and is implicated in many age-associated diseases, including Parkinson's disease and Alzheimer's disease (Dawson and Dawson 2003; Finkel and Holbrook 2000; Harman 1956). Oxidative stress can be induced by external or internal factors, such as UV radiation or the respiratory system in mitochondria. It can damage cellular macromolecules, such as nucleic acids, proteins, and lipids, which may interfere with normal cellular functions and ultimately lead to cell death (Imlay 2003). Therefore, antioxidant defense systems must have critical roles in maintaining normal cellular processes during aging. Organisms carrying mutations in genes responsible for antioxidant defense mechanisms likely shorten longevity, whereas its enhancement may extend longevity. We focus on the most studied antioxidant genes encoding superoxide dismutase (SOD), catalase, and thioredoxin in *Drosophila*.

SOD scavenges superoxide anion radicals and thus protects cells from oxidative damage. The *Drosophila* genome contains three genes *Sod1*, *Sod2*, and *Sod3*. Localization of the protein products is different; SOD1, SOD2, and SOD3 are in the cytoplasm, mitochondria, and extracellular space, respectively. SOD1 and SOD3 are copper/zinc (Cu/Zn) SODs, whereas SOD2 is a manganese (Mn) SOD.

7.1.1.1 Cytoplasmic SOD (Sod1)

Flies with a null mutation of *Sod1* are hypersensitive to paraquat, a free radical generator, and have shortened longevity (Phillips et al. 1989). The mutant flies were hypersensitive to hyperoxia, glutathione depletion, and ionizing radiation, and all these phenotypes were rescued by a wild-type *Sod1* transgene (Parkes et al. 1998a). The introduction of additional copies of the *Sod1* gene did not show a marked

increase in longevity (Seto et al. 1990). A slight rise in longevity was observed when bovine Cu/Zn SOD was expressed using the *actin5c* promoter (Reveillaud et al. 1994). In contrast, transgenic flies overexpressing human SOD1 in adult motoneurons dramatically extended longevity by up to 40% and rescued other defects of the null mutant (Parkes et al. 1998b). Sun and Tower (1999) developed an "FLP-OUT" system to overexpress a gene at desired life cycle stages with a controlled genetic background. The longevity of flies overexpressing SOD1 extended up to 48% (Sun and Tower 1999). The longevity extension by SOD1 overexpression was striking in the experiments, where control flies were relatively short-lived (Orr and Sohal 2003). Thus, the effects of SOD1 overexpression appear to be dependent on the experimental context.

7.1.1.2 Mitochondrial SOD (Sod2)

SOD2, a manganese superoxide dismutase (Mn-SOD), detoxifies superoxide radicals (O^{-}_{2}) in mitochondria. The role of this enzyme must be critical for cells to protect from oxidative damage during aging. Transgenic overexpression of Mn-SOD reduced the longevity by 4–5% (Mockett et al. 1999). There was no difference in the hydrogen peroxide-releasing rate of mitochondria, protein oxidative damage, or resistance to 100% oxygen between wild-type and flies overexpressing Mn-SOD. When Mn-SOD was overexpressed using the FLP-OUT technique, the mean longevity of flies increased by an average of 16% (Sun et al. 2002). The maximum longevity increased by 15%, but the one line showed a 37% increase. Simultaneous overexpression of catalase and Mn-SOD had no additional benefit, consistent with the previous observations that catalase is present in excess in adult flies through longevity. The RNA interference (RNAi)-mediated knockdown of *Sod2* causes mortality in young adults and enhances sensitivity to paraquat toxicity (Kirby et al. 2002). Knocking down of *Sod2* did not cause overtly harmful effects on larval and pupal development. A null mutation, $Sod2^{n283}$, was generated by imprecise excision of a *P*-element transposon inserted in the locus (Duttaroy et al. 2003). Adult flies homozygous for the mutation died within 24 h after eclosion, indicating a critical role of *Sod2* in adult survival. Flies heterozygous for the mutation ($Sod2^{n283/+}$) are sensitive to oxidative stress induced by paraquat treatment. The adult lethality of $Sod2^{n283}$ was exclusively due to the loss of *Sod2* function since a wild-type *Sod2* transgene rescued this phenotype.

7.1.1.3 Extracellular SOD (Sod3)

The *Drosophila Sod3* gene encodes a functional extracellular SOD. The physiological role of *Sod3* has been investigated using a loss of function mutant and the RNA-mediated knockdown (Jung et al. 2011). Neither the mutation nor knockdown of *Sod3* shows any apparent defects during development. However, longevity was significantly reduced in these flies at 25 and 29 °C, indicating that *Sod3* is required

for normal longevity in adult flies. Since they also show reduced viability against paraquat treatment, *Sod3* appears to play a significant role as a superoxide anion scavenger.

Sod3 is one of the highly upregulated genes in a fly model of amyloid β (Aβ) toxicity (Favrin et al. 2013). RNAi-mediated knockdown of *Sod3* improved the phenotype associated with Aβ-expressing flies, namely, climbing performance and survival. The results indicate that *Sod3* in Aβ model flies increases Aβ toxicity. Since there was no increase in catalase expression in Aβ flies, the upregulation of *Sod3* may increase toxic H_2O_2.

7.1.1.4 Catalase (Cat)

Flies with a hypomorphic mutation in *Cat* had only 14% catalase activity in the parent control flies that had average longevity (Orr and Sohal 1992). Transgenic flies overexpressing *Cat* with increased levels of catalase activity (up to 80%) showed enhanced resistance to hydrogen peroxide but did not extend longevity (Orr and Sohal 1994). The overexpression of both Cu/Zn SOD (*Sod1*) and *Cat* exhibited a one-third extension of longevity and reduced the accumulation of 8-hydroxydeoxyguanosine during aging and in response to the exposure of live flies to X-rays (Sohal et al. 1995). On the contrary, there was no significant increase in flies' longevity overexpressing *Cat* and flies co-overexpressing Cu/Zn SOD (*Sod1*) and Cat (Sun and Tower 1999). Catalase was targeted ectopically to the mitochondria matrix by fusing a leader peptide derived from ornithine aminotransferase with its N-terminus (Mockett et al. 2003). There was no impact of this targeted expression of catalase on the longevity of the flies. However, they became more resistant to exogenous hydrogen peroxide, paraquat, and cold stress (Mockett et al. 2003).

7.1.1.5 Thioredoxin (Trx-2, TrxT, dhd)

Thioredoxin (TRX) is an antioxidant molecule conserved from bacteria to humans (Arner and Holmgren 2000). The sequence containing the redox-active site, Cys-Gly-Pro-Cys-Lys, is conserved among all TRX family proteins (Holmgren 1985). It is a major cellular protein disulfide reductase carrying a conserved active site with a pair of cysteine residues, and it serves as an electron donor to enzymes, such as thioredoxin-dependent peroxide reductase (Miranda-Vizuete et al. 2000; Chae et al. 1994) and ribonucleotide reductase (Thelander and Reichard 1979; Holmgren 1985). Upon substrate reduction, two sulfhydryl (SH) groups in the active center of reduced thioredoxin, Trx-(SH)2, are converted to disulfide in the oxidized form, Trx-S2. TRX is induced by various oxidative stimuli, including UV irradiation, inflammatory cytokines, and chemical carcinogens, and plays crucial roles in regulating cellular responses such as gene expression, cell proliferation, and apoptosis (Nishinaka et al. 2001).

The *Drosophila* genome contains three TRX family genes, *Trx-2*, *TrxT*, and *deadhead* (*dhd*), all of which have a characteristic active center for TRX and show a similar extent of sequence homology to human TRX. *Trx-2* is expressed ubiquitously, whereas *dhd* and *TrxT* are sex-specific, predominantly expressed in females and males, respectively (Svensson and Larsson 2007; Svensson et al. 2003; Pellicena-Palle et al. 1997; Salz et al. 1994).

Trx-2 is one of the longevity-extending genes identified in a systematic gain-of-function screen in *Drosophila* (Seong et al. 2001a). *Trx-2* was overexpressed ubiquitously under the control of an *hsp70* promoter. Later it was shown that neural-specific overexpression of any of TRX (*Trx-2*, *TrxT*, *dhd*) is sufficient for extending longevity and improving locomotor activity in aged animals (Umeda-Kameyama et al. 2007). Besides longevity, the overexpression of *Trx-2* increases resistance to oxidative stress in adult flies (Svensson and Larsson 2007).

Studies on loss-of-function mutants are necessary to understand the role of endogenous TRX in oxidative stress resistance and longevity. Loss-of-function mutation in *Trx-2* has been generated by *P*-element imprecise excision and used for biochemical and physiological characterization (Tsuda et al. 2010b). The loss of *Trx-2* reduced longevity, hypersusceptibility to paraquat, and accumulation of protein carbonyl, an oxidative stress marker in aged animals. The mean longevity of the mutant was 36% shorter than that of wild-type flies. In addition, *Trx-2* mutants expressed high levels of antioxidative genes, such as *Sod1*, *catalase*, and *glutathione synthetase*, suggesting that they are exposed to high levels of oxidative stress.

The overexpression of any of the *Drosophila* TRX genes has been shown to suppress the accelerated neurodegeneration that occurs in the *Drosophila* Parkinson's disease model, in which the human Parkin-associated endothelin receptor-like receptor (Pael-R) is expressed in all neurons (Umeda-Kameyama et al. 2007). The Pael-R-induced phenotype includes the selective loss of dopaminergic neurons and reduced locomotor activity, and all of these were suppressed by *Drosophila* TRX as efficiently as human Parkin (Yang et al. 2003). The mechanism of suppression could be complex since TRX has a wide variety of cellular functions, including a cytoprotective effect against oxidative stress (Nakamura et al. 1994; Andoh et al. 2002), a neuroprotective activity (Hori et al. 1994), a neurotrophic activity (Endoh et al. 1993), the regulation of the stability of apoptosis signal-regulating kinase 1 (ASK1) through ubiquitination-proteasomal degradation (Liu and Min 2002), and to interact with unfolded and denatured proteins as a molecular chaperone (Kern et al. 2003). To assess the role of the redox activity of TRX in suppressing Pael-R-induced neurotoxicity in flies, redox-defective mutants, TrxT (C35A) and TrxT(D26A/K57I), have been generated. The TRX mutants could suppress the neurodegenerative phenotype, indicating that the redox activity of TRX is dispensable for inhibiting Pael-R-induced neurotoxicity. Also, the neuroprotective function of wild-type and redox-defective TRX was observed in a *Drosophila* model of Machado-Joseph disease (MJD) expressing polyglutamine (Warrick et al. 1998). Since the redox-defective TRX mutants were active as a chaperone, its activity could be necessary to suppress Pael-R or polyglutamine-induced neurotoxicity.

7.1.2 Insulin/IGF-1TOR Pathway

Insulin is an evolutionally conserved peptide hormone secreted from the pancreas and promotes glucose uptake in muscle and adipose tissue. Insulin also stimulates cell growth and differentiation and promotes the storage of glucose and lipids by stimulating amino acid uptake, protein synthesis, glycogenesis, and lipogenesis (Saltiel and Kahn 2001). The insulin/IGF-1 and target of rapamycin (TOR) pathways are among the signaling pathways that control cell and organismal growth, body size, and longevity. Dietary restriction or mutations that reduce the insulin/IGF-1/TOR signaling activity produce a small body size and extend longevity.

In *Drosophila*, a mutation-reducing body size was first identified among a collection of *P*-element insertion lines (Bohni et al. 1999). The gene was named *chico*, which means small body in Spanish. It encodes a protein similar to the vertebrate insulin receptor substrate (IRS), and *chico* mutants are less than half the size of wild-type flies, owing to fewer and smaller cells. The mutants show metabolic abnormalities such as delayed development and abnormal accumulation of lipids (Bohni et al. 1999). Then, *chico* mutants were found to be long-lived (Clancy et al. 2001).

Insulin-like receptor (*InR*) mutants were also long-lived (Tatar et al. 2001). Heteroallelic combinations of *InR* alleles were used to produce viable and dwarf adults with a substantially low level of INR kinase activity. Among four distinct alleles, only InR^{p5545}/InR^{E19} females showed extended longevity. The *InR* dwarf female flies appear to be very much affected by the endocrine system. Juvenile hormone (JH) synthesis was significantly reduced in mutant females. In the mutant dwarf flies, the triacylglycerol level is elevated fourfold, as observed in diapause *D. triauraria* mutants and dwarf *D. melanogaster* mutants for *chico* and Cu/Zn SOD activity increases twofold. The topical application of a JH analog, methoprene, to the mutant females could induce vitellogenesis and revert the long-lived phenotype to the control level. Therefore, partial defects in JH synthesis account for infertility and extended longevity (Tatar et al. 2001).

The *Drosophila* genome encodes eight insulin-like peptides (*Drosophila* insulin-like peptides1–8: Dilp1–8). In adult flies, *Dilp2*, *Dilp3*, and *Dilp5* are expressed in median neurosecretory cells in the brain. The ablation of these cells leads to increased fasting glucose levels in the hemolymph of adults, similar to that found in diabetic mammals (Broughton et al. 2005). They also exhibit increased lipid and carbohydrate storage, reduced fecundity, and reduced tolerance to heat and cold. The ablated flies show extended longevity and increased resistance to oxidative stress and starvation, implying that these ligands are involved in the insulin/IGF-1 signaling (Broughton et al. 2005). The RNAi-mediated knockdown of *Sir2*, a mammalian SIRT1 homolog, upregulates *Dilp2* and *Dilp5* expression. These genes might be involved in the mechanism of longevity extension by dietary restriction (Banerjee et al. 2012, 2013). Since the expression of *Dilp3* and *Dilp5* is upregulated in *Dilp2* knockdown individuals, there may be a compensatory mechanism among these genes (Grönke et al. 2010).

The role of intracellular components of the insulin/IGF-1/TOR pathways, such as PTEN, FOXO, TOR, 4E-BP, and S6K, has been examined for their functions to regulate longevity (Partridge et al. 2011; Kannan and Fridell 2013). We identified *wdb* and *lkb1* as longevity-extending genes through a gain-of-function screen of those already screened for the ability to reduce wing and eye sizes (Funakoshi et al. 2011). The overexpression of *wdb* reduces the level of phosphorylated AKT, while the overexpression of *lkb1* increases the level of phosphorylated AMPK and reduces the level of dephosphorylated S6K. These results suggested that *wdb*- and *lkb1*-dependent longevity extension was mediated by the downregulation of S6K, a downstream component of the insulin/IGF and TOR signaling pathways.

Tsuda et al. (2010a) provided genetic evidence that insulin-degrading enzyme (IDE) antagonizes Dilp2 signaling and the human Aβ-induced neurotoxicity in *Drosophila* Alzheimer model. IDE, a zinc metalloendopeptidase, has been implicated in the pathogenesis of both DM2 and AD (Fakhrai-Rad et al. 2000). Overexpression of *Drosophila* Ide (dIde) in IPCs reduces to 92% and 94% of control flies, respectively. When dIde or human IDE (hIDE) was misexpressed in the developing wing imaginal discs, the wing size was significantly reduced in these flies: 82% and 83% of the control, respectively. These results indicate that both *dIde* and *hIDE* negatively regulate tissue growth. Misexpression of *Dilp2* in developing wing imaginal discs increases wing size. However, the co-overexpression of *dIde* suppresses the Dilp2-induced phenotype. *Dilp2* promotes growth through the insulin receptor, InR (Brogiolo et al. 2001). Overexpression of *InR* in the wing imaginal disc also increases wing size. However, unlike those induced by *Dilp2*, the InR-induced phenotype is not suppressed by co-overexpression of *dIde*, suggesting that dIde acts upstream of InR. PTEN is a negative regulator of the insulin signal by inhibiting PI3K activity. Loss-of-function mutations in PTEN increase body size by elevating PI3K activity (Goberdhan et al. 1999). dIde overexpression did not affect the large wing phenotype caused by the PTEN mutation. These genetic experiments suggest that dIde negatively regulates the insulin signaling pathway, most likely between the Dilp2 ligand and InR.

Human IDE is capable of digesting both β-amyloid (Aβ) and the Aβ precursor protein (APP) intracellular domain (AICD) in vitro. To examine whether overexpression of *dIde* can suppress the neurotoxicity induced by Aβ in vivo, we used the *Drosophila* AD model, in which human APP and the β-site APP-cleaving enzyme (BACE) are misexpressed in photoreceptor neurons (Greeve et al. 2004). In this model, a highly organized architecture of retinal photoreceptors degenerates in an age-dependent manner. Forced expression of *dIde* or *hIDE* suppresses neuronal degeneration in this model. In addition, pan-neural overexpression of APP and BACE using *elav*-GAL4 shortens the longevity of adult flies. The reduced life span was partially rescued by forced expression of *dIde* or *hIDE*, suggesting that dIde or hIDE can inhibit the pathological processes associated with Aβ and AICD accumulation in vivo.

7.1.3 JNK Signaling Pathway

Seong et al. (2001a) identified 25 genes whose overexpression extended the longevity through a misexpression screen. Among 13 genes whose functions are known or suggested, six were related to stress resistance or redox balance. We investigated the function of *plenty of SH3s* (*POSH*) in detail. In mammals, POSH has been shown to function as a scaffold protein need to activate the JNK signal (Saitoh et al. 1998; Villafania et al. 2000; Tapon et al. 1998). It has a RING finger domain and four SH3 domains, which were conserved between *Drosophila* and mammals. Neural-specific overexpression of *POSH* extended the longevity by 14% (Seong et al. 2001b). In addition, forced expression of *POSH* during development caused various morphological abnormalities, reminiscent of ectopic activation of the JNK signal. Overexpression of *POSH* induced *puckered* (*puc*) encoding a serine/threonine protein phosphatase induced by JNK activation. POSH is also required for terminating immune response after infection through degrading TAK1, an activator of both the JNK and the Relish pathways (Tsuda et al. 2005).

The JNK signaling cascade is triggered by a variety of insults, including UV radiation and oxidative stress. Wang et al. (2003) identified downstream target genes induced by JNK signaling and demonstrated the role of JNK signaling in oxidative stress tolerance. Longevity was dramatically extended in flies heterozygous for a loss-of-function allele of *puc*, a negative regulator of JNK. The *puc*-dependent longevity extension was suppressed by a mutation of the JNK activator, hep^{1}, demonstrating that an increase in JNK signaling activity extends the longevity phenotype.

JNK promotes nuclear translocation of Foxo and induces the expression of Foxo-dependent stress response genes that promote cell-autonomous stress defense and damage repair. Wang et al. (2005) demonstrated that Foxo is required for JNK to extend longevity. JNK also antagonizes the insulin/IGF-1 signaling systemically by activating Foxo and downregulating the expression of Dilp2 in insulin-producing cells (IPCs). JNK-dependent inhibition of insulin production has been observed in low-nutrient conditions (Agrawal et al. 2016). Eiger, the *Drosophila* homolog of TNFα, is produced by fat body cells, released in the hemolymph, and activates its receptor Grindelwald locally expressed in the brain IPCs, leading to JNK-dependent inhibition of insulin production.

7.1.4 Epigenetic Mechanism

Histone modification is one of the central epigenetic mechanisms that regulate gene expression. Trimethylated histone H3 lysine 27 (H3K27me3) is repressive methylation of histone H3 established by the polycomb repressive complex (PRC) through its core catalytic subunit, the H3K27-specific methyltransferase encoded by the *E(z)* gene in flies (Jones and Gelbart 1990). Flies heterozygous for mutations in *E(z)* or

esc encoding H3 binding protein increase longevity (33% or 45% longer than control), reduce H3K27me3 levels, and increase resistance to oxidative stress and starvation (Siebold et al. 2010). Mutations in the polycomb silencing antagonist *trithorax* suppressed the increased longevity and stress resistance. Also, the moderate reduction of H3K27me3 in long-lived *E(z)* heterozygotes partially derepress direct targets of polycomb silencing. Moskalev et al. (2019) observed 22–23% life span extension in *E(z)* heterozygous mutants for both sexes and higher levels of resistance to hyperthermia, oxidative stress, and endoplasmic reticulum stress. Genome-wide transcriptome analyses identified 239 genes whose expression level was altered more than twice by *E(z)* mutation. The affected genes include those involved in carbohydrate metabolism, lipid metabolism, drug metabolism, and nucleotide metabolism.

Ma et al. (2018) observed an age-associated decrease of H3K27me3 levels in muscles and analyzed changes of epigenome profiles obtained using the ChIP followed by high-throughput DNA sequencing. There was a dramatic shift in the pattern of H3K27me3 modification in aging. The role of PRCs in aging was explored using mutations in 24 genes encoding PRC components. The majority of mutants showed mild or no effect, but those bearing esc, *E(z)*, *Pcl*, *Su(z)12* of PRC2, and *Psc* and Su(z)2 of PRC1, lived substantially longer. The combination of *Pclc421* and *Su(z)12c253*, trans-heterozygote double mutants showed the most potent effect in H3K27me3-reduction and life extension. Transcriptome analyses identified several hundreds of upregulated genes. The analysis of 63 genes with known effects on aging, including genes in insulin/IGF-1, mTOR pathways, revealed no consistent changes in the expression in PRC2 mutants. Thus they are unlikely to contribute to PRC2-dependent longevity. Gene ontology analysis revealed that the "glycolytic process" and "closely related pathways" were highlighted for genes upregulated, while the "oxidation-reduction process" was enriched for genes downregulated. LC-MS-based untargeted metabolomics also demonstrated enhanced glycolysis in long-lived PRC2 mutants. Using weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath 2008), two glycolytic genes, *Tpi* (triosephosphate isomerase) and *Pgi* (phosphoglucose isomerase), whose expressions were upregulated in different tissue types across individual long-lived PRC2 mutants. Mutations in these genes mitigate or diminish the longevity benefits of PRC2 deficiency.

Conversely, transgenic expression of *Tpi* and *Pgi* in wild-type background improved life span, locomotion, and resistance to oxidative stress. Therefore, the upregulation of glycolytic genes alone is sufficient to mimic antiaging features of PRC2 mutants. This comprehensive study underscores the mechanistic link between epigenetic, transcriptional, and metabolic processes in aging, highlighting the role of glycolysis in promoting metabolic health and longevity (Ma et al. 2018).

7.2 Epigenetic Inheritance of Longevity

Maternal diet has impacts on the metabolism and longevity in offspring. Namely, a maternal diet (high sugar) increased carbohydrate storage and decreased cholesterol storage in developing offspring, and adult offspring accumulate increased triglyceride levels when challenged with a high-sugar diet (Buescher et al. 2013). The effects can be inherited through multiple generations. *Drosophila melanogaster* will continue to be a model system to explore the mechanism underlying the transgenerational inheritance of metabolic traits.

Acknowledgments The work in our laboratory was supported in part by grants from the Ministry of Health, Labour and Welfare of Japan (MHLW); the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT); New Energy and Industrial Technology Development Organization (NEDO); the Japan Science and Technology Agency (JST); and the Japan Agency for Medical Research and Development (AMED) and a special grant from the Tokyo Metropolitan Government.

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Part IV
Metabolism: Factors Affecting Tissue Aging

Chapter 8
NAD^+ Metabolism in Aging

Sailesh Palikhe and Takashi Nakagawa

Abstract Nicotinamide adenine dinucleotide (NAD^+) is an essential molecule found in all living cells, which is involved in fundamental biological processes such as energy metabolism, DNA repair, epigenetic regulation, and neuronal axon homeostasis. Several studies have shown that tissue NAD^+ levels decrease with aging. This age-related NAD^+ decline has been associated with hallmarks of aging and the development and progression of a wide range of age-related diseases, such as metabolic disorders, cancer, and neurodegenerative diseases. Preclinical studies have largely shown that boosting NAD^+ levels by oral administration of NAD^+ precursors, such as nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN), can ameliorate age-associated pathologies and extend health span. Early clinical trial results suggest that NR and NMN are safe and bioavailable in humans and certain effectiveness has been demonstrated. Thus, NAD^+ metabolism is an exciting field whose modulation can provide beneficial effects and may improve human health.

Keywords NAD^+ · Aging · Nampt · Sirtuin · CD38 · PARP

8.1 Introduction

Nicotinamide adenine dinucleotide (NAD^+) was originally discovered as a heat-stable factor that boosts alcohol fermentation in yeast by Arthur Harden and William John Young in 1906 (Harden and Young 1906). Subsequent studies found that

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_8

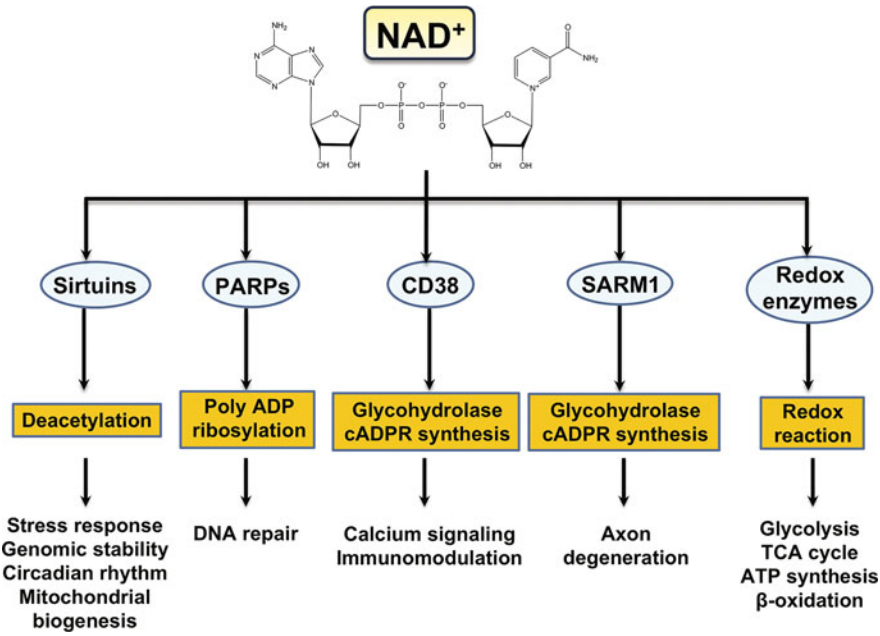

Fig. 8.1 Biological functions of NAD^+. Sirtuins, PARPs, CD38, and SARM1 use NAD^+ as a cosubstrate, while redox enzymes use NAD^+ as a coenzyme to perform various cellular functions

NAD^+ serves as a cofactor that mediates various redox reactions through the transfer of electrons between NAD^+ (oxidized form of NAD) and NADH (reduced form of NAD). Therefore, NAD^+ regulates many important metabolic pathways, such as glycolysis, fatty acid β-oxidation, the tricarboxylic acid cycle, and mitochondrial oxidative phosphorylation (Yaku et al. 2018). The importance of NAD^+ was thought to be limited to redox biology until when NAD^+ was identified as a cosubstrate for ADP-ribosylation of proteins by ADP-ribosyltransferases (Gill 1975; Kaslow et al. 1981). Since then various enzymes, such as sirtuins, poly(ADP-ribose)polymerases (PARPs), NAD^+ glycohydrolase, cyclic ADP-ribose (cADPR) synthase, DNA ligase, and NAD^+ capping enzymes, have been known to use NAD^+ as a cosubstrate (Yaku et al. 2018; Bird et al. 2016). Because of the diverse cellular functions of these enzymes, NAD^+ has important roles in critical cellular processes, such as energy metabolism, chromatin remodeling, DNA repair, inflammation, and neuronal axon homeostasis (Fig. 8.1). The discovery of its role as a cosubstrate for different cellular enzymes has reignited interest in understanding NAD^+ biology.

The deficiency of NAD^+ causes pellagra, a disease characterized by four "Ds": dermatitis, diarrhea, dementia, and death (Sydenstricker 1958). In the present world, pellagra is rare; however, decreased cellular NAD^+ concentrations occur under defined conditions, including aging and aging-related diseases (Rajman et al. 2018; Covarrubias et al. 2021). NAD^+ levels in the average healthy human adult

are maintained relatively high, ~3 g, but its levels steadily decline with age (Rajman et al. 2018). This age-related decline in NAD^+ levels has been linked strongly to aging and importantly also to the development and progression of several age-related diseases, such as obesity, type 2 diabetes, sarcopenia, and Alzheimer's disease. The decline in NAD^+ levels impairs NAD^+-dependent important cellular processes which result in cellular damage and stress (Covarrubias et al. 2021). Low levels of NAD^+ accelerate physiological aging and are thought to be one of the central phenomena for causing age-related problems (Mehmel et al. 2020). Therefore, in recent years, NAD^+ has attracted significant attention as a potential therapeutic target for treating aging and aging-associated diseases. In this chapter, we will discuss how NAD^+ is synthesized, how NAD^+ is consumed, and why NAD^+ levels decline with age, especially in mammals. We will also discuss how NAD^+ decline contributes to the development of hallmarks of aging, functional decline of various organs, and the current progress of NAD^+ precursors in preclinical and clinical studies.

8.2 NAD^+ Biosynthesis

NAD^+ is constantly consumed by the action of NAD^+-dependent enzymes in cells. In this process, the glycosidic bond between nicotinamide (NAM) and ribose moiety is broken, destroying the NAD^+ molecule (Cohen 2020). Thus, the continuous biosynthesis of NAD^+ is pivotal for maintaining its homeostatic levels in cells. In mammals, cellular NAD^+ is synthesized from a variety of dietary precursors: L-tryptophan (Trp), nicotinic acid (NA), nicotinamide riboside (NR), nicotinamide mononucleotide (NMN), and NAM (Fig. 8.2) (Yaku et al. 2018). Depending on the bioavailability of the precursors, there are three pathways for the synthesis of NAD^+ in cells: (1) the de novo pathway started from Trp; (2) the Preiss-Handler pathway utilizing NA; and (3) the salvage pathway utilizing NAM, NR, and NMN (Yaku et al. 2018). In the de novo pathway, Trp is converted to quinolinic acid (QA) through six enzymatic and nonenzymatic steps and quinolinic acid phosphoribosyltransferase (Qprt) conjugates QA with phosphoribosyl pyrophosphate to generate nicotinic acid mononucleotide (NAMN). Subsequently, NAMN is converted to nicotinic acid dinucleotide (NAAD) by NMN/NAMN adenylyltransferase (Nmnat) and then amidated to NAD^+ by NAD^+ synthetase (Yaku et al. 2018). The de novo pathway is mainly used in the liver to generate NAD^+ and supply NAM to other tissues (Liu et al. 2018). Although kidneys and macrophages are also reported to use the de novo pathway for NAD^+ synthesis, the relative contribution to systemic NAM supply is much less than that in the liver (Mehr et al. 2018; Minhas et al. 2019). In non-hepatic tissues, NAD^+ synthesis almost exclusively rely on the salvage pathway, utilizing NAM supplied from the liver and via recycling (Liu et al. 2018). In the salvage pathway, NAM is converted to NMN by nicotinamide phosphoribosyltransferase (Nampt), the rate-limiting

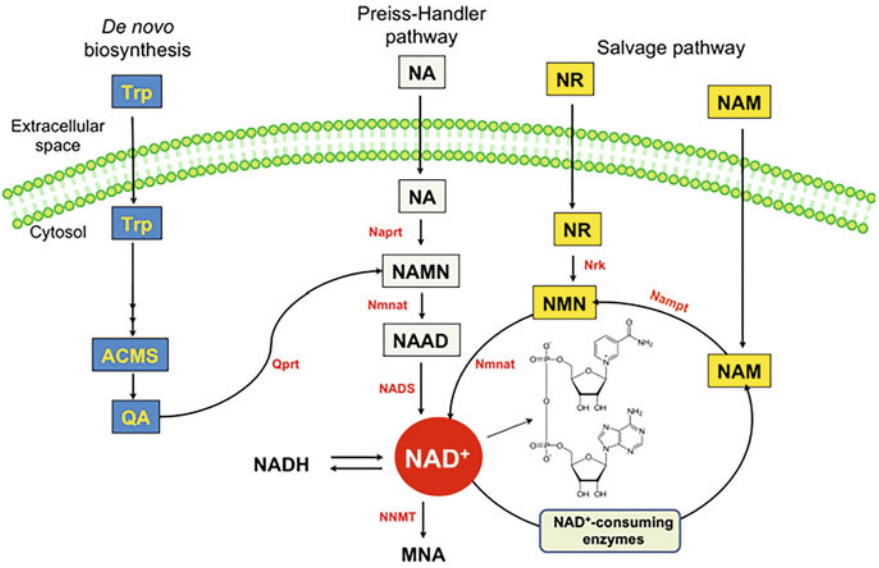

Fig. 8.2 Schematic showing NAD^+ metabolism pathway. *Trp* tryptophan, *NA* nicotinic acid, *NAM* nicotinamide, *QA* quinolinic acid, *NMN* nicotinamide mononucleotide, *NR* nicotinamide riboside, *ACMS* α-amino-β-carboxymuconate-ε-semialdehyde, *NAMN* nicotinic acid mononucleotide, *NAAD* nicotinic acid adenine dinucleotide, *NAD* nicotinamide adenine dinucleotide, *NADH* reduced form of NAD, *Nampt* nicotinamide phosphoribosyltransferase, *Nmnat* nicotinamide mononucleotide adenylyltransferase, *QPRT* quinolinic acid phosphoribosyltransferase, *NAPRT* nicotinic acid phosphoribosyltransferase, *Nrk* nicotinamide riboside kinase, *NADS* NAD^+ synthetase, *NNMT* nicotinamide *N*-methyltransferase

enzyme in this pathway. Nmnat then converts NMN into NAD^+. In the Preiss-Handler pathway, NA is converted to NAMN by nicotinic acid phosphoribosyltransferase (Naprt) which then follows the same pathway as in the de novo pathway to generate NAD^+. Naprt is highly expressed in the liver, small intestine, and kidney; however, the importance of this pathway in NAD^+ synthesis is not completely understood (Hara et al. 2007, 2003).

8.3 NAD^+ Consumption

NAD^+ is consumed mainly by three groups of enzymes: (1) deacylase in the sirtuins family; (2) ADP-ribosyltransferase (ART), including PARP; and (3) NAD^+ glycohydrolase and cADPR synthase (Okabe et al. 2019). Sirtuins are a group of NAD^+-dependent deacylases that catalyzes the removal of acetyl or acyl groups from lysine of target substrate proteins (Okabe et al. 2019). NAD^+ is cleaved between NAM and ADP-ribose, and the latter serves as an acyl acceptor, generating acyl-ADP-ribose (Cantó et al. 2015). There are seven sirtuins in mammals in different

subcellular compartments: nuclear for SIRT1, SIRT6, and SIRT7; cytoplasmic for SIRT2; and mitochondrial for SIRT3, SIRT4, and SIRT5. Sirtuins mediated deacetylation and deacylation activate various proteins which promote mitochondrial homeostasis, genomic stability, neuronal survival, stem cell rejuvenation, and prevent certain aspects of the aging process, such as neurodegeneration, loss of stem cells, and mitochondrial dysfunction (Vassilopoulos et al. 2011).

ARTs are another group of enzymes that consume NAD^+. By using NAD^+ as a donor, ARTs covalently link single or multiple ADP-ribose moieties from NAD^+ to substrate proteins, forming mono-ADP-ribosylation or poly-ADP-ribosylation (Liu and Yu 2015). ADP-ribosylation is an important protein posttranslational modification that affects DNA repair, epigenetic regulation, neurodegeneration, and aging (Liu and Yu 2015). Among the ARTs family, PARP1 and PARP2 are the most characterized ART members which play an important role in single-strand break DNA repair process. Once single-strand break DNA damage occurs, PARP1 and PARP2 accumulate in the damaged site and ADP ribosylated themselves. Auto-ADP ribosylated PARP1 and PARP2 induce base excision repair complex to start the repair process. PARP1 and PARP2 account for the vast majority of PARP activity in the cell (Michels et al. 2014). Age-associated DNA damage has been thought to be one of the causes for age-related NAD^+ decline via the activation of the PARP-mediated DNA repair process.

The third NAD^+ degrading enzyme is NAD^+ glycohydrolase, which include CD38 and SARM1 (Covarrubias et al. 2021). These enzymes cleave NAD^+ by their glycohydrolase activity to generate NAM and ADPR. Additionally, these enzymes have ADP-ribosyl cyclases activity that catalyzes the hydrolysis of NAD^+ to generate NAM and cADPR, a Ca^{2+}-mobilizing second messenger active in many cell types (Covarrubias et al. 2021). CD38 is ubiquitously expressed in many cell types (Piedra-Quintero et al. 2020). Recent research suggests that CD38 is a major consumer of NAD^+ in mammals. Evidence for this includes the observations that mice lacking CD38 or treated with the CD38 inhibitor, 78c, have elevated NAD^+ levels in multiple tissues, including liver and skeletal muscles (Camacho-Pereira et al. 2016; Tarragó et al. 2018). However, recent studies demonstrated that CD38 preferably degrades NMN than NAD^+ and indirectly reduces NAD^+ levels (Chini et al. 2020). SARM1 is a newly identified NAD^+-consuming enzyme and has been particularly shown to be involved in NAD^+ degradation in neuronal axons. SARM1 is expressed primarily in the brain (Lin et al. 2014). A new study indicated that the activity of SARM1 is regulated by NMN/NAD^+ ratio and increased NMN/NAD^+ ratio triggers the destruction of NAD^+ through SARM1 activation and promotes axon degeneration (Figley et al. 2021).

8.4 NAD^+ Levels Decline with Age

NAD^+ levels have been reported to be dramatically reduced in different human tissues, including the liver, pelvic skin, and plasma in older age (Massudi et al. 2012; Zhou et al. 2016; Clement et al. 2019). Age-dependent decline of NAD^+ levels is observed not only in humans but also in different species, such as *Caenorhabditis elegans*, *Drosophila*, and mice, suggesting that age-related NAD^+ decline is a universal phenomenon (Mouchiroud et al. 2013). Although we do not completely understand the mechanism for the decline of NAD^+ levels, it is believed that the reduced synthesis or increased consumption, or both is the cause. Several studies have shown that the expression of enzymes of NAD^+ synthesis pathways decreases during aging. Nampt expression levels decrease with age in several tissues, including adipose tissues, skeletal muscle, and pancreas (Zhu et al. 2017). Since most of the tissues in mammals use the salvage pathway to generate NAD^+, decreased Nampt expression would have a negative effect on NAD^+ levels of many tissues. Similarly, Qprt expression has also been reported to decrease in human macrophages obtained from old individuals ($\geq$65-year-old) as compared to young individuals ($\leq$35-year-old) (Minhas et al. 2019). This decline of Qprt expression was associated with an accumulation of QA and decreased abundance of NAMN, NAAD, and NAD^+, suggesting that the de novo pathway is also compromised with age (Minhas et al. 2019). Unlike the salvage pathway, the de novo pathway is utilized primarily in the liver. However, the declined de novo pathway with age can influence systemic NAD^+ levels possibly by limiting the supply of NAM (Liu et al. 2018). On the other hand, a recent study has shown that the NAD^+ synthesis flux is maintained in tissues of aged mice (25-month-old) (McReynolds et al. 2021). By using a stable-isotope tracing strategy, they showed that the NAD^+ synthesis from the de novo and salvage pathways are not impaired in different tissues of aged mice (McReynolds et al. 2021). Therefore, future studies are necessary to better understand whether NAD^+ synthesis gets impaired with age or not.

Another cause for the age-related decline of tissue NAD^+ levels is increased consumption by NAD^+-consuming enzymes. There is substantial evidence that NAD^+ consumption processes largely contribute to the age-related NAD^+ decline, particularly by PARPs and CD38 (Imai and Guarente 2014; Camacho-Pereira et al. 2016). Overactivation of PARPs in response to excess DNA damage during aging depletes cellular NAD^+ levels significantly (Imai and Guarente 2014). Indeed, the treatment by PARP1 inhibitor or PARP1 deletion in mice has increased NAD^+ levels in various tissues (Bai et al. 2011). Likewise, CD38 knockout mice show a robust increase in NAD^+ levels in various tissues, including liver, skeletal muscle, and adipose tissues, and these mice are also resistant to age-related NAD^+ decline (Camacho-Pereira et al. 2016). Additionally, treatment with CD38 inhibitor, 78c to aged mice, has been shown to restore tissue NAD^+ levels accompanied by improved age-associated pathologies (Tarragó et al. 2018). Importantly, CD38 protein levels and its activity increases dramatically with age (Camacho-Pereira et al. 2016). Thus, CD38 has been considered to be one of the major culprits for NAD^+ decline during aging.

8.5 Effects of Age-Related NAD+ Decline on Hallmarks of Aging

Aging is a multifactorial and complex process, affecting organisms at the molecular, cellular, tissue, and systemic levels (López-Otín et al. 2013). Aging is accompanied by several changes known as "hallmarks of aging," which are genomic instability, cellular senescence, telomere attrition, epigenetic alteration, deregulated nutrient sensing, mitochondrial dysfunction, and stem cell exhaustion (López-Otín et al. 2013). Age-related NAD^+ decline has been proposed as one of the major contributing factors for the hallmarks of aging. Several hallmarks of aging such as mitochondrial dysfunction, stem cell exhaustion, and genomic instability have been linked to reduced NAD^+ levels (Fang et al. 2017). For example, a decline in NAD^+ levels with age is associated with mitochondrial dysfunction in skeletal muscle of mice, while NAD^+ replenishment improved mitochondrial function by enabling mitochondrial biogenesis and mitophagy (Gomes et al. 2013; Zhang et al. 2016). Like mitochondrial function, oral administration of NAD^+ precursor, NR, promoted rejuvenation of intestinal stem cells in aged mice and reversed an impaired ability to repair gut damage induced by dextran sulfate sodium treatment (Igarashi et al. 2019). Age-related NAD^+ decline also affects DNA damage response against various cellular stressors which can lead to genomic instability. NAD^+ precursor supplementation decreased ionizing radiation-induced DNA damage and improved genomic stability in human peripheral blood mononuclear cells (PBMC) (Weidele et al. 2017). Therefore, age-related NAD^+ decline plays a pivotal role in driving molecular and cellular changes that are associated with aging.

It has been reported that more than 500 enzymatic reactions depend on NAD^+ (Rajman et al. 2018). Thus, the systemic decline of NAD^+ levels affects the activities of many enzymes. SIRT1 is one of the enzymes which activity depends on NAD^+ levels, and it has important roles in mitochondrial homeostasis (Tang 2016). A reduction in SIRT1 activity downregulates mitochondrial biogenesis, oxidative metabolism, and antioxidant defense pathways, leading to damage to complex I of the electron transport chain and a decline in mitochondrial function (Lombard et al. 2011). Therefore, during aging, inadequate NAD^+ levels in cells affect the activities of many cellular enzymes causing disturbances in cellular processes, which results in acceleration of aging.

8.6 Role of NAD+ in Age-Associated Functional Decline of Organs

Aging is often accompanied by the accumulation of deleterious biological changes, which increases an organism's vulnerability to diseases such as diabetes, hypertension, obesity, and neurodegenerative diseases (López-Otín et al. 2013). The decline of NAD^+ levels in tissues with age impairs the functions of NAD^+-dependent

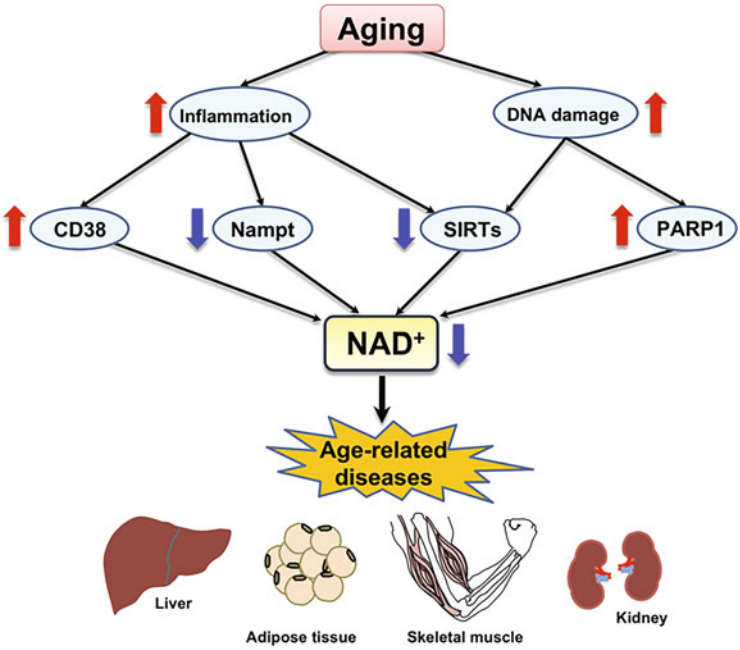

Fig. 8.3 NAD^+ decline during aging increases susceptibility to age-related diseases. Aging induces chronic inflammation and increases DNA damage in different organs, such as the liver, adipose tissue, skeletal muscle, and kidney. Inflammation increases CD38 expression and activity and reduces Nampt and SIRTs expression, whereas DNA damage increases PARP1 activation, which collectively contributes to NAD^+ decline in tissues. This NAD^+ decline compromises functions of different organs leading to age-related diseases

enzymes and thus contributes to the development and progression of age-associated diseases (Fig. 8.3). NAD^+ deficiency mainly affects tissues that need high cellular energy, such as the liver, skeletal muscle, adipose tissues, and kidneys. The importance of NAD^+ in some of the important organs is discussed below:

8.6.1 Liver

Liver is one of the metabolically active organs. Adequate NAD^+ levels are extremely important to maintain its optimal function. NAD^+-dependent signaling pathways are known to protect the liver from different pathological conditions, including fat accumulation, fibrosis, and insulin resistance, which are related to the development of fatty liver diseases, such as nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) (Ding et al. 2017). For example, SIRT1 is involved in the maintenance of mitochondrial function and fatty acid homeostasis by activating its downstream targets PGC-1α and SREBP1 (Ponugoti et al. 2010;

Miranda et al. 2014; Tang 2016). Conversely, decreased hepatic NAD^+ levels limit the activity of SIRT1 and promote liver steatosis by increasing de novo lipogenesis and inhibiting fatty acid β-oxidation (Ding et al. 2017). The decline of hepatic NAD^+ levels has been reported in various liver diseases in humans, including NAFLD, NASH, and alcohol-related liver disease (ALD) (Zhou et al. 2016; Parker et al. 2020). The decreased hepatic NAD^+ levels can promote the progression of liver diseases by limiting the NAD^+-dependent adaptive responses. These observations are further supported by the results that treatment by FK866, a Nampt-specific inhibitor, promotes lipid accumulation and hepatic steatosis in high-fat diet (HFD)-fed mice. FK866 treatment decreased protein levels of SIRT1 and phospho-AMPK, while the expressions of the lipogenic genes including SREBP1, FASN, ACC, and SCD1 were increased in the liver of HFD mice (Wang et al. 2017) Likewise, dominant-negative Nampt-overexpressing (DN-Nampt Tg) mice, in which hepatic NAD^+ levels were diminished, displayed NAFLD-like phenotypes, including lipid accumulation, chronic inflammation, and impaired insulin sensitivity in the liver. NR administration to DN-Nampt Tg mice rescued the NAFLD-like phenotypes (Zhou et al. 2016). Besides, NAD^+ boosting has been reported to increase liver regeneration capacity. After partial hepatectomy, NR-treated mice have promoted uniform liver regeneration and improved lipid metabolism (Mukherjee et al. 2017). Therefore, NAD^+ metabolism could be a therapeutic target for liver diseases.

8.6.2 Adipose Tissue

Adipose tissues are specialized forms of connective tissue which are the major sites for fat storage and also act as endocrine organs by secreting various bioactive compounds that regulate metabolic homeostasis (Hajer et al. 2008). Disruption of its function causes an imbalance in systemic metabolism and can lead to several diseases including type 2 diabetes, cardiovascular disease, hepatic steatosis, and cancers (Hajer et al. 2008). Adipose tissue is highly adaptable to environmental changes in early life due to its ability to rapidly alter its endocrine, inflammatory, and metabolic functions. However, as we age, its function starts to decline and can lead to adipose tissue dysfunction. The mechanisms responsible for adipose tissue dysfunction are multifactorial. Studies have shown that adipose tissue dysfunction is partly linked to age-related NAD^+ decline. Adipose tissue predominantly synthesizes NAD^+ via the salvage pathway, where Nampt is a major enzyme (Yamaguchi and Yoshino 2017). Both Nampt and NAD^+ levels in white adipose tissue decline during aging and aging-associated pathologies, including obesity and diabetes (Camacho-Pereira et al. 2016; Yamaguchi and Yoshino 2017). This decline of NAD^+ levels can seriously compromise the function of adipose tissues. For example, adipocyte-specific Nampt knockout (ANKO) mice have severe insulin resistance even under nonobese condition. Additionally, these mice showed severe adipose tissue dysfunction, manifested by local adipose tissue inflammation, increased

plasma free fatty acid concentrations, and decreased production of adiponectin. Remarkably, oral administration of NMN for 4–6 weeks largely restored adipose tissue NAD^+ biosynthesis and completely normalized insulin resistance in ANKO mice (Stromsdorfer et al. 2016). The decline of NAD^+ levels in adipose tissues not only affects its functional role but also interferes with the development of adipocytes. It has been recently reported that Nampt-mediated NAD^+ synthesis is required for the differentiation of preadipocytes to mature adipocytes (Okabe et al. 2020). Therefore, age-related decline of NAD^+ levels in adipose tissue can cause dysfunction of adipose tissue which can drive systemic pathophysiological processes resulting in age-related chronic disease.

8.6.3 Skeletal Muscle

Sarcopenia is defined as the loss of skeletal muscle mass and strength as a result of aging. It is largely responsible for the weight loss, weakness, and impaired locomotion observed in the elderly (Demontis et al. 2013). Although it is not clearly understood what molecular processes drive the sarcopenia, intrinsic factors like increased apoptosis, loss of proteostasis, increased mitochondrial dysfunction, and defects in Ca^{2+} homeostasis are thought to be contributing factors (Demontis et al. 2013). Recent research suggests that the age-related decline in NAD^+ levels in skeletal muscle also contributes to skeletal muscle aging. Skeletal muscle requires a high turnover of ATP to sustain contraction, facilitated by glycolysis and oxidative phosphorylation, which depend on the redox functions of NAD^+ (Frederick et al. 2016). Like many other organs, skeletal muscle NAD^+ is also largely generated via the salvage pathway as exhibited by 85% decline of NAD^+ levels in skeletal muscle-specific Nampt knockout mice (Frederick et al. 2016). Moreover, these mice had reduced ATP content, compromised energetics, and impaired muscle regeneration, which got worse with age, indicating that NAD^+ deficiency in skeletal muscle can negatively affect its development and functional role (Frederick et al. 2016). In the context of muscular dystrophy models, the *Mdx* mouse, which undergoes progressive muscle degeneration and weakness, has decreased NAD^+ and Nampt protein levels in skeletal muscle. NAD^+ repletion by NR administration improved muscle function accompanied by improved muscle energetics and reduced inflammation and fibrosis (Ryu et al. 2016). Human studies also show that NAD^+ levels are important for skeletal muscle homeostasis. Patients suffering from sarcopenia had decreased NAD^+ levels and mitochondrial bioenergetic dysfunction in skeletal muscle as compared to age-matched controls (Migliavacca et al. 2019). Furthermore, sarcopenic muscle had reduced expression of Nmnat1 and Nampt. Another study showed that the biopsy samples from vastus lateralis muscle demonstrated a approximately twofold decrease of muscle NAD^+ concentrations in mitochondrial myopathy patients. Niacin supplementation for 10 months increased muscle NAD^+ content by 2.3-fold in these patients with improved muscle strength as exhibited by an improved capacity to run or exercise and decreased frequency of muscle

cramps (Pirinen et al. 2020). These studies suggest that the depletion of NAD^+ in skeletal muscle leads to the impairment of mitochondrial function contributing to muscle dysfunction. A recent study showed that macrophage-secreted Nampt binds to CCR5, a chemokine receptor, of muscle stem cells and induces its proliferation and causes muscle regeneration (Ratnayake et al. 2021). As Nampt levels have also been shown to decline with age, the decline in muscle regeneration capacity during old age might be partly contributed by NAD^+ metabolism.

8.6.4 Kidney

The kidneys are important organs that remove harmful products and excess fluid from the body (Nitta et al. 2013). With age, the kidneys also undergo age-related structural and functional decline as manifested by low glomerular filtration rate (Nitta et al. 2013). The cause for such age-related changes in kidneys is not well understood. Several lines of evidence indicate that reduced levels of NAD^+ are involved in age-related kidney dysfunction. Renal cells require a constant supply of ATP to pump solutes across unfavorable gradients, and NAD^+ is necessary for ATP production through glycolysis and oxidative phosphorylation (Muraoka et al. 2019). Thus, age-related decline of NAD^+ levels in kidneys can predispose individuals to kidney diseases, while increased NAD^+ levels are renoprotective. A study showed that NAD^+ biogenesis is impaired during acute kidney injury (AKI). Mice with heterozygous deletion of Qprt were more susceptible to AKI. Furthermore, the supplementation of NAM reduced incidences of AKI in cardiac surgery patients (Mehr et al. 2018). Similarly, modulation of the de novo pathway was beneficial and protective in the ischemia-reperfusion injury-induced AKI model. α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD) is an enzyme in the de novo pathway which diverts upstream metabolite ACMS away from NAD^+ synthesis. Oral administration of an ACMSD inhibitor, TES-0125, increased NAD^+ levels in the kidneys and protected them against ischemia-reperfusion injury-induced AKI (Katsyuba et al. 2018). Thus, NAD^+ biosynthetic deficiency is a risk factor for AKI, and NAD^+ augmentation could be beneficial. A possible mechanism by which NAD^+ supplementation might improve outcomes in renal diseases is by activating sirtuins. NMN has been shown to protect against cisplatin-induced AKI in a SIRT1-dependent manner. Similarly, proximal tubular-specific Nampt conditional knockout mice were more susceptible to streptozotocin-induced diabetic nephropathy as manifested by high tubular damage. Conversely, proximal tubular-specific Nampt-overexpressing transgenic mice were protected against streptozotocin-induced diabetic nephropathy, and this protective effect was dependent on SIRT6 (Muraoka et al. 2019) Therefore, the augmentation of NAD^+ levels in the kidneys can protect against age-related kidney diseases by the activation of sirtuins.

8.7 NAD^+ Precursors for Restoring NAD^+ Levels in Animals and Humans

NR and NMN are the two most studied NAD^+ precursors. Administration of these molecules can restore the depleted NAD^+ levels in tissues (Ryu et al. 2016; Zhou et al. 2016). Various preclinical studies have shown that NR administration provides beneficial effects against different age-related diseases, such as metabolic and degenerative diseases. For example, co-administration of NR with HFD at a dose of 400 mg/kg/day for 12 weeks protected mice from weight gain, improved insulin sensitivity, and increased mitochondrial content in skeletal muscle and brown adipose tissue (Cantó et al. 2012). Age-related muscle loss is often associated with reduced muscle regeneration efficiency. By increasing muscle stem cell number, NR improved muscle functions and regenerative efficiency in aged mice (Zhang et al. 2016). These promising results of preclinical trials have prompted numerous clinical trials for NR. To date, multiple phase I clinical trials have been performed by various groups and have a common consensus that NR is safe and orally bioavailable. Depending on clinical trials, NR at a dose of 100 mg to 2000 mg has been used to evaluate the safety and effectiveness. NR administration in humans has been found to increase NAD^+ levels in whole blood and PBMC (Airhart et al. 2017; Martens et al. 2018). Although NR supplementation did not change NAD^+ levels in skeletal muscle, NAAD and methyl-nicotinamide levels were significantly elevated (Elhassan et al. 2019). Few clinical trials have investigated the effectiveness of NR in improving age-associated physiological decline. Oral supplementation of NR at the dose of 500 mg twice per day for 3 weeks did not improve skeletal muscle function in older people (70–80-year-old), while circulating pro-inflammatory cytokine levels were significantly reduced (Elhassan et al. 2019). Another study showed that NR oral supplementation at the dose of 500 mg twice per day for 6 weeks showed some tendency of improvement in cardiovascular functions, decreased systolic blood pressure, and improved aortic stiffness in middle-aged individuals (Martens et al. 2018). A longer treatment of NR for 12 weeks at a dose of 2000 mg per day to obese, insulin-resistant men failed to show any improvement in insulin sensitivity and whole body glucose metabolism (Dollerup et al. 2018). Although these small-scale clinical trial results do not reflect the benefits achieved in preclinical studies, some of the results are still promising. Thus, large-scale trials with longer time frames would be necessary to determine the efficacy of NR.

NMN has also shown promising results in preclinical studies in treating age-related diseases. Twelve months of oral administration of NMN to mice showed improved age-associated pathologies compared to untreated controls (Mills et al. 2016). Fertility was improved in old female mice after 4 weeks of oral administration of NMN (Bertoldo et al. 2020). However, unlike NR there have been only two clinical trials for NMN. A single-arm non-randomized intervention was conducted by single oral administration of 100, 250, and 500 mg NMN to ten healthy men. The primary objective of this clinical trial was to assess the safety of single-dose NMN. All the administered doses of NMN were safe and well-tolerated by participants without any significant adverse effects (Irie et al. 2020). Recently, the result of

another clinical trial for NMN was published, which was done in postmenopausal women with prediabetes status. In this study, NMN was given orally at a dose of 250 mg per day for 10 weeks. After 10 weeks of treatment, muscle insulin sensitivity was improved with increased NAD^+ turnover (Yoshino et al. 2021). Overall, the early human studies of NR and NMN have shown some hope for the potential use in the treatment of age-related pathological conditions. Future studies will shed light on whether NR and NMN would be a single molecule to treat multiple pathologies.

8.8 Conclusions

NAD^+ was discovered over 100 years ago; however, its importance in aging was known a few decades ago. Ever since it has attracted the attention of the scientific community as a potential therapeutic target for preventing and treating aging and aging-associated diseases. Preclinical studies have largely shown that the age-related NAD^+ decline contributes to various age-related diseases, and these diseases can be prevented or treated by the administration of NAD^+ precursors, like NR and NMN. Early clinical trial data show that these precursors are bioavailable and safe in humans; however, their effectiveness has not been studied thoroughly. We believe that future human studies will help us to understand whether NAD^+ metabolism modulation would be an ideal approach for treating multiple diseases.

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Chapter 9
Mitochondrial Dysfunction and Growth Differentiation Factor 15 in Aging

Yasunori Fujita and Masashi Tanaka

Abstract Mitochondria are central to cellular energy metabolism and are linked to a broad range of cellular processes. Mitochondria have received considerable interest as a promising target for aging-related conditions. However, the regulatory mechanisms and downstream effects of mitochondrial dysfunction in aging remain incompletely understood. In addition, although decreased mitochondrial function has been detected in aged tissues and cells, the distribution and features of cells with mitochondrial dysfunction is not fully understood. In studies of mitochondrial diseases caused by impaired respiratory chain function due to genetic mutations, growth differentiation factor 15 (GDF15) has been identified as a diagnostic biomarker. Impaired energy metabolism induces expression and secretion of GDF15 in cells with mitochondrial dysfunction, and circulating GDF15 is elevated in animal models and patients with mitochondrial diseases. Interestingly, circulating GDF15 levels strongly correlate with age in community-dwelling adults and predict all-cause mortality in older adults. Additionally, patients with age-related diseases present higher levels of circulating GDF15, which are positively associated with adverse outcomes. These findings imply a possible link between mitochondrial dysfunction, GDF15, and mechanisms underlying aging and age-related diseases.

Keywords Mitochondria · Mitochondrial diseases · GDF15 · Age

9.1 Introduction

Mitochondria are multifunctional organelles that play essential roles in cellular homeostasis. One of the most important mitochondrial functions is ATP production, which occurs through oxidative phosphorylation in the mitochondrial inner

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_9

membrane. Interestingly, mitochondria contain an organelle-specific double-stranded circular genome, referred to as mitochondrial DNA (mtDNA). Human mtDNA encodes 13 proteins, which are components of the oxidative phosphorylation complexes; 22 transfer RNAs; and two ribosomal RNAs. Mitochondria are also a central hub of cellular metabolism, contributing to an array of cellular processes (Martinez-Reyes and Chandel 2020; Spinelli and Haigis 2018). Mitochondrial dysfunction is caused by intrinsic and extrinsic factors, including genetic abnormalities, metabolic dysfunction, impaired ion homeostasis, environmental toxins, chemotherapy, and infection, which induce stress responses at both mitochondrial and cellular levels (Eisner et al. 2018). Severe mitochondrial dysfunction thus affects not only cell function but also cell survival.

Mitochondrial diseases are intractable disorders caused by defective mitochondrial oxidative phosphorylation due to genetic abnormalities (Gorman et al. 2016). Large numbers of mutations and deletions in mitochondrial and nuclear genomes are present in patients with mitochondrial diseases (Gorman et al. 2016). Patients with mitochondrial diseases present a wide range of symptoms. The pathological mechanisms of mitochondrial diseases have been studied using a unique cell culture model, referred to as cybrid cells, which are generated by fusing mtDNA-depleted cells (ρ^0 cells) and cytoplasts from patient-derived cells. In patients with mitochondrial diseases, mutated mtDNAs coexist with wild-type mtDNAs, referred to as heteroplasmy, and mutation loads vary not only between patients but also among individual cells in the same patient. Energy metabolism becomes impaired when the percentage of mutated mtDNA exceeds a certain threshold, which is typically over 50% (Gorman et al. 2016).

The connection between mitochondrial dysfunction and aging has been a topic of intensive research. However, the role of mitochondrial dysfunction in aging mechanisms has not yet been established. Mitochondrial function generally declines with age, but the affected cell types and magnitude of mitochondrial dysfunction in these cells are incompletely understood. Although animal and cell culture models of mitochondrial diseases do not fully recapitulate aging-related mitochondrial dysfunction, cybrid cells with mtDNA mutations and deletions could be useful for identifying changes that occur in cells chronically exposed to mitochondrial dysfunction during the aging process. Indeed, using the cybrid model, we identified growth differentiation factor 15 (GDF15), which is potentially linked to aging, as a marker for mitochondrial dysfunction and mitochondrial diseases.

This chapter reviews the research that identified GDF15 as a marker for mitochondrial dysfunction and mitochondrial diseases and subsequently discusses the association of GDF15 with aging and age-related diseases. Moreover, we will discuss the significance of mitochondria and GDF15 in aging mechanisms.

9.2 GDF15 as a Marker for Mitochondrial Dysfunction and Mitochondrial Diseases

9.2.1 Cybrid Cells with Pathogenic mtDNA Mutations

Cybrid cells are generated by fusion of nucleated cells with cytoplasts. In studies of mitochondrial diseases, cybrid cells have been generated to investigate the impact of pathogenic mtDNA mutations on mitochondrial respiratory function. For example, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) is one of the most common mitochondrial diseases. A large part of MELAS patients have an A-to-G mutation at nucleotide position 3243 of the mtDNA, which is located in the mitochondrial leucyl-transfer RNA gene ($tRNA^{Leu(UUR)}$) (Gorman et al. 2016). Cybrid cells harboring more than approximately 90% of mutant mtDNA exhibit decreased mitochondrial protein synthesis, leading to a significant decline in mitochondrial respiratory capacity (Chomyn et al. 1992). In addition, in studies using cybrid cells derived from patients with other mitochondrial diseases, including Leber hereditary optic neuropathy (LHON), myoclonus epilepsy with ragged-red fiber (MERRF), Kearns-Sayre syndrome, and Leigh's disease, have identified associations between mtDNA mutation or deletion and mitochondrial dysfunction, which contribute to disease pathogenesis (Wilkins et al. 2014).

9.2.2 Energy Metabolism in Cybrid Cells with Mitochondrial Dysfunction

Decreased cellular ATP levels and increased NADH/NAD^+ ratios are critical consequences of impaired mitochondrial respiration. A substantial decline in intracellular ATP levels is predictably deleterious to cell function. However, increased glycolysis can compensate for decreased mitochondrial ATP generation in most conditions. Increased NADH/NAD^+ ratio due to defective mitochondrial respiration is caused by NADH accumulation. Recently, serine catabolism was identified as a major source of NADH under impairment of mitochondrial respiration (Yang et al. 2020). According to this report, decreased consumption of NADH in the electron transport chain results in NADH accumulation, suppressing most NADH-producing pathways, including pyruvate oxidation, fatty acid oxidation, and the TCA cycle. Conversely, the serine catabolism pathway continues to produce NADH despite overaccumulation of cellular NADH due to the robust activity of methylene tetrahydrofolate dehydrogenase 2 (MTHFD2), a key redox enzyme in the NADH generation pathway.

Interestingly, one of the most deleterious effects of increased NADH/NAD^+ is the suppression of glycolytic ATP production by inhibition of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction. Therefore, decreasing the NADH/NAD^+ ratio could promote cell function and survival despite impaired mitochondrial

respiration. Pyruvate, the end-product of glycolysis, can be reduced to lactate by lactate dehydrogenase (LDH), concomitant with oxidation of NADH to NAD^+. Therefore, exogenous pyruvate could potentially suppress NADH/NAD^+ ratio increases in cells with mitochondrial dysfunction (Tanaka et al. 2007). Indeed, proliferation of ρ^0 cells is dependent on the supplementation of exogenous pyruvate (King and Attardi 1989). However, elevated lactate accelerates NADH/NAD^+ increase in cells with mitochondrial dysfunction, leading to an energetic crisis caused by impaired glycolytic flux. Consistent with these notions, metabolic analyses of cybrid cells harboring a MELAS-causing mutation demonstrated that sodium lactate significantly increased intracellular NADH/NAD^+ ratios and decreased intracellular ATP levels, while treatment with sodium pyruvate allowed for sustained energetic sufficiency despite impaired mitochondrial function (Kami et al. 2012).

9.2.3 Transcriptional Response to Impaired Energy Metabolism in Cybrid Cells

When mitochondrial respiration is compromised, distinct transcriptional programs are triggered to maintain cellular homeostasis. An early study of transcriptional profiling in cybrid cells harboring pathogenic mtDNA mutations identified that activating transcription factor 4 (ATF4) and its target genes are induced by mitochondrial dysfunction (Fujita et al. 2007). ATF4 is a key transcription factor in the mediation of the integrated stress response (ISR), which is induced by multiple stress stimuli, such as amino acid deprivation, endoplasmic reticulum (ER) stress, viral infection, and heme deficiency. Induction of downstream ISR genes has also been reported not only in other cell culture models but also in animal models and patients with mitochondrial diseases (Forsstrom et al. 2019; Quiros et al. 2017). The ISR pathway, which is activated by impaired mitochondrial respiration, can induce transcriptional activation of genes involved in biosynthesis and transport of amino acids, one-carbon metabolism, transsulfuration, and aminoacyl-tRNA biosynthesis. Although ATF4 knockdown and ISR inhibition increase mitochondrial respiration under nonstress conditions, the cell-autonomous effect of this response on mitochondrial dysfunction and energy metabolism remains incompletely understood. Importantly, exposure to sodium lactate, but not sodium pyruvate, further increases the expression of ATF4 target genes in cybrid cells harboring a MELAS-inducing mutation (Fujita et al. 2015), suggesting that ISR activation in cells with mitochondrial dysfunction could be stimulated by elevated NADH/NAD^+ ratios and/or decreased ATP levels.

9.2.4 GDF15 as a Marker for Mitochondrial Dysfunction

GDF15, a member of the transforming growth factor-β superfamily, is an important stress-responsive secretory protein. A study using cybrid cells with a MELAS-causing mtDNA mutation revealed that mitochondrial dysfunction induces GDF15 expression and secretion, which is further increased by severely impaired energy metabolism due to treatment with exogenous lactate (Fujita et al. 2015). Consistent with this finding, GDF15 expression is increased in cells exposed to respiratory chain inhibitors and in animal models of mitochondrial diseases (Forsstrom et al. 2019; Quiros et al. 2017). Furthermore, GDF15 expression levels are increased in skeletal muscle from patients with mitochondrial diseases (Forsstrom et al. 2019; Kalko et al. 2014). GDF15 is regulated by a diverse array of transcription factors, including the stress-responsive transcription factor p53 (Tan et al. 2000). Transcriptomic analysis of cybrid cells with a MELAS-causing mutation has identified that p53 and ATF4 target gene expressions are increased by exposure to lactate. In addition, p53 target genes are increased in the skeletal muscle of patients with mitochondrial myopathy (Kalko et al. 2014).

The promoter region of the GDF15 gene contains p53 biding sites, which mediate GDF15 upregulation in response to DNA damage (Tan et al. 2000). The potential role of p53-mediated GDF15 induction in the context of impaired mitochondrial respiration is as yet unknown. Currently, it has not been determined whether p53 directly regulates GDF15 in the context of impaired mitochondrial respiration. On the other hand, ATF4 knockdown suppresses GDF15 transcriptional activation induced by mitochondrial translation inhibition (Forsstrom et al. 2019). Furthermore, C/EBP homologous protein (CHOP), a downstream transcription factor of ATF4 and p38 MAPK target, directly upregulates GDF15 when mitochondrial translation is impaired (Chung et al. 2017). Thus, mitochondrial dysfunction is thought to induce GDF15 expression through ATF4 and CHOP in the ISR pathway or via the p38 MAPK pathway. Although the effects of GDF15 induction on mitochondrial dysfunction and impaired energy metabolism remain unknown, prior findings have provided sufficient evidence to support the utility of GDF15 as a potential marker for impaired energy metabolism in cells with mitochondrial dysfunction (Fig. 9.1).

9.2.5 GDF15 as a Biomarker for Mitochondrial Diseases

GDF15 circulates in body fluids such as the blood and cerebrospinal fluid under physiological conditions. Serum GDF15 levels are significantly elevated in patients with mitochondrial diseases (Fujita et al. 2015; Kalko et al. 2014; Yatsuga et al. 2015). Increased circulating GDF15 is thought to be derived from lesion tissues with mitochondrial dysfunction. Indeed, concomitant with increased serum GDF15 levels, GDF15 expression is increased in the skeletal muscle from patients with

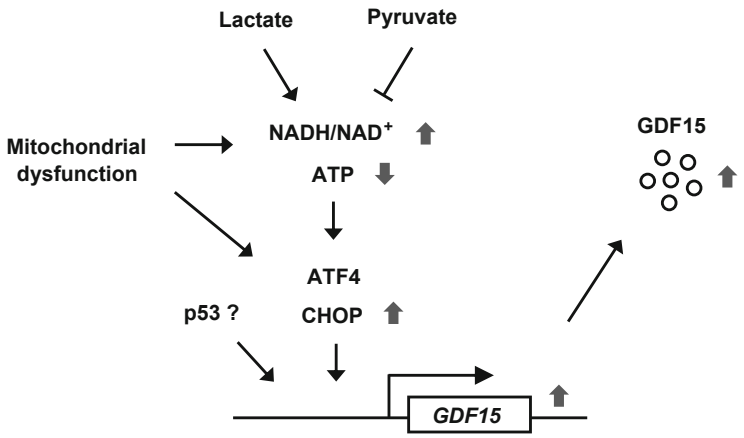

Fig. 9.1 Schematic diagram of GDF15 induction in response to impaired energy metabolism in cells with mitochondrial dysfunction. Mitochondrial dysfunction results in increased NADH/NAD^+ ratio and decreased ATP level. When mitochondrial function is impaired, the expression and secretion of GDF15 are induced through the ATF4 and CHOP-related pathways. The GDF15 promoter contains p53 binding sites, but it is unclear if p53 mediates GDF15 upregulation in response to mitochondrial dysfunction. In cells with mitochondrial dysfunction, the transcriptional response of GDF15 is enhanced by exogenous lactate but not pyruvate, concomitant with exacerbated impairments in energy metabolism

mitochondrial myopathy due to thymidine kinase 2 (TK2) mutations (Kalko et al. 2014). Additionally, GDF15 levels are correlated with disease severity in patients with mitochondrial disease (Dominguez-Gonzalez et al. 2020; Yatsuga et al. 2015). The sensitivity and specificity of GDF15 for diagnosing mitochondrial diseases are better than that of other markers such as the lactate/pyruvate ratio (Yatsuga et al. 2015). The utility of GDF15 in the diagnosis of mitochondrial diseases has been confirmed in several clinical studies (Lin et al. 2020). Moreover, GDF15 could be a potential marker for therapeutic response in patients with TK2-deficient myopathy (Dominguez-Gonzalez et al. 2020). These findings suggest that in mitochondrial diseases, serum GDF15 is a useful marker for diagnosing disease, evaluating disease severity, and monitoring therapeutic response.

9.3 GDF15 and Aging

9.3.1 Characteristics of GDF15

GDF15 is secreted into the extracellular space as a dimeric mature protein generated by proteolytic cleavage of its propeptides (Bootcov et al. 1997). Under physiological conditions, GDF15 is expressed in diverse tissues, including the placenta, prostate, lung, trachea, bladder, kidney, and choroid plexus, although its expression levels

vary in these tissues (Unsicker et al. 2013). Epithelial cells are a major cell type responsible for GDF15 expression in GDF15-expressing tissues, but the autocrine and paracrine functions of GDF15 released from epithelial cells have not been fully elucidated. In pathological conditions, GDF15 induction has been observed in tissue lesions (Lockhart et al. 2020). For example, GDF15 is significantly induced in cardiomyocytes in the ischemic area of the heart in patients with acute myocardial infarction (Kempf et al. 2006). In patients with chronic kidney disease (CDK), circulating GDF15 levels correlate with renal GDF15 expression levels (Nair et al. 2017). GDF15 expression is increased in malignant tumors from patients with several types of cancer (Koopmann et al. 2004; Welsh et al. 2001). The potential role of mitochondrial dysfunction in GDF15 induction under pathological conditions remains unknown.

9.3.2 GDF15 Functions Through Its Specific Receptor GFRAL

Recently, glial cell line-derived neurotrophic factor (GDNF) receptor alpha-like (GFRAL) was identified as a specific GDF15 receptor (Emmerson et al. 2017; Hsu et al. 2017; Mullican et al. 2017; Yang et al. 2017). Intriguingly, GFRAL expression is exclusive to the area postrema (AP) and the nucleus of the solitary tract (NTS) of the hindbrain in rodents, primates, and humans (Emmerson et al. 2017; Hsu et al. 2017; Mullican et al. 2017; Yang et al. 2017). In humans, *GFRAL* mRNA is also detectable in the testes and adipose tissue, albeit at lower levels (Mullican et al. 2017). GDF15 binding to GFRAL induces an interaction with the proto-oncogene tyrosine-protein kinase receptor Ret (RET), leading to the formation of GDF15-GFRAL-RET ternary complexes that transduce GDF15 signaling to downstream intracellular signaling pathways. The extracellular signal-related kinase (ERK), protein kinase B/Akt, and phospholipase Cγ (PLC-γ) signaling pathways have been proposed as potential downstream targets of GDF15-GFRAL-RET complexes (Mullican et al. 2017).

Animal studies have identified that the GDF15-GFRAL axis has an anorexigenic effect mediated by appetite regulation, leading to weight loss (Emmerson et al. 2017; Hsu et al. 2017; Mullican et al. 2017; Yang et al. 2017). Activation of GFRAL-expressing neurons suppresses feeding, inhibits gastric emptying, and produces a conditioned taste aversion (Sabatini et al. 2021). Several reports have identified a neuronal circuit that mediates the anorexigenic effects of the GDF15-GFRAL axis. GDF15 activates calcitonin gene-related peptide (CGRP)-expressing neurons in the lateral parabrachial nucleus and neurons in the central amygdala (Hsu et al. 2017), which contribute to anorexia under acute stress conditions such as illness, trauma, and injury (Morton et al. 2014). Additionally, GFRAL-expressing neurons project to the parabrachial nucleus (PBN) to mediate the aversive and anorexigenic effects of GDF15 (Sabatini et al. 2021). Likewise, GDF15 plays an important role in anorexia and weight loss through GFRAL-expressing neurons in the brain stem and as a part

of emergency neurocircuits. Importantly, physiological GDF15 levels do not significantly affect GFRAL-mediated appetite regulation (Klein et al. 2021). GDF15 also elicits cancer cachexia, characterized by loss of adipose tissues and skeletal muscle mass, which is mediated by adipose tissue upregulation of lipolysis-related genes through a peripheral sympathetic axis independent of anorexia (Salminen et al. 2017). Moreover, several studies have demonstrated that GDF15 affects immunity and inflammation by mediating immune cell function in the contexts of tumor development and tissue injury (Lockhart et al. 2020; Wischhusen et al. 2020). However, the precise regulatory mechanisms of GDF15 in these contexts have not yet been elucidated.

9.3.3 Correlation of Circulating GDF15 Levels with Age

The profile of circulating proteins is altered during the aging process. Plasma proteomic analysis using the SOMAscan assay, which can quantify 1301 proteins in human body fluids using aptamer-based technology, identified that circulating GDF15 levels were positively correlated with age in 240 healthy individuals aged 22–93 years and that its positive correlation with aging was the strongest among the 197 total proteins identified to be positively correlated with aging (Tanaka et al. 2018) (Fig. 9.2).

Studies focusing on mitochondria-associated proteins, including GDF15, FGF21, and humanin, also demonstrated that plasma GDF15 levels were significantly correlated with age in 693 individuals aged 21–113 years (Conte et al. 2019). A recent study that measured 2925 proteins in plasma from 4263 adults with ages ranging from 18 to 95 years using the SOMAscan assay demonstrated that the

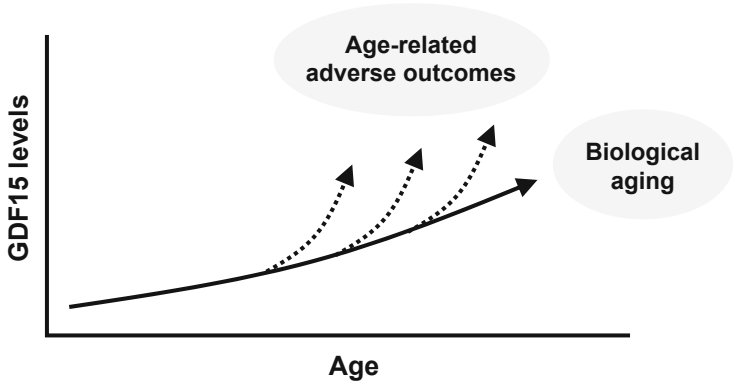

Fig. 9.2 Schematic diagram of the putative relationship between circulating GDF15 levels and biological aging and age-related adverse outcomes across the life span. Circulating GDF15 levels correlate with age, which may reflect the relative degree of biological aging and pathological changes of age-related adverse outcomes, including disease development and all-cause mortality

human plasma proteome was nonlinearly altered with age, instead exhibiting distinct waves of changes during the fourth, seventh, and eighth decades of life. This extensive proteomic profiling has also identified that GDF15 is increased across the life span (Lehallier et al. 2019). GDF15 levels also increase with age in mice (Lehallier et al. 2019).

9.3.4 GDF15 and Adverse Outcomes in Older Adults

The epidemiological significance of GDF15 in community-dwelling older adults has been evaluated in several cohort studies. Serum GDF15 levels were associated with all-cause mortality in a Swedish male cohort and twin cohort (Wiklund et al. 2010). Baseline plasma GDF15 concentrations and their changes over 5 years independently predicted all-cause mortality in elderly individuals that participated in the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study (Eggers et al. 2013). GDF15 was also reported to be associated with all-cause mortality in elderly community-dwelling individuals in the Activity and Function in the Elderly in Ulm (ActiFE Ulm) study (Rothenbacher et al. 2019). Moreover, plasma GDF15 was identified as a strong predictor of not only all-cause mortality but also of cardiovascular and non-cardiovascular mortality, in Rancho Bernardo Study participants (Daniels et al. 2011). Several epidemiological studies have therefore corroborated that GDF15 levels are associated with all-cause mortality in community-dwelling older adults.

Age-related cognitive decline is one of the most important adverse outcomes in older adults. Serum GDF15 levels were associated with cognitive performance and decline in older adults that participated in the longitudinal community-based Sydney Memory and Aging Study (MAS) (Fuchs et al. 2013). Additionally, serum GDF15 levels are inversely associated with brain gray matter volume in community-living older adults (Jiang et al. 2015). Physical capability in older adults progressively declines with age, which is associated with disability and mortality (Rantanen et al. 1999, 2003). A recent study of physical function in healthy community-dwelling adults aged 22–93 years demonstrated that elevated plasma GDF15 levels were associated with low gait speed and decreased physical performance (Semba et al. 2020). In addition, serum GDF15 levels were associated with muscle strength and lower extremity function in older patients with cardiometabolic disease (Oba et al. 2020). Overall, circulating GDF15 levels are associated with adverse outcomes in older adults living in similar environmental conditions (Fig. 9.2).

9.3.5 GDF15 and Age-Related Diseases

Many studies have assessed circulating GDF15 in patients with cardiovascular disease. Circulating GDF15 levels are elevated in patients with myocardial

infarction, chronic and acute heart failure, and atherosclerosis (Wollert et al. 2017). GDF15 is associated with adverse outcomes such as disease development, progression, and poor prognosis in cardiovascular disease (Wollert et al. 2017). An early study first identified increased GDF15 expression in infarcted heart tissues (Kempf et al. 2006), suggesting that increased circulating GDF15 levels could be attributed to localized upregulation of GDF15 in the heart. In contrast to this notion, circulating GDF15 levels do not correlate with increased infarct size and myocardial damage levels (Wollert et al. 2017). In addition, GDF15 expression was nearly undetectable in the myocardium from patients with nonischemic dilated cardiomyopathy that required left ventricular assist device support (Lok et al. 2012), implying that tissues other than the heart could be sources of GDF15 in advanced heart failure patients. Severe or advanced cardiac diseases are often accompanied by peripheral organ damage, which could elicit GDF15 induction. So far, the sources of increased circulating GDF15 in pathological conditions have not been fully elucidated, but circulating GDF15 is known to be dependent on the type of disease, its severity, and associated comorbidities.

Circulating GDF15 levels are elevated in malignancies, including prostate cancer (Bauskin et al. 2005), colorectal cancer (Brown et al. 2003), liver cancer (Liu et al. 2015), and pancreatic cancer (Koopmann et al. 2004). GDF15 has been identified as a potential prognostic marker for prostate cancer (Brown et al. 2009), colorectal cancer (Wallin et al. 2011), oral squamous cell carcinoma (Schiegnitz et al. 2012), esophageal squamous cell carcinoma (Wang et al. 2014), and glioblastoma (Shnaper et al. 2009). Given that GDF15 expression is increased in tumor specimens (Brown et al. 2003; Fisher et al. 2015; Koopmann et al. 2004; Scrideli et al. 2008), cancer cells themselves are thought to be a major source of elevated GDF15 in patients with malignancy. On the other hand, other studies have revealed that not only cancer cells but also tumor-associated macrophages express GDF15 in esophageal squamous cell carcinoma (Urakawa et al. 2015). Although further studies are required to support this link, tumor-associated macrophages could partially contribute to circulating GDF15 elevation in cancer patients.

Circulating GDF15 levels are increased in older individuals with chronic kidney disease (CKD) (Kim et al. 2019) and are associated with incident CKD and rapid decline in renal function (Ho et al. 2013). As discussed above, GDF15 levels significantly correlate with renal levels of *GDF15* mRNA in patients with CKD (Nair et al. 2017), implying that the increased circulating GDF15 in CKD patients is due, at least in part, to localized upregulation of GDF15 expression in the kidney. Interestingly, mitochondrial dysfunction has been implicated in the pathogenesis of acute kidney injury and consequent CKD (Zuk and Bonventre 2019). Mitochondrial dysfunction may contribute to circulating GDF15 elevation in these pathological conditions.

9.4 Discussion and Perspectives

Mitochondria have a pivotal role in cellular metabolism and are therefore linked to a wide array of biological processes. Thus, decreased mitochondrial function causes or contributes to disease processes at the cellular, organ, and whole body levels. Indeed, the deleterious effects of mitochondrial dysfunction have been thoroughly documented in cultured cells, animal models, and patients with mitochondrial diseases. Mitochondrial function has been reported to decline with age. However, the cells and tissues in which mitochondrial dysfunction occurs have not been fully identified, particularly in the context of specific age-related pathologies. The detection of mitochondrial dysfunction is dependent not only on its magnitude but also the relative proportion of cells with mitochondrial dysfunction in tissue samples. A small subset of cells with mitochondrial dysfunction could be present in tissues and organs in which mitochondrial dysfunction has yet to be detected. However, depending on their function, small populations of cells with mitochondrial dysfunction could have minimal effects on tissue or organ function. Nonetheless, given the fact that aging is a long-term process, impaired mitochondrial function in a relatively small proportion of cells could potentially affect surrounding cells in a chronic and progressive manner.

To fully understand the significance of mitochondria in aging mechanisms, it would be important to identify age-related mitochondrial changes and their causes and consequences. In most mitochondrial diseases, the critical change is a decline in respiratory chain activity, which is caused primarily by genetic mutations and deletions. The major consequence of mitochondrial dysfunction in this context is cellular energy deficiency responsible for impaired cell function or cell death. In animals and humans, mtDNA mutations accumulate with age in several tissues (Kauppila et al. 2017). However, whether aging-related mtDNA mutations lead to changes in respiratory chain activity and further adverse consequences remain unknown.

There is increasing evidence that mitochondria are linked to inflammation (Jang et al. 2018). mtDNAs released from mitochondria to the cytosol activate the inflammasome (Jang et al. 2018), which is speculated to contribute to age-related chronic inflammation. Whether this process indeed occurs with age and the mechanism underlying mtDNA release remain unclear. Interestingly, mitophagy, a mitochondrial quality control system, decreases in aged rodent tissues (Jang et al. 2018), implying that this functional decline could contribute to the accumulation of damaged mitochondria. However, potential age-related mitochondrial changes and their causes remain incompletely understood. In addition, studies demonstrating a direct association between damaged mitochondria and age-related tissue and organ functional declines are still limited.

Prior studies, ranging from cultured cells to patients with mitochondrial diseases, have indicated that mitochondrial dysfunction induces GDF15 expression, which causes increased circulating GDF15 levels. In addition, human studies have demonstrated that circulating GDF15 levels strongly correlate with age. These findings

raise the possibility that increased circulating GDF15 with age is indicative of increased cells with mitochondrial dysfunction. However, whether GDF15-expressing cells progressively accumulate across the life span remains unknown. Additionally, given that GDF15 can be induced by other stress signals, GDF15 expression is not indicative only of mitochondrial dysfunction. To determine the association between mitochondrial dysfunction and GDF15 during the aging process, novel methods and technologies that precisely and sensitively detect mitochondrial dysfunction in tissues and organs at the single-cell level will be necessary. Nonetheless, GDF15 is at present one of the most useful markers for mitochondrial dysfunction.

Accumulating evidence demonstrates that circulating GDF15 levels predict all-cause mortality in community-dwelling older adults, are elevated in age-related diseases, and are associated with their severity. This supports the notion that GDF15 is a potential marker for biological aging and pathological changes in older adults, including mitochondrial dysfunction.

In conclusion, although mitochondrial dysfunction and GDF15 are closely linked to aging, their roles in aging mechanisms have not been fully elucidated. Mitochondria will continue to attract considerable interest in elucidating the mechanisms of aging.

Acknowledgments The authors would like to thank Drs. Ito M, Kojima T, Yatsuga S, and Koga Y for their contribution to the related works.

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Chapter 10
Sirtuins and Metabolic Health

Jing Xu and Munehiro Kitada

Abstract Aging is an inevitable process accompanied by the degradation of organisms. The pathogenesis and progression of aging-related diseases such as diabetes, obesity, and neurodegenerative diseases are closely related to increased oxidative stress, dysfunction of mitochondrial biogenesis, activation of inflammation, and impairment of autophagy. The sirtuin family (SIRT1-7) mainly consists of antiaging molecules that function as nicotinamide adenine dinucleotide (NAD^+)-dependent enzymes with deacetylase activity. Sirtuins have antioxidative and anti-inflammatory effects, and they improve mitochondrial function and restore impaired autophagy in multiple tissues and organs, including the brain, liver, kidney, and heart. A series of studies have demonstrated that interventions based on activating sirtuins, including calorie restriction, and drug interventions may confer protection against aging-related disorders. Sirtuins may become potential targets for the treatment of aging-related metabolic diseases in the future.

Keywords Sirtuin · Aging · Oxidative stress · Inflammation · Autophagy

10.1 Introduction

Aging is a universal process characterized by the degradation of the physiological functions of tissues and organs, inevitably leading to death (López-Otín et al. 2013). Aging is accompanied by a decline in nicotinamide adenine dinucleotide (NAD^+) levels, leading to cellular oxidative stress, mitochondrial dysfunction, inflammation,

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_10

Table 10.1 Characteristics of mammalian sirtuins

Sirtuins	Function	Location	Characteristics of sirtuins knocked down in animal models
SIRT1	Deacetylation	Nucleus and cytoplasm	Death in perinatal period (Lee et al. 2008)
SIRT2	Deacetylation	Cytoplasm and nucleus	Insulin resistance and overweight (Lantier et al. 2018)
SIRT3	Deacetylation	Mitochondria	Hyperacetylation of mitochondrial protein, decrease in oxygen consumption, and increase of oxidative stress (Lombard et al. 2007; Jing et al. 2011)
SIRT4	ADP-ribosyltransferase	Mitochondria	Age-related insulin resistance (Anderson et al. 2017)
	Deacetylase		
	Lipoamidase		
SIRT5	Deacetylase	Mitochondria	Dysregulation of ammonia detoxification in the urea cycle (Nakagawa et al. 2009)
	Desuccinylase		
	Demalonylase		
SIRT6	Deacetylation	Nucleus and cytoplasm	Severe metabolic defects, early death (Mostoslavsky et al. 2006)
SIRT7	Deacetylation	Nucleus and cytoplasm	Short life spans, heart hypertrophy, cardiomyopathy (Vakhrusheva et al. 2008), and chronic hepatic steatosis (Shin et al. 2013)

and autophagy dysregulation and apoptosis, which contribute to the development and progression of multiple metabolic-related diseases, including diabetes, cancer, and neurodegenerative diseases (Kitada et al. 2013).

The silent information regulator (SIR) 2 protein was first identified in yeast in 1984 (Shore et al. 1984) and subsequently found to promote longevity in yeast (Kaeberlein et al. 1999). From bacteria to mammals, SIR2 is highly conserved in structure and function. The mammalian SIR2 orthologs are named sirtuins (SIRT1–7) and mainly exert NAD^+-dependent deacetylation effects (Table 10.1). SIRT1, SIRT6, and SIRT7 are mainly expressed in the nucleus, where they present deacetylation effects on histones to regulate gene transcription and modification. These nuclear sirtuins shuttle from the nucleus to the cytoplasm and participate in metabolic regulation (Chang and Guarente 2014). SIRT2 is mainly expressed in the cytoplasm and regulates the cell cycle via deacetylation (Inoue et al. 2007). SIRT3, SIRT4, and SIRT5 are predominantly expressed in mitochondria, where they regulate metabolic enzymes and energy production (Haigis and Guarente 2006). In contrast to other sirtuins, SIRT4 plays a crucial role in insulin secretion through ADP-ribosylation-mediated glutamate dehydrogenase (GDH) inhibition (Fig. 10.2) (Argmann and Auwerx 2006). Although SIRT5 participates in the urea cycle by deacetylating carbamoyl phosphate synthetase 1 (CPS1) (Fig. 10.1) (Nakagawa et al. 2009), it also functions as a demalonylase and desuccinylase, which can demalonylase glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and desuccinylate pyruvate kinase isozymes M2 (PKM2) that participate in glycolysis

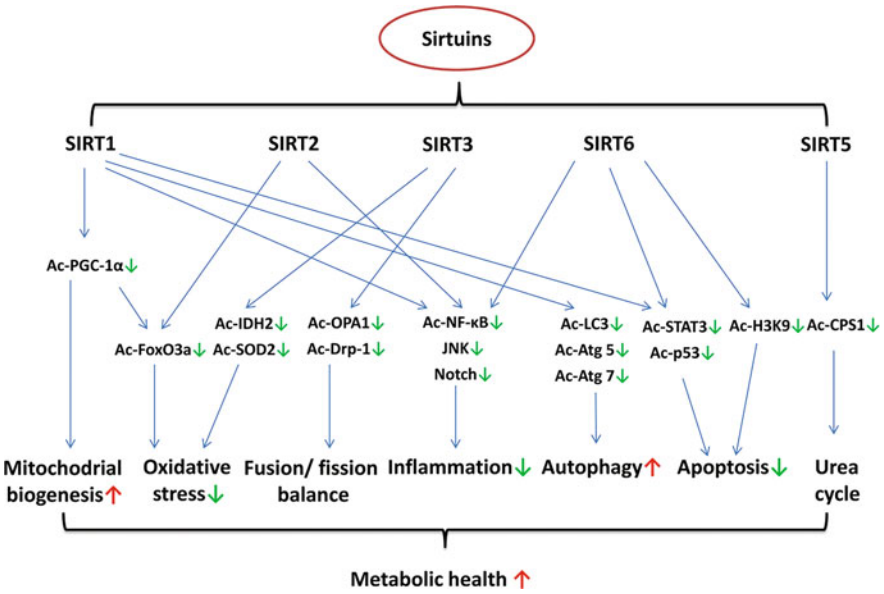

Fig. 10.1 Deacetylation of sirtuins on the pathogenic factors of metabolic health. Aging-related diseases are closely related to the dysfunction of mitochondrial biogenesis, increased oxidative stress, imbalance of mitochondrial fusion and fission, activation of inflammation, and impairment of autophagy. Sirtuins are considered antiaging molecules. Most sirtuins, especially SIRT1, SIRT2, SIRT3, SIRT5 and SIRT6, exhibit predominately deacetylated effects. Deacetylating substrates through these mechanisms activates sirtuins, possibly benefiting metabolic health by improving mitochondrial biogenesis, ameliorating oxidative stress, maintaining fusion and fission balance, suppressing inflammation, restoring impaired autophagy and mitophagy, alleviating apoptosis, and balancing the urea cycle

(Du et al. 2011; Nishida et al. 2015) (Fig. 10.2). Knocking out some nuclear sirtuins has lethal effects. SIRT1$^{-/-}$ mice die in the perinatal period (Lee et al. 2008), and SIRT6$^{-/-}$ mice have severe metabolic defects and die at the age of 4 weeks (Mostoslavsky et al. 2006). Although knockout of other sirtuins does not affect survival, it may cause a variety of metabolic abnormalities. SIRT2$^{-/-}$ mice exhibit insulin resistance and are overweight (Lantier et al. 2018). SIRT3$^{-/-}$ mice show hyperacetylation of mitochondrial proteins, a decrease in oxygen consumption, and an increase in oxidative stress (Lombard et al. 2007; Jing et al. 2011). SIRT4$^{-/-}$ mice exhibit age-related insulin resistance (Anderson et al. 2017). SIRT5$^{-/-}$ mice exhibit impaired ammonia detoxification in the urea cycle and suppression of glycolysis (Nakagawa et al. 2009; Nishida et al. 2015). SIRT7$^{-/-}$ mice present with a shorter life span and develop heart hypertrophy, cardiomyopathy (Vakhrusheva et al. 2008), and chronic hepatic steatosis (Table 10.1) (Shin et al. 2013). Sirtuins are considered antiaging molecules with NAD^+-dependent deacetylation activity and participate in a series of metabolic processes, including apoptosis, oxidative stress, mitochondrial dysfunction, inflammation, and impairment of autophagy (Hall et al. 2013). Therefore, sirtuins may be key targets for preventing the development and progression of aging-related metabolic diseases.

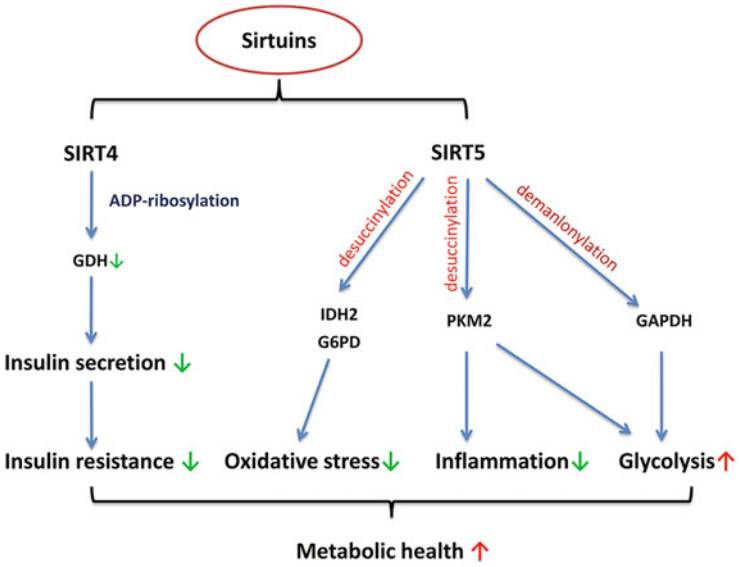

Fig. 10.2 Effects of sirtuins in addition to deacetylation. SIRT4 functions as an ADP-ribosylase to suppress the activity of glutamate dehydrogenase (GDH), further inhibiting insulin secretion and conferring protection against insulin resistance. SIRT5 predominately functions as a demalonylase and desuccinylase, which can demalonylase glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and desuccinylate pyruvate kinase isozyme M2 (PKM2) that participates in glycolysis and desuccinylate isocitrate dehydrogenase 2 (IDH2) and glucose-6-phosphate dehydrogenase (G6PD) to ameliorate oxidative stress. Desuccinylation of PKM2 can also suppress the secretion of inflammatory cytokines

10.2 Sirtuins in Oxidative Stress and Mitochondrial Biogenesis (Fig. 10.1)

Oxidative stress contributes to the progression of aging-related diseases. Reactive oxygen species (ROS) are products of cellular adaptation to elevated levels of cellular stress. Mitochondria are major sources of superoxide metabolism. The production of ($O_2^{\bullet -}$) superoxide depends on adenosine triphosphate (ATP) consumption in metabolic processes, including the tricarboxylic acid (TCA) cycle and respiratory chain in mitochondria. Increased levels of $O_2^{\bullet -}$ may lead to a decrease in the bioavailability of nitric oxide (NO) (Wang et al. 2018a). Superoxide dismutases (SODs) constitute a class of enzymes that scavenge superoxides and includes Cu/ZnSOD (SOD1), MnSOD (SOD2), and ECSOD (SOD3, extracellular SOD) (Indo et al. 2015). MnSOD is localized in mitochondria and converts $O_2^{\bullet -}$ into hydrogen peroxide (H_2O_2), and then, H_2O_2 can be converted into H_2O via glutathione peroxidase (GPX) and peroxiredoxins (PRXs) (Wang et al. 2018a). An

imbalance in the production and inhibited scavenging of ROS induced by mitochondrial dysfunction may result in oxidative stress (Indo et al. 2015; Kitada et al. 2020).

The antioxidant effect of sirtuins predominantly depends on their NAD^+-degrading deacetylated activity. SIRT1 is the most extensively studied sirtuin and is prominently involved in mitochondrial biogenesis and ROS scavenging through its deacetylation of multiple oxidant-related substrates. Peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) is the most widely studied substrate of SIRT1, which regulates mitochondrial biogenesis, induces oxidative phosphorylation gene expression, and suppresses oxidative stress in multiple organs and tissues (Tang 2016). SIRT1 directly deacetylates PGC-1α or further regulates peroxisome proliferator-activated receptor α (PPARα) via PGC-1α deacetylation to increase mitochondrial fatty acid oxidation, further protecting cells in skeletal muscle and white adipose tissue against insulin resistance (Gerhart-Hines et al. 2007). In different tissues and mammalian cells, SIRT1 regulates members of the Forkhead box O (FoxO) transcription factor family, a regulator of insulin signaling, longevity, and oxidative stress. Previous studies showed that SIRT1 deacetylates the expression of several antioxidant genes, including SOD2, PRX3, and PRX5, via the FOXO3a/PGC-1α complex (Brunet et al. 2004; Olmos et al. 2013). The effect of SIRT1 on the FoxO family is regulated by AMP-activated protein kinase (AMPK). In turn, the regulation of mitochondrial function by AMPK is also affected by SIRT1 (Canto et al. 2009; Price et al. 2012). Additionally, SIRT2 can deacetylate FoxO3a and then activate the transcription of SOD2 in response to oxidative stress in the white adipose tissue and kidney (Wang et al. 2007). In adipocytes, overnutrition-induced activation of hypoxia suppresses SIRT2 activity, leading to the acetylation of PGC-1α and ultimately resulting in the dysregulation of fatty acid oxidation and mitochondrial biogenesis (Krishnan et al. 2012). Multiple studies have demonstrated that SIRT3 deacetylates mitochondrial antioxidant enzymes, including SOD2 (Qiu et al. 2010; Tao et al. 2010; Ogura et al. 2018) and isocitrate dehydrogenase 2 (IDH2) (Someya et al. 2010), to suppress oxidative stress and protect against aging-related disorders. Moreover, SIRT3 deacetylates optic atrophy1 (OPA1) and dynamin-related protein 1 (Drp1), contributing to the balance of mitochondrial fusion and fission processes in response to stress and acute injury of the heart and kidney (Samant et al. 2014; Morigi et al. 2015). As a nuclear sirtuin, SIRT6 deacetylates histone H3 lysine 9 (H3K9) to suppress glycolysis, increase mitochondrial respiration (Zhong et al. 2010), and inhibit oxidative damage by coactivating nuclear factor erythroid 2-related factor 2 (NRF2) (Pan et al. 2016). SIRT6 can also deacetylate PGC-1α and then decrease gluconeogenesis, involving glucose 6-phosphate (G6P) and phosphoenolpyruvate carboxykinase (PEPCK), to inhibit hepatic gluconeogenesis (Dominy et al. 2012). In contrast to that of other sirtuins, the antioxidative effect of SIRT5 is dependent on the desuccinylation of IDH2 and glucose-6-phosphate dehydrogenase (G6PD), which increases ROS scavenging (Zhou et al. 2016).

10.3 Sirtuins in Inflammation (Fig. 10.1)

Aging-related chronic diseases, including diabetes, obesity, cardiovascular disease, and neurological diseases, are accompanied by chronic low-grade inflammation (Candore et al. 2010). Accumulated evidence has demonstrated an increase in the expression of inflammatory cytokines, including the interleukin (IL) family and tumor necrosis factor alpha (TNFα), and activation of inflammatory signaling, particularly the nuclear factor-kappa B (NF-κB) pathway, in the development of aging-related metabolic disorders (Candore et al. 2010; Mendes et al. 2017).

Sirtuins are involved in regulating multiple inflammatory pathways. SIRT1 directly suppresses inflammatory signaling and inflammatory cytokines, including TNFα, IL-1β, and IL-6, by deacetylating the NF-κB p65 subunit at Lys310, and protects against diabetic kidney diseases (Chen et al. 2002; Yeung et al. 2004; Kitada et al. 2011; Ruijie Liu et al. 2014). SIRT1 also suppresses other inflammatory pathways, such as c-Jun N-terminal kinase (JNK) and signal transducer and activator of transcription 3 (STAT3), which protect against kidney injury (Yoshizaki et al. 2010; Chen et al. 2015; Hong et al. 2018). SIRT2 can also deacetylate NF-κB p65 to reduce inflammatory responses in immune cells (Lo Sasso et al. 2014). The deacetylation of H3K9 in the nucleus by SIRT6 activation has been confirmed to have anti-inflammatory effects by suppressing multiple inflammatory signaling pathways, including the NF-κB, JNK, and Notch signaling pathways, in the liver, adipose tissue, and kidney (Kawahara et al. 2009; Xiao et al. 2012; Liu et al. 2017). In contrast to that of other sirtuins, the anti-inflammatory effect of SIRT5 is dependent on the desuccinylation of PKM2, which suppresses the expression of IL-1β in macrophages (Fig 10.2) (Wang et al. 2017).

10.4 Sirtuins in Autophagy (Fig. 10.1)

Autophagy is a lysosomal degradation process by which cells remove damaged organelles and excessive proteins (Rubinsztein et al. 2011). Autophagy is mainly regulated by nutritional status and is involved in the progression of aging-related diseases. Autophagy is impaired under overnutrition conditions, such as obesity and diabetes, but activated under nutritional deficiencies, such as starvation and calorie restriction (CR) (Mizushima and Komatsu 2011; Rubinsztein et al. 2011). Therefore, sirtuins may participate in the regulation of autophagy in the pathogenesis of aging-related diseases.

The study of the sirtuin family in the regulation of autophagy mainly focuses on the role of SIRT1. SIRT1 is essential for the activation of starvation-induced autophagy. Previous studies have shown that SIRT1 can deacetylate nuclear microtubule-associated proteins 1A/1B light-chain 3 (LC3) and autophagy-related (Atg) 5 and 7 to induce autophagy (Lee et al. 2008; Huang et al. 2015). Moreover, accumulating data have shown that the AMPK-mediated nutrient sensing pathway

also participates in the regulation of autophagy by activating SIRT1, which is involved in the pathogenesis of diabetic nephropathy and Parkinson's disease (Kitada et al. 2011; Wu et al. 2011; Ou et al. 2014; Han et al. 2016; Li et al. 2019). In contrast to other sirtuins, SIRT3 is essential for maintaining cardiac autophagy and mitophagy by deacetylating Foxo3a and increasing Parkin expression to protect against diabetic cardiomyopathy (Yu et al. 2017). SIRT6 induces autophagic flux in macrophages to reduce foam cell formation (He et al. 2017).

10.5 Sirtuins in Apoptosis (Fig. 10.1)

Apoptosis is an inevitable programmed death accompanied by the aging process. From yeast to mammalian animal models, sirtuins have been confirmed to exert antiapoptotic effects and are linked to the survival of various cells and tissues. Previous studies have shown that SIRT1 may improve DNA damage, promote the survival of cultured neuronal cells (Luo et al. 2001; Vaziri et al. 2001), and inhibit high-glucose-induced apoptosis in proximal renal tubular cells (Wang et al. 2016) through the deacetylation of p53. SIRT1 also alleviates oxidative stress and high-glucose-induced apoptosis of podocytes and cells in db/db mice via the deacetylation of FoxO3a (Hasegawa et al. 2008) and STAT3 (Ruijie Liu et al. 2014). SIRT3 (Yu et al. 2017; Zhang et al. 2017), SIRT4 (Shi et al. 2017), SIRT5 (Liu et al. 2013; Wang et al. 2018b), and SIRT6 (Fan et al. 2019) also exert antiapoptotic effects in rodent and cell models of diabetic chronic complications.

10.6 Interventions Targeting Sirtuins

Based on these antioxidative stress and anti-inflammatory and antiapoptotic effects, as well as their crucial roles in improving mitochondrial biological biogenesis and enhancing autophagy, sirtuins may become the focus of intervention research and potential targets for the treatment of aging-related diseases (Table 10.2).

CR has been confirmed to be an effective intervention to extend longevity and slow aging progress from yeast to rodents (Lin et al. 2000; Haigis and Guarente 2006). This effect is, in part, required for the activation of sirtuins. CR ameliorates inflammation via SIRT1-mediated NF-κB p65 deacetylation (Kitada et al. 2011), enhances mitophagy to confer protection against hypoxia-induced tissue damage via the SIRT1/Fox3a axis (Kume et al. 2010), suppresses oxidative stress via SIRT3-mediated SOD and IDH2 deacetylation (Qiu et al. 2010; Someya et al. 2010), and increases the levels of SIRT6 to maintain glucose homeostasis and lipid metabolism (Kanfi et al. 2008; Kim et al. 2010), which may improve the treatment of aging-related diseases, including diabetes, obesity, and hearing loss.

Given the effects of CR, some reagents mimicking the effects of CR have been under investigation. Resveratrol, a natural product derived from grapes, blueberries,

Table 10.2 Interventions targeting on sirtuins

Interventions	Enzymes	Targeting proteins	Effects
Calorie restriction	SIRT1	NF-κB p65	Anti-inflammation
		Fox3a	Antioxidative stress
	SIRT3	SOD, IDH2	Antioxidative stress
	SIRT6		Maintain glucose homeostasis and lipid metabolism
Resveratrol	SIRT1	PGC-1α	Improve mitochondrial biogenesis
		NF-κB	Anti-inflammation
		AMPK	Restore autophagy
Metformin	SIRT1	AMPK/PGC-1α	Antioxidative stress
		AMPK/FoxO1	Restore autophagy
ACIAR	SIRT3		Ameliorate mitochondrial dysfunction

and raspberries, functions as a SIRT1 agonist. Resveratrol activates the SIRT1/PGC-1α axis to improve mitochondrial function (Lagouge et al. 2006, 2016), ameliorate oxidative stress (Zhang et al. 2019a,b), suppress inflammation via the SIRT1/NF-κB pathway (Yeung et al. 2004), and restore impaired autophagy via AMPK/SIRT1 signaling (Wu et al. 2011; Guo et al. 2013; Zhao et al. 2017), which protects against aging-related metabolic disorders.

Metformin is a classic first-line medication for the treatment of type 2 diabetes, as recommended in multinational guidelines. Numerous studies have shown that metformin has multiple benefits beyond glycemic control, which may be mainly dependent on AMPK/SIRT1 activation. Previous studies have shown that metformin can suppress high-glucose-induced oxidative stress by activating the AMPK/SIRT1 signaling pathway (Zheng et al. 2012) and can enhance autophagy via the AMPK/SIRT1/FoxO1 pathway (Ren et al. 2020) and the AMPK/SIRT1/PGC-1α axis (Li et al. 2019). Moreover, metformin protects against high-glucose-induced cardiomyocyte apoptosis by activating SIRT3 (Zhang et al. 2017). Another AMPK agonist, ACIAR, can activate SIRT3 function, ameliorating cisplatin-induced mitochondrial dysfunction in acute kidney injury (Morigi et al. 2015).

10.7 Perspectives

In this section, we highlight the effects of sirtuins in aging-related metabolic diseases. The development and progression of aging-related diseases such as diabetes, obesity, and neurological diseases are closely related to the activation of oxidative stress, inflammation, impairment of autophagy, and dysregulation of mitochondrial biogenesis (Chan 2006; López-Otín et al. 2013). A number of previous studies have confirmed that activating sirtuins, especially SIRT1, SIRT3, and SIRT6, can alleviate oxidative stress and inflammation and improve mitochondrial

biogenesis, conferring further protection against aging-related metabolic disorders. Some classic drugs, including metformin and some compounds currently being researched, have also proven to improve the treatment of aging-related disorders by activating sirtuins in cells and animal models. These results indicate that sirtuins may become potential targets for the treatment of metabolism-related diseases. Although the antiaging effects of sirtuins have been confirmed in cell and rodent models of aging-related diseases, whether sirtuin agonists can be translated into clinical treatments for humans still needs to be further researched and confirmed in the future.

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Chapter 11
Autophagy in Aging and Longevity

Shuhei Nakamura, Tatsuya Shioda, and Tamotsu Yoshimori

Abstract Macroautophagy (autophagy) is an evolutionally conserved cytoplasmic degradation system in which varieties of materials are sequestered by a double membrane structure, called autophagosome, and delivered to the lysosomes for the degradation. Due to the wide varieties of targets, autophagic activity is essential for cellular homeostasis and survival. Accumulating evidences suggest that the activity of autophagy decreases with age, whereas several interventions which induce activation of autophagy promote longevity and prevents age-related diseases. Here we summarize recent progress regarding the role of autophagy in animal aging and life span regulation.

Keywords Autophagy · ATGs · Longevity · Rubicon · MML-1/Mondo · HLH-30/TFEB · Autophagosome · Lysosome

11.1 Overview of Autophagy

Autophagy is a conserved lysosomal degradation essential for cellular homeostasis and stress resistance. Autophagy can be classified into three distinct types depending how cytoplasmic materials are delivered to lysosomes: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Among these, this review particularly focuses on macroautophagy, since its roles and regulation in aging and age-related diseases are well documented. Macroautophagy, hereafter referred to as autophagy, is a catabolic process targeting wide varieties of cellular contents. Autophagy occurs at basal level in normal condition but is accelerated by several stresses such as starvation, accumulation of abnormal proteins, organelle damage, and pathogen infection. During autophagy, a small cisterna, called isolation membrane (also called isolation membrane or phagophore), elongates and surrounds a portion of cytoplasm to form a double-membraned structure, called the

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_11

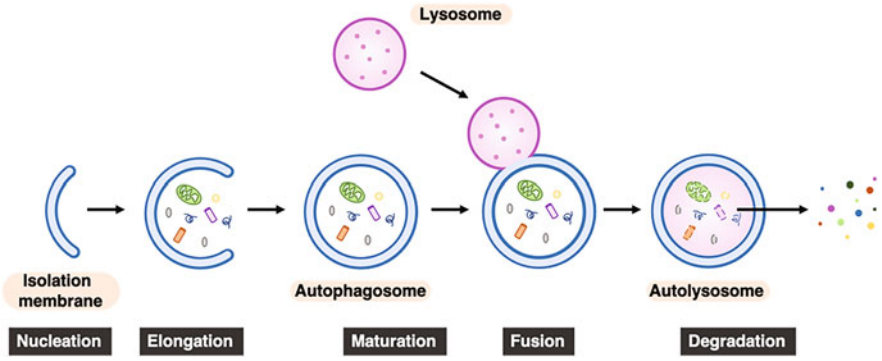

Fig. 11.1 Overview of autophagy. Upon induction of autophagy by stress, cytoplasmic materials are sequestered by a double-membraned structure, called an autophagosome. These autophagosomes fuse with lysosomes to become autolysosomes, in which the sequestered cargos are degraded and recycled for the maintenance of cellular homeostasis. Autophagy can be divided into several steps: formation of the isolation membrane (nucleation), elongation of the isolation membrane (elongation), completion and transport of the autophagosome (maturation), docking and fusion between autophagosome and lysosome (fusion), and degradation of the cargos inside the autolysosome (degradation)

autophagosome. Autophagosomes are then transported and fuse with lysosomes to form autolysosomes for the digestion of sequestered contents (Fig. 11.1). Autophagy is originally considered to be a bulk and nonselective degradation system. But subsequent studies show autophagy selectively degrades cargos and by doing so contribute to the intracellular homeostasis. Many cargos such as damaged mitochondria, damaged lysosomes, invading bacteria, lipid droplet, and aggregated proteins are selectively sequestered and degraded by specific selective autophagy, called mitophagy, lysophagy, xenophagy, lipophagy, and aggrephagy, respectively. During autophagy, several autophagy-related (ATG) genes are engaged sequentially in a highly regulated manner. Genetic studies in yeast have identified more than 30 ATG genes that are required for autophagy, most of which are conserved from yeast to mammals. Essential ATG genes are organized into at least six functional groups that allow for the nucleation, elongation, maturation, and fusion of the autophagosome. These functional groups are the Atg1/ULK initiation complex, the class III PI3 kinase nucleation complex, Atg9 vesicles, the phosphatidylinositol 3-phosphate (PI3P)-binding Atg18/Atg2 complex, the Atg5-Atg12 conjugation system, and the Atg8/LC3-PE (Atg8/LC3-phosphatidylethanolamine) conjugation system. The first step of autophagy initiates from the activation of Atg1/ULK complex, which leads to the formation of isolation membrane. The next step involves membrane nucleation by the class III Vps34/PI3 kinase nucleation complex (consisting of Vps34, Atg6/Beclin1, Atg14L, and Vps15/p150) via the production of PI3P, to start the formation of a double-membrane structure, isolation membrane (or phagophore). In mammals, the isolation membrane originates from the endoplasmic reticulum (ER), the mitochondrial contact site, and from others including the Golgi, endosomes, and plasma membrane (Chan and Tang 2013; Hamasaki et al. 2013). To start elongation, the

isolation membrane recruits the PI3P-binding complex consisting of Atg18/WIPI and Atg2, which regulates the distribution of Atg9, a transmembrane protein that has been proposed to deliver lipids to the isolation membrane and the growing autophagosome. During the next step, the isolation membrane expands into a double-membrane structure called the autophagosome. Autophagosome elongation is dependent on two ubiquitin-like conjugation systems, the Atg5-Atg12 conjugation system and the Atg8/LC3-PE. In Atg5-Atg12 conjugation system, Atg7 and Atg10 (E1- and E2-like enzymes, respectively) conjugate Atg12 to Atg5, and this complex associates with Atg16. Then, the Atg12–Atg5 conjugate promotes the conjugation of phosphatidylethanolamine (PE) to cytosolic Atg8/LC3, which is formed by the cleavage of the ubiquitin-like protein Atg8/LC3 by the protease Atg4. During this process, PE-conjugated LC3 associates with the autophagosomal membrane, and therefore LC3 is most commonly used as an experimental marker of autophagosomes (Fujita et al. 2008; Kabeya 2000; Mizushima and Levine 2010). The autophagosome eventually matures into a closed cargo-containing vesicle, which then fuses with the lysosome to become the autolysosome, and its contents are finally degraded for recycling. Autophagosome-lysosome fusion step is mediated by HOPS complex, phosphoinositides, Rab proteins, and SNEREs. In addition, autophagosome lysosome fusion step is negatively regulated by Rubicon which comprises different class III PI3K complex including Beclin1, UVRAG, Vps34, and Vps15 (Matsunaga et al. 2009). The detailed molecular mechanism of autophagosome formation and autophagosome-lysosome fusion is summarized in recent specific review paper (Nakamura and Yoshimori 2017; Nakatogawa 2020). As described in the following section, recent genetic evidence indicates that autophagy has a crucial role in the regulation of animal life span. The basal level of autophagic activity is elevated in many longevity paradigms, and importantly its activity is required for life span extension. On the other hand, the activity of autophagy decreases with age in many organisms. Pharmacological treatments have been shown to extend life span through the activation of autophagy, indicating autophagy could be a potential and promising target to modulate animal life span.

11.2 Activation of Autophagy Is One of the Convergent Mechanisms of Animal Longevity

Aging represents the functional deterioration of an organism. For a long time, aging is not considered as a tightly regulated process. During last twenty decades, the evolutionally conserved molecular mechanisms which delay animal aging and extend life span have been identified using several model organisms, including yeast, worms, fly, and mice. These pathways, for instance, include reduced insulin/IGF-1 signaling, dietary restriction, reduced mTOR signaling, germline removal, and reduced mitochondrial respiration. Extensive efforts to identify the downstream mechanism in each longevity pathway reveals that numerous but

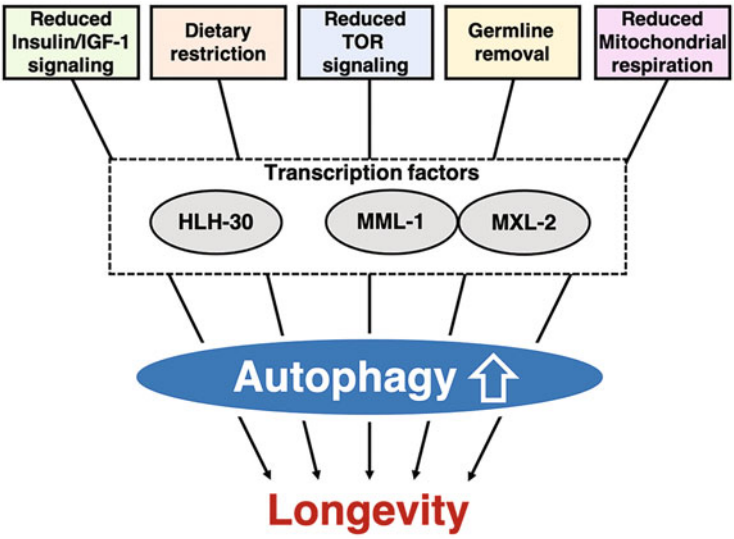

Fig. 11.2 Activation of autophagy is one of the convergent mechanisms of several longevity pathways. Several transcription factors such as HLH-30/TFEB and MML-1/Mondo are commonly activated in multiple longevity pathways. These regulate autophagic activity and extend its life span

different sets of factors or biological processes mediate in each longevity pathways, although some factors work in common. Notably, recent studies suggest that autophagy is one of the convergent downstream mechanisms of all these longevity paradigms. The activity of autophagy is elevated by several transcription factors in long-lived animals and is required for their longevity (Fig. 11.2, Table 11.1).

Reduced insulin/IGF-1 signaling has been shown to extend the life span in several species (Kenyon 2010). The first connection between autophagy and longevity has been reported in this insulin/IGF-1 signaling pathway in *C. elegans* (Meléndez et al. 2003). In long-lived *daf-2* (encoding *C. elegans* insulin/IGF-1 receptor) mutants, autophagy activity is elevated, as reflected by increased autophagic vesicles by electron microscopy and GFP::LGG-1 (a homolog of LC3 in *C. elegans*) puncta, a *C. elegans* autophagosome marker. Importantly, RNAi knockdown of *bec-1/Beclin1* shortens *daf-2* life span, indicating that the activity of autophagy is essential for *daf-2* longevity. Reduction of insulin/IGF-1 signaling pathway extends the life span in *Drosophila* and mice as well. In *Drosophila*, life span extension with deletion of the insulin receptor substrate chico was completely abrogated by the knockdown of Atg5 (Bjedov et al. 2020). Moreover, human centenarian has mutations in this pathway, suggesting that this longevity pathway seems to be conserved up to human. The exact mechanisms of autophagic activation in *daf-2* mutants are unclear, but they could include posttranslational and transcriptional regulation. For instance, the catalytic subunit of the energy regulator AMPK (AAK-2 in *C. elegans*) is essential for life span extension in *daf-2* mutants (Apfeld et al. 2004), and it regulates autophagy in both *C. elegans* and mammals (Egan et al. 2011). It is possible that

Table 11.1 Genetic modulation of autophagic activity which leads to longevity

Procedure for lifespan extension	Model organisms	Phenotypes	Correlation between autophagy and longevity	References
Reduced insulin/IGF-1 signaling pathway (*daf-2* KO)	*C. elegans*	Lifespan extension, enhanced autophagy	*bec-1* KD abolished DAF-2-mediated lifespan extension	Meléndez et al. (2003)
Dietary restriction (DR)	*C. elegans*	Lifespan extension, enhanced autophagy	*bec-1*, *atg-7*, *unc-51*, or *vps-34* KD abolished DR longevity Intestine-specific *atg-18* or *lgg-1/lgg-2* KD abolished DR longevity	Jia et al. (2007), Hansen et al. (2008), Tóth et al. (2008), Gelino et al. (2016)
Reduced TOR signaling	*C. elegans*	Lifespan extension, enhanced autophagy	*bec-1 or hlh-30* KD abolished the longevity by reduced TOR signaling	Hansen et al. (2008), Lapierre et al. (2013)
Reduced mitochondrial respiration (*atp-3* KD)	*C. elegans*	Lifespan extension, enhanced autophagy	*bec-1, unc-51*, or *atg-18* KD abolished ATP-3-mediated longevity	Tóth et al. (2008)
Germline removal	*C. elegans*	Lifespan extension, enhanced autophagy	*bec-1*, *vps-34*, *lgg-1*, *unc-51, atg-18, hlh-30, or mml-1* KD abolished germline-mediated longevity Intestine-specific *atg-18* KD abolished germline-mediated longevity	Lapierre et al. (2011), Chang et al. (2017), Lapierre et al. (2013), Nakamura et al. (2016)
Overexpression of HLH-30/TFEB	*C. elegans*	Lifespan extension, enhanced autophagy	No data	Lapierre et al. (2013)
Hexosamine pathway activation (*gfat-1* KO)	*C. elegans*	Lifespan extension, enhanced autophagy	*atg-18* KD abolished GFAT-1-mediated longevity	Denzel et al. (2014)
Frataxin silencing (*frh-1* KO)	*C. elegans*	Lifespan extension, enhanced mitophagy	*sqst-1*/p62 KD abolished FRH-1-mediated longevity	Schiavi et al. (2015)
Overexpression of KLF-3	*C. elegans*	Lifespan extension, enhanced autophagy	*bec-1* KD abolished *klf-3*-mediated longevity	Hsieh et al. (2017)
Overexpression of HSF-1	*C. elegans*	Lifespan extension, enhanced autophagy	*unc-51, bec-1*, or *lgg-1* KD abolished HSF-1-mediated longevity	Kumsta et al. (2017)
Knockdown of *xpo-1*	*C. elegans*	Lifespan extension, enhanced autophagy	*atg-7* or *atg-18* KD abolished XPO-1 mediated longevity	Silvestrini et al. (2018)

(continued)

Table 11.1 (continued)

Procedure for lifespan extension	Model organisms	Phenotypes	Correlation between autophagy and longevity	References
Mutation of mir-83	*C. elegans*	Lifespan extension, enhanced autophagy	No data	Zhou et al. (2019)
Low temperature	*C. elegans*	Lifespan extension, enhanced autophagy	*bec-1* KD abolished the lifespan extension at low temperature	Chen et al. (2019)
Muscle-specific overexpression of dFOXO	*D. melanogaster*	Lifespan extension, enhanced proteostasis at least in part via autophagy	No data	Demontis et al. (2010)
Reduced muscle activin signaling	*D. melanogaster*	Lifespan extension, enhanced autophagy	Atg8a KD abolished the longevity by reduced muscle activin signaling	Bai et al. (2013)
Knockdown of AcCoAS	*D. melanogaster*	Lifespan extension, enhanced autophagy	No data	Eisenberg et al. (2014)
Midlife Drp1 induction	*D. melanogaster*	Extension of healthy lifespan, enhanced mitophagy	Atg1 KD abolished Drp1-mediated longevity	Rana et al. (2017)
Overexpression of SQST-1/p62	*C. elegans* *D. melanogaster*	Lifespan extension, enhanced autophagy/mitophagy	*lgg-1* KD abolished *sqst-1*-mediated longevity (*C. elegans*) Atg1 KD abolished p62-mediated longevity (*D. melanogaster*)	Kumsta et al. (2019), Aparicio et al. (2019)
Knockdown of *rub-1*/Rubicon	*C. elegans* *D. melanogaster*	Lifespan extension, enhanced autophagy	Neuron-specific *atg-18* KD abolished RUB-1-mediated longevity	Nakamura et al. (2019)
Overexpression of Sestrin in intestinal stem cells	*D. melanogaster*	Lifespan extension, enhanced autophagy	No data	Lu et al. (2020)
Overexpression of Atg5	Mice	Lifespan extension, enhanced autophagy	No data	Pyo et al. (2013)
Beclin1(F121A)knock-in mutation	Mice	Lifespan extension, enhanced autophagy	No data	Fernández et al. (2018)

Ampk/aak-2-regulated autophagy contributes to life span, since AMPK overexpression is sufficient to increase the longevity of *Drosophila* in an *Atg1/Ulk1/unc-51*-dependent manner (Ulgherait et al. 2014). *daf-2* mutants also displays lower expression of key autophagy-related genes. They require a master regulator of autophagy and lysosomal biogenesis, *hlh-30/TFEB*, for their long life span, to display nuclear-localized HLH-30 and have elevated levels of several autophagy-related and lysosomal genes (Lapierre et al. 2013). HLH-30 translocates to the nucleus of intestinal cells following knockdown of mTOR and *daf-2* (Lapierre et al. 2013). Since *mTor* RNAi inhibition in *daf-2* mutants do not extend *C. elegans* life span in an additive manner (Vellai et al. 2003), they mediate life span extension through at least partially overlapping mechanisms. What is the autophagy cargo relevant for longevity conferred by reduced insulin/IGF-1 signaling? A recent study suggested that mitophagy is induced in *daf-2* mutants because mitochondria accumulate upon *bec-1* and mitophagy gene inhibition and *daf-2* mutants require mitophagy genes, including adaptor protein *Bnip3/dct-1*, the E3 ligase *Park/pdr-1*, and the kinase *pink-1* for full life span extension (Palikaras et al. 2015).

Dietary restriction is one of the most prominent ways to slow aging and extend the life span in many species. Dietary restriction was first observed to slow down aging in rat about 100 years ago. Since then the beneficial effects to extend life span was confirmed in numerous species including yeast, worms, fly, fish, dogs, mice, and apes (Mair and Dillin 2008). Multiple molecular mechanisms have been proposed to mediate the effect of dietary restriction on longevity, including TOR and insulin/IGF-1 signaling. The life span of the budding yeast *S. cerevisiae* can be measured by two methods: replicative life span (RLS) and chronological life span (CLS). Both RLS and CLS can be modulated in *S. cerevisiae* by reducing nutrients in the growth media (Smith et al. 2007). One method to induce dietary restriction is by amino acid limitation, which has been shown to extend CLS and also induce autophagy (Alvers et al. 2009a). Similarly, the inhibition of the nutrient sensor mTOR by rapamycin (a compound discovered in a soil bacterium on the Easter Island Rapa Nui) increases CLS and autophagy, and autophagy genes are required for rapamycin to extend life span (Alvers et al. 2009b). However, the role of autophagy in yeast aging seems complex. Intriguingly deletion of only ATG15, but not other autophagy genes tested, blocks RLS extension induced by glucose limitation (Tang et al. 2008) which is another method of dietary restriction in yeast. Several models of dietary restriction exist in *C. elegans* (Greer and Brunet 2009), including *eat-2* mutants, which carry an acetylcholine receptor mutation that impairs pharyngeal pumping and reduces food intake. *eat-2* mutants show increased numbers of GFP::LGG-1 in hypodermal seam cells. The longevity of *eat-2* mutants are also abolished when several autophagy genes including *unc-51/ULK1*, *bec-1/Beclin1*, *vps-34*, *atg-18*, and *atg-7* are inactivated (Hansen et al. 2008; Jia and Levine 2007). In *eat-2* animals, some autophagy genes are transcriptionally induced by several transcription factors, including *hlh-30*, *pha-4*, and *nhr-62* (Hansen et al. 2008; Heestand et al. 2013; Lapierre et al. 2013). Recently it has been shown that intestinal autophagy is essential for life span extension during dietary restriction (Gelino et al. 2016).

How these transcription factors contribute to activation of autophagy and longevity in spatial and temporal manners need to be clarified in future study. Similar to yeast, in *C. elegans*, life span extension induced by dietary restriction may be at least partly mediated through TOR, because TOR inhibition in *eat-2* mutants does not further extend life span (Hansen et al. 2007). In line with this, similar to dietary-restricted worms, the inhibition of TOR extends life span in a transcription factor *pha-4*- or *hlh-30*-dependent manner (Lapierre et al. 2013; Sheaffer et al. 2008). In *Drosophila*, rapamycin treatment results in a modest life span extension, and this effect requires the autophagy gene *Atg5* (Bjedov et al. 2010), suggesting that the reduction of TOR extends the life span in *Drosophila* at least partially through autophagy similar to yeast and worms. Rapamycin extends mammalian life span and ameliorates neurodegeneration and osteoarthritis in mice (Harrison et al. 2009; Li et al. 2014). Other groups also confirmed the positive effect of rapamycin on the life span in mice using different genetic backgrounds (Lamming et al. 2013). However, the contribution of autophagy to these mice is unclear.

Reproduction is negatively correlated with longevity in many species. Removal of germline stem cells by laser microsurgery or genetic mutation extends life span in *C. elegans* and *Drosophila*. In worms, temperature-sensitive mutant, *glp-1(e2141)*, which encodes *C. elegans* Notch receptor shows the reduction of germline stem cells and life span extension. It has been shown that the numbers of GFP::LGG-1 puncta are increased in germline-deficient *glp-1* animal and autophagy genes are essential for their longevity (Lapierre et al. 2011). In germline-deficient animal, several transcription factors including *hlh-30*, *mml-1/mxl-2*, and *pha-4* have been shown to induce autophagy genes (Lapierre et al. 2011, 2013; Nakamura et al. 2016). Interestingly, intestine-specific knockdown of autophagy genes abolishes *glp-1* longevity, while it is not the case in *daf-2* mutants, indicating critical differences of autophagy regulation in individual tissues between conserved longevity paradigms (Chang et al. 2017). *glp-1* animals have increased lipase activity, and *lipl-4* is required for *glp-1* animals to live long (Wang et al. 2008). *Lipl-4* overexpression increases autophagy and life span, and this animal requires autophagy gene for longevity (Lapierre et al. 2011). These studies indicate lipid turnover by autophagy is essential for longevity.

The free radical theory proposes that aging is the cumulative result of oxidative damages to cells and tissues over time. These molecular damages are caused by reactive oxygen species (ROS) which is generated primarily from mitochondrial respiration. Although oxidative damages increase with age, it is still unclear if this is indeed a causative effect to organism aging. Importantly, reduced mitochondrial respiration is known to extend the life span of many organism from yeast to mice (Hur et al. 2010; Kirchman et al. 1999). In worms, the reduction of electron transport chain components extends life span, when they are inhibited during larval stages. Several mitochondrial mutants including ubiquinone synthetase mutant *clk-1* and iron-sulfur mutant *isp-1* also show longevity. Larval inhibition of autophagy genes (*vps-34*, *atg-18*, and *lgg-1*) specifically shortens the life span of *clk-1* and *isp-1* mutants (Lapierre et al. 2013; Tóth et al. 2008). Consistent with a role for autophagy, these mutants display increased numbers of GFP::LGG-1 punctae in the hypodermal

cells during larval stage L3 (Lapierre et al. 2013). Frataxin is a nuclear-encoded mitochondrial protein involved in the biogenesis of iron-sulfur (Fe-S) cluster-containing proteins and also involved in the function of the mitochondrial respiratory chain. Partial depletion of *frh-1* has been shown to increase autophagic activity and extends the life span of wild-type animals, but not *bec-1* mutants (Schiavi et al. 2013). Moreover, a recent report showed that the longevity of *frh-1* mutants requires mitophagy genes for its longevity (Schiavi et al. 2015).

In addition to the role of autophagy in longevity, the loss of autophagic activity has been shown to cause premature aging phenotypes in many species. An unbiased screening for genes involved in chronological life span in yeast identified several short-lived mutants which have mutation in macroautophagy genes (Matecic et al. 2010). Decreased life span is also observed in *C. elegans Atg1/unc-51*, *Atg7*, *Atg18*, and *Beclin1/bec-1* loss of function mutants (Tóth et al. 2008). Similar findings are reported in *Drosophila* as well (Simonsen et al. 2008). Although whole-body knockout of Atg genes in mice leads to postnatal death, conditional tissue-specific knockouts of Atg7 or Atg5 show several age-associated phenomena including aggregation of inclusion bodies in neurons, accumulation of lysosomes containing lipofuscin pigments, disorganized mitochondria, increased protein oxidation, and decreased muscle mass (Rubinsztein et al. 2011).

11.3 Autophagic Activity Declines with Age

Autophagic activity is known to decrease with age in several species (Chang et al. 2017; Chapin et al. 2015; Del Roso et al. 2003; Donati et al. 2001; Uddin et al. 2012). Interestingly, the study using centenarians shows the general increase of autophagy genes (Xiao et al. 2018) and also increased circulating Beclin1 (Emanuele et al. 2014). Based on these correlations between autophagy and aging, it is reasonable to test if the forced activation of autophagy suffices to extend animal life span (Table 11.1). Indeed, the overexpression of HLH-30/TFEB, a master regulator of autophagy and lysosomal biogenesis, extends worm life span (Lapierre et al. 2013). Consistent with this, the inhibition of HLH-30/TFEB nuclear export or the treatment of HLH-30/TFEB agonists have been recently shown to extend the life span in worms and mitigate metabolic syndromes in mice (Silvestrini et al. 2018; Wang et al. 2017). In addition, ATG5 overexpression or Beclin1 gain of function in mice extends life span (Fernández et al. 2018; Pyo et al. 2013). Moreover, the neuronal overexpression of Atg8 or mild upregulation of Atg1 is sufficient to extend life span in *Drosophila* (Bjedov et al. 2020; Simonsen et al. 2008). Although the molecular mechanism underlying age-dependent autophagic decline has remained elusive, the recent study suggests that age-dependent accumulation of autophagy negative regulators; Rubicon is one of such mechanisms (Matsunaga et al. 2009; Nakamura et al. 2019). Rubicon is increased in *C. elegans*, *Drosophila*, and mouse tissues such as the liver and kidney, and importantly knockdown of Rubicon increases life span in an autophagy-dependent manner and/or ameliorate several age-associated phenotypes,

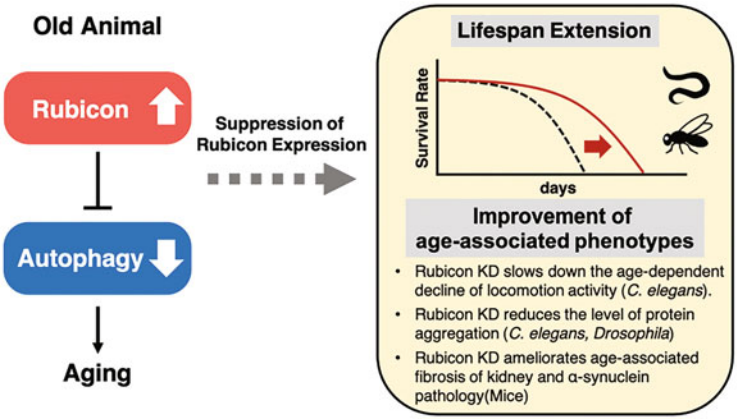

Fig. 11.3 The increase of Rubicon is a signature of aging and autophagic decline. Rubicon expression increases with age. This causes a decrease in autophagic activity, which curtails life span and leads to aging. Knockdown of Rubicon increases life span and improves age-associated phenotypes in many species

such as kidney fibrosis and α-synuclein pathology in these animals (Fig. 11.3). Specific microRNA is also involved in the autophagic decline across tissues during aging in *C. elegans* (Zhou et al. 2019b). *mir-83* is upregulated by the transcription factor hsf-1/HSF1 in the intestine during aging and transported across tissues. *mir-83* disrupts autophagy in intestines and muscles by downregulating lysosomal calcium channel cup-5/TRPML1 essential for the induction of autophagy. Lysosomal morphology and activity decrease age in *C. elegans* and are regulated in many longevity pathways (Sun et al. 2020), which partly explain autophagic decline with age. In addition, a key autophagy-negative regulator, the mTOR activity, has been shown to increase over time in some mouse tissues, which might also affect age-dependent autophagic impairment (Baar et al. 2016). On the other hand, another upstream regulator of autophagy, the AMPK activity, is constant during aging, but AMPK activation by an activator, such as AICAR or exercise, is blunted by aging (Reznick et al. 2007).

11.4 Autophagy and Age-Related Neurodegenerative Diseases

Autophagy is essential to prevent many age-associated diseases. Among them autophagy activity in neuronal cells is particularly essential, since neuronal cells are post-mitotic and they cannot segregate and dilute the proteotoxic damage. Therefore, proteostasis function by autophagic activity is essential to prevent the accumulation of several aggregation-prone proteins which lead to neurodegeneration. Indeed, the impairment of the autophagy pathway is involved

in many age-associated neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson disease (PD) (Hara et al. 2006; Komatsu et al. 2006; Mizushima et al. 2008).

AD involves the deposition of extracellular β-amyloid (Aβ) plaque and intracellular neurofibrillary tangles containing hyperphosphorylated tau. Both Aβ and Tau are known to be substrates of autophagy. The loss of autophagy by conditional knockout of ATG7 in mouse brain leads to phosphor-tau accumulation (Inoue et al. 2012). On the other hands, autophagic activation decreases tau levels (Krüger et al. 2012). The heterozygous deletion of Beclin1 increases both intracellular and extracellular Aβ (Pickford et al. 2008). The accumulation of autophagic vacuoles is observed in AD neurons (Nixon et al. 2005), suggesting that autophagy is impaired in this pathological condition.

PD is characterized by the accumulation of α-synuclein. α-synuclein overexpression impairs autophagy due to mislocalization of ATG9 (Winslow et al. 2010). Accumulation and propagation of α-synuclein aggregation in the brain can be suppressed by autophagy activation by an autophagy-negative regulator, Rubicon (Nakamura et al. 2019). The loss of function of Parkin, an E3 ubiquitin ligase, and PINK1, a mitochondrial protein kinase, causes autosomal-recessive and sporadic juvenile onset PD (Kitada et al. 1998; Valente et al. 2001, 2004). Parkin and PINK1 are known to regulate mitophagy to clear the damaged mitochondria in the cell culture. PINK1 recruits Parkin on damaged mitochondria to induce mitophagy. However, PINK1 and Parkin knockout mice fail to develop PD-associated phenotypes (Gautier et al. 2008; Palacino et al. 2004), suggesting that PD could be caused by other mechanism.

11.5 Molecular Mechanism Regulating Autophagy and Longevity

In many cases, autophagic activation at the transcript level seems essential for longevity. Several autophagy and lysosomal genes are regulated by different transcription factors. The bHLH transcription factor, TFEB originally identified as a master regulator of lysosomal biogenesis, is subsequently shown to regulate autophagy and fat metabolism (Sardiello et al. 2009; Settembre et al. 2011, 2013). TFEB is known to be negatively regulated by nutrient sensor mTOR. At nutrient-rich condition, TFEB is phosphorylated on lysosome. Phosphorylated TFEB is bound to 14-3-3 and is mainly localized on cytosol. Upon starvation, TOR becomes inactivated, and TFEB is then dephosphorylated and translocated in the nucleus to initiate the transcription of target genes (Martina et al. 2012; Settembre et al. 2012). The dephosphorylation of TFEB is mediated by calcineurin activated upon calcium efflux through TRPML1 (Medina et al. 2015). The *C. elegans* homolog of TFEB, HLH-30, has been shown to regulate genes involved in autophagy and lysosomal function. Essentially, HLH-30 is translocated to the nucleus by inhibition of insulin/IGF-1 signaling, mitochondrial respiration, TOR signaling, translation, and germline

removal and is required for their longevity (Lapierre et al. 2013). Moreover, the overexpression and activation of *hlh-30* is sufficient to extend the life span of wild-type animals (Lapierre et al. 2013). These results indicate that HLH-30/TFEB is a master transcription factor downstream of many longevity pathways possibly through the transcriptional activation of target genes involved in autophagy and lysosomal function.

Other bHLH transcription factor complex, MML-1/MXL-2, has been identified as a novel regulator of longevity (Johnson et al. 2014; Nakamura et al. 2016). MML-1/MXL-2 belongs to Myc and Mondo family member and their homologs, MondoA/MLX or ChREBP/MXL functions as a glucose sensor. MML-1/MXL-2 is required for the longevity conferred by germline removal, reduced insulin/IGF-1 signaling, reduced mitochondrial respiration, and reduced TOR signaling in *C. elegans*. Interestingly, the inhibition of MML-1/MXL-2 impairs HLH-30 nuclear localization and activation of autophagy in germline less long-lived animals, *glp-1*. This is partly through the regulation of *lars-1*, a positive regulator of TOR signaling. Interestingly, in *glp-1*, MML-1/MXL-2 and HLH-30 mutually regulated each other. Comprehensive transcriptome analysis reveals that they have many shared target genes including lysosomal genes but also have preferential targets. Some autophagy genes including *atg-2/ATG2*, *atg-9/ATG9*, and *epg-9/ATG101* are preferentially regulated by MML-1/MXL-2, while *unc-51/ULK1* and *lgg-1/LC3* are regulated by HLH-30. Thus, they might distribute the responsibilities to reinforce autophagy and longevity in germline less animals.

In *C. elegans*, *Drosophila*, and mouse, the reduction of insulin/IGF-1 signaling ultimately activates DAF-16/FOXO function and extends life span. DAF-16 could regulate autophagy partly through regulating autophagy and lysosomal genes (Li and Zhang 2016). Consistent with this, the overexpression of DAF-16 increases the number of autophagosomes during bacterial infection (Jia et al. 2009). However, although *daf-2* and *daf-16* double mutants do not show longevity, these mutants still have increased numbers of autophagosomes (Hansen et al. 2008). Possibly other factors compensate the activity of autophagy, or DAF-16 regulates autophagy at other timing. Interestingly, DAF-16 interacts with HLH-30 and cooperates target gene expression, promoting stress resistance and longevity (Lin et al. 2018). Other forkhead transcription factor, PHA-4/FOXA, binds to the promoter region of *unc-51/Ulk1*, *bec-1/Becn1*, and *lgg-1/LC3* which work in the early stage of autophagosome formation and upregulates these genes in worms, leading to autophagic activation. *pha-4* is required for the longevity by mTOR inhibition, germline removal, and calorie restriction through the activation of autophagy (Hansen et al. 2008; Lapierre et al. 2011).

11.6 Intervention of Aging via Modulating Autophagy

In addition to the abovementioned rapamycin, several pharmacological treatments have been shown to extend animal life span and health span through the activation of autophagy (Table 11.2). Administration of a natural polyamine, spermidine, is

Table 11.2 Pharmacological modulation of autophagic activity which leads to longevity

Chemical	Model organisms	The effect of autophagy	The lifespan extension was abolished by	References
Resveratrol	*C. elegans*	Enhanced autophagy	Knockdown of *bec-1*	Morselli et al. (2010)
GlcNAc	*C. elegans*	Lifespan extension, enhanced autophagy	No data	Denzel et al. (2014)
α-ketoglutarate	*C. elegans*	Increased LGG-1/LC-3 puncta	No data	Chin et al. (2014)
Metformin	*C. elegans*	Lifespan extension, increased mRNA levels of ATGs	No data	Chen et al. (2017)
Tomatidine	*C. elegans*	Median lifespan extension, enhanced mitophagy	No data	Fang et al. (2017)
Ikarugamycin	*C. elegans*	Lifespan extension, enhanced autophagy	No data	Wang et al. (2017)
Salicylic acid	*C. elegans*	Lifespan extension, enhanced autophagy	Knockdown of *bec-1* or *hlh-30*	Shamalnasab et al. (2018)
Nordihydroguaiaretic acid	*C. elegans*	Median lifespan extension, enhanced autophagy	No data	Tezil et al. (2019)
Myo-inositol	*C. elegans*	Lifespan extension, enhanced mitophagy	Knockdown of *pink-1*	Shi et al. (2020)
4,4′-dimethoxychalcone	*C. elegans* *D. melanogaster*	Lifespan extension, enhanced autophagy	Knockdown of *atg-5* (*C. elegans*) Knockdown of Atg7 (*D. melanogaster*)	Gutierrez et al. (2019)
Spermidine	*C. elegans* *D. melanogaster* Mice	Lifespan extension, enhanced autophagy	Knockdown of *bec-1* (*C. elegans*) Knockdown of Atg7 (*D. melanogaster*)	Eisenberg et al. (2009, 2016)
Rapamycin	*C. elegans* *D. melanogaster* Mice	Lifespan extension, enhanced autophagy	Knockdown of Atg5 or Atg1 (*D. melanogaster*)	Bjedov et al. (2010), Schinaman et al. (2019)
Urolithin A	*C. elegans* Mice	Lifespan extension, enhanced mitophagy	Knockdown of *bec-1, vps-34*, or *pink-1*	Ryu et al. (2016)

beneficial for the health in a number of species and extends the life span of yeast, worms, flies, and mice (Eisenberg et al. 2009, 2016). The survival of cultured mammalian cells is also promoted by treatment with spermidine, and this is accompanied by epigenetic hypoacetylation of histone H3 via the inhibition of histone acetyltransferase activity. This, in turn, correlates with the transcriptional upregulation of multiple autophagy-related genes, including *Atg5* and *Lc3/ATG8/lgg-1/2* (Eisenberg et al. 2009). In keeping with this observation, spermidine fails to extend the life span of *C. elegans* subjected to *bec-1* RNAi, whereas it increases the expression of DsRed::LGG-1 (Eisenberg et al. 2009) in a *sir-2*-independent fashion (Morselli et al. 2011). In flies, spermidine alters the expression of autophagy markers, protects against age-induced memory loss in an autophagy-dependent manner and extends the life span in an *Atg7*-dependent manner (Gupta et al. 2013). Collectively, these data suggest that the positive effects of spermidine on health and longevity are mediated, at least in part, via autophagy induction.

Resveratrol is a naturally occurring polyphenolic compound found in grapes and an activator of the NAD-dependent histone deacetylase sirtuin (SIRT1). Administration of resveratrol is known to extend the life span of several model organisms (Park et al. 2013). The life spans of yeast, worms, and flies can be extended by the overexpression and/or pharmacological activation of SIRT1, and the life span of mice is extended by brain-specific overexpression of SIRT1 (Giblin et al. 2014). Especially, the life span extension in *C. elegans* seems to be dependent on autophagy since resveratrol fails to extend the life span of *bec-1*(*RNAi*)-treated animals. Additionally, resveratrol increases DsRed::LGG-1 levels in wild-type animals but not in *SIR-2.1* loss-of-function mutants (Morselli et al. 2010). These observations are in agreement with findings in mammalian cells, where the pharmacological activation of SIRT1 by resveratrol treatment stimulates autophagic flux. In contrast, SIRT1 −/− fibroblasts show suppressed autophagy during starvation and show elevated acetylation of key autophagy protein (Lee et al. 2008). Thus, SIRT1 promotes autophagy via the deacetylation of proteins involved in the autophagy pathways. Recent evidence suggests that Beclin1 is acetylated by p300 and deacetylated by SIRT1 (Sun et al. 2015). Acetylated Beclin1 recruits Rubicon, leading to the inhibition of autophagosome maturation, and SIRT1 might promote autophagy through deacetylation of Beclin1. SIRT1 is also known to co-immunoprecipitate with ATG5, ATG7, and LC3 and deacetylate these in vitro, and these interactions could be also essential for autophagy regulation (Lee et al. 2008).

Urolithin A as a first-in-class natural compound that induces mitophagy both in vitro and in vivo following oral consumption. In *C. elegans*, urolithin A prevents the accumulation of dysfunctional mitochondria with age and extends life span (Ryu et al. 2016). Likewise, Urolithin A prolongs normal activity during aging in *C. elegans*, including mobility and pharyngeal pumping, while maintaining mitochondrial respiratory capacity. These effects are observed in rodents, where Urolithin A improves exercise capacity in two different mouse models of age-related decline of muscle function, as well as in young rats.

Tomatidine, a natural compound abundant in unripe tomatoes, inhibits age-related skeletal muscle atrophy in mice. Recent study shows that tomatidine

extends life span and healthspan in *C. elegans* (Fang et al. 2017). Tomatidine improves many *C. elegans* behaviors related to healthspan and muscle health, including increased pharyngeal pumping, swimming movement, and reduced percentage of severely damaged muscle cells. Microarray, imaging, and behavioral analyses reveal that tomatidine maintains mitochondrial homeostasis by modulating mitochondrial biogenesis and PINK-1/DCT-1-dependent mitophagy. A detailed analysis shows tomatidine induces mitochondrial hormesis by mildly inducing ROS production, which in turn activates the SKN-1/Nrf2 pathway and possibly other cellular antioxidant response pathways, followed by increased mitophagy. This mechanism occurs in *C. elegans*, primary rat neurons, and human cells.

The biguanide metformin extends healthspan and life span in several models including *C. elegans* and mice and the risk of dementia in humans (Barzilai et al. 2016). Metformin has pleiotropic roles and inhibits mitochondrial respiration, mTOR, and activates AMPK, leading to the activation of autophagy (Song et al. 2015; Xie et al. 2011). Whether metformin-mediated life span extension requires autophagy activity needs to be clarified.

11.7 Conclusion

Accumulating evidence suggest that the activation of autophagy is one of the common mechanisms of many longevity paradigms. Moreover, the forced activation of autophagy suffices to extend life span and ameliorates age-associated phenotype in model organism, implying autophagy activation is one of the promising methods to delay human aging. However, actual roles of autophagy contributing to longevity need to be clarified further. It is also critical to establish the method to measure autophagic activity in human. In addition, recent evidence also suggests that autophagy activation in some context becomes detrimental rather than beneficial. For instance, too much upregulation of autophagy genes becomes rather detrimental (Bjedov et al. 2020) and elevated autophagy shortens life span when the mitochondria permeability increases (Zhou et al. 2019a). The neuronal inhibition of some autophagy genes during post reproductive period extends life span (Wilhelm et al. 2017). In addition, autophagy inhibition is necessary in adipocyte for proper function (Yamamuro et al. 2020). Further studies to reveal tissue and timing-specific roles of autophagy are required to make autophagy modulation a promising antiaging therapy in the next decades.

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Chapter 12
Sarcopenia: Current Topics and Future Perspective

Minoru Yamada, Kaori Kinoshita, Shosuke Satake, Yasumoto Matsui, and Hidenori Arai

Abstract Sarcopenia, which is an age-related loss of skeletal muscle mass and muscle weakness, is a disease that is currently attracting attention due to its high prevalence and significant impact on adverse health outcomes. Several international working groups have reported diagnostic criteria for sarcopenia, and it is now common to diagnose sarcopenia based on skeletal muscle mass, muscle strength, and physical performance. As countermeasures, resistance exercise and protein (amino acid) intake are recommended, as these have been shown to increase skeletal muscle mass, increase muscle strength, and improve physical function. We expect that more substantial basic and clinical research will be conducted in the future so that more appropriate management strategies for sarcopenia can be implemented.

Keywords Sarcopenia · Muscle mass · Muscle strength · Exercise · Nutrition

12.1 What Is Sarcopenia?

Sarcopenia is a term coined by Rosenberg in 1989, combining the Greek words "sarx," meaning muscle, and "penia," meaning loss (Rosenberg 1989). When this term was first proposed, it meant only a decrease in skeletal muscle mass, but since the European Working Group on Sarcopenia in Older People (EWGSOP) reported

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_12

new diagnostic criteria in 2010 (Cruz-Jentoft et al. 2010), it has become common to define sarcopenia as a combination of skeletal muscle loss and muscle weakness/physical function decline. We organized the Asian Working Group (AWGS) for Sarcopenia in 2013 and proposed an algorithm specific for Asian people in 2014 (Chen et al. 2014). A new ICD-10-CM disease code for sarcopenia (M62.84) was established in October 2016, and many countries have accepted sarcopenia as a disease entity. Recently, the importance of muscle quality in addition to quantity and strength as skeletal muscle indicators has been indicated, and the understanding of sarcopenia is gradually changing with the progression of research.

12.2 How to Diagnose Sarcopenia

There are several international diagnostic criteria for sarcopenia, including the EWGSOP2 (Cruz-Jentoft et al. 2019), AWGS 2019 (Chen et al. 2020), International Working Group on Sarcopenia (IWGS) (Fielding et al. 2011), and the Foundation for the National Institutes of Health Sarcopenia Project (FNIH) (Studenski et al. 2014) criteria (Table 12.1). All of the criteria include measurements of skeletal muscle mass and physical function. However, muscle strength assessment is not included in the IWGS criteria.

In terms of appendicular skeletal muscle mass, the method of correcting muscle mass using the square of the height or body mass index (BMI) has been used. Although there is currently no consensus on which correction method is more appropriate (Cruz-Jentoft et al. 2019; Chen et al. 2020), we recently reported that low muscle mass with either height-squared or BMI correction was associated with adverse outcomes among Japanese older adults (Kinoshita et al. 2021b). The same study suggested that height-squared correction is more likely to miss sarcopenia associated with obesity, whereas BMI correction is more likely to miss sarcopenia associated with thinness (Kinoshita et al. 2021b). In addition, our study indicated that the cutoff value for low muscle mass with BMI correction in Asians may be lower than that the FNIH criterion (Moon et al. 2016; Kinoshita et al. 2021a), and the accumulation of evidence is necessary to establish an optimal cutoff value.

Table 12.1 Definitions of sarcopenia according to the different research groups

	Skeletal muscle mass	Physical function	Muscle strength
EWGSOP	○	○	○
AWGS	○	○	○
IWGS	○	○	
FNIH	○	○	○

EWGSOP European Working Group on Sarcopenia in Older People, *AWGS* Asian Working Group for Sarcopenia, *IWGS* International Working Group on Sarcopenia, *FNIH* Foundation for the National Institutes of Health Sarcopenia Project

Although physical function indicators are included in many criteria, they were positioned as outcome indicators in the FNIH criteria and as severity assessment indicators in the EWGSOP2 criteria. Walking speed is a typical physical function assessment, but the AWGS 2019 criteria also includes the five times sit-to-stand test and the Short Physical Performance Battery (SPPB) as physical function measures and proposes cutoff values based on evidence from Asian populations.

12.3 AWGS 2019

Here, we take the most recent criteria, the AWGS 2019 criteria, as an example and outline its contents. AWGS published a consensus report (AWGS 2019) (Chen et al. 2020), which was a revised version of the diagnostic criteria reported in 2014 (Chen et al. 2014). Since there are many older people with or at risk of sarcopenia in Asian countries with aging populations, the AWGS 2019 proposed the use of community or primary care settings in addition to clinical settings to diagnose sarcopenia (Fig. 12.1). The diagnostic indicators are muscle strength, physical function, and skeletal muscle mass, and sarcopenia is determined by low skeletal muscle mass plus low muscle strength or low physical function. The reference values for each of these are determined by data from representative cohort studies in Asian countries and by expert consensus (Table 12.2).

The flow of the AWGS 2019 guidelines in the clinical setting is described below. The process begins with the detection of patients with possible sarcopenia based on

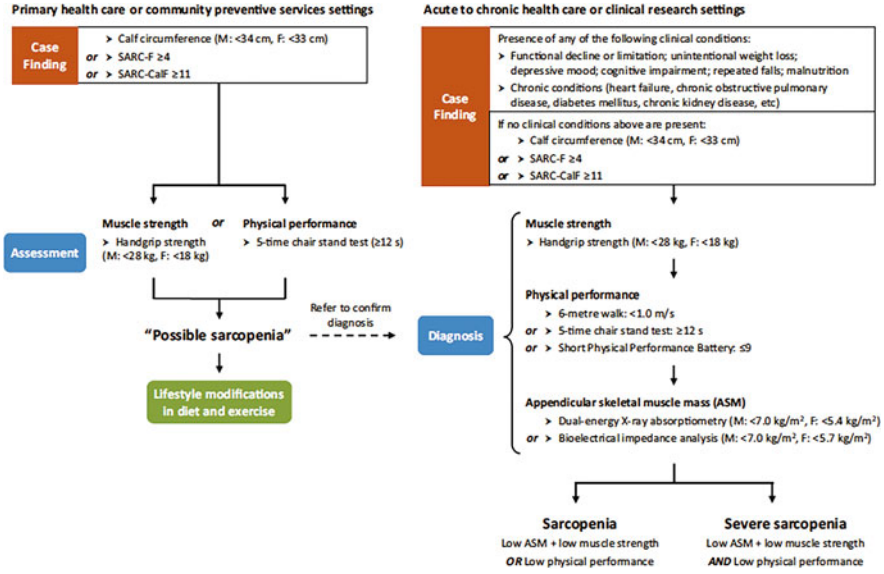

Fig. 12.1 Diagnostic algorithm of sarcopenia in AWGS 2019 (Chen et al. 2020)

Table 12.2 Cutoff values for diagnosing sarcopenia according to the AWGS 2019 criteria

	Men	Women
Calf circumference	<34 cm	<33 cm
SARC-F	≧4	
Grip strength	<28 kg	<18 kg
5 times sit-to-stand test	≧12 s	
Gait speed	<1.0 m/s	
Short Physical Performance Battery	≦9	
Skeletal muscle index		
Bioelectrical impedance	<7.0 kg/m^2	<7.0 kg/m^2
Dual-energy X-ray absorptiometry	<7.0 kg/m^2	<5.4 kg/m^2

clinical symptoms and case finding. The clinical symptoms include functional decline and weight loss, whereas case finding tests include calf circumference, SARC-F and SARC-CalF. This is followed by measurements of muscle strength (grip strength), physical function (gait speed, 5 times sit-to-stand test, and SPPB), and skeletal muscle mass (dual-energy X-ray absorptiometry (DXA) and bioelectrical impedance (BIA)). Skeletal muscle mass loss is a requirement for diagnosing sarcopenia, and sarcopenia is determined when muscle weakness or physical function decline is observed. If all three indicators of skeletal muscle mass loss, muscle weakness, and physical function decline are present, the patient is judged to have severe sarcopenia. In community or primary care settings, grip strength and five times sit-to-stand tests should be performed for the diagnosis of possible sarcopenia after case finding with calf circumference measurements or SARC-F/SARC-CalF. Thus, the diagnosis of sarcopenia is widely applicable in almost all clinical settings based on the AWGS 2019 criteria.

12.4 Utilization of Phase Angle

In recent years, with the increase in the use of BIA to measure skeletal muscle mass, the phase angle (PhA), which is calculated from reactance and resistance measured by BIA, has become the focus of attention. PhA is thought to reflect the physiological functioning level of the cell, and in unhealthy cells, the reactance at the cell membrane is reduced, resulting in a lower PhA. It has been shown to be related to the quantity and quality of skeletal muscle, muscle strength, and physical function (Basile et al. 2014; Yamada et al. 2017, 2019) and has been reported to be useful in identifying sarcopenia (Di Vincenzo et al. 2021). Characteristically, PhA, like muscle strength, changes sensitively with training (Dos Santos et al. 2016) and is thought to be useful as an outcome measure for determining the effectiveness of interventions. Although PhA is not included in the diagnostic algorithm for sarcopenia, further evidence could support the inclusion of PhA as a criterion for sarcopenia.

12.5 Prevalence of Sarcopenia

The prevalence of sarcopenia varies widely by age, definition, and study setting. Reports that have determined age-specific prevalence rates in community-dwelling older adults have shown that the prevalence of sarcopenia increases with age, especially after the age of 75 years (Yamada et al. 2013; Kitamura et al. 2021). In addition, a systematic review examining the prevalence of sarcopenia in community-dwelling older adults using each diagnostic guideline reported a 12.9% prevalence by the EWGSOP criteria and AWGS criteria, a 9.9% prevalence by the IWGS criteria, and an 18.6% prevalence by the FNIH criteria (Mayhew et al. 2019). Furthermore, a systematic review examining the prevalence by setting showed that the prevalence was 11% in males and 9% in females among community-dwelling older people, 51% in males and 31% in females among nursing home residents, and 23% in males and 24% in females among inpatients (Papadopoulou et al. 2020). Thus, although the prevalence varies somewhat according to diagnostic criteria and setting, the high prevalence of sarcopenia is noteworthy and indicates the need for widespread measures.

12.6 Etiology of Sarcopenia

A variety of factors are thought to be associated with sarcopenia, including inactivity and poor nutrition, as well as few exercise units, chronic inflammation, increased oxidative stress, and increased insulin resistance (Fig. 12.2) (Dickinson et al. 2013). In this context, inactivity and poor nutrition are variable factors, and there is ample room for intervention.

12.7 Genetics of Sarcopenia

Although extensive studies on the genetic basis of muscle quality and quantity have been performed to date, there is insufficient evidence to demonstrate a causal relationship with sarcopenia, suggesting that the multifactorial mechanisms underpinning muscle regulation may not be reducible to one single gene or gene variant. Pratt et al. conducted a systematic review demonstrating that the alpha-actinin (*ACTN3*), angiotensin-converting enzyme (*ACE*), and vitamin D receptor (*VDR*) genotypes and ten DNA polymorphisms were significantly associated with muscle phenotypes (Pratt et al. 2019). Further studies need to be conducted to elucidate the causal relationship of specific genotypes or gene polymorphisms to predict the development of sarcopenia in humans. In addition, microRNAs are promising candidates for sarcopenia research because they are involved in the proliferation, differentiation, and stem cell renewal of skeletal muscle and the aging-related loss of

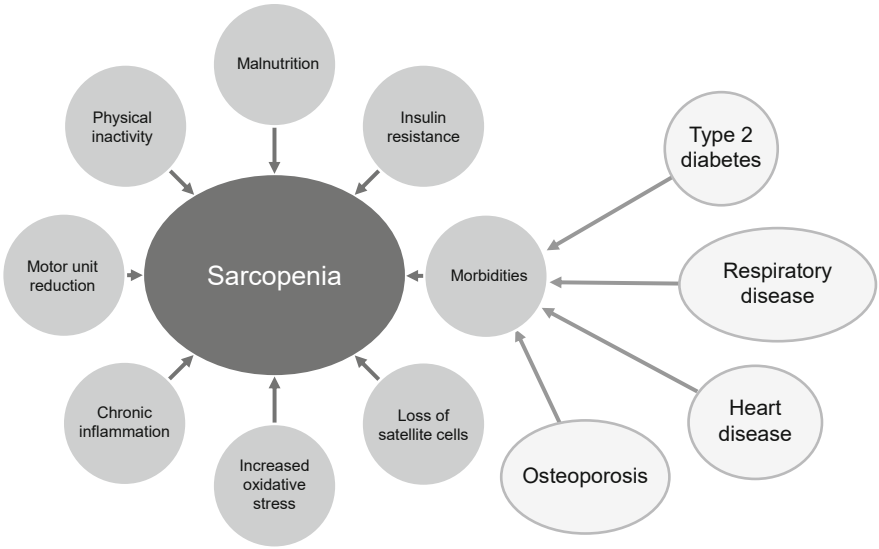

Fig. 12.2 Factors associated with sarcopenia

muscle mass (Jung et al. 2019). Jung et al. conducted a comprehensive review on the role of microRNAs in skeletal muscle aging and highlighted their potential as biomarkers or therapeutic targets (Jung et al. 2019).

12.8 Prognosis of Sarcopenia

Sarcopenia is known to be strongly associated with the development of subsequent adverse health outcomes, such as falls, fractures, hospitalization, disability, and death (Fig. 12.3) (Bianchi et al. 2016; Beaudart et al. 2017; Liu et al. 2017; Uemura et al. 2019; Yeung et al. 2019; Zhang et al. 2020; Huang et al. 2021). It is characterized not only by falls, fractures, and disability, which are directly affected by muscle weakness, but also by outcomes such as hospitalization and death.

12.9 Relationship Between Sarcopenia and Disease

Although sarcopenia is an age-related disease, many people with comorbidities are likely to develop sarcopenia. Among them, so-called secondary sarcopenia caused by diseases, such as type 2 diabetes, respiratory disease, cardiovascular disease, and osteoporosis, can develop (Fig. 12.3) (Pacifico et al. 2020). In particular, osteoporosis is strongly associated with sarcopenia; previous studies have shown that

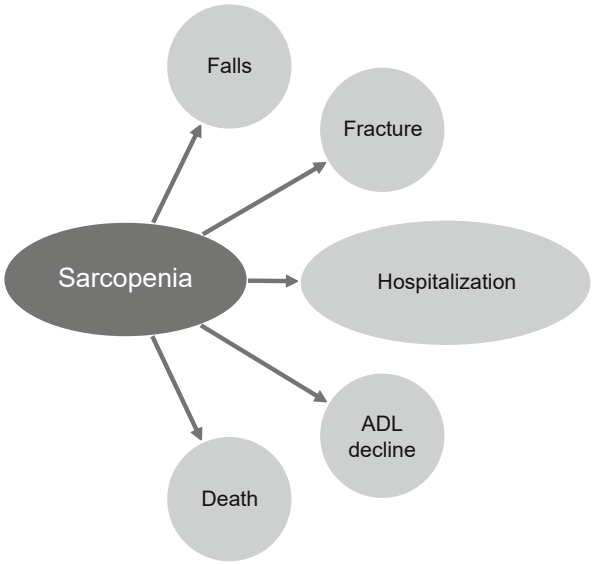

Fig. 12.3 Prognosis of sarcopenia

sarcopenia and osteoporosis often coexist (Huo et al. 2015; Drey et al. 2016), and the concept of osteosarcopenia has been proposed (Fagundes Belchior et al. 2020). Recently, the association between cognitive decline and sarcopenia has also been indicated (Chang et al. 2016), but since both are affected by aging, their interpretation needs careful consideration.

12.10 Macroscopic Features of Age-Related Changes in Skeletal Muscle

It is obvious that skeletal muscles undergo age-related changes. Various studies have shown that skeletal muscle mass shows a gradual decline among total body muscle mass starting at approximately 40–50 years of age (Fig. 12.4) (Speakman and Westerterp 2010; Jackson et al. 2012; Yamada et al. 2014), and in sarcopenic individuals, the amount of decline deviates from that associated with the normal aging process. However, such age-related changes do not occur in the same way in all skeletal muscles, of which there are more than 400 in the whole body; a study on age-related changes in muscle strength over a 10-year period showed that muscle weakness was more pronounced in the lower limbs than in the upper limbs in both men and women (Hughes et al. 2001). A study that included the trunk showed that so-called antigravity muscles were more likely to be affected (Fig. 12.5) (Vitasalo et al. 1984). In particular, among the anti-gravity muscles, relatively large muscle groups located near the body surface have a common characteristic of containing a large number of type 2 fibers and are prone to age-related changes. Among them, the

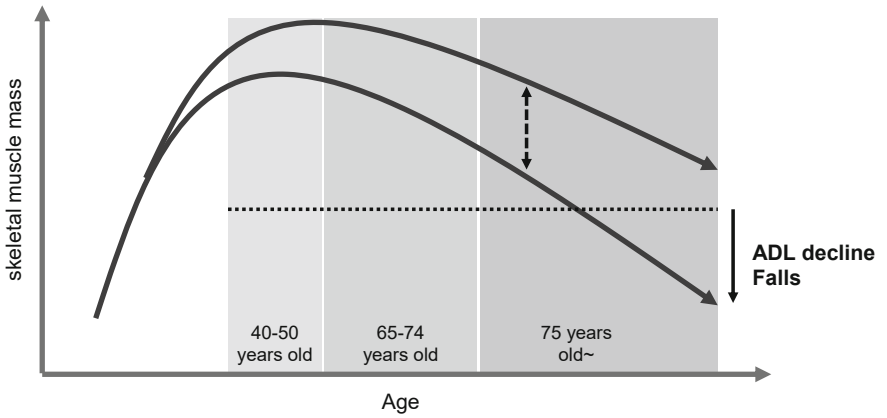

Fig. 12.4 Age-dependent changes in skeletal muscle

quadriceps, which is a representative anti-gravity muscle, is an especially major indicator of age-related changes in skeletal muscle. In our study, the quadriceps muscle showed greater age-related changes than the other muscles in the thigh region, and such changes were more pronounced in males than in females (Kasai et al. 2015). In a study using CT cross-sections in the same cohort, the quadriceps cross-sectional area was found to be associated with lower extremity muscle strength just as much as or more than the skeletal muscle mass index (SMI) obtained by DXA (Tsukasaki et al. 2020). Moreover, the muscle cross-sectional area, which represents muscle mass, as well as CT values, which represent muscle quality, were independently associated with muscle strength (Mizuno et al. 2021). Similarly, in a recent study in frailty-clinic outpatients assessed with CT cross-sectional images of the quadriceps muscle, muscle cross-sectional area was more closely related to muscle strength, whereas CT values were more closely related to physical function (Oba et al. 2021).

12.11 Microscopic Features of Age-Related Changes in Skeletal Muscle

Age-related changes in skeletal muscle naturally occur in the muscle fibers. Age-related changes in muscle fibers have two characteristics: (1) a decrease in the number of muscle fibers and (2) a decrease in the cross-sectional area of muscle fibers (Lexell 1995). The latter is a common feature of disuse muscular atrophy, while the former is a characteristic unique to age-related changes that are not observed in disuse muscular atrophy (Table 12.3) (Kanazawa et al. 2017). In addition, although muscle fibers can be broadly classified into type 1 fibers and type 2 fibers, it has been shown that type 2 fibers are more susceptible to the effects of aging, whereas type 1 fibers are relatively easy to maintain (Fig. 12.6) (Lexell

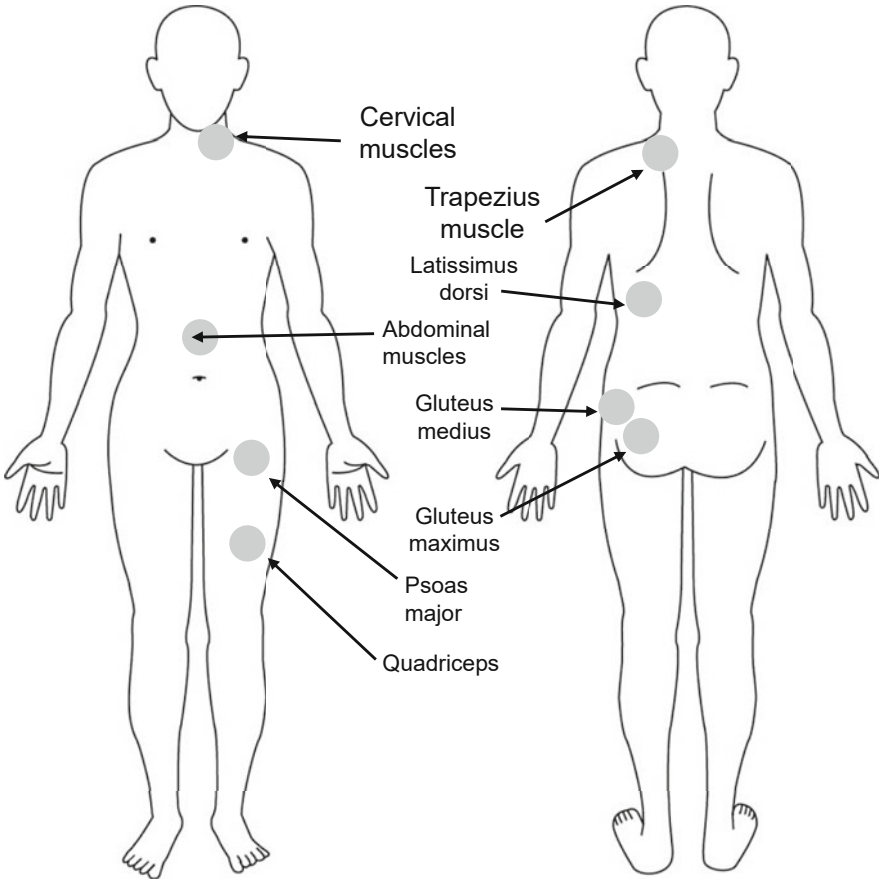

Fig. 12.5 Muscles that tend to be influenced by age

Table 12.3 Characteristics of sarcopenia caused by aging and disuse

	Age-related sarcopenia	Disuse-related sarcopenia
Number of muscle fibers	↓	→
Cross sectional area of muscle fibers	↓	↓

1995). One of the reasons for this difference is thought to be the influence of satellite cells. Satellite cells are considered to be involved in the repair and hypertrophy of myofibers, and it has been shown that satellite cells in type 2 fibers decrease with age, whereas satellite cells in type 1 fibers are unaffected (Verdijk et al. 2007). While changes in muscle fiber type are a factor pertaining to muscle quality, other micro changes related to muscle quality include increased accumulation of intra- and intermuscular fat (Goodpaster et al. 2001; Therkelsen et al. 2016), increased muscle fibrosis (Brack et al. 2007), and increased fat indicated by lower CT values on CT images (Aubrey et al. 2014).

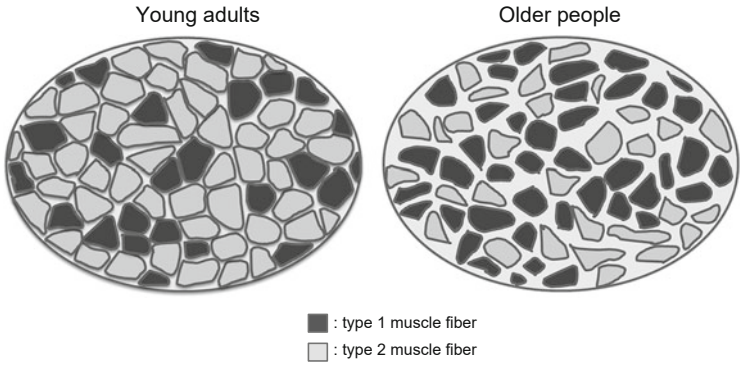

Fig. 12.6 Age-related changes in type 1 and 2 muscle fibers in human cells (image of cross-section of skeletal muscle on electron microscopy)

12.12 Prevention of Sarcopenia

There are few studies that consider the development of sarcopenia as an outcome, and it is not clear whether various interventions can prevent the development of sarcopenia. Several interventional studies have examined the effects of exercise and protein intake on skeletal muscle mass and strength; however, the outcomes were not limited to the occurrence of sarcopenia. A meta-analysis showed that resistance exercise increased muscle strength and skeletal muscle mass in healthy older people (Borde et al. 2015). On the other hand, a meta-analysis examining the effects of protein intake on muscle strength and skeletal muscle mass in healthy older subjects did not find any advantage of protein intake (Ten Haaf et al. 2018). In many cases, healthy older people are able to consume sufficient amounts of protein from their daily diet, and it is thought that the significance of additional protein is unlikely to affect outcomes.

12.13 Interventions for Sarcopenia

Exercise, protein (essential amino acids) intake, and a combination of these interventions have been shown to be effective for the treatment of sarcopenia. Resistance exercise has been shown to be effective for the treatment and prevention of sarcopenia and has been shown to improve skeletal muscle mass, muscle strength, and physical function (Yoshimura et al. 2017; Arai et al. 2018; Bao et al. 2020). In addition, it is known that protein intake, which is not significant in the prevention of sarcopenia, has a significant effect on skeletal muscle in sarcopenic subjects (Komar et al. 2015).

12.14 How to Provide Resistance Training

When resistance exercise is performed for the purpose of preventing or treating sarcopenia, the following two points should be carefully considered. The first is awareness of the number of repetitions as well as the amount of load, and the second is the continuation of exercise.

In general, when resistance exercise is performed for the purpose of muscle strengthening, it is considered ideal to perform it with a high load of 70–80% of the maximum performance. However, in recent years, it has been shown that muscle protein synthesis and muscle strengthening effects can be obtained by performing many repetitions with low loads rather than high loads when the target population is older people (Van Roie et al. 2013; Agergaard et al. 2017). This evidence is important when prescribing exercise for older adults with various risk levels, and the fact that increased repetitions, even at low loads, can help counteract sarcopenia is important information.

The effects of resistance exercise are not permanent and disappear in a relatively short period of time. Studies with a 12-week resistance exercise period followed by a 24-week rest period showed that muscle strength and muscle mass gained through resistance exercise were almost halved after 12 weeks and almost completely lost after 24 weeks (Fig. 12.7) (Taaffe et al. 2009; Zech et al. 2012; Yasuda et al. 2014). This trend was also observed for muscle quality, and it has been shown that muscle density and phase angle, which are indicators of muscle quality, improve with training and worsen with the cessation of training (Taaffe et al. 2009; Dos Santos

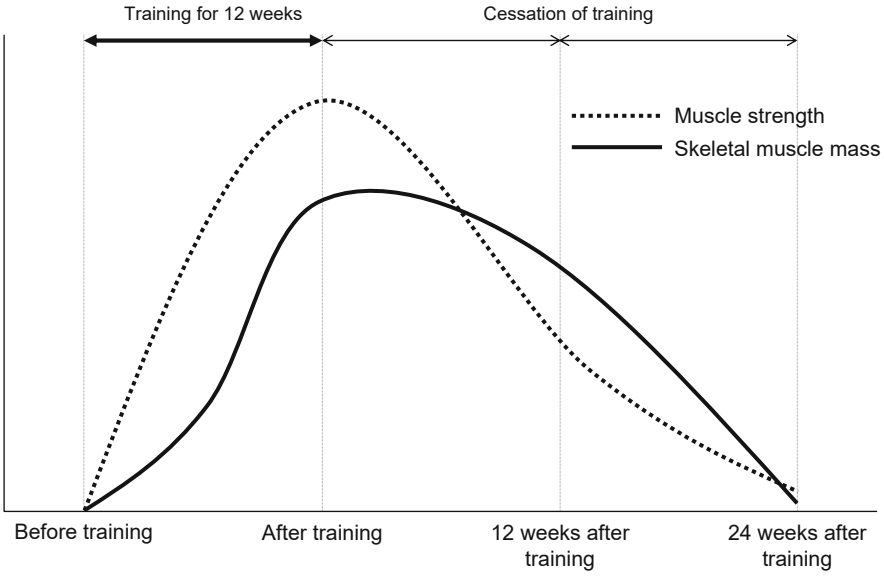

Fig. 12.7 Effects of resistance exercise on muscle strength and skeletal muscle mass

et al. 2016). Therefore, it is important for patients to continue exercise and to promote awareness of and behavioral changes focusing on low-load, high-repetition exercise as described above for a long period of time.

12.15 How to Provide Amino Acids and Protein for Persons with Sarcopenia

Although amino acid and protein intake have been demonstrated to be useful in the treatment of sarcopenia, the method of intake also needs to be examined. In general, amino acid/protein intake immediately after exercise is considered beneficial, but this method is not necessarily optimal for older subjects. It is known that muscle protein synthesis is accelerated 1–2 h after exercise in both young and older people in a similar manner (Kumar et al. 2009). However, when protein is ingested, more time is needed to increase the amino acid concentration in the blood, and it peaks after approximately 1 h in young people and 3 h in older people (Milan et al. 2015). In other words, protein intake immediately after exercise contributes to the promotion of postexercise muscle protein synthesis in young people, but it is difficult to contribute to the promotion of postexercise muscle protein synthesis in older people, even if protein is consumed immediately after exercise.

Against this background, in recent years, there has been a renewed emphasis on the need to maintain a balance between three daily meals. This is based on the idea that by maintaining a uniform protein intake in all three meals, the amino acid concentration in the blood will be maintained above a certain level throughout the day. In general, protein intake at breakfast tends to be inadequate and increases gradually with lunch and dinner (Paddon-Jones et al. 2015). Therefore, if protein is to be provided as a supplement, it is considered important to fortify protein in the morning. Muscle protein synthesis is more likely to decrease not only in the morning but also when protein intake is uneven among the three meals than when it is uniform (Paddon-Jones et al. 2015). Therefore, in the case of sarcopenia prevention, the current protein balance among the three meals should be examined, and the same daily protein intake should be maintained. In the case of treatment, the daily protein intake should be increased while maintaining a balance among the three meals.

12.16 Pharmacological Treatment of Sarcopenia

Although a variety of drugs for sarcopenia, including myostatin/ActR2 signaling inhibitors, exercise mimetics, anabolic hormones, and natural compounds, have been investigated for sarcopenia treatment, none have been approved for clinical use. For example, one novel approach targets the myostatin/activin type II receptor (ActRII) pathway to induce hypertrophy of skeletal muscles, with the expectation of

improved functional ability (Lach-Trifilieff et al. 2014). Bimagrumab (BYM338) is a fully human monoclonal antibody that can block ligand binding and promote the differentiation of human myoblasts (Rooks and Roubenoff 2019). However, no significant improvement in skeletal muscle function in old sarcopenic patients was shown in a preclinical study (Rooks et al. 2020). Further drug development for the treatment of sarcopenia is warranted because a significant number of sarcopenic patients cannot perform resistance training or achieve appropriate nutritional intake, as suggested by the recommendation.

12.17 Conclusions

Here, we summarized the current status of the concept, pathogenesis, diagnosis, and epidemiology of and intervention methods for sarcopenia. Sarcopenia is still a relatively new concept, and only recently has clinical research been actively pursued. Therefore, there are limits to what is known, and many unknowns remain in this field. We hope that an interdisciplinary approach to sarcopenia will contribute to global aging research by consolidating knowledge from around the world.

Acknowledgments This study was supported in part by Research Funding for Longevity Sciences from the National Center for Geriatrics and Gerontology, Japan (grant number 20–57).

Conflicts of Interest None.

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Chapter 13
Osteoporosis and Cellular Senescence in Bone

Takashi Kaito and Yuichiro Ukon

Abstract Osteoporosis is a very common disorder among the elderly. As society continues to age, the socioeconomic burden associated with pharmaceutical therapies and the treatment of osteoporotic fractures is likely to increase dramatically. The present standard pharmaceutical treatment is an antiresorptive agent, which is less than optimal as it can attenuate bone formation in addition to suppressing osteoclastic bone resorption.

Recent evidence has clarified that senescent cells and the senescence-associated secretory phenotype (SASP) in the bone microenvironment play significant roles in age-related osteoporosis and has suggested that the clearance of senescent cells from bone could emerge as a novel treatment strategy. In this review, we summarize the latest knowledge on bone cell senescence and the related possible treatment strategies for osteoporosis.

Keywords Bone · Senescence · Osteoporosis · Osteoblast · Osteocyte · Osteoclast

13.1 Introduction

Osteoporosis is a systemic chronic disease characterized by low bone mass and fragility. As life expectancy increases, so does the burden of osteoporotic fracture (Johnell and Kanis 2006). Current pharmacological treatments include antiresorptive agents (bisphosphonates, estrogen and selective estrogen-receptor modulators, and anti-receptor activator of nuclear factor κB ligand antibody) and anabolic agents (parathyroid hormone and related fragments [PTH1-34] and anti-sclerostin antibody) (Ukon et al. 2019). Both of these treatment approaches have drawbacks; however, the antiresorptive agents are associated with decreased bone formation (coupling), while the beneficial effects of the anabolic agents diminish with time. Recent scientific advances have clarified the relationship between osteoporosis and

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_13

cellular senescence and suggested that using "senolytic drugs" to eliminate senescent cells or otherwise inhibiting the senescence-associated secretory phenotype (SASP), which is correlated with local and systemic inflammation, have potential as novel treatment strategies for osteoporosis (Farr et al. 2016, 2017).

13.2 Role of Bone Cells and Age-Related Changes

Bone is a dynamic organ: as osteoclasts continuously resorb old bone, osteoblasts continually form new bone, in a delicately balanced process of "remodeling." Osteocytes, which are terminally differentiated osteoblasts, control bone homeostasis by sending signals to both osteoclasts and osteoblasts. Bone remodeling helps maintain the quality of skeletal bone by rebuilding any areas with micro-damage or fracture and changing the shapes of bones in response to mechanical stress (Fig. 13.1).

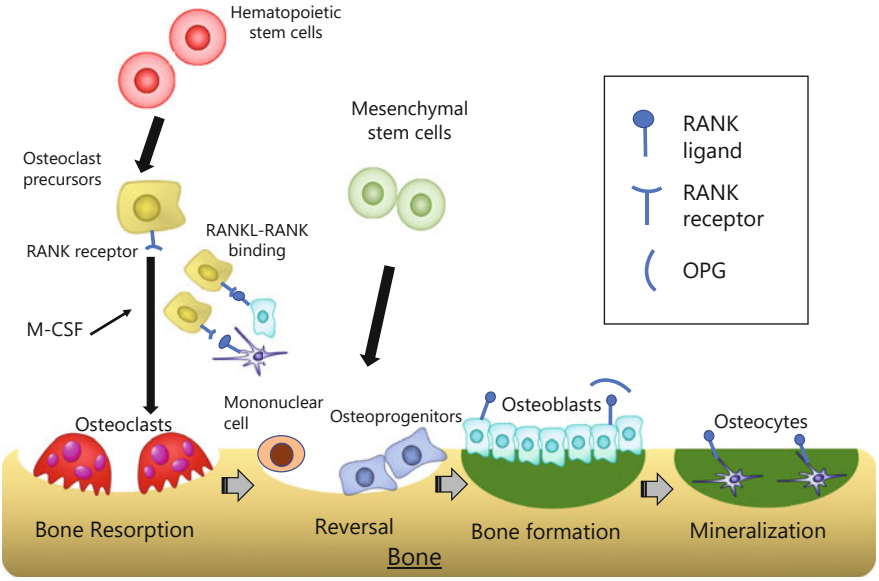

Fig. 13.1 Bone remodeling. Bone remodeling occurs by three sequential phases. In bone resorption phase, osteoclasts resorb old bone tissue. In the next reversal phase, mononuclear cells gather on the absorbed bone surface to prepare osteogenic microenvironment for the bone formation phase. In bone formation phase, osteoprogenitor cells attaches on the surface and differentiate into osteoblasts. Osteoblast are embedded in bone matrix and become osteocytes. Both osteoblasts and osteocytes express receptor activator of nuclear factor κB (RANK) ligand. Osteoprotegerin (OPG) is the decoy receptor for RANK ligand. Hematopoietic stem cells differentiate into osteoclast by the stimulation of macrophage colony stimulating factor (M-CSF) and RANKL. OPG bind and neutralize RANKL

With aging, the balance between bone formation and bone resorption is disrupted as the rate of bone resorption overtakes that of bone formation. These age-related changes in the remodeling balance vary by gender. Men tend to exhibit a steady decrease in bone formation, whereas women exhibit higher rates of bone turnover as postmenopausal estrogen deficiency leads to increases in both bone formation and osteoclastic resorption (Seeman 2002). In both genders, however, prolonged imbalance in bone remodeling leads to bone loss and osteoporosis. Osteoporotic bone contains lower proportions of osteogenic progenitor cells and increased proportions of adipose tissue in the bone marrow. Bone marrow contains both mesenchymal and hematopoietic stem cells; the mesenchymal stem cells (MSCs) are sources of osteoblasts, adipocytes, and chondrocytes. Studies have shown that a shift in the number of MSCs committed to adipocyte formation leads to the increased bone marrow adipose tissue (Justesen et al. 2001; Sui et al. 2016).

13.3 Cellular Senescence in the Bone Microenvironment

Cellular senescence, defined as irreversible and permanent cell cycle arrest, is triggered by a variety of mechanisms including dysfunctional telomeres, DNA damage, aberrant activation of oncogenes or loss-of-function of tumor suppressor genes, oxidative stress, and other genotoxic stresses such as chemotherapy (Hayflick and Moorhead 1961). These stimuli cause cellular senescence mainly via the p53/p21 and p16/pRB pathways (Khosla et al. 2020).

In addition to ceasing cell division, senescent cells exhibit a flattened and enlarged morphology in vitro and secrete pro-inflammatory cytokines, growth factors, and matrix-remodeling factors. This phenomenon, known as the senescence-associated secretory phenotype (SASP), can cause chronic inflammation and cancer development by affecting the paracrine system and potentially other tissues or organs. The changes associated with the SASP vary depending on cell type, tissue, stimuli, and even the duration of senescence (Basisty et al. 2020).

13.4 Bone Phenotype in Animal Models of Accelerated Senescence

13.4.1 DNA Damage

One of the inducers of senescence is DNA damage. The DNA repair protein known as excision repair cross-complementary group 1 (ERCC1) plays an essential role in the DNA excision pathway. Ercc1$^{-/\Delta}$ mice, which have one null and one hypomorphic ERCC1 allele, have been widely used as a progeroid mouse model because the cellular senescence and SASP markers in 4- to 5-month-old Ercc1$^{-/\Delta}$

mice are comparable to those in naturally aged mice (Niedernhofer et al. 2006). Ercc1$^{-/\Delta}$ mice develop age-dependent progressive osteoporosis due to reduced osteoblastic bone formation and enhanced osteoclastic bone resorption. Bone marrow stromal cells (BMSC) from Ercc1$^{-/\Delta}$ mice have compromised osteogenic differentiation and enhanced SASP, leading to increased secretion of inflammatory cytokines such as IL-6 and TNF-α and increased expression of RANKL. In addition, Ercc1$^{-/\Delta}$ mice have elevated levels of the DNA damage marker γH2AX and the cellular senescence marker p16^{INK4a} at the primary spongiosa of the tibia.

Notably, inhibition of NFκB-mediated signaling reverses bone senescence in Ercc1$^{-/\Delta}$ mice, suggesting that bone senescence in this model of progeroid DNA damage is mediated at least in part by increased NFκB signaling (Chen et al. 2013). These findings in Ercc1$^{-/\Delta}$ mice indicate that DNA damage plays significant roles in age-related osteoporosis.

13.4.2 Telomere Shortening

Another inducer of senescence is telomere shortening. Telomere length is maintained by the enzyme telomerase (Chen et al. 2013). Telomerase-deficient mice (Terc$^{-/-}$) demonstrate age-dependent bone loss after 8 weeks of age; this is caused by reduced osteoblastic bone formation and slightly increased osteoclastic bone resorption, which is supposed to be caused by the proinflammatory microenvironment in the bones of Terc$^{-/-}$ mice. Wang et al. have demonstrated that telomere-mediated defects in osteoblast differentiation are associated with increased p53/p21 expression (Wang et al. 2012). These data suggest an association between telomere dysfunction and age-related bone loss.

13.5 Cellular Senescence in Bone

In vivo cellular senescence in bone was first demonstrated by Farr et al. (2016) who used cell sorting to investigate the existence of cellular senescence and SASP in naturally aged wild-type mice. They found that p16^{INK4a} expression as quantified by qPCR was significantly higher in all senescent cells in the bone microenvironment, including osteoblasts, progenitors of osteoblasts, and osteocyte myeloid cells, while p21 expression was significantly higher in senescent osteocytes and p53 expression was significantly higher in senescent osteocytes and myeloid cells. Additionally, they measured 36 established SASP markers in their respective cell populations and found that 26 of the 36 SASP factors associated with senescent osteocytes and 23 of the 36 SASP factors associated with senescent myeloid cells. In contract, the SASP factors in senescent osteoblasts and osteoblast progenitors showed only slight changes compared to respective non-senescent cell populations. Finally, they analyzed human bone samples from elderly and young women and found that both

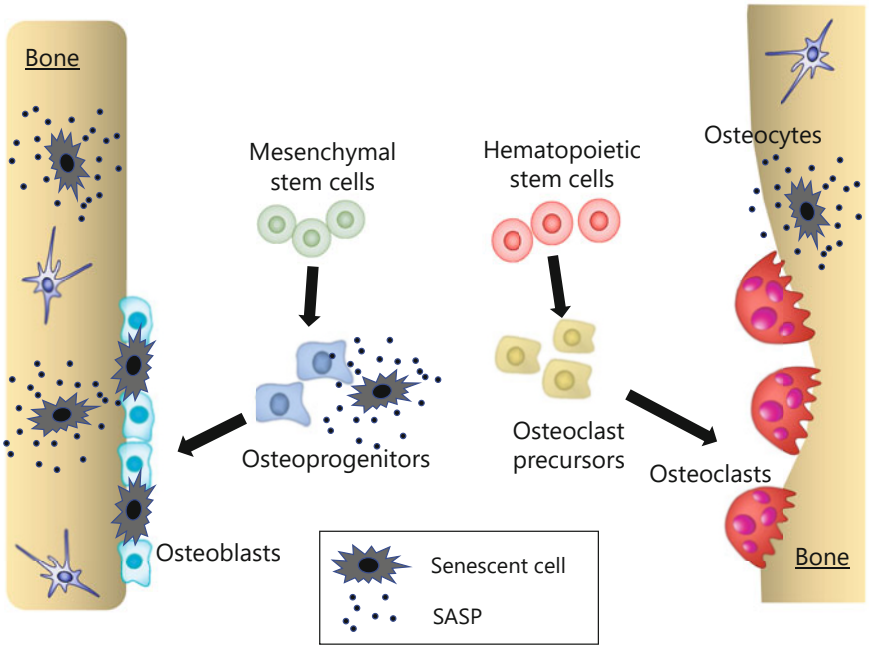

Fig. 13.2 Senescent cells in bone microenvironment. In bone microenvironment, cellular senescence is observed in osteoprogenitors, osteoblasts, osteocytes. Among them, SASP factors were demonstrated predominately in osteocytes. Inflammatory cytokines in SASP factors from osteocytes, osteoprogenitors enhance osteoclastic bone resorption

$p16^{INK4a}$ and p21 expression as well as 12 of the 36 SASP factors were significantly higher in bone from elderly women. Collectively, these results suggest that senescent osteocytes play a pivotal role in age-related osteoporosis in both mice and humans (Farr et al. 2016). Opinions are divided regarding $p16^{INK4a}$ expression in osteoblast progenitors, and Kim et al. have reported that p21 and SASP but not $p16^{INK4a}$ are elevated in osteoprogenitors (Kim et al. 2019) (Fig. 13.2).

13.6 Elimination of Senescent Cells in Bone Using Transgenic Mice

Recent studies have clarified that the elimination of senescent cells can extend life span and reverse the effects of age-related diseases. INK-ATTAC (Baker et al. 2011) and p16-3MR (Demaria et al. 2014) are two representative transgenic mice in which the elimination of $p16^{INK4a}$-positive cells can be induced. In INK-ATTAC mice, caspase-8 is located downstream of the $p16^{INK4a}$ promoter, and

caspase-8 transcription by the administration of AP20187 can induce apoptosis only in cells expressing $p16^{INK4a}$.

In INK-ATTAC mice, the elimination of up to 30% of all senescent bone cells enhances osteoblastic bone formation and reduces osteoclastic bone resorption, leading to increased bone volume and better bone microstructure (Farr et al. 2017).

In p16-3MR mice, in which herpes simplex virus thymidine kinase (HSV-tk) is located downstream of the $p16^{INK4a}$ promoter, the phosphorylation of ganciclovir (GCV) by HSV-tk triggers the transformation of ganciclovir into a DNA polymerase inhibitor, leading to cell death. GCV was administered to p16-3MR mice between either 12 and 24 or 20 and 26 months of age to investigate whether senescent bone cell removal can prevent and reverse age-related bone loss. However, GCV administration did not change the p16 expression levels in osteocytes of these mice. In contrast, senescent osteoclast progenitors were eliminated, but this had no effect on age-related bone loss (Kim et al. 2019).

13.7 Senolytic and Senomorphic Approaches to Treating Osteoporosis

These "elimination of senescent cells" strategy have been tried to apply to human in the form of senolytic drugs, which disable senescent cell antiapoptotic pathways (SCAPs) and induce apoptotic cell death of senescent cells.

Zhu et al. have demonstrated that the periodic administration of a combination of dasatinib and quercetin (D + Q) can eliminate senescent cells from naturally aged mice and prevent age-related disorders (Zhu et al. 2015). Farr and Khoslo have reported that D + Q administration leads to significant improvement in bone mass and strength as well as increased osteoblast counts and bone formation rates and decreased osteoclast counts and endocortical bone resorption rates in 20-month-old male mice. In an attempt to suppress the SASP (a senomorphic approach), they also administered JAK inhibitor (roxolitinib) to 22-month-old mice and observed a significant reduction in osteoclast counts with no change in osteoblast counts (Farr et al. 2017).

In contrast, ABT-263, another senolytic drug, led to bone loss and impaired osteoprogenitor function in aged mice although it effectively removed senescent hematopoietic stem cells and muscle stem cells in naturally aged mice and radiation-induced senescence-accelerated mice (Chang et al. 2016). The use of Bcl-xl inhibitors as senolytics is largely limited by their on-target dose-limiting platelet toxicity, though recent studies have attempted to reduce their off-target effects (He et al. 2020). The beneficial roles of cellular senescence in the physiological bone microenvironment must be clarified to prevent off-target effects of senolytic drugs.

13.8 Summary

Pharmacologic elimination of senescent cells and suppression of SASP is a promising treatment strategy for osteoporosis. Estrogen deficiency, which is caused by an independent mechanism, remains to be treated separately. In addition, the multi-organ off-target effects of the elimination of senescent cells and suppression of SASP should be better clarified in clinical trials before this new strategy is offered as a standard option for the prevention and treatment of osteoporosis.

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Chapter 14
Aging and Chronic Kidney Disease Viewed from the FGF-Klotho Endocrine System

Makoto Kuro-o

Abstract Endocrine fibroblast growth factors (FGFs) require Klotho proteins as an obligate co-receptor to bind to and activate FGF receptor tyrosine kinases. FGF23 is a bone-derived hormone secreted in response to phosphate intake and acts on renal tubules that express αKlotho to increase urinary phosphate excretion per nephron and maintain the phosphate balance. FGF21 is secreted from the liver upon fasting and acts on the suprachiasmatic nucleus in the brain where βKlotho is expressed to induce responses to stress, including activation of the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system. FGF21 is regarded as an antiaging hormone, because mice overexpressing FGF21 live longer than wild-type mice. Both FGF23 and FGF21 start increasing since early stages during the course of chronic kidney disease (CKD) progression. The increase in FGF23 compensates for decrease in the functional nephron number by increasing phosphate excretion per nephron, thereby maintaining phosphate homeostasis. However, FGF23-induced increase in phosphate concentration in the renal tubular fluid damages tubular cells and triggers interstitial fibrosis. In addition, FGF23 causes decrease in the serum level of active vitamin D followed by increase in parathyroid hormone, leading to mineral and bone disorders (CKD-MBD). The increase in FGF21 is necessary to survive CKD, because CKD mice lacking FGF21 exhibit poorer prognosis than wild-type CKD mice. However, FGF21-induced activation of the sympathetic nervous system results in blood pressure dysregulation. Thus, pathophysiology of CKD can be viewed as adverse effects associated with adaptive responses of the FGF-Klotho endocrine system to maintain survival and phosphate homeostasis.

Keywords Fibroblast growth factor-23 (FGF23) · Fibroblast growth factor-21 (FGF21) · αKlotho · βKlotho · Chronic kidney disease (CKD) · Calciprotein particle (CPP)

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_14

14.1 Discovery of the *Klotho* Gene

The *klotho* gene was identified as the gene mutated in a mouse strain that exhibited premature aging (Kuro-o et al. 1997). The founder of this strain was a transgenic mouse carrying exogenous transgene inserted at the chromosome 5. Although expression of the transgene was not detectable in this strain, mice homozygous for the transgene developed complex aging-like symptoms after weaning, including growth arrest, multiple organ atrophy (gonads, thymus, skin, fat, etc.), vascular calcification, cardiac hypertrophy (Faul et al. 2011), sarcopenia, osteopenia (Kawaguchi et al. 1999), emphysematous lung, hearing disturbance (Kamemori et al. 2002), cognition impairment (Nagai et al. 2003), frailty, and premature death around 2 months of age. Because these phenotypes were observed only in homozygotes for the transgene, we hypothesized that the insertional mutation caused by chromosomal integration of the transgene might have disrupted a putative "aging-suppressor gene," which we named Klotho after a Greek goddess who spins the thread of life.

We found that approximately ten copies of the transgene were integrated in tandem at the 5′-flanking region of an unknown gene at that time, which later turned out to be the *klotho* gene (Kuro-o et al. 1997). As the mutated allele (*kl*) was a severe hypomorphic allele, *kl/kl* mice barely expressed the *klotho* gene. The *klotho* gene encodes a single-pass transmembrane protein and is expressed only in a few cell types in wild-type mice, including distal tubular epithelial cells in the kidney, choroid plexus epithelial cells in the brain, and chief cells in parathyroid glands (Ben-Dov et al. 2007). The fact that *kl/kl* mice exhibited disorders in tissues that did not express the *klotho* gene endogenously raised the possibility that a humoral factor (s) might mediate the function of the *klotho* gene. The extracellular domain of Klotho has weak homology to the family 1 glycosidases. However, it was not clear whether Klotho protein would have glycosidase activity, because the two amino acids critical for the enzymatic activity (Asn and Glu) and thus conserved in all the family 1 glycosidases were replaced with other amino acids (Asp and Ala) (Kuro-o et al. 1997).

14.2 Klotho Protein Function

The clue to identification of the Klotho protein function was the report on mice lacking fibroblast growth factor-23 (FGF23) (Shimada et al. 2004b). FGF23 is a member of the fibroblast growth factor (FGF) family, but unlike the other FGF family members that function as paracrine and autocrine factors, it functions as an endocrine factor (hormone) (Goetz et al. 2007). FGF23 had been known to function as a bone-derived "phosphaturic" hormone that promoted urinary phosphate excretion (Shimada et al. 2004a). In response to phosphate intake, FGF23 is secreted from the bone (osteocytes/osteoblasts) and acts on the kidney to suppress phosphate

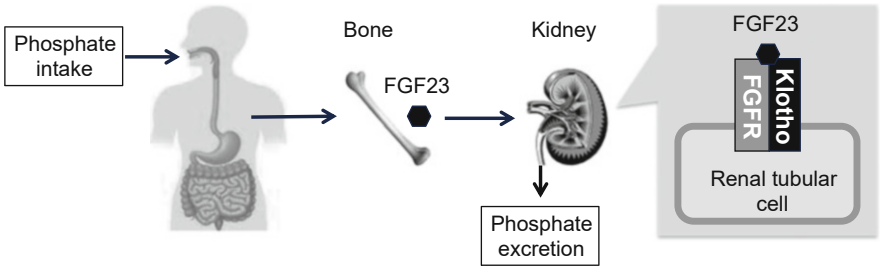

Fig. 14.1 The FGF23-αKlotho endocrine axis. FGF23 functions as a phosphaturic hormone to balance between phosphate intake and phosphate excretion

resorption in proximal tubules and increase phosphate excretion per nephron, thereby maintaining the phosphate balance (Fig. 14.1) (Kuro-o 2019). Considering the phosphaturic activity of FGF23, mice lacking FGF23 were predicted to suffer from impaired excretion of phosphate into urine, resulting in phosphate retention. As expected, mice lacking FGF23 developed hyperphosphatemia and vascular calcification (Shimada et al. 2004b). However, they unexpectedly developed aging-like phenotypes, including growth arrest, multiple organ atrophy (gonads, thymus, skin, fat, etc.), sarcopenia, osteopenia, frailty, and premature death around 2 months of age (Shimada et al. 2004b). These phenotypes were reminiscent of those observed in *kl/kl* mice. In addition, we had known that *kl/kl* mice developed hyperphosphatemia. These observations prompted us to hypothesize that FGF23 and Klotho might function in the same signaling pathway involved in maintenance of phosphate homeostasis.

At that time, FGF23 was supposed to use FGF receptor tyrosine kinases as its cognate receptor. However, the affinity of any FGF receptor (FGFR) isoforms to FGF23 was too low ($K_D > 220$ nM) to bind to FGF23 at its physiological concentration (~1 pM) (Yu et al. 2005). Hence, identity of the physiological FGF23 receptor had been unknown. The answer was that FGF23 required Klotho to bind to FGFRs. We found that Klotho forms constitutive binary complexes with FGFR1c, FGFR3c, and FGFR4. FGF23 binds to the FGFR-Klotho complexes with high affinity and activates the canonical FGF signaling pathway that culminates phosphorylation of FGFR substrate-2 (FRS2α) and its downstream target extracellular signal-regulated kinases (ERK1/2) (Kurosu et al. 2006). This finding was confirmed later in other laboratories (Urakawa et al. 2006). The fact that Klotho functions as the obligate co-receptor for FGF23 explains why *kl/kl* mice and mice lacking FGF23 exhibited identical phenotypes.

14.3 Discovery of the FGF-Klotho Endocrine Axes

After the discovery of the *klotho* gene, search for the GenBank database identified another gene encoding a single-pass transmembrane protein with ~40% amino acid identity with Klotho and was named βKlotho (Ito et al. 2000). Since then, Klotho was renamed as αKlotho to avoid confusion. Like αKlotho, βKlotho forms complexes with FGFR1c and FGFR4, which is expressed predominantly in adipose tissues and in the liver, respectively (Kurosu et al. 2007). Thus, the βKlotho-FGFR1c complex is expressed in adipose tissues, whereas the βKlotho-FGFR4 complex is expressed in the liver.

In the FGF family, FGF19 and FGF21 also function as endocrine factors besides FGF23. FGF19 is secreted from the intestinal epithelium upon feeding, reaches the liver via the portal circulation, and binds to the βKlotho-FGFR4 complex expressed on hepatocytes to induce metabolic responses to feeding, including suppression of bile acid synthesis and promotion of protein and glycogen synthesis (Inagaki et al. 2005; Kir et al. 2011; Potthoff et al. 2011). On the other hand, FGF21 is secreted from hepatocytes upon fasting and binds to the βKlotho-FGFR1c complex expressed on adipocytes to induce metabolic responses to fasting, including lipolysis (Inagaki et al. 2007; Ogawa et al. 2007; Potthoff et al. 2009). These three FGFs (FGF19, FGF21, and FGF23) are collectively designated as endocrine FGFs and different from the other FGF family members in that they function not as growth factors but as hormones regulating multiple metabolic processes and that they require Klotho family proteins to bind to their cognate FGFRs (Table 14.1) (Kuro-o 2019).

In 2018, crystal structure of the FGFR1c-αKlotho-FGF23 ternary complex and the βKlotho-FGF21 complex was solved (Chen et al. 2018; Lee et al. 2018). As given its namesake, αKlotho protein send out a long "thread" termed the receptor binding arm with intrinsically disordered structure. Once it captures FGFR and takes a fixed structure, a groove is created between αKlotho and FGFR into which FGF23 fits (Fig. 14.2) (Kuro-o 2018a).

14.4 Phosphate and CKD

Phosphorus is one of the six elements essential for life (H, C, N, O, S, P), but it has never drawn much attention in the medical field until quite recently. The situation has changed since hyperphosphatemia was identified as a major mortality risk for

Table 14.1 The three FGF-Klotho endocrine axes

Endocrine FGFs	Secreted upon	Secreted from	Target organs	Receptor	
				FGFR	Klotho
FGF19	Feeding	Intestine	Liver	4	β
FGF21	Fasting	Liver	Fat, Brain	1c	β
FGF23	Phosphate intake	Bone	Kidney	1c, 3c, 4	α

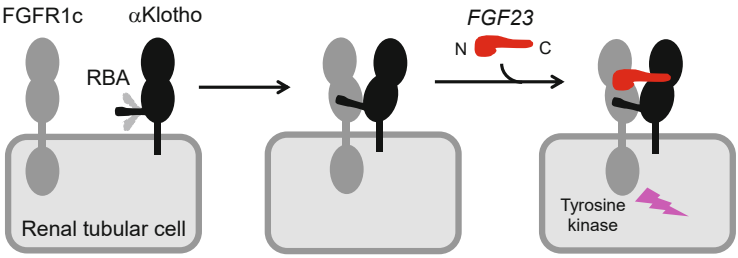

Fig. 14.2 Structure of αKlotho. The N-terminal portion and the C-terminal portion of FGF23 face to FGFR1c and αKlotho, respectively. *RBA* receptor binding arm

Table 14.2 Stages of chronic kidney disease (CKD)

CKD stage	1	2	3	4	5
eGFR (mL/min/1.73 m^2)	≥90	89–60	59–30	29–15	<15

patients with chronic kidney disease (CKD) (Block et al. 1998; Ganesh et al. 2001). CKD is a new disease entity established a few decades ago and defined as any abnormality of the kidney structure and/or function lasting for 3 months or longer (Webster et al. 2017). CKD ensues in patients not only with renal disorders (e.g., chronic glomerulonephritis and polycystic kidney disease) but also with disorders causing renal complications (e.g., diabetes and hypertension). Therefore, CKD is very prevalent in the aging society, affecting more than 10% of the total population (Levey et al. 2005; Hill et al. 2016). Once CKD progresses to renal failure, renal replacement therapy (dialysis or renal transplantation) becomes necessary, which burdens healthcare worldwide. However, many CKD patients die from cardiovascular events before developing renal failure. Regardless of the underlying disorders, CKD progression can be viewed as a process of progressive loss of functional nephrons. CKD is classified from stage 1 (early stage) through stage 5 (end stage) based on the estimated glomerular filtration rate (eGFR) in clinical settings (Table 14.2).

Among several hormones that are increased or decreased during CKD progression, FGF23 is the first to move (Isakova et al. 2011). Serum FGF23 levels start elevating as early as stage 2–3. FGF23 increases phosphate excretion per nephron and compensates for the decrease in the nephron number to maintain the balance between phosphate intake and excretion. Besides functioning as a phosphaturic hormone, FGF23 also functions as a counter-regulatory hormone for active vitamin D (1,25-dihydroxyvitamin D_3). FGF23 lowers serum levels of active vitamin D through downregulating renal expression of 1α-hydroxylase necessary for its synthesis and upregulating renal expression of 24-hydroxylase necessary for its degradation (Shimada et al. 2004a). The decrease in active vitamin D induces secretion of parathyroid hormone (PTH), because a robust negative feedback loop exists between active vitamin D and PTH, leading to secondary hyperparathyroidism. Serum phosphate levels start increasing in stage 4–5, when the residual nephron number

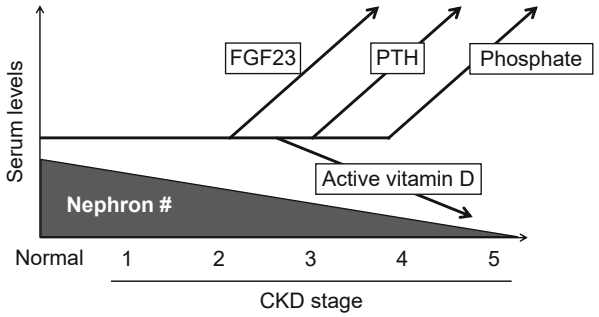

Fig. 14.3 Pathophysiology of CKD-MBD. An increase in FGF23 indicates that the phosphate intake is in excess relative to the residual nephron number

becomes too low to balance phosphate excretion with phosphate intake. In this way, disturbed mineral metabolism characterized by high FGF23, low active vitamin D, high PTH, and high phosphate ensues in this order during the course of CKD progression, which is designated as CKD-MBD (mineral-bone disorder) (Fig. 14.3). Thus, CKD-MBD can be viewed as a result of an effort to maintain phosphate homeostasis against decrease in the nephron number during CKD progression (Kuro-o 2019).

In rodents, it was reported that renal tubular damage and interstitial fibrosis ensued when phosphate load excreted per nephron exceeded ~1.0 μg/day (Haut et al. 1980). Although the mechanism by which increase in phosphate excretion per nephron damages the kidney remains to be determined, it has been postulated that increased phosphate load per nephron should elevate phosphate concentration in the renal tubular fluid to trigger formation of tiny calcium phosphate precipitations, which might induce tubular damage (Lau 1989). Regardless of the mechanism, renal tubular damage should reduce the nephron number and demand further increase in FGF23 to maintain the phosphate balance unless phosphate intake is reduced. Thus, the increase in FGF23 compensates for the decrease in the nephron number and is indispensable for maintaining the phosphate homeostasis. However, it induces renal tubular damage and fibrosis to trigger a deterioration spiral leading to further nephron loss and acceleration of CKD progression (Kuro-o 2019).

In humans, healthy adults on regular diet excrete ~1.0 g of phosphate into urine. The nephron number in humans is approximately one million per kidney on average (Denic et al. 2017). Therefore, phosphate excretion per nephron is estimated as 0.5 μg/day. The nephron number is decreased with age as a part of the aging process. It has been reported that the nephron number of the elderly people in their 60s or 70s is approximately 50% less than that of the young people in their 20s (Denic et al. 2017). Unless phosphate intake is reduced with age, the amount of urinary phosphate excretion does not change. Therefore, phosphate excretion per nephron can reach 1.0 μg/day in the elderly people and may accelerate kidney aging. In humans, age-associated renal pathology characterized by tubular damage, interstitial inflammation and fibrosis, and glomerulosclerosis is universally observed even in "healthy" individuals and recognized as "aging kidney" (O'Sullivan et al. 2017).

Hence, the amount of phosphate taken in from the daily diet potentially contributes to aging kidney in the elderly people.

14.5 Phosphate Accelerates Aging

As described above, *kl/kl* mice exhibit premature aging. The fundamental abnormality in *kl/kl* mice is disturbed phosphate homeostasis (phosphate retention) caused by the inability to induce phosphaturia in response to phosphate intake due to insensitivity to FGF23. Provided that phosphate retention is the cause of premature aging in *kl/kl* mice, restoration of the phosphate balance by restricting dietary phosphate intake should rescue *kl/kl* mice from premature aging. Indeed, most of the aging-like phenotypes were alleviated when *kl/kl* mice were placed on low phosphate diet (Morishita et al. 2001; Stubbs et al. 2007). These observations have led us to the notion that phosphate accelerates aging.

The signs and symptoms of *kl/kl* mice resemble those of patients with renal failure. Like *kl/kl* mice, patients with renal failure suffer from vascular calcification, cardiac hypertrophy, sarcopenia, osteopenia, frailty, and high mortality. They also exhibit hyperphosphatemia, hyper-FGF23-emia, and loss of renal αKlotho expression. Furthermore, dietary phosphate restriction and administration of phosphate binders improve their clinical outcomes. Because of the striking similarity to *kl/kl* mice that have been established as a mouse model of accelerated aging, renal failure patients have been viewed as a clinical model of accelerated aging (Stenvinkel and Larsson 2013). However, a fundamental difference exists between them. The cause of phosphate retention in renal failure patients is abolition of renal function in general. In contrast, the cause of phosphate retention in *kl/kl* mice is inability to excrete phosphate into urine. Their renal function is otherwise normal. In fact, *kl/kl* mice have normal serum creatinine levels and never develop renal failure. The complex signs and symptoms displayed by renal failure patients have been collectively designated as uremia and believed to be caused by accumulation of multiple "uremic toxins" that should have been excreted into urine. However, the fact that renal failure patients and *kl/kl* mice develop very similar pathophysiology suggests that phosphate may be the most important uremic toxin.

14.6 Calciprotein Particles (CPPs)

As the mechanism by which phosphate accelerates aging, we have proposed "the CPP theory of aging" (Kuro-o 2018b, 2019, 2020). CPPs are mineral-protein complex composed of solid-phase calcium phosphate and serum protein fetuin-A and dispersed as colloids in the blood. Because the blood is supersaturated regarding calcium and phosphate ions, even a slight and transient increase in blood phosphate levels can trigger precipitation of amorphous calcium phosphate. However, the

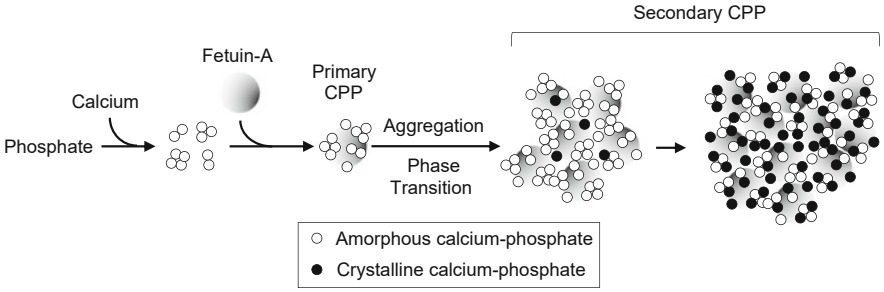

Fig. 14.4 Formation of calciprotein particles (CPPs). Formation of CPPs is a physicochemical process that progresses spontaneously over time

calcium phosphate precipitates never grow and occlude capillaries, because these precipitates are adsorbed by fetuin-A and prevented from growing into large crystals. As a result, a fetuin-A molecule laden with tiny amorphous calcium phosphate precipitates is generated, which is termed primary CPPs. Primary CPPs spontaneously undergo self-aggregation and phase transition of calcium phosphate from the amorphous phase to the crystalline phase to become secondary CPPs (Fig. 14.4) (Kuro-o 2019; Jahnen-Dechent et al. 2020). Primary CPPs function physiologically as a potent inducer of FGF23 expression and secretion (Akiyama et al. 2019), whereas secondary CPPs have the activity that induces cell death in cultured vascular endothelial cells (Di Marco et al. 2008) and renal epithelial cells (Kunishige et al. 2020). CPPs are endocytosed and transported to lysosomes. Accumulation of CPPs in lysosomes increases their luminal pH, which disturbs lysosomal function and autophagic flux, leading to vulnerability to oxidative stress and cell death (Kunishige et al. 2020). CPPs also induce calcification in cultured vascular smooth muscle cells (Reynolds et al. 2004; Ewence et al. 2008; Sage et al. 2011) and innate immune responses in cultured macrophages as if they were a pathogen (Smith et al. 2013). As indicated by a coined word "inflammaging," chronic inflammation is known to accelerate aging (Franceschi et al. 2000). In addition, recent clinical studies demonstrated that serum CPP levels were associated with clinical parameters for vascular calcification and inflammation (coronary artery calcification scores, aortic pulse wave velocity, hs-CRP, etc.) (Hamano et al. 2010; Smith et al. 2012). Considering the pathogenic activity of secondary CPPs, the correlation observed in these clinical studies may not merely correlation but causation. Phosphate, once precipitated with calcium to become CPPs, may behave like a pathogen that induces chronic noninfectious inflammation and cell damages, eventually accelerating aging.

14.7 CPPs and Lipoproteins

In mammals, insoluble materials are adsorbed by specific serum proteins and dispersed in the blood as colloids to be transported between organs. Lipids are adsorbed by apoproteins and dispersed in the blood as lipoproteins to be eventually stored in adipose tissues. However, when mistargeted to arteries, atherosclerosis ensues. Likewise, calcium phosphate are adsorbed by fetuin-A and dispersed in the blood as CPPs to be eventually stored in the bone. However, when mistargeted to arteries, vascular calcification ensues. Thus, the two distinct types of arteriosclerosis, atherosclerosis and vascular calcification, can be sublated as a disorder caused by mistargeting of insoluble materials, lipids, and calcium phosphate, respectively. In addition, ectopic accumulation of lipids in the liver and skeletal muscles induces fatty liver and insulin resistance, leading to metabolic syndrome. Likewise, CPPs in extraosseous tissues and extracellular fluid induce cell damage and chronic inflammation, potentially leading to acceleration of aging (Table 14.3).

14.8 Secreted αKlotho

The extracellular domain of αKlotho is clipped on the plasma membrane by membrane-anchored secretases and released into the extracellular space (Chen et al. 2007; Bloch et al. 2009). The secreted αKlotho exerts multiple functions independently of FGF23 as a humoral factor. It regulates cell surface abundance of several ion channels and transporters. Secreted αKlotho is reported to interact with particular sugar chains of transient receptor potential cation channel subfamily V member 5 and 6 (TRPV5, TRPV6) (Chang et al. 2005; Cha et al. 2008; Alexander et al. 2009) and renal outer medullary potassium channel (ROMK1) (Cha et al. 2009) to prevent them from being internalized, thereby increasing cellular calcium import and potassium export, respectively. It still remains controversial whether secreted αKlotho functions as a lectin (sugar-binding protein) that binds to any specific sugars or an enzyme that hydrolyzes any specific glycosidic bonds in glycans of these glycoproteins. On the other hand, secreted αKlotho reduces the cell surface abundance of TRPC6 through blocking phosphoinositide-3-kinase-dependent exocytosis of TRPC6 (Xie et al. 2012). Sodium-dependent phosphate co-transporters (Npt2a) are downregulated by secreted αKlotho through promoting their endocytosis and

Table 14.3 Disorders caused by mistargeting of colloids containing insoluble materials

Insoluble materials	Lipid	Calcium phosphate
Protein	Apoprotein	Fetuin-A
Colloids	Lipoprotein	CPP
Storage	Fat	Bone
Disorders	Atherosclerosis	Vascular calcification
	Metabolic syndrome	Aging

degradation (Hu et al. 2010). In addition, secreted αKlotho inhibits activity of several growth factors, including insulin-like growth factor-1 (IGF1) (Kurosu et al. 2005), Wnt (Liu et al. 2007; Zhou et al. 2013), and transforming growth factor-β1 (TGFβ1) (Doi et al. 2006), through distinct mechanisms. Secreted αKlotho inhibits the intracellular IGF1 signaling pathway at the level of receptor tyrosine phosphorylation (Kurosu et al. 2005). The activity of secreted αKlotho that inhibits Wnt signaling depends on its ability to directly bind to Wnt (Liu et al. 2007). Secreted Klotho also binds to the type-II TGFβ receptor (TGFβR2) and inhibits TGFβ1 binding to TGFβR2 (Doi et al. 2006). Of note, transgenic mice that overexpress αKlotho were reported to live longer than wild-type mice (Kurosu et al. 2005). These transgenic mice had higher serum levels of secreted αKlotho than wild-type mice. The ability of secreted αKlotho to inhibit IGF1 signaling might contribute to the extended life span, because adequate suppression of the somatotroph endocrine axis (the growth hormone-IGF1 axis) has been shown to extend life span in various experimental animals (Kenyon 2001).

14.9 FGF21-βKlotho Endocrine System

FGF21 was originally identified as a hormone secreted from hepatocytes upon fasting and bound to the FGFR1c-βKlotho complex expressed on adipose tissues to induce metabolic responses to fasting (Kharitonenkov et al. 2005; Inagaki et al. 2007; Ogawa et al. 2007). Shortly thereafter, it was reported that FGF21 also had the ability to attenuate responsiveness of hepatocytes to growth hormone (GH) (Inagaki et al. 2008). Thus FGF21 can induce not only metabolic responses resembling those induced by calorie restriction (CR) but also resistance to GH. CR and GH resistance are known to suppress aging and extend life span in various experimental animals (Kenyon 2001; Guarente 2013). In fact, transgenic mice that overexpress FGF21 live much longer than wild-type mice (Zhang et al. 2012). Thus, FGF21 can be regarded as an "antiaging hormone." However, overexpression of FGF21 was associated with various adverse effects, including disturbed circadian rhythm and overactivation of the sympathetic nervous system and the hypothalamus-pituitary-adrenal axis (Bookout et al. 2013). Recent studies have demonstrated that these adverse effects were dependent on FGF21 acting in the central nervous system.

FGF21 can cross the blood-brain barrier and act on neurons in the suprachiasmatic nucleus (SCN) where βKlotho is expressed (Bookout et al. 2013). As SCN is the center of circadian rhythm, overexpression of FGF21 in mice disturbed circadian behavior with reduced activity in the dark phase and promoted torpor, which is a short-term hibernation-like state with low body temperature to avoid energy expenditure (Inagaki et al. 2007). In addition, activation of SCN by FGF21 stimulated production of corticotropin-releasing hormone (CRH) in the hypothalamus. CRH stimulates the hypothalamus-pituitary-adrenal axis to elevate circulating corticotropin levels and activates the sympathetic nerve system. Thus,

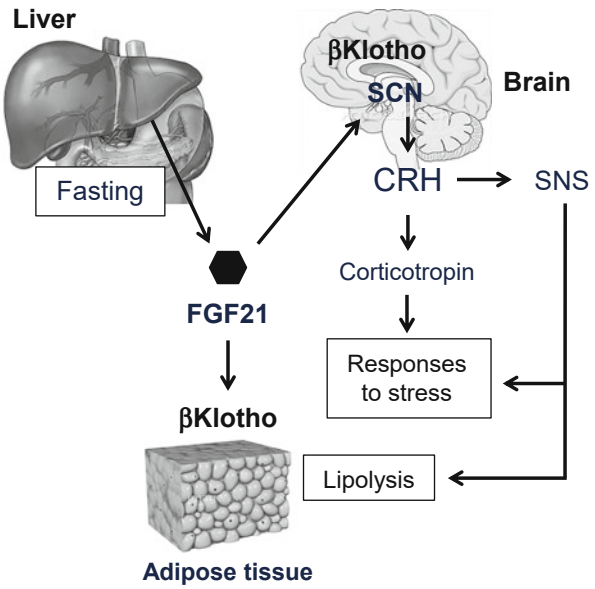

Fig. 14.5 The FGF21-β Klotho endocrine axis. FGF21 activates lipolysis not only directly through acting on adipose tissues but also indirectly through activating the sympathetic nerve system (SNS)

FGF21 can be regarded as a stress-responsive hormone (Fig. 14.5) (Bookout et al. 2013).

14.10 FGF21 and CKD

Besides FGF23, serum FGF21 levels start increasing since early stages in CKD patients (Lin et al. 2011). In a mouse model of CKD (uninephrectomy followed by high phosphate diet feeding), FGF21 is increased significantly as well (Nakano et al. 2019). We hypothesized that the increase in FGF21 might be a response to stress caused by CKD and thus required to survive CKD. To test this hypothesis, we introduced CKD to mice lacking FGF21 (*Fgf21*$^{-/-}$ mice) and wild-type mice and compared their survival curves. As expected, *Fgf21*$^{-/-}$ mice showed poorer prognosis than wild-type mice (Fig. 14.6), indicating that FGF21 is necessary to survive CKD (Nakano et al. 2019). We next asked if the increased FGF21 in CKD might induce adverse effects similar to those observed in FGF21 overexpressing transgenic mice. To determine the circadian rhythm of blood pressure in mice, we placed a catheter in the internal carotid artery and monitored their arterial blood pressure continuously over 2 days under the conscious and unrestricted condition using a telemetry system. Although CKD mice showed a normal circadian rhythm in blood pressure (high in nighttime and low in daytime), they showed significantly enhanced blood pressure fluctuation when compared with non-CKD mice (Fig. 14.7a, b) (Nakano et al. 2019). The enhanced blood pressure fluctuation was attributed to augmentation of the blood pressure elevating response during physical activity,

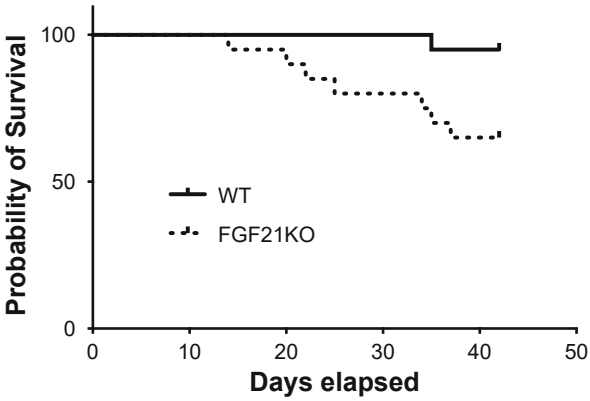

Fig. 14.6 Survival curves of CKD mice. FGF21 knockout mice (FGF21 KO, $N = 20$) and wild-type mice (WT, $N = 20$) were uninephrectomized at 8 weeks of age, placed on high phosphate diet containing 2.0% inorganic phosphate at 12 weeks of age, and then censored at 18 weeks of age. $p = 0.017$ by log-rank test. (Modified from reference Nakano et al. 2019)

which was associated with increase in sympathetic nerve activity and reciprocal decrease in parasympathetic nerve activity. Importantly, the CKD-induced blood pressure dysregulation was not observed in *Fgf21*$^{-/-}$ mice (Fig. 14.7c, d). In addition, administration of FGF21 alone induced the similar blood pressure dysregulation in non-CKD mice (Fig. 14.7e, f). These findings indicated that FGF21 was necessary and sufficient to induce the blood pressure dysregulation observed in CKD mice (Nakano et al. 2019). This may be also applicable to humans, because CKD patients universally exhibit activation of sympathetic nerve system and augmentation of the blood pressure elevating response during physical activity (Downey et al. 2017).

14.11 Concluding Remarks

There have been two mainstreams of aging research. One is to investigate the mechanism of longevity evolutionarily conserved among yeasts, nematodes, flies, mice, and primates, leading to the notion that adequate calorie restriction extends life span in various experimental animals (Guarente 2013). The other mainstream is to investigate the mechanism of cell senescence, which revealed the fact that accumulation of senescent cells accelerates aging at the organismal level (Baker et al. 2011). Based on these findings, calorie restriction mimetics (Ingram et al. 2006) and senolytic drugs (drugs that kill senescent cells selectively) (Kirkland et al. 2017) are expected to extend life span in humans. However, even if clinical trials are successful and these medicines indeed extend the average life span, the healthcare system in the aging society can get worse unless the health span extension predominates over the life span extension. By freeing ourselves from these two mainstreams and pursuing the mechanism of aging specific to higher organisms, we may be able to develop a new antiaging medicine. The FGF-Klotho endocrine system is unique to vertebrates and may become a novel target of aging research aiming at extension of health span.

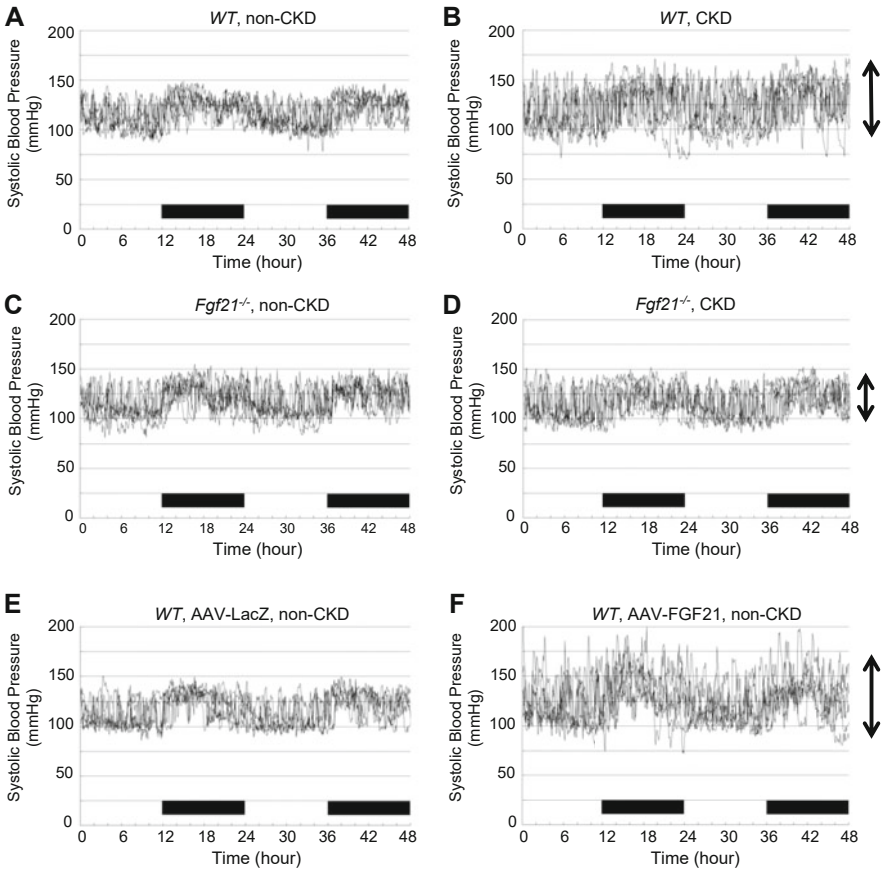

Fig. 14.7 Effects of FGF21 on blood pressure in mice. Blood pressure was measured continuously for 2 days using a telemetry system. Each panel represents overlapped line charts of systolic blood pressure from six mice. The black bars indicate the nighttime. The double arrows indicate the average amplitude of fluctuation of systolic blood pressure. CKD mice were prepared as described in Fig. 14.6. Non-CKD mice were prepared by sham-operation at 8 weeks of age and placed on regular diet containing 0.35% inorganic phosphate throughout the experimental period. (**a**) Wild-type non-CKD mice. (**b**) Wild-type CKD mice. (**c**) *Fgf21*$^{-/-}$ non-CKD mice. (**d**) *Fgf21*$^{-/-}$ CKD mice. (**e**) Wild-type non-CKD mice injected with an adeno-associated virus vector (AAV) expressing LacZ to serve as a control for (**f**). (**f**) wild-type non-CKD mice injected with AAV expressing FGF21. (Modified from reference Nakano et al. 2019)

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Chapter 15
Aging Biomarker SMP30 into a New Phase of Vitamin C and Aging Research

Akihito Ishigami

Abstract Proteomics has provided comprehensive information in terms of aging in general and, in particular, regarding age-associated molecules, including senescence marker protein-30 (SMP30). This factor has been deemed one of the best candidates for elucidating the mechanism underlying senescence, as assessed by our group on performing functional analyses of multiple organs. We propose that the SMP30-knockout murine strain, in which SMP30 is completely absent, is the most useful model for understanding human aging. Deleting SMP30 results in the absence of an enzyme responsible for synthesizing vitamin C, a well-known antioxidant. Further research on the biological functions of SMP30 will provide valuable tools for treating or offsetting the deleterious effects of aging in humans.

Keywords Aging · Apoptosis · Ascorbic acid · Calcium homeostasis · Gluconolactonase · Organophosphatase · Proteomic analysis · SMP30 · Vitamin C

15.1 Introduction

Notably, the degree of aging varies among individuals, even when presenting identical chronological age. Additionally, the degree of aging tends to gradually amplify with increasing age. Understanding our aging degree could help control the subsequent aging progress maximally and achieve healthy aging. However, to date, no reliable aging index that can precisely evaluate the degree of aging has been reported. Therefore, it is necessary to continue exploring aging biomarkers that can objectively and accurately evaluate the degree of aging. This review article introduces the aging biomarker, senescence marker protein-30 (SMP30), first discovered by our research group. Moreover, we describe our investigations and reveal that the long-term depletion of vitamin C (L-ascorbic acid) accelerates aging in SMP30-knockout mice.

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_15

15.2 Discovery of Age-Associated Protein SMP30

In 1991, to determine molecular abnormalities during the aging process, we surveyed age-associated changes in soluble proteins identified in rat livers using proteomic analysis. Accordingly, we detected and isolated a novel rat liver protein, which, on initial calculation, presented a molecular weight of 30 kDa according to commercially available molecular weight markers. It was observed that the expression level of this protein decreased with aging independent of androgen levels; we named this protein SMP30 (Fig. 15.1) (Fujita et al. 1992b). This designation of SMP30 was accurate until, at more sensitive resolutions, the mass of the SMP30 molecule was found to be 34 kDa. However, the assigned name was maintained.

The next step was to prepare an antiserum against SMP30, employed to localize SMP30. Accordingly, this protein was most prominently identified in the liver and kidneys among the various organs assessed (Fig. 15.2) (Ishigami et al. 2003). Subsequently, we isolated and characterized two cDNA clones encoding rat SMP30 (Fujita et al. 1992a). The open reading frame, consisting of 897 bp, encoded 299 amino acids. The estimated molecular weight and pI of the deduced polypeptides were 33,387 and 5.1, respectively. Genomic Southern hybridization analysis showed that SMP30 is widely conserved among higher animals. Meanwhile, a computer-assisted homology analysis of nucleic acid and protein databases revealed no marked homology with other known proteins. Therefore, SMP30 appears to be a novel protein. Additionally, we cloned human SMP30 and documented an 88.6% homology with rat SMP30 (Fujita et al. 1995). The results of regional mapping using a panel of 11 rodent-human somatic hybrids indicated that the gene is located in the p11.3-q11.2 segment of the X chromosome (Fujita et al. 1995). Analysis of the murine genomic clone revealed that the SMP30 gene was organized into seven exons and six introns, spanning approximately 17.5 kb (Fujita et al. 1996).

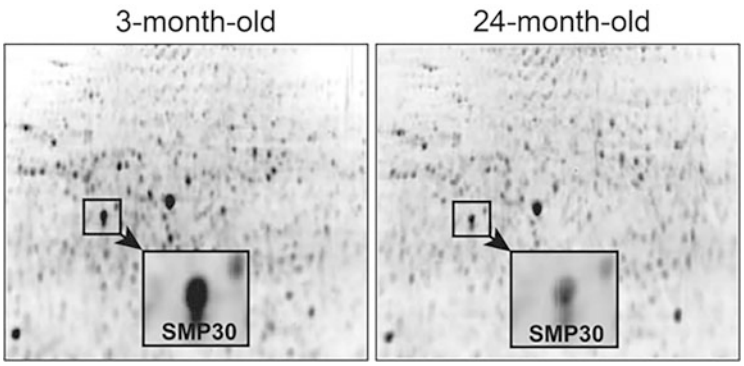

Fig. 15.1 Proteomic profile of SMP30 in soluble liver proteins from 3-month- and 24-month-old rats. SMP30 was first calculated to possess a molecular mass of 30 kDa. However, it was later found to present a molecular mass of 34 kDa. Amounts of this protein decrease with aging in an androgen-independent manner. *SMP30* senescence marker protein-30

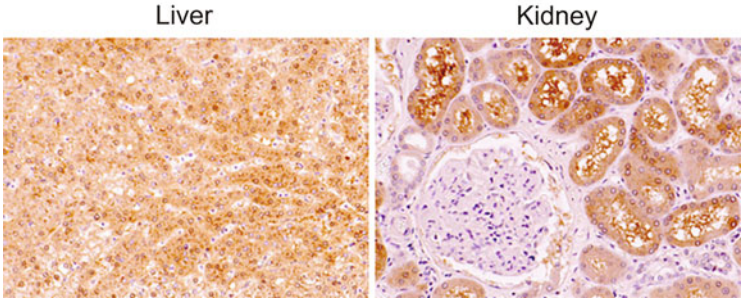

Fig. 15.2 SMP30 expression in the human liver and kidney. Liver, SMP30 is expressed in parenchymal cells. Kidney, SMP30 is abundant in proximal tubular cells. Localization of SMP30 at the brush border is prominent. *SMP30* senescence marker protein-30

Accumulated genomic information revealed that the SMP30 gene is highly conserved among numerous animal species, with this finding expanded to include nonvertebrates (Goto 2000; Nakajima and Natori 2000). These results indicated the critical biological functions of SMP30.

15.3 Functional Analysis of SMP30

At the point of SMP30 discovery, no functional domain was recognized in the entire amino acid sequence. Subsequently, another research group reported a calcium-binding protein identified with SMP30. However, our purified rat SMP30 failed to demonstrate calcium-binding activity (Kondo et al. 2004).

We further examined the potential function of SMP30 in calcium homeostasis (Fujita et al. 1998; Inoue et al. 1999). Our results established that HepG2 (HepG2/SMP30), a human hepatoma cell line, expressed large amounts of SMP30 after transfection with human cDNA. We then investigated the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$) and the Na^{+}-independent Ca^{2+} efflux from these cells following extracellular ATP stimulation. Although stimulation with ATP induced a transient increase in $[Ca^{2+}]i$ in both HepG2/SMP30 and mock-transfected HepG2 cells, the rate of $[Ca^{2+}]i$ decreased after that peak was enhanced twofold following transfection with human SMP30 cDNA. Correspondingly, Ca^{2+} efflux significantly increased in transfected HepG2/SMP30 cells when compared with mock transfectants. On inducing cell death by Ca^{2+} ionophore treatment, SMP30 transfectants showed greater survival than mock transfectants. These results suggest that SMP30 regulates $[Ca^{2+}]i$ by modulating Ca^{2+}-pump activity in plasma membranes. Therefore, downregulation of SMP30 during aging may contribute to the deterioration of cellular functions.

Ultrastructural studies by scanning electron microscopy revealed numerous microvilli covering the surfaces of HepG2/SMP30 cells, whereas few microvilli were observed in control HepG2 cells (Ishigami et al. 2005). Transmission electron

microscopy revealed groups of HepG2/SMP30 cells with bile canaliculi and specialized adhesion contacts, including tight junctions and desmosomes, at intracytoplasmic membranes. However, in controls, units of only two cells were observed, lacking any specialized adhesion junctions. Moesin (Takeuchi et al. 1994), which plays a crucial role in the formation of microvilli structures, and ZO-1 (Ando-Akatsuka et al. 1999), concentrated in tight junctions and adherence junctions located at the apical end of epithelial cells, are known to be accumulated in microvilli and tight junctions, respectively. The intensity of moesin and ZO-1 staining in the contact regions of each cell was markedly higher in HepG2/SMP30 cells than in control cells. Moreover, moesin was stained in more interior areas, which corresponded with the microvilli of bile canaliculi. Bile canaliculi with microvilli were formed at the apical ends of HepG2/SMP30 cells. These results indicate that SMP30 has an important physiological function as a participant in cell-to-cell interactions, implying that SMP30 downregulation during the aging process contributes to the deterioration of cellular interactivity.

15.4 SMP30 as an Organophosphatase

In 1999, a report on the function of SMP30 was presented by Billecke and colleagues (Billecke et al. 1999), which characterized a novel soluble protein from the mouse liver as possessing enzymatic activity, i.e., hydrolysis of diisopropyl phosphorofluoridate (DFP). This molecule was found to hydrolyze sarin, soman, and tabun, compounds that have been commonly employed in terror attacks in Japan. However, it lacked paraoxonase (PON) and arylesterase activity toward paraoxon and phenyl acetate, respectively. Subsequent amino acid sequencing of the purified DFPase revealed that it was identical to SMP30 (Little et al. 1989). Thus, SMP30 was classified as an enzyme. However, the substrates employed were artificial chemicals developed after World War I.

We further characterized the organophosphatase nature of SMP30 (Kondo et al. 2004). Despite the sequence similarity between SMP30 and serum PON, the inability of SMP30 to hydrolyze PON-specific substrates such as paraoxon, dihydrocoumarin, γ-nonalactone, and δ-dodecanolactone indicate that SMP30 is distinct from the PON family. The livers from normal mice demonstrated readily detectable DFPase activity, whereas no such enzyme activity was observed in the livers of mice lacking SMP30. Moreover, mice hepatocytes lacking SMP30 were far more susceptible to DFP-induced cytotoxicity than those from normal mice. This phenomenon accounts for the functional decrease in detoxification observed in elderly individuals.

15.5 SMP30 Homolog in Fireflies

The first report of a natural substrate for SMP30 was presented by Gomi et al. (Gomi et al. 2002; Gomi and Kajiyama 2001), identifying an SMP30 homolog designated as the luciferin-regeneration enzyme (LRE) in fireflies (*Photinus pyralis*). LRE converts oxyluciferin to luciferin via an intermediate substance. The deduced amino acid sequence based on cDNA analysis showed a maximum of 39% identity with the insect anterior fat protein (AFP) and mammalian SMP30. However, only 1% LRE is expressed in the lanterns of fireflies. Despite this possible link to LRE, the precise function of SMP30 in the human body remains elusive.

15.6 SMP30 Deficiency

Originally, SMP30 was discovered owing to its decreased expression with progressive aging. If this decrease is long-lasting, SMP30 deficiency in animal models can be regarded as an ultimate decrease approaching zero. To elucidate the effect of this SMP30 decrease with aging, we introduced a null mutation of the SMP30 gene into the germline of mice (Ishigami et al. 2002).

Although these mutant (SMP30-knockout) mice were viable and fertile, SMP30-knockout mice presented a lower body weight and shorter life span than the wild-type controls (Fig. 15.3) (Ishigami et al. 2002, 2004). The mean survival time of these knockout mice was approximately 6 months. In contrast, wild-type mice survived for approximately 24 months. Although phenotypical analysis during the life span of SMP30-knockout mice revealed no apparent abnormalities, immediate postmortem examination revealed atrophy in almost all abdominal organs. Thus, aging progressed at approximately four times the speed in SMP30-knockout mice when compared with the controls.

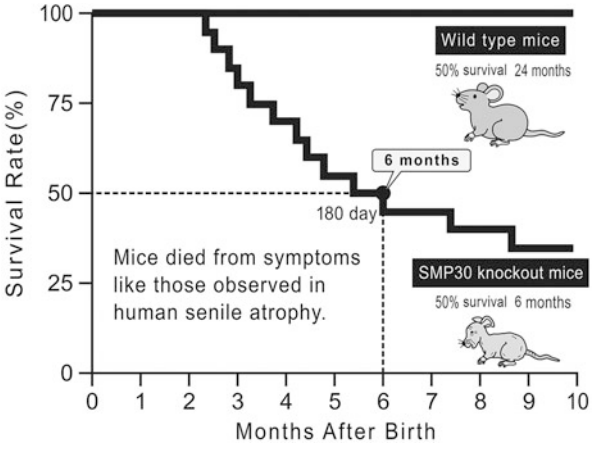

Fig. 15.3 The life span of SMP30-knockout mice is shortened, demonstrating a survival time of 50%, i.e., 180 days (6 months), in 20 animals compared with wild-type mice. *SMP30* senescence marker protein-30

Under an electron microscope, hepatocytes from SMP30-knockout, but not wild-type mice, displayed numerous lipid droplets at 12 months of age, with abnormally enlarged mitochondria with indistinct cristae and enlarged lysosomes filled with electron-dense bodies (Ishigami et al. 2004). In liver specimens from SMP30-knockout mice, the number of lipid droplets visible around the central vein markedly increased in size and amount with aging. In SMP30-knockout mice, biochemical analysis of neutral lipids, i.e., total hepatic triglyceride and cholesterol, showed approximately 3.6- and 3.3-fold higher levels, respectively, than those from age-matched wild-type mice. Moreover, levels of total hepatic phospholipids from SMP30-knockout mice were approximately 3.7-fold higher than those of their wild-type counterparts.

Following thin-layer chromatography analysis, phosphatidylethanolamine, cardiolipin, phosphatidylcholine, phosphatidylserine, and sphingomyelin were found to be accumulated in lipid extracts from SMP30-knockout mice livers. Indeed, this abnormal lipid metabolism could underlie the shortened life span of SMP30-knockout mice.

We then investigated the tissue susceptibility to cytokine-induced apoptosis using primary cultured hepatocytes, as SMP30 could rescue cells from cell death induced by a calcium influx, using a calcium ionophore as previously described (Fujita et al. 1998; Inoue et al. 1999). In SMP30-knockout mice, hepatocytes were more susceptible to apoptosis induced by tumor necrosis factor-α (TNF-α) and actinomycin D (ActD) than hepatocytes from wild-type mice. Furthermore, the TNF-α/ActD-induced caspase-8 activity in hepatocytes derived from SMP30-knockout mice was twofold greater than that in matched cells from wild-type mice. In contrast, no significant difference was observed in TNF-α/ActD-induced NF-κB activation in hepatocytes derived from wild-type versus SMP30-knockout mice; this indicates that SMP30 is not associated with TNF-α/ActD-induced NF-κB activation.

Moreover, deletion of the SMP30 gene enhanced susceptibility to another apoptosis inducer. After treating SMP30-knockout mice with sublethal doses of anti-Fas antibodies, marked liver injury was observed in SMP30-knockout mice but not in wild-type mice (Ishigami et al. 2002). Collectively, these results demonstrate that SMP30 protects cells from apoptosis and other cell injuries.

15.7 SMP30 Is a Gluconolactonase (GNL)

In 2004, we noted an amino acid sequence homology between rat SMP30 and two bacterial gluconolactonases (GNL: EC 3.1.1.17) derived from *Nostoc punctiforme* and *Zymomonas mobilis* (Kanagasundaram and Scopes 1992), respectively, based on data retrieved from the public genome database of the National Center for Biotechnology Information (NCBI). In subsequent biochemical studies, we identified SMP30 as the lactone-hydrolyzing enzyme GNL in animal species (Kondo et al. 2006). SMP30 purified from rat liver showed lactonase activity toward the aldonolactones, D- and L-glucono-γ-lactone, D- and L-gulono-γ-lactone, and D- and

L-galactono-γ-lactone, necessitating for Zn^{2+} or Mn^{2+} as a cofactor. Furthermore, in SMP30-knockout mice, no GNL activity was detected in the liver. Accordingly, we concluded that SMP30 is a unique GNL in the liver. However, the first report regarding the discovery of GNL in higher animal species did not provide an in-depth description of its substrate molecules (Brodie and Lipmann 1955).

15.8 SMP30-Knockout Mice Are Unable to Synthesize Vitamin C

Humans, monkeys, and guinea pigs cannot systemically synthesize vitamin C owing to several genetic mutations in the enzyme L-gulono-γ-lactone oxidase (GLO), located toward the end of the vitamin C synthetic pathway (Fig. 15.4). However, mice possess no mutation in GLO and can, therefore, synthesize vitamin C. SMP30/GNL is an enzyme with one GLO located forward from the terminus. Therefore, we assumed that SMP30-knockout mice were unable to synthesize vitamin C in vivo. Accordingly, to confirm our hypothesis, we performed a nutritional study using a vitamin C-deficient diet. SMP30-knockout mice fed a vitamin C-deficient diet failed to thrive, with reduced body weight, as well as symptoms typical of scurvy such as bone fractures and a decrease in bone density attributed to imperfect construction of

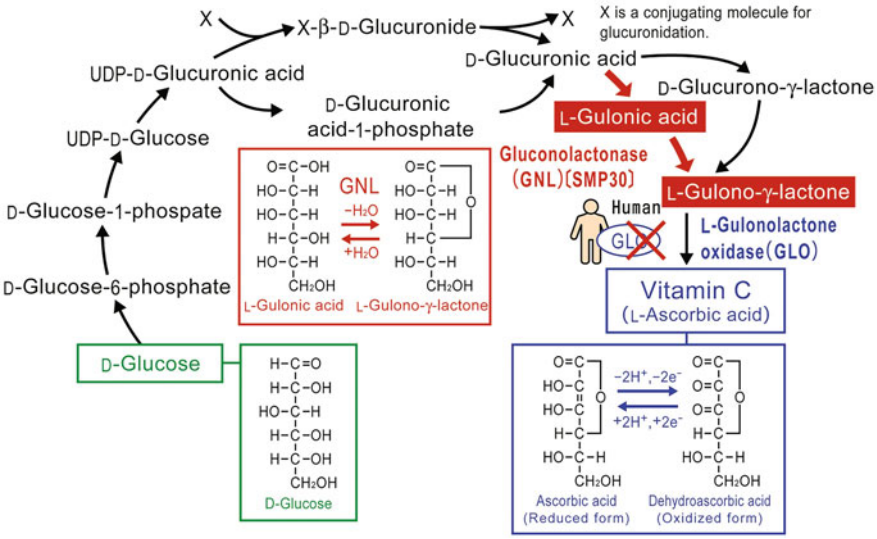

Fig. 15.4 Vitamin C biosynthetic pathway. The pathway from D-glucose to L-gulonic acid is shared with that of early steps in the uronic acid cycle. X is a conjugating molecule for glucuronidation. SMP30 is a gluconolactonase (GNL), which catalyzes L-gulonic acid into L-gulono-γ-lactone. In humans, L-gulonolactone oxidase (GLO) is absent owing to mutations. *SMP30* senescence marker protein-30

the collagen fiber and rachitic rosary (an abnormality in rib cartilage formation) (Kondo et al. 2006). Moreover, SMP30-knockout mice died 135 days after initiating the vitamin C-deficient diet. At the time of death, vitamin C levels in the livers and kidneys were <1.6% of those detected in wild-type control mice. This finding that SMP30-knockout mice developed scurvy-like symptoms when fed a vitamin C-deficient diet verified the pivotal role of SMP30 in vitamin C biosynthesis. Moreover, by employing SMP30-knockout mice, we demonstrated an alternative in vivo pathway for vitamin C synthesis involving D-glucurono-γ-lactone (Fig. 15.4), although the flux is relatively marginal (Kondo et al. 2006).

15.9 Vitamin C Deficiency Accelerates Aging

As SMP30-knockout mice died of scurvy when fed a vitamin C-deficient diet, it would be incorrect to state that vitamin C deficiency promotes aging. It should be noted that while scurvy is a disease, aging is not. Aging refers to a gradual decrease in physiological functions over time. However, our SMP30-knockout mice died early (as measured by survival times), approximately four times faster than their wild-type counterparts (Fig. 15.3), and this observation was documented before SMP30 was identified as a GNL. During initial investigations, no scurvy-like symptoms were observed in the SMP30-knockout mice, presumably because the autoclaved mouse chow contained ~55 mg/kg of vitamin C. We now know that this amount of vitamin C is extremely limited to maintain normal tissue levels; in fact, each mouse consumed approximately 2.5% of vitamin C per day of dietary needs. However, we can state that aging progressed approximately four times faster than normal in these knockout mice, as they received restricted vitamin C over a prolonged period. Thus, these results indicate that vitamin C restriction accelerates aging. To date, there has been no scientific evidence for this conclusion despite the conventional wisdom that vitamin C possesses an antiaging effect. To our knowledge, this is the first report systematically demonstrating that vitamin C deficiency can decrease life span by employing SMP30-knockout mice.

15.10 Rough Estimation of Vitamin C Level That Accelerates Human Aging

In humans, the recommended daily vitamin C intake is approximately 100 mg (~5 or 6 strawberries) according to the "Japanese dietary reference intake of vitamin C" published by the Japan Ministry of Health, Labor and Welfare. Based on our experimental results using SMP30-knockout mice, long-term consumption of 2.5 mg (2.5% of 100 mg) of vitamin C accelerates aging. However, this calculation does not apply when vitamin C intake stops briefly, that is, for several days, as the

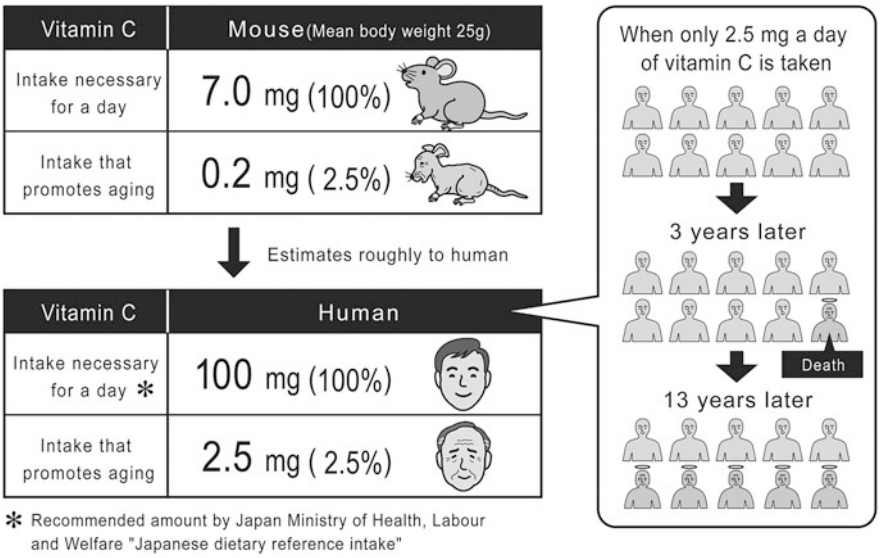

Fig. 15.5 Rough estimate of vitamin C intake that accelerates aging in humans

healthy body is known to accumulate a requisite supply of vitamin C. Aging accelerates only in the long term when as little as 2.5 mg of vitamin C is consumed per day over a period of approximately 3 years, after which a vitamin C deficiency can kill one in ten individuals according to our rough estimate (Fig. 15.5). Moreover, half of these vitamin C-deprived individuals can be estimated to die in approximately 13 years. However, this theoretical value applies only to experimental results in the SMP30-knockout mice and cannot be directly extrapolated to humans.

15.11 Currently Available Findings Using SMP30-Knockout Mice

To date, we have reported that SMP30-knockout mice that experienced a prolonged vitamin C deficiency demonstrated an increased rate of pulmonary emphysema (Koike et al. 2010, 2014; Sato et al. 2006), susceptibility to ultraviolet radiation-induced cataracts (Ishikawa et al. 2012), epidermal atrophy, and excessive ultraviolet B-induced skin pigmentation (Sato et al. 2012), as well as a decrease in skin collagen content and hair growth (Arai et al. 2009), with reduced levels of noradrenaline and adrenaline in adrenal glands (Amano et al. 2014). Moreover, vitamin C deficiency reportedly increases superoxide formation in the brain (Kondo et al. 2008, 2014; Sato et al. 2008) and protein oxidation levels in the liver, with estimated protein carbonyls formed by the oxidation of arginine, lysine, threonine, and proline (Amano et al. 2014; Sato et al. 2014). Enhanced expression of sodium-dependent

vitamin C transporter (SVCT) 1 and SVCT2, as well as increased vitamin C uptake in the liver (Amano et al. 2010), can be observed. We also reported that insufficient vitamin C intake during gestation induces abnormal cardiac dilation in fetal and neonatal SMP30-knockout mice, indicating that a diet supplying an adequate amount of vitamin C is essential to provide optimal conditions for fetal and neonatal health (Kawahori et al. 2020; Kishimoto et al. 2013).

15.12 Perspectives of Vitamin C and Aging Research Using SMP30-Knockout Mice

It is well-established that vitamin C cannot be synthesized within the human body. Therefore, SMP30-knockout mice can be considered a closely related model animal to humans. In both species, the potent antioxidant effect of vitamin C efficiently eliminates reactive oxygen species. As the correlation between aging and the levels of reactive oxygen species is well-established, our experimental results from SMP30-knockout mice are an extremely reliable predictor of related conditions in humans. Our results indicate the necessity of adequate vitamin C consumption from fresh fruits and vegetables to avoid a life-shortening deficiency.

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Part V
Aging Brain: Cognitive Decline, Synaptic Plasticity

Chapter 16
Age-Related Memory Impairments Are Caused by Alterations in Glial Activity at Old Ages

Motomi Matsuno and Minoru Saitoe

Abstract As people grow older, their memory decreases, a phenomenon known as age-related memory impairment (AMI). AMI is increasing as a social problem, and it severely reduces the quality of life of elderly people. AMI has been studied in various animals other than humans, including mice, rats, and *Drosophila*. Here we review studies of AMI in *Drosophila*. In particular, AMI in *Drosophila* consists of an age-dependent alteration in neuron-glial signaling. This prevents glia from providing the necessary support for neuronal signaling at old ages. Alterations in neuron-glial signaling are also associated with AMI in mammals, suggesting possible methods of improving memory in the elderly.

Keywords Drosophila · Age-related memory impairment · Neuron-glia interaction · Glutamate signaling · PKA · Pyruvate carboxylase

16.1 Associative Memory in *Drosophila* and the Effects of Aging on Memory

The fruitfly, *Drosophila melanogaster*, can learn and form memories (Tully and Quinn 1985). For example, flies can form Pavlovian olfactory associations where they learn to associate specific odors with either pain or rewards. For aversive conditioning, flies are exposed to two odors, known as conditioned stimuli (CS). One odor is paired with painful electrical shocks and is called the CS+. The second odor is not paired with shocks and is called the CS−. Flies are first exposed simultaneously to the CS+ and electrical shocks for 1 min. They are then exposed to the CS− for 1 min without electrical shocks. During conditioning, flies learn to associate the CS+, but not the CS−, with pain. Flies can be tested to determine whether they learned this association by placing them in a T-maze where they are allowed to choose between the CS+ and CS− odors. Immediately after training,

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_16

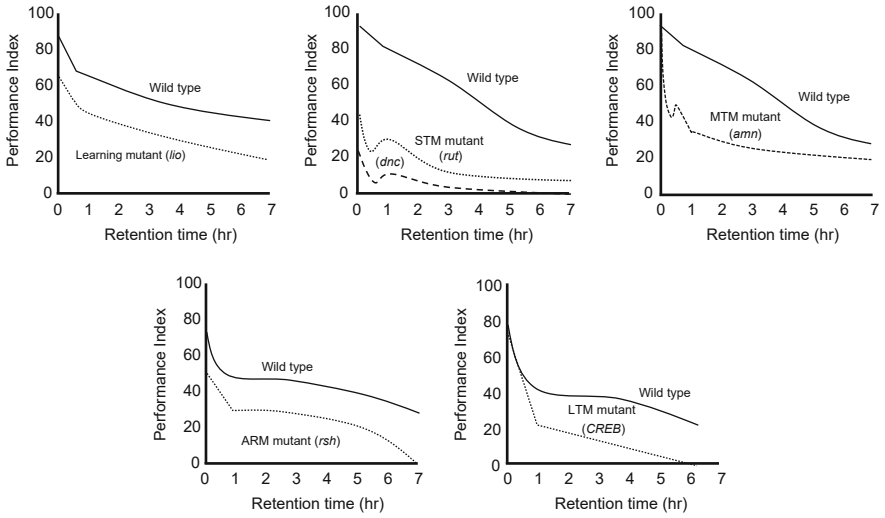

Fig. 16.1 Memory retention curves of learning and memory mutants

most flies overwhelmingly choose the CS− odor. Memory can be measured at different time points by testing the flies at different times after training.

Memory formed after a single cycle of conditioning decays within approximately 24 h. However, similar to humans and other animals, flies can form longer memories if they are trained multiple times (Tully et al. 1994). If flies are conditioned multiple times with appropriate rest intervals between each conditioning (spaced training), they form long-term memory (LTM) which lasts for over 7 days. If flies are conditioned multiple times without rest intervals between trainings (massed training), they form increased amounts of a memory known as anesthesia-resistant memory (ARM), which lasts up to 3 or 4 days after training. Thus, similar to memory in other animals, memory in flies consists of various different types. LTM is distinct from shorter forms of memory because it requires de novo gene expression and protein synthesis. This characteristic is likely what allows LTM to be sustained for so long. While ARM is also a longer-lasting memory form, it does not require de novo gene expressions and protein synthesis and does not last as long as LTM. However, ARM is a robust memory form that is resistant to cold-shock anesthesia. Memory produced immediately after training is fragile and can be easily forgotten if flies are subsequently exposed to a stress such as cold shock anesthesia. However, if trained appropriately, over time memory is consolidated into more robust forms including ARM and LTM (Tully et al. 1994).

Flies can be tested for memory at various time points after conditioning. At early time points, memory is high (flies strongly prefer the CS− over the CS+), but this memory decreases over time. By plotting memory scores as a function of time after training, we can generate a memory retention curve (Figs. 16.1 and 16.3a). Young wild-type flies have a characteristic retention curve (Fig. 16.1, wild-type, Fig. 16.3a, 1-day-old), while various learning and memory mutants as well as old flies have

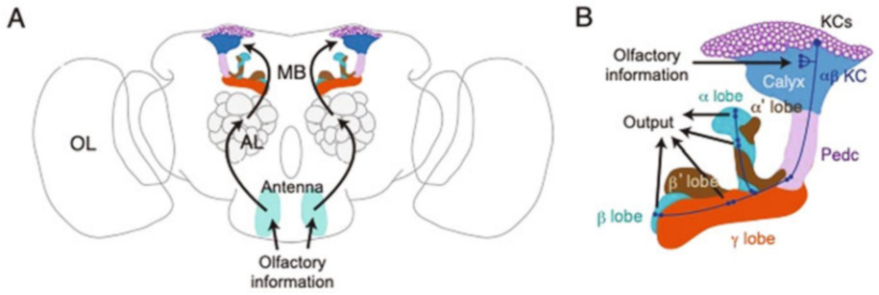

Fig. 16.2 Structure of fly brain. (**a**) Olfactory information pathway showing how odor information is conveyed from the antennae to the mushroom bodies. (**b**) The structure of mushroom bodies. Kenyon cells (KCs) of the MB receive olfactory information in the calyx and send axons to the MB lobes

retention curves with different shapes. From these differently shaped retention curves, different memory mutants can be classified as mutants that affect short-term memory (STM), middle-term memory (MTM), anesthesia-resistant memory (ARM), and long-term memory (LTM) (Fig. 16.1). Genes affecting STM include *rutabaga* (*rut*) (Livingstone et al. 1984), which encodes a cAMP-producing adenylyl cyclase (AC) and *dunce* (*dnc*), which encodes a cAMP-degrading phosphodiesterase (PDE) (Dudai et al. 1976). MTM-related genes include *amnesiac* (*amn*), which encodes a neuropeptide (Feany and Quinn 1995) and *DC0*, which encodes the catalytic subunit of PKA (Skoulakis et al. 1993). A mutation specifically affecting ARM is found in the *radish* (*rsh*) which encodes a protein of unknown function (Folkers et al. 2006), and LTM-related genes include genes encoding transcription factors such as *CREB* (Yin et al. 1994, 1995), *Notch* (Ge et al. 2004; Presente et al. 2004), and *repo* (Matsuno et al. 2015).

Odor and shock information are thought to become associated in the mushroom bodies (MBs), bilateral brain structures required for memory formation (Fig. 16.2). The MB consists of about 2000 Kenyon cells (KCs). Each KC projects axons into either the αβ, α′β′, or γ lobes of the MBs, and each of these lobes play distinct roles in formation, retention, and recall of different memory types (Dubnau et al. 2001; Krashes et al. 2007; McGuire et al. 2001). Further supporting the importance of the MBs in memory, many memory-related genes including *rutabaga*, *dunce*, *DC0*, and CREB are preferentially expressed and function in the MBs (Han et al. 1992; Hirano and Saitoe 2013; Nighorn et al. 1991).

Flies are able to form associations between odors and rewards as well as associations between odors and pain. In this case, the CS+ odor is paired with a sugar reward instead of electrical shocks. This type of training is known as olfactory reward conditioning and results in a preference, rather than an avoidance, of the CS+. Similar to formation of aversive memory, formation of appetitive memories also requires activity of the MBs. Besides olfactory conditioning, courtship conditioning, in which male flies learn to suppress courtship activity after being rejected by mated females, has been used to assess associative memory in *Drosophila*.

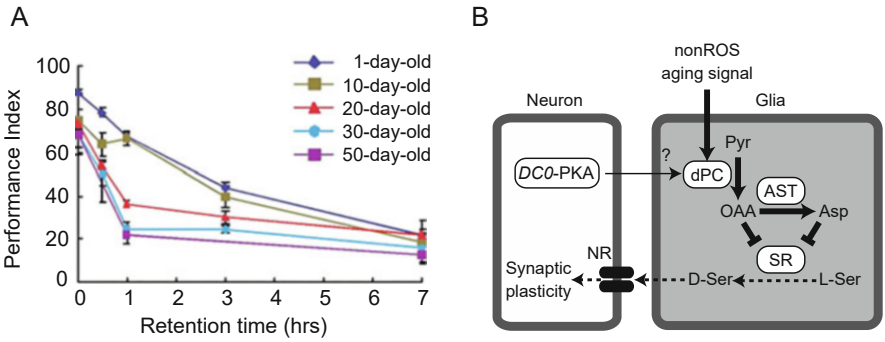

Fig. 16.3 Age-related impairments in MTM and working model. (**a**) Memory retention curves of aged flies (from Tamura et al. 2003). Similar to *amn* mutants, memory decay in old flies is significant at 1 h after training. (**b**) Working model of MTM impairments. Certain aging signals increase expression of dPC, leading to suppression of D-Ser production. *Pyr* pyruvate, *OAA* oxaloacetate, *Asp* aspartate, *AST* aspartate transaminase, *SR* serine racemase, *NR* NMDA receptor

However, both appetitive and courtship behaviors are altered upon aging, making it difficult to study these behaviors in old flies (Tonoki et al. 2020). Therefore, most AMI studies in flies have used the aversive olfactory conditioning paradigm.

Although mechanisms that regulate organismal life span have been identified, it had been unclear (1) whether brain aging, including AMI, is also caused by specific biological mechanisms, or (2) whether AMI is caused by a nonspecific deterioration of memory processes upon aging. Studies in *Drosophila* have addressed these questions and found that (1) AMI is not a nonspecific disruption of memory but rather a specific impairment of two memory phases, MTM and LTM (Mery 2007; Tamura et al. 2003), and (2) brain aging and organismal aging can be separated, since life span and aging can be accelerated or delayed without affecting AMI onset (Hirano et al. 2012), and suppression of AMI can be achieved without affecting life span (Yamazaki et al. 2007).

16.2 Age-Related Impairments in MTM

Comparing the memory retention curves of old flies (20-days-old and older) to curves of young flies (1-days-old), we can see that aged flies show a significant decrease in memory at 1 h after training (Fig. 16.3a). Although old flies also have decreases in 0-h (3 min) and 7-h memory, these decreases are relatively minor compared to the decrease that occurs between 30 min and 3 h, a time window encompassed by middle-term memory (MTM). In addition, memory retention curves of old flies resemble those of *amn* mutants, which are defective for MTM (Fig. 16.1). Further, memory retention curves of old *amn* mutants are not significantly different from curves of young *amn* mutants, indicating that *amn* mutants are

already defective for the memory phase affected by aging. Altogether, these results indicate that aging causes a specific reduction in MTM.

The *amn* gene encodes several neuropeptides, one of which is PACAP (pituitary adenylate cyclase-activating polypeptide). *amn* gene products are expressed in a pair of dorsal paired medial (DPM) neurons, which project their axons to the MB, and synaptic output from DPM neurons is essential for MTM formation (Waddell et al. 2000). Tonoki et al. showed that artificial activation of DPM neurons restores MTM in old flies (Tonoki and Davis 2012). These results suggest that aging causes changes in DPM activity, which alters activity of protein kinase A (PKA) in the MBs, decreasing MTM.

Since AMI exerts differential effects on different memory phases, this suggests that AMI is caused by changes to specific biochemical pathways involved in specific memory phases instead of a general decrease in health and brain activity. Based on this idea, T Tamura and D Yamazaki in our lab performed genetic screening to identify specific mutants that affect AMI. They found that a heterozygous mutant of the *DC0* gene (*DC0*/+), which encodes the PKA catalytic subunit, does not show significant memory impairment even at 20 days of age and older. *DC0* is preferentially expressed in the MBs, and expression of the *DC0* transgene in the MBs of *DC0*/+ flies restores AMI, while overexpression impairs MTM even in young flies (Yamazaki et al. 2007). These results indicate that AMI is accelerated by increasing PKA activity in the MBs and delayed by inhibiting PKA activity. However, we did not observe a change in PKA activity upon aging, suggesting that PKA regulates activity of another protein whose activity changes during aging, causing AMI.

Yamazaki et al. (2014) next used proteomics to identify a protein whose expression is both regulated by PKA and changes upon aging. They found that amounts of the *Drosophila* homologue of pyruvate carboxylase (dPC) increase upon aging and are reduced in *DC0*/+ mutants. Furthermore, heterozygous mutants of *dPC* (*dPC*/+) do not show decreased MTM upon aging, suggesting that age-dependent increases in dPC are responsible for inhibiting MTM at old ages.

In both *Drosophila* and mammals, dPC is expressed in glial cells rather than in neurons. Glia express serine racemase (SR) which converts L-serine to D-serine, a coactivator of NMDA-type glutamate receptors. Thus, one important role of glial cells is to help neuronal glutamate signaling. Interestingly, pyruvate carboxylase synthesizes oxaloacetate (OAA) from pyruvate, and OAA and aspartate have been shown to inhibit serine racemase (Dunlop and Neidle 2005; Strisovsky et al. 2005). This suggests that an age-dependent increase in dPC inhibits production of D-serine, thus inhibiting glutamate signaling in old flies. Consistent with this idea, amounts of D-ser are reduced in brains of old flies compared to young flies. In contrast, high amounts of D-ser are maintained in the brains of old *DC0*/+ and *dPC*/+ flies, in which AMI is suppressed. Finally, impairments in MTM are rescued when old flies are fed D-Ser. These results suggest that increased expression of dPC inhibits glial D-serine synthesis, resulting in decreased activity of NMDA receptor signaling, causing AMI in old flies (Fig. 16.3b). Life span is normal in both *dPC*/+ and *DC0*/+ mutant flies, indicating that PKA and dPC regulate AMI independent of organismal aging.

Independently from glial D-serine production, age-dependent defects in MTM have also been associated with decreases in autophagy. Sigrist's group has shown that feeding aged flies spermidine or overexpressing Odc-1, an enzyme involved in polyamine synthesis, in the MB of old flies rescues age-dependent defects in MTM. They demonstrate that this suppression of AMI is strictly dependent on autophagy (Gupta et al. 2013).

16.3 Age-Related Impairments in LTM

Among longer-lasting consolidated memory forms, aging adversely affects LTM, but not ARM (Fig. 16.4a) (Mery 2007). Similar to age-related defects in MTM, defects in LTM are also caused by glial cell dysfunction. However, age-related defects in LTM occur in old *DC0*/+ flies, and administration of D-Ser does not rescue age-related LTM impairments, indicating that defects in MTM and LTM are caused by different mechanisms.

LTM formation requires de novo transcription and translation in both neurons and glia. Transcription factors such as c-Fos and CREB must be activated in neurons, while the transcription factor, Repo, must be activated in glia. Furthermore, in order to increase Repo activity at appropriate times, neurons must communicate with glia. This communication occurs through a homophilic cell-adhesion molecule, Klingon

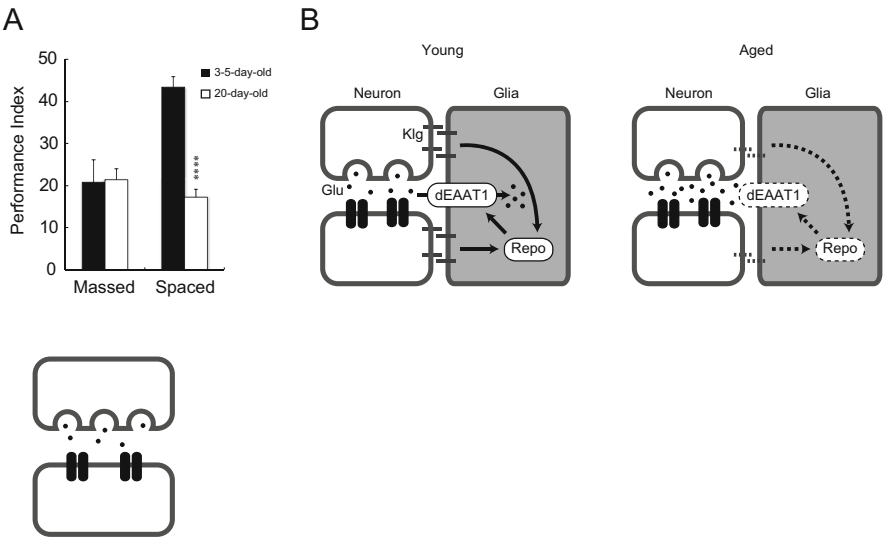

Fig. 16.4 Age-related impairments in LTM and working model. (**a**) While memory formed in aged flies after spaced training is disrupted, memory after massed training is not. (**b**) Working model of LTM impairments. Due to decreased expression of Klg, Repo-dependent expression of dEAAT1 is suppressed in aged flies. This leads to excess glutamate signaling

(Klg). Klg is expressed in both glia and neurons and is thought to mediate interactions between these cell types. Spaced training increases expression of Klg, which in turn increases expression of Repo. Both *repo* and *klg* mutants are defective for LTM. These results suggest that neuronal activity induces glial Repo-dependent gene expression through a Klg-dependent process (Matsuno et al. 2009, 2015). What genes do glia need to express for LTM? One Repo target gene whose expression increases after spaced training is *dEAAT1*, the *Drosophila* homologue of an excitatory amino acid transporter. dEAAT1 regulates neuronal glutamate signaling by clearing extracellular glutamate from synaptic sites. Knocking down of *dEAAT1* in glia impairs LTM suggesting that formation of LTM requires a decrease in extracellular glutamate after spaced training (Matsuno et al. 2019).

Expression of both *klg* and *repo* decreases in aged flies. This prevents increased expression of *dEAAT1* after spaced training in aged flies. Artificially increasing *repo* expression in glial cells rescues LTM in old flies as well as in *repo* and *klg* mutants. LTM in old flies can also be rescued by increasing expression of *dEAAT1* or by pharmacological treatment with memantine, an NMDA receptor antagonist, or riluzole, a drug that inhibits glutamate activity after spaced training (Matsuno et al. 2019). From this data, we hypothesized that in aging flies, *dEAAT1* expression is not sufficiently increased in glial cells after spaced training. This results in excessive glutamate signaling which inhibits LTM.

LTM is stored in α/β-KCs in the MB (Miyashita et al. 2018; Pascual and Preat 2001). In addition, after spaced training, increased Ca^{2+} responses to the CS+ odor are observed in the α/β-lobes (Yu et al. 2006). These altered Ca^{2+} responses are referred to as an LTM trace. In aged flies, LTM traces are not observed (Tonoki and Davis 2015). Synaptic output from DPM neurons is required for LTM as well as MTM, but synaptic contacts between DPM cells and the MBs are abolished during aging. These findings suggest that the age-related decreases in synaptic transmission between DPM neurons and MBs maybe a cause of LTM deficits in old flies (Tonoki and Davis 2015). However, it has not been confirmed yet whether artificially increasing synaptic transmission between DPM neurons and MB can rescue age-related LTM impairments.

TORC2 is a serine/threonine kinase and mediates the rapamycin-insensitive, TOR2-unique pathway. It has been reported that TORC2 activity is reduced in aged flies, and pharmacological enhancement of this activity rescues LTM in old flies (Johnson et al. 2015). TORC2 induces actin polymerization, which is required for synaptic structural changes. Thus, it has been proposed that LTM defects develop during aging due to a decrease in actin polymerization caused by reduced TORC2 activity. On the other hand, actin polymerization in the MBs promotes the forgetting of ARM (Gao et al. 2019). One prominent model for *Drosophila* memory is that ARM and LTM function antagonistically (Isabel et al. 2004). In this model, formation of ARM inhibits the formation of LTM, and formation of LTM inhibits ARM formation. This suggests that an age-dependent decrease in TORC2 enhances ARM, which in turn impairs LTM. The higher amounts of TORC2 in young flies favor actin polymerization and increased LTM at the expense of ARM. If this were the case, however, ARM should be increased by aging. We and others do not observe a

decrease in ARM in old flies, but we also do not observe an increase. Thus far, the linkage between age-related declines in the Klg-Repo-dEAAT1 pathway decreased TORC2 activity, and the absence of LTM trace formation is not clear. However, the combination of a simple brain structure, well-developed anatomical genetics, and the ability to genetically manipulate the activity of various neurons in *Drosophila* should reveal connections soon.

16.4 Defects in Neuron-Glia Interactions in Mammalian Models

The relationship between functional and structural changes in glial cells and age-related cognitive declines has been studied in mammals as well as in *Drosophila*, although in mammals, various other causes, such as age-related mitochondrial dysfunction and dysregulation of energy metabolism, increased oxidative stress and DNA damage, autophagy dysfunction, stress dysregulation, and increased inflammatory responses, have also been linked to age-related cognitive dysfunction (Mattson and Arumugam 2018). Recent transcriptome analyses suggest that glial cells are the first cells to undergo gene expression changes upon aging. In the aged human brain, the majority of gene expression changes is observed in glial cells rather than neurons (Soreq et al. 2017). During aging, glial cells undergo morphological and functional changes (microglia have fewer protrusions, astrocytes less support synaptic function, and oligodendrocytes have a reduced ability to form myelin sheaths) that reduce their neuroprotective role (Salas et al. 2020). Guerra-Gomes et al. suggest that Ca^{2+} signaling in astrocytes is responsible for memory impairment in aged mice (Guerra-Gomes et al. 2018). Astrocytes regulate synaptic plasticity and form part of the blood-brain barrier structure. Guerra-Gomes et al. created knockout mice that lack IP3R2 in astrocytes and examined age-related synaptic function and memory impairment. Interestingly, while aged wild-type mice exhibited synaptic and memory deficits, these deficits were suppressed in aged IP3R2 KO mice. This suggests that Ca^{2+} release from internal Ca^{2+} stores via IP3R2 is responsible for AMI. Notably, age-related changes in morphology and gene expression of glial cell are brain site-specific; the surface area and volume of GFAP-positive astrocytes are increased in the hippocampus, and astrocyte- and oligodendrocyte-specific genes expression is altered in the hippocampus and substantia nigra (Rodriguez et al. 2014; Soreq et al. 2017). These area specific changes may explain why synaptic and neuronal dysfunction occur preferentially in certain brain regions and why only certain types of learning are affected in the elderly.

The glial dysfunction we observe in aged *Drosophila* may also cause AMI in mammals. Amounts of D-serine in the hippocampus are significantly reduced in the hippocampus of aged rats that display cognitive declines, and induction of LTP in aged rats is restored by administration of D-serine (Turpin et al. 2011). Moreover, LOU/C/Jail rats which maintain high D-serine levels into old ages, do not show

cognitive impairments when compared to control rats. In line with these results, it has been reported that D-Serine administration promotes spatial learning, problem solving, and learning ability in the elderly (Avellar et al. 2016).

Defects in LTM are thought to be caused by the inability of old *Drosophila* to induce dEAAT1 expression after spaced training. These defects can be rescued by feeding flies riluzole or memantine after spaced training. Similarly, expression of the mammalian glutamate transporter, GLT1, is decreased in the hippocampus of aging rats and riluzole restores both GLT1 expression and spatial memory in these rats (Brothers et al. 2013). In AD patients with dementia, GLT1 expression in the entorhinal cortex is decreased compared to AD patients without dementia, and increased extracellular glutamate levels are thought to exacerbate symptoms (Hoshi et al. 2018). Memantine, an NMDA receptor antagonist, is thought to inhibit glutamate excitotoxicity induced by Alzheimer's disease (Parsons et al. 1999). Memantine is a selective NMDA receptor inhibitor whose affinity is lower than MK801. Interestingly, like Mg^{2+}, the inhibitory effect of memantine is dependent on membrane potential (Rammes et al. 2008). These properties allow memantine, unlike MK801, to protect neurons without impairing synaptic plasticity or learning and memory.

Studies in *Drosophila* and mammals have shown that administration of D-Ser and memantine to aged flies and mice markedly improves declining learning and memory. These findings indicate that AMI is reversible and suggest that fundamental therapeutics for AMI may be achieved by developing techniques to restore the normal function of glial cells.

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Chapter 17
Critical Roles of Glial Neuroinflammation in Age-Related Memory Decline

Tatsuhiro Hisatsune

Abstract Memory capacity gradually declines with age, but the cause of this decline is surprisingly unclear. Through studies of aging animals and animal models of neurodegenerative disorders, it has been noted that glial neuroinflammation is a central cause of age-related memory decline. In particular, using mouse models of vascular dementia and Alzheimer's disease, we have found that chronic glial inflammatory reactions in the hippocampal formation are responsible for memory decline and that the suppression of the glial neuroinflammatory reactions can induce the prevention of memory decline. In addition, these neuroinflammatory reactions also caused the inhibition of hippocampal neurogenesis, which is one of the typical prototypes of age-related memory decline. Chronic glial inflammatory reactions consist of astrocytic hyper-activation as well as microglial inflammatory responses, which produce cytotoxic molecules of superoxide radicals as well as inflammatory chemokines. As a translational study, we have also tested the effect of the anti-inflammatory molecules of histidine-containing dipeptide (imidazole-dipeptide) in Alzheimer's model mouse as well as elderly human subjects and found that the long-term treatment of the dipeptide preserved memory function in both animal model and elderly individuals, probably due to the suppression of the glial inflammatory reactions. These observations may suggest that glial neuroinflammation is central to the decline of memory performance and that quenching of this inflammatory response may improve age-related memory decline.

Keywords Memory · Dementia · Histidine-containing dipeptide · Newborn neuron · Adult hippocampal neurogenesis · Neurovascular unit · Neuroinflammation · Mild cognitive impairment · Alzheimer's disease

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_17

17.1 Introduction

Age-related memory decline is a universal phenomenon in all animal species, from nematode, fruit fly, mouse, and human (Hof and Mobbs 2001; Jagust 2013); however, the mechanism behind this memory decline is surprisingly unclear. There is no doubt that there is a general mechanism for the aging of brain function throughout animal species, but in the sense of animal evolution, since the advent of vertebrates, animals have had a closed vascular system that assist all physiological functions of all the cell types that make up the body. Therefore, the aging of the blood system can be considered a significant contributor to the aging of brain function, as supported by using heterochronic parabiosis experiment (Villeda et al. 2011) and subsequent studies (Horowitz et al. 2020; Smith et al. 2020). As a long-time belief, it had been thought that the decline in brain function was mainly due to the loss of neurons, but recent studies have strongly suggested that the dysregulation of neuronal network function due to glial neuroinflammatory reactions in combination with degeneration of neurovascular unit which composed of vascular endothelial cells, pericytes, and glial cells in vertebrate animals (Heneka et al. 2015; Heneka 2020), rather than the loss of neurons. Therefore, if we can eliminate the cause of the neuroinflammation related to neurovascular unit dysfunction, neuronal function should recover and brain function must improve.

Age-related memory decline has been also reported in many animal species including nematode and fruit fly. It has been pointed out that dysfunction of glial cells is involved in the alteration of neuronal cells in both nematode (Frakes et al. 2020) and fruit fly (Matsuno et al. 2019), although these animal species do not possess closed vascular systems. We have been investigating the mechanism of memory decline in aging animals of mammalian species such as rodents and monkeys. In the brain of the aged macaque monkeys, there is a reduction of adult hippocampal neurogenesis, together with enhanced gliosis (Aizawa et al. 2009, 2011). In the mouse model of Alzheimer's disease, the maturation of newborn neurons in the dentate gyrus of the hippocampus was inhibited due to the enhanced gliosis, and the pharmacological suppression of this gliosis recovered the rate of neurogenesis and memory performance (Matsuda and Hisatsune 2017). In addition, we have observed the preservation of memory performance in a mouse model for vascular dementia by the pharmacological treatment with an antagonist for a purinergic receptor, P2Y1, expressed in activated astrocytes to suppress glial inflammation after the middle cerebral artery occlusion (Chin et al. 2013). When memory function is impaired in aged animals, the alteration in glial cell types is observed in the neural circuit, which controls memory performance in the nervous system of the aged animals.

In elderly human individuals, the decline in memory function has been pointed out ubiquitously and is sometimes recognized as mild cognitive impairment (MCI), which is a precursor stage to dementia (Petersen et al. 1999). MCI patients have subjective complaints of forgetfulness, but their general cognitive function is normal, and they do not have dementia. In MCI, about half develop dementia within

5 years. Most of the rest remain in the MCI state, but a few may return to a healthy cognitive normal state, called "revert" (Roberts et al. 2014). Improving the decline of memory function in the elderly may lead to the prevention of the onset of dementia. If we can understand the mechanism of memory decline, it will be possible to restore memory function by restoring impaired neuronal network function and restoring the normal functioning of neural circuits. In other words, removing the causes of impaired neuronal function will lead to the regeneration of neural function. Neuroinflammation can be one of the causes that induce memory decline in MCI as well as Alzheimer's disease (Heneka et al. 2015). Therefore, the control of neuroinflammation shall become a strong strategy to inhibit the decline in cognitive as well as memory functions even in elderly humans. MCI, a condition in which memory function is mildly impaired, can be broadly classified according to the type of dementia, such as MCI due to Alzheimer's disease or MCI due to vascular dementia (Albert et al. 2011). In the studies of brain tissues from people who died with normal cognitive function, it has been suggested similar changes seen in Alzheimer's disease (senile plaques and neurofibrillary changes), and also brain tissue changes related to cerebrovascular disease (infarct foci and encephalitis). These cellular events occurring in elderly human individuals could be a cause to induce neuroinflammation, which may be one of the causes of brain aging.

17.2 Age-Related Cognitive Decline in Animal Models

17.2.1 Cognitive Declines in Animal Model for Vascular Dementia

To investigate the molecular mechanism of brain dysfunction, we conducted a mildly impaired memory function in a mouse model of cerebral infarction and examined the changes that occur in the brain (Chin et al. 2013). By occluding the middle cerebral artery, extensive foci of infarction are formed in the brain, mainly in the striatum. In the hippocampus, which is involved in memory, there are almost no infarct foci, but there is an inflammatory response with activation of astrocytes and microglia. In this mouse model of cerebral infarction, neural activity is increased in the hippocampal formation (Nochi et al. 2012). In addition, the proliferative potential of neural stem cells is transiently increased, and the over-proliferation of neural stem cells was suppressed by diazepam (Nochi et al. 2013). In mice in which the purine receptor P2Y1 molecule, which is involved in the inflammatory response of the brain, was knocked out, occlusion of the middle cerebral artery did not cause a decline in memory function. It was suggested that the P2Y1 receptor molecule associated with cerebral inflammation is included in the mechanism of memory impairment as well as neuroinflammation in the mouse model of cerebral infarction (Chin et al. 2013), supported by the results from the mouse model of Alzheimer's disease (Reichenbach et al. 2018) (Fig. 17.1).

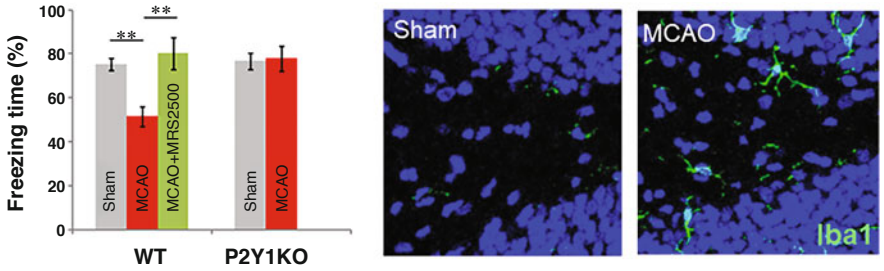

Fig. 17.1 Influence of brain inflammation on impaired memory function. *Left*: Memory function is impaired in a mouse model of middle cerebral artery occlusion (MCAO) but is avoided by treatment with MRS2500, an antagonist for P2Y1 receptors. In addition, P2Y1 knockout mice do not show memory impairment even after induction of cerebrovascular injury. *Right*: Inflammatory response in the brain in the hippocampus after MCAO. The dentate gyrus region is shown. Iba1 is a marker molecule for microglial cells. $^{**}p < 0.01$, Bar: 50 μm (Chin et al. 2013)

17.2.2 Memory Declines in Mouse Model for Alzheimer's Disease

To investigate the mechanism of memory decline in a mouse model for Alzheimer's disease, we have utilized APP and presenilin (Psen) double transgenic model mice (Kaneko et al. 2017), in which it takes more than 12 months after birth to show the accumulation of senile plaques and memory impairment. In addition, we have been also studying memory decline in a mouse model of Alzheimer's disease with diabetes (Herculano et al. 2013; Matsuda and Hisatsune 2017). This mouse model can induce memory impairment in a shorter period (6 months) than the conventional Alzheimer's model mouse. This model mouse is created by administering a high-fat diet to an Alzheimer's disease model mouse. After the mice became 4 months old, we started feeding them a high-fat diet containing 32% fat. Eight weeks after the start of the high-fat diet, we conducted a memory test as well as histochemical evaluations. Using this model, we investigated the ameliorative effect of several food components with antioxidant and anti-inflammatory effects on the decline in memory function. The results showed that carnosine (a type of imidazole dipeptide), an anti-inflammatory ingredient, had an ameliorating effect on memory decline (Herculano et al. 2013). Histologically, the vascular damage with RAGE accumulation seen in the model mice disappeared after the administration of carnosine (Fig. 17.2). These results suggest that inflammatory reactions in the brain are one of the mechanisms by which memory function declines and that the decline in memory function may be avoided by suppressing these inflammatory reactions.

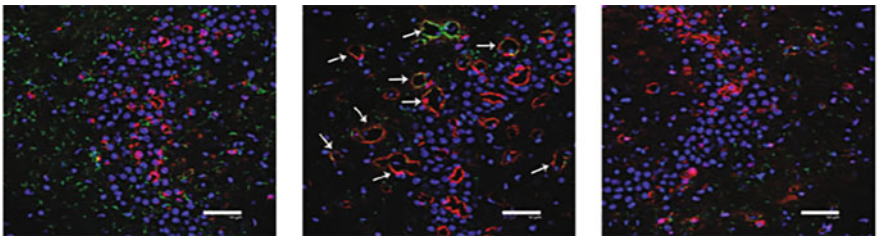

Fig. 17.2 Vascular inflammation on memory impairment in an AD model mice. Vascular damage findings with RAGE accumulation in the hippocampus of a mouse model of Alzheimer's disease (**a**, control, **b**, AD mouse). Administration of the anti-inflammatory molecule resulted in the disappearance of cerebrovascular lesions in the CA3 region of the hippocampus (**c**). RAGE (*green*): receptor for advanced glycation end product (reflecting inflammatory response in cerebral blood vessels), CD31 (*red*): a marker of vascular endothelial cells. Bar: 50 μm (Herculano et al. 2013)

17.3 Critical Roles of Neuroinflammation in Age-Related Cognitive declines

17.3.1 Glial Neuroinflammation

We studied the histological changes that occur in the brain tissue of the App/Psen Alzheimer's disease mouse model, in which memory, as well as cognitive functions, are impaired. In the case of memory impairment, neuroinflammation with activation of microglia and astrocytes was observed in the hippocampus. Treatment with rivastigmine, a cholinesterase inhibitor used in the treatment of Alzheimer's disease, suppressed glial activation and the production of the pro-inflammatory cytokines IL-1β and TNF-α, thereby avoiding the decline in memory function (Matsuda and Hisatsune 2017). Shi et al. have also shown that microglial activation was involved in Tau phosphorylation in the brain of human ApoE4 knock-in P301S mice (Shi et al. 2017). Using the App/Psen Alzheimer's disease mouse model, Venegas et al. (2017) showed that ASC specks (inflammasomes) released by microglia expanded the accumulation of amyloid plaques and impaired memory function and that knockout of ASCs reduced amyloid plaques and improved memory function. Microglia, macrophage-like cells in the brain, are thought to be involved in the decline of memory function by interfering with neuronal activity through the production of inflammatory cytokines and inflammasomes during the progression of Alzheimer's disease.

In the brains of mouse models of Alzheimer's disease with impaired memory function, astrocytes (stellate glial cells) are also activated along with microglia and contribute to increased neuroinflammation (Kaneko et al. 2017). They have also reported that astrocytes are activated and neuroinflammation is increased in the hippocampus of a mouse model of cerebrovascular dementia with impaired memory function and that knockout of the P2Y1 receptor suppresses astrocyte activation and improves impaired memory function (Chin et al. 2013). Reichenbach et al. showed that P2Y1 expression in astrocytes was increased around amyloid plaques in the

brains of Alzheimer's disease patients and mouse models and that administration of a P2Y1 antagonist returned neural activity to normal (Reichenbach et al. 2018). Goetzl et al. have developed a method to isolate astrocyte-derived exosomes from plasma samples of Alzheimer's disease patients and found that inflammatory cytokines and complement molecules were increased in the patient samples (Goetzl et al. 2018). Increased neuroinflammation by astrocytes as well as microglia is involved in the progression of Alzheimer's disease, and the suppressive regulation of the glial neuroinflammation could be a target to ameliorate cognitive decline occurred in the age-related neurodegenerative condition such as Alzheimer's disease and vascular dementia.

17.3.2 Neuroinflammation at the Cerebrovascular Unit

In the hippocampus of diabetic Alzheimer's disease model mouse, in which feeding a high-fat diet to a mouse model of Alzheimer's disease can induce diabetic Alzheimer's disease, we observed abnormal histopathological findings in brain capillaries with an accumulation of RAGE (Herculano et al. 2013), as well as glial neuroinflammation. In the hippocampus of aged Alzheimer's disease mice over 18 months of age, these abnormalities in brain capillaries were also detected (Kaneko et al. 2017). Administration of an anti-inflammatory dipeptide alleviated the degenerative cellular changes at the neurovascular unit which is composed of glial neuroinflammation as well as an impaired neurovascular component of brain micro-capillary pericytes and endothelial cells (Herculano et al. 2013; Kaneko et al. 2017). Zlokovic and his colleagues have elegantly demonstrated that the ApoE4 molecule acts on pericytes to increase the permeability of the cerebrovascular barrier in a mouse model of Alzheimer's disease (Bell et al. 2012) and brain tissue from patients with Alzheimer's disease (Halliday et al. 2016). Neuroinflammation, which reduces cerebrovascular function and inhibits amyloid drainage in the brain, is contributing to the worsening of Alzheimer's disease.

The hallmarks of Alzheimer's disease are amyloid plaques and neurofibrillary changes; recent genomic studies have also shown that neuroinflammation is one of the factors that accelerate neurodegeneration. GWAS (genome-wide association studies) analysis of genetic predisposition to Alzheimer's disease has identified 20 new genes, and about half of which are closely related to neuroinflammation, including TREM2, CD33, and HLA-DRB1/5 locus (Lambert et al. 2013; Strooper and Karran 2016). TREM2 is a rare mutant that increases the risk of Alzheimer's disease nearly threefold. TREM2 dysfunction leads to decreased mTOR signaling and impaired energy metabolism in microglia and also induces an increase in autophagosomes. TREM2-deficient Alzheimer's disease mice treated with cyclocreatine treatment of TREM2-deficient Alzheimer's disease mice with cyclocreatine improved microglial energy metabolism and the pathological picture associated with neurodegeneration (Ulland et al. 2017). Successful control of

neuroinflammation is expected to lead to new therapeutic strategies for Alzheimer's disease.

17.4 Age-Related Cognitive Decline in Elderly Individuals

A randomized controlled trial has been conducted to determine whether inflammation in the brain plays a role in memory decline in elderly humans and whether reducing the inflammatory response can improve memory decline. In our cohort study, participants were randomly divided into two groups and given the test formula or a placebo (Hisatsune et al. 2016). The test formula contained the imidazole dipeptide molecules anserine and carnosine in a ratio of 3:1. Anserine is a dipeptide similar to carnosine, which is found in poultry. Before and after the intake of the formula, various memory function tests (e.g., Wechsler memory test—delayed recall of logical memory task) were conducted in a double-blind manner, and MRI measurements were taken to investigate structural and functional changes in the brain (Rokicki et al. 2015; Ding et al. 2018). We also measured the levels of several inflammatory cytokines in the blood to investigate the systemic inflammatory response (Hisatsune et al. 2016; Katakura et al. 2017). Memory function was significantly improved in the test group with anti-inflammation treatment compared to the placebo group. Thus, we confirmed reproducibility regarding the preservation of memory function in elderly individuals, which had been originally observed in animal model studies (Herculano et al. 2013; Kaneko et al. 2017). Based on the results of MRI cerebral blood flow analysis, it was observed that the test formula improved cerebral blood flow reduction, supported by the results of resting-state functional MRI analysis (Rokicki et al. 2015). The results of blood tests showed that the amount of IL-8 in the blood of the test diet group decreased, suggesting that the degree of systemic inflammatory response improved after the intake of the anti-inflammatory molecules (Hisatsune et al. 2016). Subsequently, we have also performed clinical studies by recruiting MCI individuals to assess the effect of these anti-inflammatory molecules and found that the treatment with either imidazole dipeptide mixture (750 mg anserine and 250 mg carnosine per day) or anserine (500 mg per day) alone improved the dysfunction of cognitive performance (Masuoka et al. 2019; Masuoka et al. 2021a). In addition, we have also obtained the beneficial effect of anti-inflammatory molecules in daily food from community-dwelling cohort studies (Sakurai et al. 2020, 2021). These results of the human study strongly suggest that inflammatory reactions in the brain are one of the mechanisms by which memory function declines due to aging and that suppressing these inflammatory reactions may improve the state of brain circuits related to memory, thereby avoiding the decline in memory function (Masuoka et al. 2021b).

17.5 Conclusion

In this chapter, I mentioned that neuroinflammation is a major cause of age-related memory decline. Neuroinflammation based on abnormal activation of glial cells is a phenomenon that is also found in all animal species including nematode and fruit fly and must be universal. On the other hand, increased neuroinflammation via the neurovascular system is a phenomenon peculiar to animal species having a closed vascular system. The fact that memory function declines, centered on hippocampal degeneration, is observed with aging, which seems to be a unique phenomenon limited to mammals, and glial neuroinflammation is a central cause to induce memory decline in mammals.

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Chapter 18
Central Mechanisms Linking Age-Associated Physiological Changes to Health Span Through the Hypothalamus

Akiko Satoh

Abstract The hypothalamus, which is located on the ventral side of the brain, is composed of several areas, called nuclei. Each hypothalamic nucleus implements a distinct role in several physiological traits. Growing evidence suggest molecules/signaling pathways including sirtuin, mechanistic target of rapamycin (mTOR), and nuclear factor-κB (NF-κB), specific areas of the hypothalamus (the dorsomedial/lateral hypothalamus, mediobasal hypothalamus, and arcuate nucleus), neural stem cells, and temperature-sensitive neurons within the hypothalamus play a role in the regulation of mammalian longevity. These previously identified molecules/pathways and cell types in the hypothalamus have some common function in physiological traits. Therefore, further elucidation of common mechanisms by which the hypothalamus controls one or more physiological traits during aging through multiple aging/longevity regulators will help us to uncover novel integrative mechanisms underlying aging, longevity, and age-associated pathophysiology.

Keywords Brain · Hypothalamus · Aging · Longevity · Sirtuin · NF-κB · mTOR · Neural stem cells · Thermoregulation · Metabolism · Circadian behaviors · Sleep-wake patterns

18.1 Introduction

The brain is a part of the central nervous system and divided into four main parts, the brainstem, cerebellum, diencephalon, and cerebrum, which integrates information from entire organs/tissues in our body and coordinates body functions (Ludwig et al. 2021). The diencephalon is a small part of the brain and located deep within the brain

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_18

containing epithalamus, thalamus, subthalamus, and hypothalamus. The hypothalamus is an ancient area of the brain (Lemaire et al. 2021; Saper and Lowell 2014; Xie and Dorsky 2017; Zhou et al. 2020), and is a very small proportion of it (0.3−0.5% of the whole brain in both mice and humans), and is composed of several areas, called nuclei. Each hypothalamic nucleus implements a distinct role in several vital physiological traits including appetite, thermoregulation, sleep-wake cycle, circadian rhythm, and autonomic nervous system. Dilman has already documented the potential role of the hypothalamus in the regulation of aging/longevity in 1971 (Dilman 1971). The hypothalamus responds to several environmental stimuli, and the capacity of these responses might decline with advanced age, resulting in the acceleration of aging and subsequently the development of age-associated diseases. Consistent with this idea, several studies show that rodent models with brain- or hypothalamus-specific gene manipulation significantly extend or shorten their life span (Table 18.1), implicating the brain, in particular, the hypothalamus as one of critical parts in our body that controls mammalian longevity (Liu et al. 2021; Satoh et al. 2017).

18.2 Molecules and Signaling Pathways in the Hypothalamus that Control Mammalian Longevity

18.2.1 Sirtuin

Sirtuin is a nicotinamide adenine dinucleotide^{+} (NAD^{+})-dependent deacylase (e.g., deacetylase, desuccinylase, demaloynylase, deglutarylase, long-chain deacetylase, ADP-ribosyltransferase). Numerous studies demonstrate that overexpression of sirtuin extends life span in several experimental organisms, whereas deletion of sirtuin shortens life span (Chang and Guarente 2014; Newman et al. 2012; Satoh et al. 2017). Therefore, sirtuin is an evolutionally conserved regulator of life span. In mammals, there are seven sirtuin family proteins, named Sirt1 to Sirt7. Among them, gain-of-function of Sirt1 or Sirt6 promotes lifespan extension in mouse models (Kanfi et al. 2012; Satoh et al. 2013).

For Sirt1, hypothalamic-specific gain-of-function models have been reported for mammalian longevity. For instance, brain-specific *Sirt1*-overexpressing transgenic (BRASTO) mice show lifespan extension in both males and females (Satoh et al. 2013) (Table 18.1). The log-hazard ratio reveals that a slope is the same but the intercept is varied between genotypes, suggesting that BRASTO mice live longer than wild-type mice by delaying the onset of aging without changing the pace of aging. In fact, BRASTO mice do display a delayed onset of aging phenomena including low physical activity and poor sleep quality as defined by low delta power. Old BRASTO mice also maintain youthful mitochondrial function in skeletal muscle compared to old control mice. In addition, mice with knockdown of *Sirt1*, its

Table 18.1 Longevity study using rodent models with brain-specific gene manipulation

Strain	Models	Promoter	Sex	Median or mean life span	Maximum life span	Reference
FVB/N	αMUPA	αA-crystallin	Female	Extension	Extension	Miskin and Masos (1997)
C57BL/6	UCP2 Tg brain	Hypocretin	Male	Extension	Extension	Conti et al. (2006)
			Female	Extension	Extension	
C57BL/6	bIrs2-KO	Nestin	Combined	Extension (Het)	Extension	Taguchi et al. (2007)
			Combined	Extension (Homo)	Extension	
C57BL/6	bIGF1RKO	Nestin	Combined	Extension	No extension	Kappeler et al. (2008)
			Male	Extension	No extension	
			Female	Extension	No extension	
Sprague-Dawley	Npy-Tg	Npy	Female	Extension	Extension	Michalkiewicz et al. (2003)
C57BL/6	N/Ikbkb$^{lox/lox}$	Nestin	Combined	Extension	Extension	Zhang et al. (2013)
C57BL/6	MBH-IκB-α	Synapsin promoter-directed lentiviral	Male	Extension	Extension	Tang et al. (2015), Zhang et al. (2013)
C57BL/6	MBH-IKK-β	Synapsin promoter-directed lentiviral	Male	Shorten	Shorten	Tang et al. (2015), Zhang et al. (2013)
C57BL/6	BRASTO	Prion	Combined	Extension	Extension	Satoh et al. (2013)
			Male	Extension	A trend of extension	
			Female	Extension	Extension	
C57BL/6	MBH-TK1	Sox2 promoter directed lentiviral	Male	Shorten	Shorten	Zhang et al. (2017)
C57BL/6	MBH-dnIκB-α-htNSCs implantation	–	Male	Extension	Extension	Zhang et al. (2017)
C57BL/6	Rictor$^{Nkx2.1-/-}$	Nkx2-1	Combined	Shorten	No data	Chellappa et al. (2019)

molecular partner *Nk2 homeobox 1* or its downstream gene *orexintype 2 receptor* (Satoh et al. 2010) within the dorsomedial and lateral hypothalamus (DMH and LH, respectively) mimic age-associated low physical activity and poor sleep quality. Remarkably, such aging phenomena are ameliorated by overexpression of *Sirt1* in the DMH (Satoh et al. 2013). Thus, maintaining Sirt1 signaling in the DMH might be crucial to delay the aging process and to extend our health span. As a note, whole-body *Sirt1*-overexpressing transgenic mice do not extend life span (Herranz et al. 2010). This discrepancy of longevity phenotypes between BRASTO and whole-body *Sirt1*-transgenic mice indicates that Sirt1 has organ-specific effects for longevity. Indeed, the level of Sirt1 is increased by dietary restriction in the cerebral cortex and hippocampus, but not in the cerebellum and midbrain (Chen et al. 2008). Moreover, another line of BRASTO mice, which does not have a DMH/LH-dominant overexpression of *Sirt1* in the brain, displays no change in life span (Satoh et al. 2013). Together, DMH (and/or LH)-specific activation of Sirt1 is necessary to promote beneficial effects against age-associated physiological changes and lifespan extension.

Although the function of brain Sirt6 is not fully elucidated, a number of studies have revealed the role of Sirt6 in longevity control. Whole-body *Sirt6*-overexpressing transgenic mice show lifespan extension in males, but not in females (Kanfi et al. 2012). In addition, haploinsufficiency of Trp53 (p53) extends life span in short-lived *Sirt6*-knockout mice (Mostoslavsky et al. 2006), suggesting that Sirt6-p53 signaling controls life span (Ghosh et al. 2018). In addition, in rodents, the capacity of the Sirt6 protein to promote DNA double-strand break (DSB) repair is higher in long-lived species than short-lived species (Tian et al. 2019). Furthermore, *Sirt6*-knockout mice show a great activity of long interspersed element-1 (LINE1), and the suppression of LINE1 by reverse-transcriptase inhibitors extends life span and improves body function of *Sirt6*-knockout mice (Simon et al. 2019). These results indicate that Sirt6 functions at a cellular level, compared to the fact that Sirt1 regulates aging and longevity through neuronal function in specific brain regions.

18.2.2 Mechanistic Target of Rapamycin

The mechanistic target of rapamycin (mTOR), a serine/threonine protein kinase, is one of the evolutionarily conserved longevity pathways, in addition to previously discussed sirtuins (Lopez-Otin et al. 2013; Pan and Finkel 2017). mTOR assembles into two complexes, mTORC1 and mTORC2. mTORC1 integrates numerous environmental and hormonal stimuli to control anabolic processes including ribosomal biogenesis, protein translation, stress-responsive transcription, and autophagy. mTORC2 plays an important role in cytoskeletal organization and insulin/PI3K signaling (Kennedy and Lamming 2016; Wullschleger et al. 2006; Zhou and Huang 2011). Rapamycin forms a complex with FK506-binding protein 12, leading to inhibit the activity of mTOR.

Systemic suppression of mTOR signaling promotes lifespan extension. In mice, deletion of mTOR signaling pathway component ribosomal S6 protein kinase 1 (S6K1) leads to lifespan extension in females (Selman et al. 2009). *S6k1*-knockout female mice also display improvements in several physiological and pathological aspects such as motor and neurological function, general activity, exploratory behavior, and bone volume. The administration of rapamycin results in lifespan extension in mice, mainly through mTORC1 inhibition (Lamming et al. 2012; Papadopoli et al. 2019). There is a study which showed the effect of hypothalamic mTOR signaling in longevity. Opposite to mTORC1 inhibition, mice lacking mTORC2 signaling, by the knockout of *Rictor*, in the hypothalamus promote adverse effects in metabolism and shortened life span. Moreover, hypothalamic-specific *Rictor*-knockout mice show low physical activity and increased susceptibility to diet-induced obesity through hyperphagia (Chellappa et al. 2019). Only chronic administration of rapamycin can inhibit mTORC2 in some cell lines or tissues (Schreiber et al. 2015). Therefore, specific inhibition of mTORC1 would reduce side effects of rapamycin in brain function, and developing new rapamycin analogs that selectively inhibit mTORC1 is highly motivated (Schreiber et al. 2019).

18.2.3 Nuclear Factor-κB

The nuclear factor-κB (NF-κB) family of transcription factors plays important roles in inflammation, immune responses, cell proliferation, differentiation, survival, and organogenesis (Cildir et al. 2016; Yu et al. 2020). NF-κB is activated by canonical and noncanonical pathways through different signaling components that differ in their biological functions. The activation of canonical NF-κB pathway is usually quick and transient, influenced by multiple stimuli, and regulates the expression of proinflammatory genes (Yu et al. 2020), while the noncanonical NF-κB pathway is activated by differential cell surface receptors, cytoplasmic adaptors, and NF-κB dimers (Cildir et al. 2016). As for notable differences between these two pathways, IκB kinase (IKK) β and NF-κB essential modulator are absolutely necessary in the canonical pathway, whereas IKKα is required in the noncanonical pathway (Dejardin et al. 2002; Senftleben et al. 2001).

The manipulation of NF-κB signaling in the mediobasal hypothalamus (MBH) affects age-associated physiological changes and life span in mice. Hypothalamic IKKβ/NF-κB is activated along with aging (Zhang et al. 2013) and overnutrition such as high-fat diet (Zhang et al. 2008), leading to inflammation. Mice with MBH-specific expression of IκB-α, which negatively regulates the canonical NF-κB pathway (Morotti et al. 2017; Perkins 2012), promote lifespan extension, whereas mice with MBH-specific expression of IKKβ, which activates NF-κB signaling, shorten life span (Zhang et al. 2013) (Table 18.1). In addition, activation of NF-κB signaling decreases transcription gonadotropin-releasing hormone (GnRH) transcription, and intracerebroventricular injection of GnRH ameliorates reductions in muscle endurance, the size of muscle fiber, skin dermal thickness,

neurogenesis, and cognition. These results suggest that the canonical NF-κB signaling pathway in the MBH plays an important role in systemic aging and longevity through suppression of hypothalamic inflammation. It would be of great interest to investigate the role of noncanonical NF-κB pathway in mammalian longevity.

18.2.4 Hypothalamic Stem Cells

In mice and humans, adult neurogenesis occurs at limited regions of the brain including the hippocampal dentate gyrus, subventricular zone/olfactory bulb, hypothalamic arcuate nucleus (Arc)/median eminence, striatum/substantia nigra, prefrontal cortex, and amygdala (Gage 2019). Numerous studies reveal that generation of new neurons in these areas serves an important role in various physiological functions such as cognition, mood and anxiety, stress response, sexual/mating behavior, metabolism, and weight homeostasis (Jurkowski et al. 2020).

The hypothalamus is one of well-studied brain regions where adult neurogenesis occurs (Jurkowski et al. 2020; Kostin et al. 2021). The level of adult neurogenesis significantly declines with advanced age in the hypothalamus as well as other brain regions in mice (Zhang et al. 2017). In humans, although age-associated decline in hippocampal neurogenesis is still debatable, its level decreases accompanied with cognitive decline (Tobin et al. 2019). It has been reported that MBH-specific depletion of hypothalamic neural stem/progenitor cells (htNSCs) display age-associated physiological changes including low levels of locomotion, muscle endurance, coordination, treadmill, and cognition and shorten life span (Zhang et al. 2017) (Table 18.1). Remarkably, the implantation of htNSCs, expressing dominant-negative IκB-α that helps survival of htNSCs, into the MBH promotes lifespan extension (Table 18.1). As for mechanisms, htNSCs regulate the secretion of exosomes and exosomal microRNAs. The levels of these exosomal microRNAs in the cerebrospinal fluid significantly decline with age, suggesting that htNSCs control age-associated pathophysiology through hypothalamic microRNAs (Zhang et al. 2017). Cellular senescence, defined by increases in p15, p16Ink4a, and senescence associated-β-galactosidase staining, and a decrease in DNA repair proteins, occurs in NSCs both in vitro and in vivo (Bedrosian et al. 2021; Daniele et al. 2016; Nicaise et al. 2019, 2020). A recent study demonstrated that long noncoding RNA (IncRNA) Hnscr, which stabilizes Y-box binding protein 1, is highly expressed in young htNSCs but is decreased during the aging process (Xiao et al. 2020). Depletion of *Hnscr* in htNSCs accelerates the senescence of htNSCs and promotes aging-related physiological symptoms (Xiao et al. 2020). Although further studies are necessary to investigate whether such regulatory systems are also applicable to other NSCs, maintaining proper amount of neural stem cells (NSCs) within the MBH is indeed beneficial to maintain body health.

18.2.5 Temperature-Sensitive Neurons

The hypothalamus governs the homeostatic response to maintain body temperature. It has been a long-standing question whether thermoregulation is associated with the regulation of aging and longevity. Dietary restriction decreases core body temperature in mice, monkeys, and humans (Lane et al. 1996; Redman et al. 2018; Tabarean et al. 2010; Weinert and Waterhouse 2007). Notably, differential body temperature under dietary restriction is prominent during the rest period (the dark phase in humans, and the light time in rodents) (Redman et al. 2018; and our unpublished data). At the same time, the alteration of core body temperature with age is varied in humans (Kenney and Munce 2003; Waalen and Buxbaum 2011). Despite its complexity, the National Institutes of Aging-Funded Baltimore Longitudinal Study of Aging found that participants with lower median body temperatures live substantially longer than those with higher body temperatures (Roth et al. 2002).

Studies using mouse models indicate a potential link between thermoregulation and longevity. Brain-specific *uncoupling protein 2 (Ucp2)*-overexpressing transgenic mice display a lowered core body temperature and increase energy efficiency by lowering the temperature within the hypothalamus. These *Ucp2*-overexpressing transgenic mice show longer life span compared to control mice in both males and females, suggesting that chronic lowered core body temperature might lead to prolonged life span (Conti et al. 2006) (Table 18.1). A recent study found hypothalamic metabolic variations under dietary restriction-mediated hypothermia are induced by citrulline–nitric oxide cycle and leucine enkephalin (Guijas et al. 2020). On the other hand, under low nutrient condition such as fasting, both long-lived BRASTO and control mice reduce body temperature, but the level of body temperature in BRASTO mice is higher than control mice, whereas *Sirt1*-knockout mice further decrease their body temperature (Satoh et al. 2010). In addition, old BRASTO mice display higher nighttime body temperature compared with old controls (Satoh et al. 2013). Thus, flexible adaptation of our body temperature to environmental stimuli might be critical to delay the aging process and to extend health span. If this is true, *Ucp2*-overexpressing transgenic mice might display greater responses in body temperature under fasting or other circumstances.

18.2.6 Neurons in the Arc

Glucose homeostasis links to our body health and may be involved in aging/longevity control. The Arc of the hypothalamus plays a critical role in glucose metabolism and contains glucose sensing neurons, neuropeptide Y (Npy)/agouti-related protein (Agrp) neurons, and Pomc/cocaine amphetamine-regulated transcript (Cart) neurons. Among them, it has been reported that hypothalamic-specific *Npy*-overexpressing transgenic rats live longer than control rats (Michalkiewicz et al. 2003) (Table 18.1). Moreover, *Npy*-knockout mice totally abrogate dietary

restriction-induced lifespan extension, revealing that Npy in the Arc is necessary to promote beneficial effects of dietary restriction (Chiba et al. 2014). Of note, distribution of Npy receptors and its responsiveness to dietary restriction seems a bit different between mice and rats (Dumont et al. 1998). Detailed mechanisms of Npy neurons in aging and longevity still remain elucidated.

One of the important roles of the hypothalamus is humoral secretion that could also be linked to longevity control. Growth hormone (GH) and insulin/insulin-like growth factor-1 (IGF-1) signaling are identified as longevity pathways by using Ames and Snell dwarf mice (Brown-Borg et al. 1996; Flurkey et al. 2002). Brain-specific *Igf-1 receptor*-knockout mice and brain-specific *insulin receptor substrate 2*-knockout mice display lifespan extension (Kappeler et al. 2008; Taguchi et al. 2007) (Table 18.1). Thus, suppression of GH and insulin/IGF-1 signaling within the brain increases life expectancy. In addition, *Gh-releasing hormone (Ghrh) receptor*-mutant mice show lifespan extension (Flurkey et al. 2001). The GHRH, which stimulates GH secretion from the anterior pituitary, is mainly secreted from neuronal populations within the Arc. Therefore, GHRH-secreting neurons in the Arc might be a central regulator promoting the effect of GH/IGF-1 signaling pathway in longevity.

18.3 Potential Common Mechanisms of Aging and Longevity via the Hypothalamus

As described, a number of signaling pathways, molecules, and cellular populations within the hypothalamus have been reported to control mammalian longevity. How do these multiple factors come together in lifespan extension? One possibility is that longevity is regulated through the common physiological changes in metabolism, circadian rhythm, and sleep-wake patterns which are primarily modulated by hypothalamic function (Fig. 18.1).

Metabolism. Sirt1, mTOR signaling, NF-κB signaling, and htNSCs in the hypothalamus have a role in systemic metabolism. Mice with deletion of *Sirt1* in Pomc

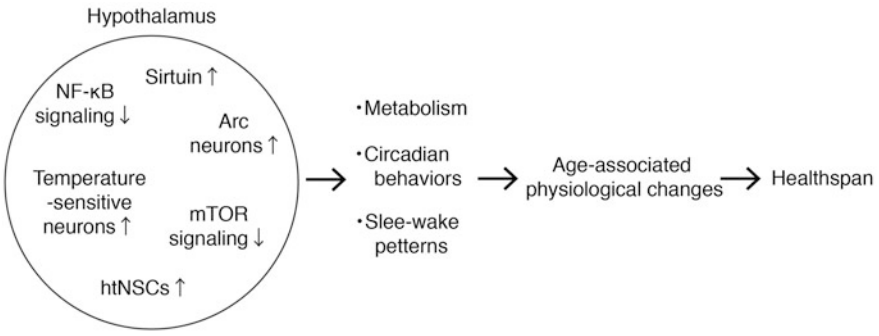

Fig. 18.1 The hypothalamus regulates health span through common physiological control

neurons localized in the Arc display a defect in brown adipose tissue-remodeling through downregulation of sympathetic nervous tone under high-fat diet (Ramadori et al. 2010). Steroidogenic factor-1 (SF-1)-specific deletion of *Sirt1* in the ventromedial hypothalamus promotes insulin resistance and develops dietary type 2 diabetes mellitus, while overexpression of *Sirt1* in SF-1 neurons protects against diet-induced obesity (Ramadori et al. 2011). It has been reported that intracerebroventricular injection of leucine increases hypothalamic mTOR signaling and decreases food intake and body weight, while intracerebroventricular rapamycin blocks its effect on food intake (Cota et al. 2006). On the other hand, genetic deletion of *Tsc1* in Pomc neurons, leading to chronic activation of mTOR signaling specifically in Pomc neurons, results in dysregulation of Pomc neurons and hyperphagic obesity (Mori et al. 2009). These results suggest that the effect of mTOR signaling in food intake might differ between each subtype of hypothalamic neurons. Moreover, htNSC-specific IKKβ/NF-κB activation leads to depletion and impairment of neuronal differentiation of htNSCs, and to the development of obesity and prediabetes (Li et al. 2012). Overnutrition promotes hypothalamic neuroinflammation in overnutritional diseases (Cai and Khor 2019). Therefore, metabolic defects and hypothalamic neuroinflammation induced by hypothalamic NF-κB activation reciprocally exacerbate physiological outcomes. On the other hand, some long-lived mouse models display adverse metabolic effects such as insulin resistance, hyperinsulinemia, and hyperleptinemia (Kappeler et al. 2008; Taguchi et al. 2007). Whether improvement of glucose metabolism is necessary to promote lifespan extension still needs to be elucidated, but exercise and appropriate diet would definitely result in delaying the onset or progression of the hallmarks of aging (López-Otín et al. 2016).

Circadian behaviors. Sirt1 in the hypothalamus mediates circadian behaviors. Knockdown of *Sirt1* in the DMH and LH promotes lowered physical activity and body temperature during the active period (the dark phase) (Satoh et al. 2013). Intrinsic circadian period is longer in brain-specific *Sirt1*-knockout mice, as well as old mice, while shorter in brain-specific *Sirt1*-overexpressing transgenic mice (Chang and Guarente 2013). Given that neurons in the suprachiasmatic nucleus (SCN), well-known as our body's master clock, generate intrinsic circadian oscillation (Welsh et al. 2010), Sirt1 controls the proper function of SCN neurons in circadian oscillation, possibly through molecular machinery of clock genes such as *Bmal1* and *Clock*. Indeed, Sirt1 binds Clock-Bmal1 in a circadian manner and promotes the deacetylation and degradation of Per2 (Asher et al. 2008). mTOR also affects the circadian clock in the SCN (Cao et al. 2013; Liu et al. 2018; Ramanathan et al. 2018). In addition, transplantation of fetal SCN into adult hamsters promotes increased locomotion and lifespan extension (Hurd and Ralph 1998). Therefore, maintaining SCN function might prolong our health span (Acosta-Rodríguez et al. 2021), and its effect may be mediated via longevity-related molecules/cells or signaling pathways. Furthermore, not only the SCN itself but communication between the SCN and Arc also organizes physiological rhythms. Microcuts removing SCN-Arc interconnectivity in Wistar rats result in a loss of rhythmicity in locomotor activity, corticosterone, and body temperature during constant dark

conditions (Buijs et al. 2017). Consistently, a recent study demonstrates that Kisspeptin 1-expressing neurons in the Arc control daily timing of food intake, along with the circadian regulation of locomotor activity, sleep, and core body temperature (Padilla et al. 2019). Since a deficit in circadian rhythms could control various physiological traits including metabolism, maintaining the central circadian clock would provide beneficial effects against age-associated physiological changes.

Sleep-wake patterns. Growing evidence suggests that hypothalamic Sirt1, mTOR signaling, and NSCs regulate sleep-wake patterns. Besides Sirt1 signaling in the DMH (and/or LH) regulating delta power which was described in Sect. 18.2.1, DMH-specific knockdown of *PR-domain containing factor 13*, a potential downstream gene of Sirt1, in mice display low delta power (Satoh et al. 2015), suggesting that Sirt1/Prdm13 signaling in the DMH is involved in sleep control. Knockout mouse model involving the inactivation of *Tsc1* in neurons and astrocytes decreases REM sleep and impairs sleep-wake differentiation between light and dark phases through upregulation of mTOR activity and orexin expression in the hypothalamus (Zhang et al. 2020). Such abnormal sleep is rescued by the administration of rapamycin (Zhang et al. 2020). In addition, chronic infusion of an antimitotic agent cytosine-β-D-arabinofuranoside, known as an AraC, into the lateral ventricle suppresses hypothalamic cell proliferation and neurogenesis, and leads to sleep-wake instability (sleep fragmentation) in young mice (Kostin et al. 2019). Finally, recent studies demonstrated that activation of neuronal nitric oxide synthase-expressing glutamatergic neurons in the preoptic area of the hypothalamus or optogenetic stimulation of galanin neurons in the ventrolateral preoptic area of the hypothalamus decreases body temperature and increases NREM sleep (Harding et al. 2018; Kroeger et al. 2018), whereas ablation of galanin neurons in the lateral preoptic area of the hypothalamus increases core body temperature and promotes sleep fragmentation (Ma et al. 2019). Thus, the relationship between sleep control and thermoregulation is of great interest not only for physiological function but also for the control of aging and longevity.

18.4 Perspective

Recent studies of single-cell genomics, transcriptomics, and proteomics elucidate the diversity and responsiveness of hypothalamic cells under several physiological conditions including aging (Armand et al. 2021; Campbell et al. 2017; Chen et al. 2017; Mickelsen et al. 2019; Moffitt et al. 2018; Ximerakis et al. 2019). These studies definitively help us to identify specific cell types, neuronal populations, and neuronal networks that are significantly altered with aging and might be involved in the process of aging. Deep analyses of hypothalamic function during aging will shed light on mechanisms underlying the process of aging and longevity in mammals. Finally, since aging is one of the most critical risk factors for various types of diseases and symptoms, determination of biological age in people, even those who are the same chronological age, would be useful to define healthy aging and possibly

for prediction of life span (Xia et al. 2017). Several studies have identified a number of molecular biomarkers such as DNA methylation (Horvath 2013), telomere length (Mather et al. 2011), immune function (Alpert et al. 2019), advanced glycosylation end products (Krištić et al. 2014), cellular senescence (Wang and Dreesen 2018), and noninvasive biometrics such as three-dimensional facial morphology (Chen et al. 2015) and frailty index (Kim et al. 2017). It would be of great interest to evaluate whether phenotypic signatures of brain function, in particular, hypothalamic function might be a good biomarker of aging in humans.

Acknowledgments I deeply appreciate Dr. Sean Johnson for his insights and comments for this manuscript. This work is supported by grants JSPS KAKENHI (17H07417, JP18H03186, 20K21780), the Japan Agency for Medical Research and Development (JP20gm5010001s0604), the Sleep Medicine Foundation, the Takeda Science Foundation, and the Research Fund for Longevity Sciences from the NCGG (28–47).

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Part VI
Aged Brain: Neurodegenerative Diseases

Chapter 19
PET Imaging of Amyloid and Tau in Alzheimer's Disease

Nobuyuki Okamura and Ryuichi Harada

Abstract Alzheimer's disease (AD) is characterized by the extracellular deposition of amyloid-β (Aβ) plaques, intracellular accumulation of hyperphosphorylated tau protein, and neuronal loss. Recent advances in the development of positron emission tomography (PET) radiotracers have clarified the neuropathological changes in the living brain. Amyloid PET tracers such as [^{11}C]PiB, [^{18}F]flutemetamol, [^{18}F]florbetapir, [^{18}F]florbetaben, and [^{18}F]NAV4694 are able to detect the presence or absence of Aβ pathologies in the early stage of AD. Recent amyloid PET studies have demonstrated that Aβ accumulation is one of the earliest events in AD that precedes cognitive decline and that the amount of Aβ reaches a plateau upon the onset of dementia. Amyloid PET is essential for the development of new therapeutic strategies aimed at reducing Aβ burden and preventing the onset of dementia. After successful clinical application of amyloid PET tracers, several tau PET tracers, including [^{18}F]flortaucipir and [^{18}F]MK-6240, have been developed and introduced in clinical research. These tracers are highly sensitive to paired helical filaments of tau but are less sensitive to non-AD tau deposits. As expected from postmortem studies, the amount and spatial extent of tau tracer retention are strongly associated with the clinical severity and phenotype of dementia. Tau PET could thus provide spatiotemporal information on the progression of tau pathology and facilitate accurate diagnosis and severity assessment of dementia when used in conjunction with amyloid PET.

Keywords Alzheimer's disease · Amyloid-β · Imaging · Positron emission tomography (PET) · Tau

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_19

19.1 Introduction

Alzheimer's disease (AD) is the leading cause of dementia in the elderly. Although the clinical diagnosis of AD has been based on progressive impairment of episodic memory and executive function, the confirmation of its diagnosis still relies on postmortem examination of specific neuropathologies in the brain. The pathological hallmarks of AD are deposition of senile plaques (SPs) and neurofibrillary tangles (NFTs), which consist of amyloid-β (Aβ) and tau proteins, respectively. Aβ is a polypeptide of 39–43 amino acids derived from proteolytic cleavage of the amyloid precursor protein (APP). The amyloid cascade hypothesis is a dominant theory of AD pathogenesis (Hardy and Higgins 1992). In this hypothesis, Aβ deposition is considered the main event that leads to the deposition of tau protein, neurodegeneration, and cognitive decline. Recent positron emission tomography (PET) studies have shown that Aβ accumulation is one of the earliest events in AD that precedes cognitive decline and that the amount of Aβ reaches a plateau by the time the clinical symptom of dementia appears (Krishnadas et al. 2021; Okamura et al. 2009). These findings strongly suggest that Aβ is a key molecule in the etiology of AD and that the accumulation of Aβ triggers subsequent neurodegenerative processes. Tau is a microtubule-associated protein that is abundant in the axons of neurons. Six isoforms of tau are generated by alternative mRNA splicing of a single gene transcript. They are classified as 3-repeat (3R) and 4-repeat tau (4R) proteins based on the number of microtubule-binding domains. NFTs are aggregates of hyperphosphorylated tau proteins called paired helical filaments (PHFs), which are composed of equal amounts of the 3R and 4R tau isoforms. Although Aβ plaques may play a key role in AD pathogenesis, the amount of NFTs correlates better with the severity of cognitive impairment in patients with dementia (Nelson et al. 2012). Furthermore, the spatial distribution of tau deposits strongly influences the clinical phenotype of dementia (Phillips et al. 2018), suggesting that the accumulation of tau in neurons affects the neurodegenerative process more directly than Aβ.

Biomarkers of Aβ, tau, and neurodegeneration have been developed in the past few decades and incorporated into the diagnostic criteria for AD. Jack et al. proposed a new biomarker-based classification called the ATN system, which was designed to characterize individuals in the AD continuum (Jack et al. 2016). AD biomarkers are divided into three binary classes: "A" represents Aβ biomarkers including amyloid PET or Aβ1-42 concentration in cerebrospinal fluid (CSF), "T" represents tau biomarkers including tau PET or CSF phospho-tau, and "N" represents neurodegeneration biomarkers including structural MRI, FDG-PET, and CSF total tau. Assessment of ATN status in asymptomatic individuals will provide important insights into underlying AD pathogenesis and an opportunity for recruitment into secondary prevention trials using disease-modifying therapies. This review focuses on two major biomarkers of "A" and "T": amyloid PET and tau PET.

19.2 Amyloid PET Imaging

Currently, it is widely believed that amyloid PET is the most reliable biomarker for Aβ pathology in the living brain. In this technique, the density of Aβ deposits can be measured using a specific radiotracer and a PET scanner. Several β-sheet binding compounds have been developed for PET imaging of Aβ deposits since the beginning of the century (Furumoto et al. 2007). The chemical structures of amyloid PET tracers are shown in Fig. 19.1. The first tracer for imaging amyloid was [^{18}F]FDDNP, which binds to both SPs and NFTs (Agdeppa et al. 2001). Several ^{11}C-labeled tracers, including [^{11}C]BF-227 and [^{11}C]SB-13, have also been developed as amyloid PET tracers and have successfully differentiated AD patients from normal individuals (Kudo et al. 2007; Verhoeff et al. 2004). The most widely used ^{11}C-labeled tracer is [^{11}C]PiB, which is a derivative of the amyloid-binding dye thioflavin-T (Klunk et al. 2004). In vitro binding studies demonstrated that PiB binds to synthetic Aβ fibrils and Aβ-rich AD brain homogenates with high affinity (Mathis et al. 2003). An autoradiographic study using AD brain tissues also showed specific binding of PiB to fibrillar Aβ deposits (Klunk et al. 2004). Human PET studies have clearly distinguished patients with AD from healthy elderly individuals (Klunk et al. 2004). Significant PiB retention was detected in the broad neocortical areas of the AD brain. The density of in vivo PiB binding was highly correlated with regional Aβ deposition in postmortem brains. Although [^{11}C]PiB can create high-contrast images, the short half-life of ^{11}C ($t_{1/2}$ = 20 min) requires an on-site cyclotron and thus limits its routine clinical use. Therefore, several ^{18}F-labeled PET tracers with a half-life of 110 min have been developed to overcome the limitations of ^{11}C-labeled tracers. Today, one thioflavin-T derivative ([^{18}F]flutemetamol) and two stilbene derivatives ([^{18}F]florbetapir and [^{18}F]florbetaben) are commercially available as

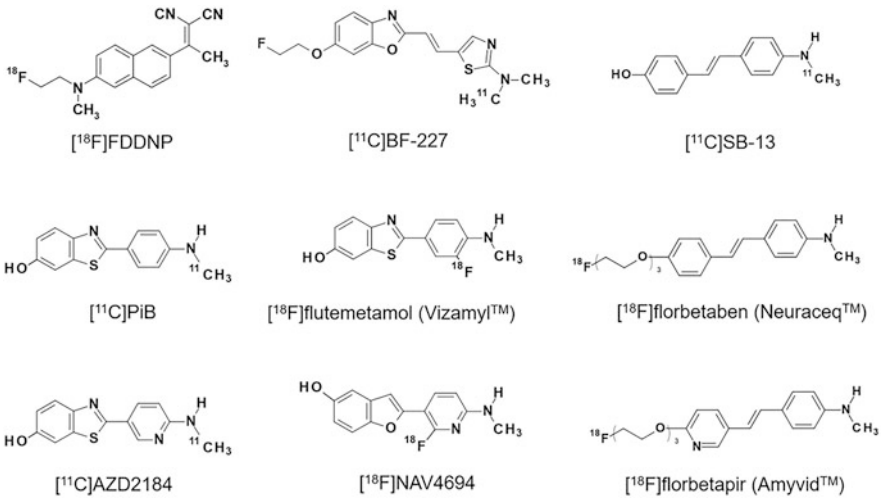

Fig. 19.1 Chemical structures of amyloid PET tracers

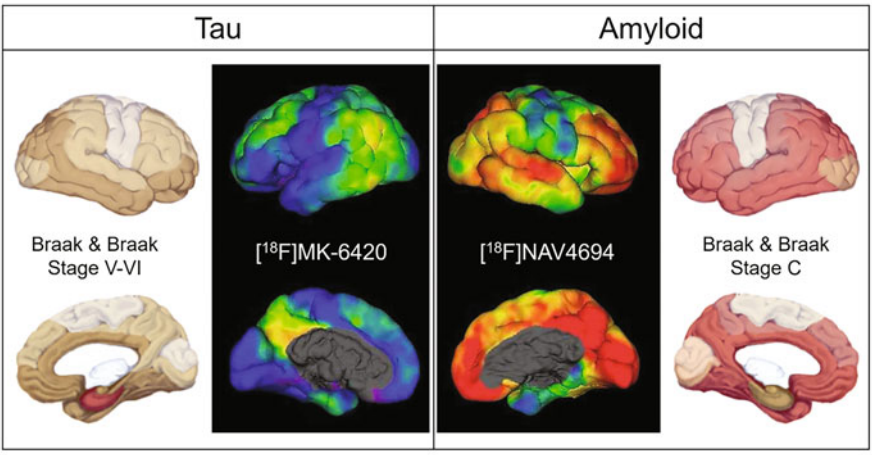

Fig. 19.2 Amyloid PET image using [^{18}F]NAV4694, tau PET image using [^{18}F]MK-6240, and Braak staging of amyloid and tau (Courtesy of Professors Christopher Rowe and Victor Villemagne, Austin Heath, Melbourne, Australia)

^{18}F-labeled amyloid PET tracers (Krishnadas et al. 2021). All three tracers bind with high affinity to Aβ fibrils and truly depict the distribution of Aβ deposits in the brain. Regional binding of these tracers is strongly correlated with PiB retention and Aβ burden measured during autopsy (Ikonomovic et al. 2020). These amyloid PET tracers show white matter retention even in the Aβ-negative population, which reflects tracer binding to myelin (Catafau and Bullich 2015). Lipophilic ^{18}F-labeled tracers tend to show a greater amount of binding in white matter than [^{11}C]PiB. In the clinical assessment of Aβ burden, tracer uptake in white matter may interfere with the visual interpretation of PET images. Thus, each pharmaceutical company developed a training program for users of ^{18}F-labeled PET tracers to improve the accuracy of amyloid PET visual reading. Following the development of three ^{18}F-labeled amyloid tracers, [^{18}F]NAV4694 (AZD4694) was newly designed to reduce the nonspecific binding to white matter and improve the signal-to-noise ratios of the other ^{18}F-labeled amyloid tracers (Juréus et al. 2010). A head-to-head comparison of [^{11}C]PiB and [^{18}F]NAV4694 showed a similar dynamic range of neocortical tracer retention in AD and identical low nonspecific binding in the white matter (Rowe et al. 2013).

In sporadic AD patients, all these amyloid tracers show a similar pattern of retention in the neocortex and are able to clearly differentiate AD patients from healthy elderly people. The spatial pattern of tracer retention was consistent with that of fibrillar Aβ deposition in the postmortem AD brain (Fig. 19.2). In AD brains, tracer retention is diffusely observed in the frontal, temporal, parietal, and cingulate cortices yet relatively spared in the sensorimotor, occipital, and mesial temporal cortices where the density of Aβ deposits is low. The regional distribution of tracer retention in carriers of familial AD mutations is different from that in sporadic AD

patients. In carriers of familial AD mutation, amyloid tracer retention is prominently elevated in the caudate nuclei (Tentolouris-Piperas et al. 2017). In a phase III study of ^{18}F-labeled amyloid tracers, visual reads of PET images had high sensitivity and specificity for the assessment of Aβ burden (Collij et al. 2021). Thus, amyloid PET can be utilized as an established biomarker of Aβ pathology in the human brain. Previous studies have shown little or no correlation between Aβ burden and cognitive impairment in AD in contrast to the good correlation of NFTs with cognition (Nelson et al. 2012). These findings suggest that Aβ plaques detected by amyloid PET do not directly cause cognitive impairment, at least in the symptomatic stage of AD.

In the clinical setting, amyloid PET is useful for the differential diagnosis of dementia. Aβ pathology is not frequently detected in cases of frontotemporal lobar degeneration (FTLD), including behavioral variant frontotemporal dementia, semantic dementia, and progressive non-fluent aphasia. Thus, amyloid PET can be used to differentiate between FTLD and AD (Krishnadas et al. 2021). However, positive amyloid PET scans are observed in some neurodegenerative conditions such as dementia with Lewy bodies (DLB). Previous studies have shown that more than half of DLB cases have cortical Aβ plaques (Quigley et al. 2011). Therefore, amyloid PET should not be used to differentiate between DLB and AD. The criteria for the appropriate use of amyloid PET recommend that it should only be used if the suspected diagnosis is AD, but the diagnosis remains uncertain after comprehensive evaluation by a dementia expert and the information on the presence or absence of Aβ pathology is expected to increase diagnostic certainty and alter management (Johnson et al. 2013). Appropriate conditions include (1) patients satisfying core clinical criteria for possible AD because of unclear clinical presentation, due to either an atypical clinical course or an etiologically mixed presentation, and (2) patients with progressive dementia and an atypically early age of onset (usually defined as $\leq$65 years of age). Negative findings on amyloid PET exclude the presence of AD-related pathology. Amyloid PET has been reported to be negative in 20% of persons referred for a clinical diagnosis of AD (Krishnadas et al. 2021), suggesting that AD is often clinically misdiagnosed without the use of Aβ biomarkers.

Aβ deposition is a gradual process that precedes tau deposition, neurodegeneration, and the onset of dementia by approximately two decades (Fig. 19.3). Therefore, amyloid PET can be used to assess Aβ pathology in the early stages of AD. Mild cognitive impairment (MCI) is a heterogeneous clinical syndrome. Amyloid PET can identify individuals with MCI due to AD pathology (prodromal AD) and predict the progression to AD dementia in the near future. More than half of MCI subjects have been reported to show a high Aβ burden in amyloid PET. Amyloid-positive MCI cases show a higher rate of progression to AD dementia (Okello et al. 2009; Shao et al. 2010; Villemagne et al. 2011). Substantial Aβ deposits have been reported in the postmortem brains of non-demented older adults (Price et al., 2009). Amyloid PET studies have shown neocortical accumulation of Aβ before the onset of cognitive decline and neurodegenerative changes in AD (Mintun et al. 2006; Rowe et al. 2007). Amyloid PET positivity parallels the prevalence of low memory scores and dementia in the general population, predating

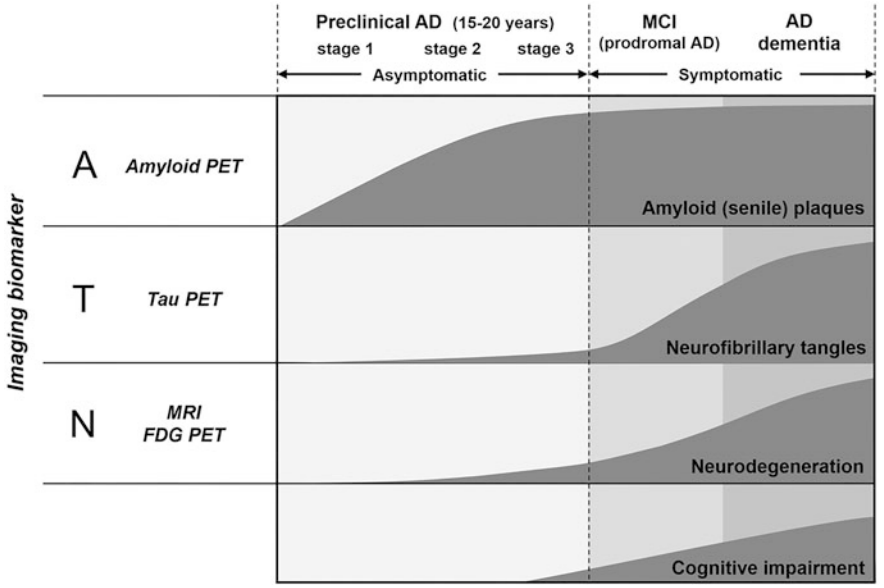

Fig. 19.3 Chronological changes in amyloid, tau, neurodegeneration, and cognitive impairment

it by 10–30 years (Jansen et al. 2018; Sperling et al. 2011). Longitudinal PET studies have estimated that it takes 12 years for normal levels to reach the threshold of amyloid PET positivity and another 20 years to reach the average amyloid levels observed in established AD patients (Villemagne et al. 2013). The ApoE genotype is known to be a major genetic risk factor for sporadic AD. Amyloid PET studies have clarified that the Aβ burden in cognitively normal elderly people is associated with the ApoE ε4 gene dose (Ossenkoppele et al. 2015; Reiman et al. 2009; Villemagne et al. 2011). Although postmortem studies have suggested a weak association between Aβ and cognition in the symptomatic stage of AD, amyloid PET studies have shown an association between Aβ and low memory performance in the asymptomatic stage of AD (Chételat et al. 2011; Jansen et al. 2018). As observed in MCI, Aβ deposition is associated with prognosis in cognitively unimpaired elderly people (Villemagne et al. 2011). Cognitive decline in cognitively unimpaired individuals was more rapid in amyloid-positive subjects than in amyloid-negative subjects (Donohue et al. 2017). Normal elderly individuals with Aβ pathology were classified as having preclinical AD, which includes cognitively normal individuals with abnormal Aβ biomarkers (stage 1); those with abnormal Aβ and neurodegeneration markers (stage 2); and those with abnormal Aβ, neurodegeneration, and subtle cognitive changes (stage 3) (Sperling et al. 2011). Persons with preclinical AD showed a greater progression rate (11% for stage 1, 26% for stage 2, and 56% for stage 3) to symptomatic AD than normal individuals (Vos et al. 2013). Therefore, amyloid PET is considered useful for the screening of elderly individuals who are at a high risk of progression to dementia. However,

appropriate use criteria for amyloid PET do not recommend the use of amyloid PET in asymptomatic individuals who are at risk of AD because a disease-modifying treatment has not yet been established (Johnson et al. 2013).

Amyloid PET is necessary for the development of new therapeutic strategies that aim to reduce the Aβ burden in the brain. Previous PET studies have shown the reduction of Aβ deposition after the treatment with an anti-Aβ antibody. Treatment with bapineuzumab for 78 weeks reduced cortical ^{11}C-PiB retention compared to both baseline and placebo (Rinne et al. 2010). Treatment with aducanumab also reduced brain Aβ plaques as measured by florbetapir PET in a dose- and time-dependent manner (Sevigny et al. 2016). Amyloid PET can thus assist in the evaluation of treatment efficacy in clinical trials of anti-amyloid therapies. Although the successful reduction of Aβ burden has been demonstrated in clinical trials of these anti-Aβ drugs, most of the drugs failed to stop the progression of cognitive decline. These failures imply that Aβ may not be an optimal therapeutic target to prevent the progression of AD. However, many researchers believe that anti-amyloid therapy is effective when the treatment starts, before irreversible neuronal damage occurs. For this reason, recent clinical trials have targeted cognitively unimpaired individuals at risk of developing sporadic AD and autosomal dominant AD individuals without cognitive dysfunction (Panza et al. 2018). In these clinical trials, amyloid PET has been used to confirm Aβ deposition for participant registration and to evaluate the treatment efficacy of anti-Aβ drugs.

19.3 Tau PET Imaging

Tau pathology is another neuropathological hallmark of AD, which follows a stereotyped spatiotemporal pattern that begins in the transentorhinal cortex, spreads to the entorhinal and hippocampal cortices then to the lateral temporal lobes, and finally reaches the association and primary sensory cortices. Contrary to Aβ pathology, tau pathology is closely associated with neuronal loss and cognitive impairment in AD. In addition, tau pathology is also found in the brains of non-AD tauopathies, including FTLD, corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and chronic traumatic encephalopathy (CTE). Therefore, tau PET imaging would provide spatiotemporal information on the progression of tau pathology associated with cognition in the living brain and facilitate accurate diagnosis of dementia using a combination of amyloid PET, precise assessment of disease severity and therapeutic efficacy, and patient enrollment for disease-modifying therapeutic trials.

Many tau PET tracers have been developed by researchers over the past 10 years (Fig. 19.4). In general, tau PET ligands recognize the cross β-sheet structure of PHFs that are composed of hyperphosphorylated tau. Tau aggregates in tauopathies contain different isoform compositions: 3R and 4R tau in AD and CTE, predominant 4R tau in CBD and PSP, and 3R tau in Pick's disease. Cryo-electron microscopy (Cryo-EM) revealed the atomic structure of PHFs derived from AD brain tissues.

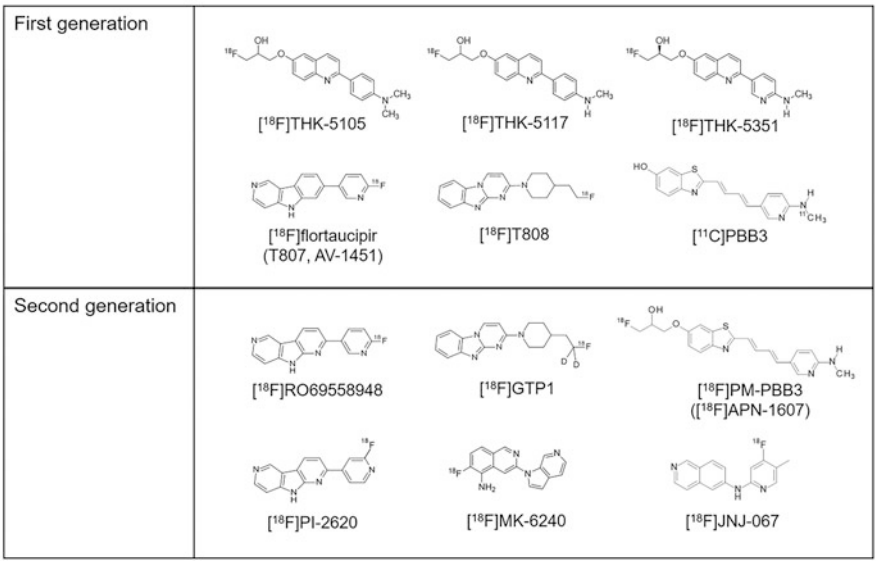

Fig. 19.4 Chemical structures of tau PET tracers

Although Cryo-EM analysis of PHFs identified the binding sites of tau PET tracers on PHFs and straight filaments (SFs) (Shi et al. 2021), the binding nature of tau PET tracers remains unclear because tau PET tracers do not completely share binding sites. Cryo-EM analyses have also revealed the atomic structure of tau aggregates from Pick's disease, CBD, and CTE. These results suggest that tau protein adopts a unique fold in each disease, suggesting difficulty in developing isoform-specific tau PET tracers.

We have developed several ^{18}F-labeled 2-arylquinoline derivatives ([^{18}F]THK-5105, [^{18}F]THK-5117, and [^{18}F]THK-5351) for imaging tau pathology in vivo and tested them in humans (Harada et al. 2018a, b; Okamura et al. 2013, 2014, 2018). The optimized derivative, [^{18}F]THK-5351, showed prominent tracer retention in the inferior temporal cortex of patients with AD, with preferable pharmacokinetics and metabolism in humans (Harada et al. 2016). [^{18}F]THK-5351 also showed elevated tracer retention in sites susceptible to tau pathology in non-AD tauopathies such as CBD and PSP (Ishiki et al. 2017; Kikuchi et al. 2016). However, [^{18}F]THK-5351 also showed prominent tracer retention in the basal ganglia of healthy control brains, which is called "off-target binding." The first generation of tau PET tracers includes the [^{18}F]THK series, the first approved tau PET tracer flortaucipir ([^{18}F]AV-1451 or [^{18}F]T-807), and [^{11}C]PBB3. These tracers suffered from off-target binding to the basal ganglia and choroid plexus. The basal ganglia are frequent sites of tau aggregates in non-AD tauopathies. The choroid plexus was near the hippocampus, and tau deposition occurred in the early phase. Therefore, the off-target binding of radiotracers in these regions may interfere with the interpretation of PET images. Further studies identified the off-target binding substrate of [^{18}F]THK-5351 as

monoamine oxidase-B (MAO-B) (Ng et al. 2017). This finding was confirmed by neuropathological validation studies in patients with AD and PSP who underwent antemortem [^{18}F]THK-5351 PET scans and postmortem examination (Harada et al. 2018a, b; Ishiki et al., 2018). Currently, [^{18}F]THK-5351 has limited utility as a biomarker of tau pathology. However, MAO-B may be a promising target for imaging reactive astrogliosis. In fact, [^{18}F]THK-5351 retention has been detected in the frequent sites of astrogliosis in various neurodegenerative diseases (Okamura et al. 2018). Therefore, we developed a selective and reversible ^{18}F-labeled MAO-B PET tracer, [^{18}F]SMBT-1, for imaging astrogliosis in the brain, through the compound optimization of [^{18}F]THK-5351 (Harada et al. 2021). As for [^{18}F]flortaucipir, MAO-A, MAO-B, and neuromelanin have been identified as putative off-target binding substrates. However, it remains unclear whether these molecules contribute to the off-target binding of [^{18}F]flortaucipir in vivo.

To overcome these limitations, second-generation tau PET tracers have been developed by pharmaceutical companies (Leuzy et al. 2019). Among them, [^{18}F]MK-6240, which was originally developed by Merck, is the most promising tau PET tracer. [^{18}F]MK-6240 binds to tau aggregates with high affinity and selectivity without significant off-target binding (Lohith et al. 2019; Walji et al. 2016). Although most second-generation tau tracers do not bind to non-AD tau aggregates, [^{18}F]PI-2620 and [^{18}F]PM-PBB3 ([^{18}F]APN-1607) have been reported to detect non-AD tau aggregates (Kroth et al. 2019; Tagai et al. 2021). However, comparative autoradiography of second-generation tau tracers demonstrated that most tau tracers, including [^{18}F]PI-2620, showed no interaction with tau aggregates in PSP (Yap et al. 2021). This result was consistent with that of the [^{18}F]RO948 PET study in AD and other neurodegenerative diseases (Leuzy et al. 2020), but not with the results of the [^{18}F]PI-2620 PET study (Brendel et al. 2020). Recently, a novel class compound, [^{18}F]CBD-2115, was reported to bind to non-AD tau aggregates with high affinity, although clinical PET studies have not yet been performed using it (Lindberg et al. 2021). Further validation studies are required to confirm the ability of tau PET tracers to detect non-AD tau aggregates.

Tau PET recapitulates the Braak stages of neurofibrillary tau accumulation that follows stereotyped spatiotemporal patterns (Pascoal et al. 2020; Scholl et al. 2016; Schwarz et al. 2016). Autopsy validation studies have been performed on various patients, including AD and non-AD patients, who underwent antemortem [^{18}F]flortaucipir PET scans (Lowe et al. 2020; Soleimani-Meigooni et al. 2020). A large autopsy validation study of antemortem flortaucipir PET demonstrated that [^{18}F]flortaucipir PET could detect advanced tau pathology (Braak stage V-VI) (Fleisher et al. 2020). However, [^{18}F]flortaucipir PET showed low sensitivity for the detection of early tau pathology (Braak stages I–IV). Based on these results, flortaucipir was approved by the US Food and Drug Administration (FDA) to estimate the density and distribution of aggregated tau neurofibrillary tangles in adult patients with cognitive impairment. Autopsy validation studies also suggested that [^{18}F]flortaucipir PET could not be used in the evaluation of non-AD tauopathies, including CTE (Mantyh et al. 2020; Soleimani-Meigooni et al. 2020). Primary age-related tauopathy (PART) is pathologically defined as the presence of

AD-type tau pathology in the medial temporal lobe in the absence of significant amyloid deposition. Although tau PET is expected to detect age-related tau pathology in PART, autopsy validation studies of [^{18}F]flortaucipir suggested low sensitivity for the detection of tau in PART. [^{18}F]MK-6240 seems to be more sensitive than flortaucipir in detecting early tau pathology. The retention of [^{18}F]MK-6240 in the transentorhinal cortex was greater in amyloid-negative (~37%) and amyloid-positive (~68%) cognitively unimpaired elderly people than in those with [^{18}F] flortaucipir (Pascoal et al., 2020). Further autopsy studies of antemortem PET using [^{18}F]MK-6240 are warranted to confirm its sensitivity in detecting early tau pathology.

Abnormal neocortical tau tracer retention has been rarely observed in the absence of neocortical Aβ deposition (Dore et al. 2021; Jack et al. 2019; Pontecorvo et al. 2017), suggesting that Aβ deposition in the neocortex could be a trigger for the spread of tau from the medial to the lateral temporal lobe. Cross-sectional studies have demonstrated that tau PET retention is associated with the degree of cognitive impairment (Johnson et al. 2016; Ossenkoppele et al. 2016). Longitudinal tau PET studies have also demonstrated that the elevation of cortical tau increased in amyloid-positive subjects but not in amyloid-negative subjects (Pontecorvo et al. 2017). Baseline changes in tau PET were significantly associated with changes in brain atrophy and cognitive performance (La Joie et al. 2020; Pontecorvo et al. 2017). The rate of tau accumulation is increased in females and younger amyloid-positive subjects, as well as in subjects with a higher baseline tau load (Smith et al. 2020). The regional distribution of tau accumulation correlates well with glucose hypometabolism in patients with atypical AD, such as those with posterior cortical atrophy, and mirrors the clinical and neuroanatomical variability in patients with AD. This suggests that tau aggregation is closely linked to the patterns of neurodegeneration and clinical manifestations of AD (Ossenkoppele et al. 2016).

Tau PET has been used in clinical trials for disease-modifying therapy. In the phase 2 clinical trial of donanemab, a humanized antibody for pyroglutamate Aβ, [^{18}F]flortaucipir PET was used to exclude patients with high levels of tau deposition because anti-amyloid treatments are considered to show limited efficacy in advanced stage patients with high tau burden (Mintun et al. 2021). The authors also investigated the effect of donanemab on brain tau load as a secondary outcome. Although donanemab did not affect global tau load, a greater reduction in tau was observed in the frontal and temporal lobes. A clinical trial of the anti-Aβ antibody aducanumab also demonstrated a reduction in tau accumulation using [^{18}F]MK-6240. These results suggest that tau PET could be a powerful tool for understanding the effect of amyloid targeting therapeutics on disease progression as well as pathophysiology in the AD spectrum. In the future, tau PET is expected to play an important role in the clinical trial of anti-tau antibodies (Roberts et al. 2020).

19.4 Conclusions

Amyloid and tau PET imaging have been widely used for the assessment of Aβ and tau pathology in the living brain and have contributed toward greater diagnostic accuracy of dementia and better monitoring of the progression of AD-related pathology. These imaging techniques and forthcoming disease-modifying therapies will contribute to the eradication of dementia in the elderly.

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Chapter 20
Presenilin/γ-Secretase in the Pathogenesis of Alzheimer's Disease

Taisuke Tomita

Abstract A genetic study of familial cases of Alzheimer's disease (AD) has revealed that amyloid-β protein (Aβ) is an important pathogenic protein in AD. Aβ is derived from its precursor protein APP by sequential cleavages mediated by β- and γ-secretases. Presenilin (PS), which is one of the major familial AD-linked genes, is a catalytic subunit of the γ-secretase. γ-Secretase is an atypical protease complex composed of four membrane proteins; PS, nicastrin, anterior pharyngeal defective 1 (Aph-1), and presenilin enhancer 2 (Pen-2). Familial AD-linked genetic mutations in PS genes augment the production of the longer form of Aβ, which is an aggregation-prone peptide. However, the development of inhibitors for γ-secretase has been discontinued so far due to side effects. In contrast, the studies of γ-secretase led to new enzymology of intramembrane proteolysis of the transmembrane domains. Furthermore, a novel class of compounds called γ-secretase modulators has been developed. In this article, we discuss the current understanding of the basic and therapeutic aspects of PS/γ-secretase.

Keywords Alzheimer's disease, Amyloid-β, γ-Secretase, Intramembrane proteolysis, Presenilin, Protease

20.1 Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder among dementia. In the AD brain, neurofibrillary tangle and senile plaque are found as pathological characteristics. Tangles are intercellular aggregates of the phosphorylated tau proteins, and the senile plaque is mainly composed of amyloid-β protein (Aβ). Several studies suggest that the deposition of Aβ starts the pathological process of AD, and tau aggregation links to neuronal cell death (Knopman et al. 2021; Long and Holtzman 2019; Selkoe and Hardy 2016). Aβ is an aggregation-prone peptide

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_20

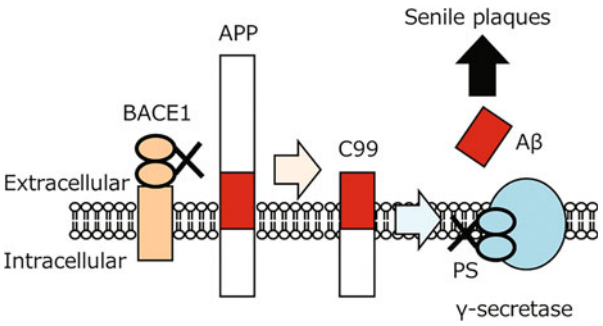

Fig. 20.1 Schematic representation of amyloidogenic pathway of APP

comprised of around 40 amino acid residues, which is a part of a single-pass transmembrane protein, amyloid β precursor protein (APP). APP is processed by non-amyloidogenic pathway or Aβ-generating amyloidogenic pathway (Kikuchi et al. 2017). In the non-amyloidogenic pathway, APP is first cleaved by ADAM10 metalloprotease at the mid position of Aβ. In the amyloidogenic pathway, APP is processed at its juxtamembrane region by β-secretase, BACE1 (Fig. 20.1). This cleavage generates C-terminal stub C99, which is then cleaved by γ-secretase to release Aβ. γ-Secretase-mediated cleavages of C83 and C99 occur at different positions to generate heterogeneity in the C-terminal length of p3 and Aβ, although the pathological significance of p3 remains controversial.

Genetic and biochemical analyses highlight the pathological importance of the heterogeneity of the C-terminal length of Aβ. The C terminus of the deposited Aβ proteins in the patients' brains mainly contains Aβ42 (Iwatsubo et al. 1994). However, under the physiological condition, neurons secrete Aβ40 as a major species, and only ~10% of secreted Aβ is Aβ42 (Asami-Odaka et al. 1995; Suzuki et al. 1994). The clue for this complex mystery was provided from the research on the biophysical property of Aβ. The longer isoforms Aβ42 and Aβ43 showed a highly aggregation-prone character compared to Aβ40. Moreover, familial AD (FAD)-linked genetic mutation on APP gene located at the C-terminal side of Aβ induced the overproduction of Aβ42 in the cultured cells (Jarrett et al. 1993). Supporting this result, the biological fluids (i.e., cerebrospinal fluids, plasma) derived from FAD patients contain a higher concentration of Aβ42 (Kosaka et al. 1997). Furthermore, Aβ42 and Aβ43 are predominantly deposited in not only the FAD brain but also the brains of Down syndrome and sporadic AD cases (Iwatsubo et al. 1995). Finally, transgenic mice expressing FAD mutation carrying APP developed the amyloid plaques (Games et al. 1995). These data indicate that the production rate of aggregable Aβ, which is determined by the cleavage of γ-secretase, impacts on the pathological process of AD.

20.2 Identification of Presenilins as an Important Factor for γ-Secretase

After the identification of FAD-linked mutation in the APP gene, genetic studies revealed that APP mutations are minor mutations in the FAD. By enormous efforts on the search for the gene responsible for FAD, two presenilin (PS) genes (*PSEN1* on chromosome 14 and *PSEN2* on chromosome 1 were identified (Levy-Lahad et al. 1995; Rogaev et al. 1995; Sherrington et al. 1995). These genes encode unknown membrane proteins showing high homology. However, identification of high Aβ42 amount in the plasma of *PSEN1*-mutation carriers implicated that PS is involved in the γ-secretase (Scheuner et al. 1996). Then, several laboratories reported that FAD-linked mutations in *PSEN* genes increase the production of a toxic and highly aggregation-prone Aβ42 in cultured cells and transgenic mouse models (Borchelt et al. 1996; Citron et al. 1997; Duff et al. 1996; Tomita et al. 1997). In contrast, cells lacking *Psen1* and *Psen2* genes showed decreased Aβ production and the accumulation of C-terminal stubs of APP (Herreman et al. 2000; De Strooper et al. 1998), suggesting that PS was a crucial factor for the γ-secretase-mediated cleavage.

Since PS was a novel protein without a known conserved sequence for proteases, there has been much debate about its function. Also, enzymatically, γ-secretase is a unique protease with completely unknown characters; first, the γ-secretase cleavage site locates within the transmembrane domain (TMD) of APP, suggesting that γ-secretase undergoes proteolysis within the membrane. Second, γ-secretase cleaves multiple sites within the same substrate to generate heterogeneity in the products (i.e., Aβ40 and Aβ42). On the other hand, the utilization of small-molecule compounds that inhibit γ-secretase activity has contributed greatly to the research. Photoaffinity labeling experiments are used to identify a compound as a fish hook to its target protein. Different research groups reported that the protein that binds to the transition-state analog-type inhibitor is PS (Esler et al. 2000; Li et al. 2000). Moreover, mutations in the aspartates of TMD6 and 7 of PS suppressed the Aβ production (Wolfe et al. 1999). At the same time, several polytopic proteins with catalytic aspartates in the conserved GxGD motif within TMDs have been identified as a novel class of proteases (Steiner et al. 2000; Weihofen et al. 2002). These supported the notion that PS belongs to a novel intramembrane cleaving GxGD-type protease family.

However, recombinant PS proteins did not show protease activity. Rather, the γ-secretase activity in vitro was detected in the high-molecular-weight fractions of the mild detergent-solubilized membrane fraction, suggesting that other factors are required for the γ-secretase activity. Advances in the genetic and biochemical analyses resolved these issues. Nematodes and fruit fries are excellent animal models in genetic studies, and PS is conserved in both animals. Intriguingly, loss of function in PS genes resulted in the loss of Notch signaling not only in these animals but also mouse (Levitan and Greenwald 1995; Struhl and Greenwald 1999; Wong et al. 1997). Notch is a well-studied transmembrane protein involved in the development and cell-to-cell communication. Cell biological studies revealed that Notch signaling

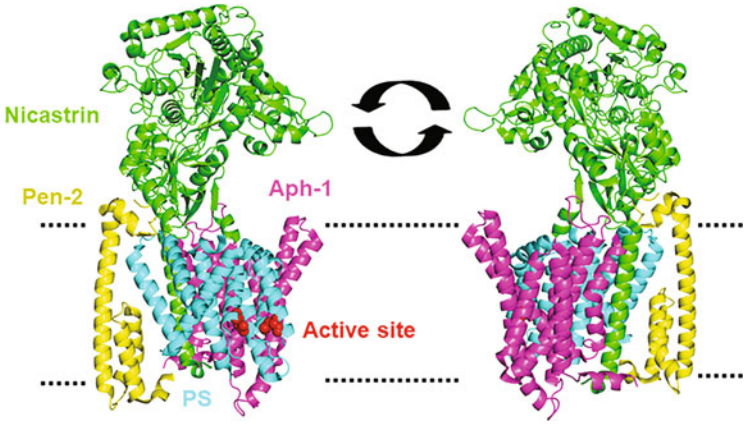

Fig. 20.2 Structure of γ-secretase complex. Each subunit is shown in a different color, with the active center aspartic acid shown as a red residue. A 180 °C rotation on the lipid bilayer indicated by the dotted line is also shown

is mediated by intramembrane proteolysis by an unknown mechanism (Kopan et al. 1996). Then, various studies were conducted using the Notch signal as an indicator. Also, protein chemical analysis of the purified γ-secretase had been performed. Finally, nicastrin (Nct), anterior pharynx defective-1 (Aph-1), and presenilin enhancer-2 (Pen-2) were identified as components of γ-secretase, and co-expression of PS with these three cofactors is sufficient for the reconstitution of γ-secretase activity (Edbauer et al. 2003; Kimberly et al. 2003; Takasugi et al. 2003). Reconstituted γ-secretase containing PS carrying FAD-linked mutation showed a robust increase in the Aβ42 production. Altogether, these data concluded that the γ-secretase is a membrane protein complex, and PS is a catalytic subunit (Fig. 20.2).

20.3 Regulation of γ-Secretase Activities as a Potential Therapeutic Strategy

Concerning several results of AD genetics, inhibiting the activity of BACE1 and γ-secretase to decrease the production of Aβ is a major strategy. Before the identification of PS as a γ-secretase component, several γ-secretase inhibitors have been identified by cell-based screening and combinatorial chemistry. However, these inhibitors block the cleavage of not only APP but other γ-secretase substrates, including Notch. Phase 3 clinical trials of semagacestat and avagacestat were discontinued because of increased incidences of skin cancer and worsening of cognitive function (Coric et al. 2015; Doody et al. 2013). The molecular basis of these adverse effects remains unclear, and the repression of Notch signaling might be involved. Then, other compounds called γ-secretase modulators (GSMs) were highlighted to alter Aβ production without affecting Notch signaling. GSMs reduce

the aggregation-prone longer Aβ species (i.e., Aβ40, Aβ42) and increase the production of the shorter Aβ species (i.e., Aβ37, Aβ38) (Mekala et al. 2020). Enzymological and structural analyses revealed that GSM directly targets PS and allosterically activates its proteolytic activity (Cai et al. 2017, 2019; Imai et al. 2019; Ohki et al. 2011, 2014; Takahashi et al. 2003; Takeo et al. 2014). In preclinical animal studies, GSMs successfully reduced the Aβ deposition in the AD model mouse. However, currently, the reduced Aβ production is not a prime therapeutic strategy against AD. Several phase 3 clinical trials of BACE1 inhibitors failed to show the therapeutic benefit in the cognitive functions of mild-to-moderate AD patients, despite the reduction of CSF Aβ and limited but significant reduction of brain plaques as assessed by amyloid PET (Hampel et al. 2020). These data suggest that, to reduce amyloid burden efficiently, secretase inhibition should be started at a very early stage and/or another approach would be required. In fact, anti-aggregated Aβ antibodies (i.e., aducanumab, gantenerumab, lecanemab, donanemab) successfully reduced the amyloid deposition in AD patients (Mintun et al. 2021; Ostrowitzki et al. 2017; Sevigny et al. 2016; Swanson et al. 2021). And recently aducanumab has been approved by FDA under the accelerated approval pathway, which provides patients with a serious disease earlier access to drugs.

20.4 Intramembrane Proteolysis and Presenilin Structure

γ-Secretase is a membrane-embedded protease that hydrolyzes the substrate inside the membrane. Before studies on PS, no structurally related protease was discovered, and the mechanism of intramembrane proteolysis was a complete mystery. Therefore, the structural biology of PS is of great interest not only from the viewpoint of drug discovery but also from the viewpoint of enzymology. However, crystallization of the fully activated enzyme is considered difficult because there are more than 19 TMDs in the assembled γ-secretase complex. Therefore, indirect approaches such as cysteine scanning and cryo-electron microscopy (cryo-EM) analysis of γ-secretase complexes have been performed (Cai and Tomita 2020). We investigated the water accessibility of specific amino acid residues of γ-secretase in a membrane-embedded state by the substituted cysteine accessibility method (SCAM). SCAM enables us to analyze the hydrophilicity of the introduced cysteine residues in the target protein using sulphydryl reagents. SCAM is a powerful approach to obtain structural information of membrane proteins without purification/solubilization (Tomita 2017). Using SCAM, we revealed that PS1 harbors a hydrophilic "catalytic pore" structure that is formed within the membrane (Sato et al. 2006, 2008; Takagi et al. 2010; Tominaga et al. 2016). Of note, such a novel structure has been confirmed by a cryo-EM study at the atomic level (Bai et al. 2015; Lu et al. 2014).

Recent structural analyses by cryo-EM techniques have demonstrated the intramembrane cleavage process in detail (Yang et al. 2017). Notably, the atomic structures of the γ-secretase-Notch and γ-secretase-C83 complexes demonstrated that the α-helical substrate (i.e., TMD) is unwound from the C terminus to form an

intermolecular antiparallel β-sheet between the substrate and PS (Yang et al. 2019; Zhou et al. 2019). This dynamic structural change of the substrate within the γ-secretase can well explain the fact that the hydrolysis reaction occurs within the lipid bilayer, a hydrophobic environment, within the transmembrane region with a generally stable α-helix structure. Furthermore, cryo-EM studies unveiled several critical regions that might be involved in the intramembrane proteolysis not only in PS but also in other γ-secretase components (Yang et al. 2021). Nevertheless, the structural biology of γ-secretase will be useful for further elucidating the cleavage mechanism of this atypical protease and for identifying various compounds that regulate the activity of the enzyme.

20.5 Perspectives

Research on FAD-linked genes has revealed the importance of Aβ in the pathogenesis of AD. The discovery and functional analysis of PS led to the identification of γ-secretase complex, whose molecular nature was previously unknown, and to the development of new enzymology of intramembrane proteolysis. Unfortunately, the γ-secretase inhibitors did not lead to the development of a therapeutic drug for AD. However, γ-secretase research did lead to the identification and study of allosteric modulators. Also, advances in chemical biology and structural biology have unveiled the molecular mechanisms of this atypical intramembrane proteolysis. Currently, the development of antibody drugs to remove accumulated Aβ, including Aducanumab, is ongoing. Beyond that, in the trend toward the prevention of AD, the approach of safe reduction of Aβ production from the very early stage is likely to be effective based on genetic studies of FAD. Thus, it is important to continue to study PS/γ-secretase from various approaches.

Acknowledgments The author would like to thank previous and current laboratory members for their contribution to this research, and Drs. Takeshi Iwatsubo (The University of Tokyo), Kei Maruyama (Saitama Medical University), Takaomi C. Saido (RIKEN), and Raphael Kopan (Cincinnati Children's Hospital Medical Center) for longtime support for the author's research. This work was supported by Grants-in-Aid for Scientific Research (A) (19H01015) from the Japan Society for the Promotion of Science (JSPS), Strategic Research Program for Brain Sciences (JP21dm0207073) from the Japan Agency for Medical Research and Development and Moonshot R&D–MILLENNIA Program (JPMJMS2024) from Japan Science and Technology Agency.

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Chapter 21
Amyloid-β in Brain Aging and Alzheimer's Disease

Hiroki Sasaguri and Takaomi C. Saido

Abstract During aging, the brain milieu changes in a multitude of ways, resulting in a decline of physiological functions and increased susceptibility to disease. One of the major age-related pathological changes in the brain is the accumulation of amyloid-β (Aβ) peptides as plaques. The appearance of pathologic Aβ species is believed to trigger Alzheimer's disease (AD), one of the principal neurodegenerative diseases and the leading cause of dementia, of which aging is the most important risk factor. Accumulation of Aβ in the brain is caused by an imbalance in Aβ kinetics that may arise from aging-related changes in degradation or clearance mechanisms. Aβ is degraded by enzymes such as neprilysin and is removed from the brain via numerous pathways including glial phagocytosis, transport across the blood-brain barrier, interstitial fluid bulk flow, and cerebrospinal fluid absorption. The functional activity of many of these systems declines with aging. Elevated levels of pathogenic Aβ species result in the emergence of senile plaques in the neuropil or as cerebral amyloid angiopathy in blood vessels. In addition, soluble Aβ oligomers appear prior to amyloid fibril formation and have detrimental effects on neuronal functions. To elucidate the pathomechanisms underlying AD, we have previously developed new mouse models that precisely recapitulate amyloid pathology without overexpressing disease-relevant molecules. Here we provide an overview of the catabolism, anabolism, and clearance of Aβ, the characteristics of amyloid pathologies in the human brain, and recent advances in AD research, including new technologies and animal models.

Keywords Senile plaque · CAA · APP · BACE1 · γ-Secretase · Neprilysin · Aβ transport · ApoE · APP knock-in mice · Marmoset · Nonhuman primate model

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_21

21.1 Introduction

Aging is characterized by a decline in physiological functions after a period of maturation; this decline occurs as a result of accumulating damage to molecules, cells, and organs (Fontana et al. 2010). In the brain, aging manifests as a decline in learning and memory, attention, decision-making speed, cognitive performance, sensory perception, and motor coordination. Aging also increases vulnerability to disease and is in fact one of the most significant risk factors for many diseases in the brain, including neurodegenerative diseases such as Alzheimer's disease (AD). One of the major age-related pathological changes in the brain is the accumulation of amyloid-β (Aβ) peptides in the neuropil, otherwise known as "'senile plaques."' Braak et al. evaluated amyloid and tau pathology in 2332 brains independent of their underlying diseases (Braak et al. 2011) and found that amyloid plaques first appeared in the neocortex from 30 to 40 years of age. Plaques were already positive in 4% of cases in their 40s. The frequency and severity of amyloid pathology increased with age, with 75% of cases being positive in their tenth decade. Amyloid pathology is one of the most important pathological hallmarks of AD and precedes the clinical onset of dementia by more than two decades (Bateman et al. 2012). Several lines of evidence support the concept that an imbalance between the production and clearance of Aβ leads to the accumulation of toxic Aβ species as an initiating factor in AD; this is termed the amyloid hypothesis (Fig. 21.1). We will review here aspects of the catabolism, anabolism, and clearance of Aβ, and the characteristics of amyloid pathologies in the brain. Recently, significant technological advances, including high-throughput expression analyses such as spatial transcriptomics, and disease modeling in animals, have been made. We will also review recent progress in research on amyloid pathologies and AD utilizing these new tools.

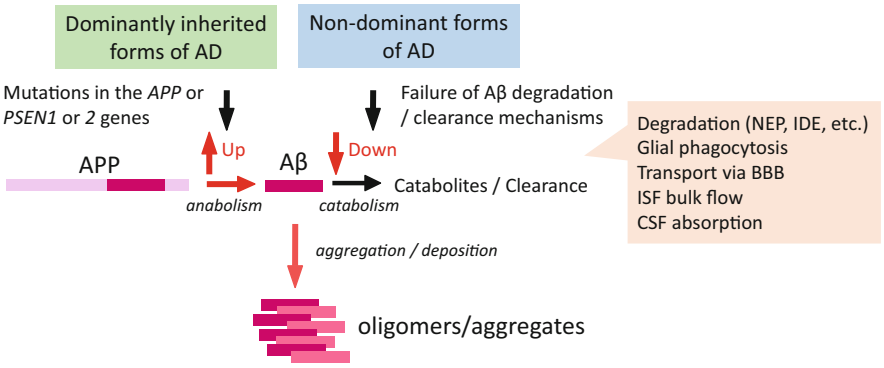

Fig. 21.1 Aβ homeostasis determined by production and degradation. The steady-state Aβ level is determined by the APP concentration, the rate of Aβ production, the rate of degradation, and transport

21.2 Catabolism, Anabolism, and Clearance of Aβ

Aβ is generated from amyloid precursor protein (APP; a type I membrane protein) through proteolytic cleavages (Fig. 21.2). APP is first cleaved by β-secretase (a type I transmembrane aspartic protease also termed β-site APP-cleaving enzyme 1 or BACE1) (Hampel et al. 2021), following which the C-terminal part of APP (CTF-β) is further digested by γ-secretase. γ-Secretase is a membrane-associated complex consisting of the following four proteins: presenilin 1 or 2 (PS1/2), nicastrin, Aph1, and Pen2 (Saido 2003; Hardy and Selkoe 2002). γ-Secretase first cleaves CTF-β by an endopeptidase-like cleavage (ε-cleavage) to produce the long forms of Aβ species, $Aβ_{48}$ and $Aβ_{49}$. Subsequently, γ-secretase trims these long Aβs by a carboxypeptidase-like cleavage (γ-cleavage) to generate smaller Aβ species of various lengths (Tomita and Iwatsubo 2013). The most common isoforms of Aβ in the brain are $Aβ_{40}$ (<80%) and $Aβ_{42}$ (10%), the latter being more toxic, more prone to aggregation, and more resistant to degradation (Zheng et al. 2012). If APP is processed by α-secretase, the resulting carboxyl-terminal fragment is subsequently cleaved by γ-secretase to produce non-amyloidogenic fragments. In addition, another APP processing pathway, η-secretase processing, produces Aη-α and Aη-β peptides (Willem et al. 2015). The η-secretase pathway is an alternative pathway followed when BACE1 is inhibited, resulting in increased Aη-α activity and reduced neuronal activity.

Several pathways are known to be involved in the clearance of Aβ: enzymatic degradation and cellular uptake, phagocytosis mediated by glial cells, transport across the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB), interstitial fluid (ISF) bulk flow, and cerebrospinal fluid (CSF) absorption into the circulatory and lymphatic systems (Tarasoff-Conway et al. 2015).

Aβ-degrading enzymes include neprilysin (NEP) (Iwata et al. 2001; Farris et al. 2003, 2007), insulin-degrading enzyme (IDE) (Farris et al. 2003; Miller et al. 2003), and kallikrein-related peptidase 7 (KLK7) (Kidana et al. 2018), as well as

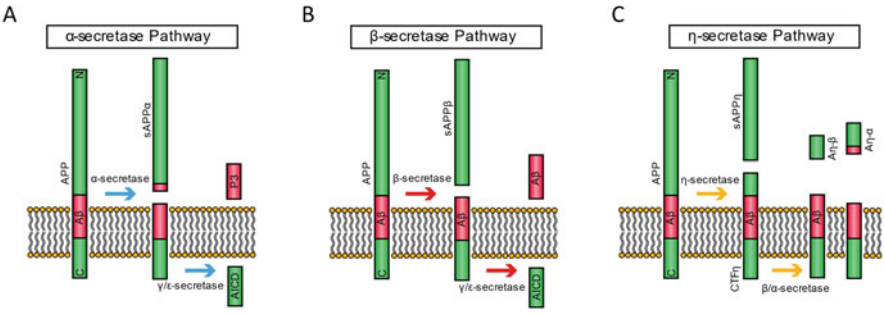

Fig. 21.2 APP processing pathways. APP is localized to the membrane and is cleaved either by α-secretase (**a**), β-secretase (BACE1), or η-secretase (**c**). Digestion by γ-secretase following BACE1-cleavage generates amyloidogenic Aβ peptides (**b**)

endothelin-converting enzyme (ECE)1/2 (Eckman et al. 2001), angiotensin-converting enzyme (ACE) (Hu et al. 2001), cathepsin D (Yamada et al. 1995; Hamazaki 1996), urokinase-type plasminogen activator (Sasaki et al. 1988), and matrix metalloendopeptidase-9 (Carvalho et al. 1997). NEP, which is a membrane-bound protein, was originally discovered in renal brush border membranes (Wong-Leung and Kenny 1968). Later, it was found that NEP was identical to enkephalinase, which participates in the proteolytic inactivation of enkephalins (Malfroy et al. 1978). In the brain, NEP plays a major role in degrading opioids and other neuropeptides (Matsas et al. 1983; Turner et al. 2001). NEP is abundant in the striatonigral pathway (Barnes et al. 1988) and hippocampus (particularly in the dentate gyrus molecular layer and the dentate gyrus granule cell layer), while in the cortex, it is present in layers 2/3 and 5 (Iwata et al. 2001). We detected NEP as a major Aβ degrading enzyme in vivo (Iwata et al. 2000, 2001). Genetic deletion of NEP in mice decreased the degradation of both exogenously administered Aβ and endogenous Aβ (Iwata et al. 2001), resulting in a twofold increase in the Aβ levels in the brains of NEP-knockout mice. In addition, we found that genetic deletion of NEP in an AD mouse model significantly increased Tris-insoluble Aβ and significantly exacerbated amyloid pathology in the brain (unpublished data). IDE is a zinc metalloendopeptidase that was originally implicated in insulin metabolism (Duckworth 1979) and later reported to hydrolyze multiple peptides, including glucagon, atrial natriuretic factor, transforming growth factor-α, β-endorphin, amylin, and APP intracellular domain (AICD) peptides in addition to Aβ (Duckworth et al. 1998; Selkoe 2001). In mice, a genetic deficiency of IDE increases $A\beta_{42}$ levels by 1.4-fold (Miller et al. 2003). KLK7, a member of the KLK protein family, was originally identified as an inflammation-induced proteolytic enzyme in the skin. KLK7 is able to cleave the hydrophobic core motif of Aβ fibrils, thereby attenuating their neurotoxicity in vitro (Shropshire et al. 2014). KLK7 is produced from astrocytes in the brain, and ablation of *Klk7* exacerbated the thioflavin S-positive Aβ pathology in AD model mice (Kidana et al. 2018). ECE1 and 2 are transmembrane metalloproteases and belong to the same family as NEP. ECE1 and 2 are detected primarily in vascular endothelial cells where they process pro-endothelin-1, a potent vasoconstrictive peptide produced in vascular endothelial cells, but are also present in neuronal cells in the brain (Masaki 2004). Knockout of ECE1/2 in AD model mice leads to a 1.2−1.3-fold increase in Aβ, which indicates that ECE1 and 2 are potentially Aβ-degrading enzymes in vivo (Eckman et al. 2003). ACE is a membrane bound zinc-dependent dipeptidyl carboxypeptidase that converts angiotensin I (Ang I) to Ang II, which plays roles in maintaining blood pressure, body fluid regulation, and sodium homeostasis (Guy et al. 2005). Genetic studies in humans have provided evidence for a potential relationship between ACE and AD (Kehoe et al. 1999; Elkins et al. 2004). Moreover, it has been shown in vitro that ACE exhibits significant inhibitory effects on the aggregation, deposition, and cytotoxicity of Aβ (Hu et al. 2001) and that purified ACE can efficiently cleave $A\beta_{42}$ to $A\beta_{40}$ (Zou et al. 2007). In agreement with this, it has been shown that ACE degrades endogenous $A\beta_{40}$ and $A\beta_{42}$ in cell culture (Hemming and Selkoe 2005). However, we previously showed that the knockout of ACE has no effects on Aβ levels in AD model mice (Iwata et al. 2001). These Aβ-degrading enzymes are mainly expressed by neurons, but glial cells also produce them, particularly under

pathological conditions (Ries and Sastre 2016). In addition to the abovementioned enzymatic degradation, extracellular Aβ can be degraded by glial phagocytosis mediated by microglia and astrocytes (Tarasoff-Conway et al. 2015; Ries and Sastre 2016).

One possible mechanism that causes Aβ accumulation in the brain is the age-related decline of NEP expression (Iwata et al. 2001, 2002; Hellström-Lindahl et al. 2008; Wang et al. 2003; Yasojima et al. 2001a, b). Interestingly, the amount and activity of NEP decline with age, especially in the outer molecular layer and the polymorphic cell layer of the hippocampal dentate gyrus, such that Aβ levels consequently increase in these brain areas (Iwata et al. 2002). The outer molecular layer of the dentate gyrus receives nerve projections from the entorhinal cortex via the perforant pathway. This pathway is markedly affected in AD, and pathological changes in this region are closely linked to cognitive impairment early in AD (Iwata et al. 2002). Additionally, NEP mRNA levels are decreased in the hippocampi and temporal cortices of early AD patients, whereas these levels are unchanged in the cerebellum (Yasojima et al. 2001a, b). Because AD pathology primarily involves the hippocampus and association cortices and spares the cerebellum, these data suggest a close relationship between region-specific declines of NEP activity and accumulation of amyloid in aging and AD.

In addition to enzyme- and glial cell-mediated degradation, Aβ is cleared from the brain by transport across the BBB and BCSFB, as well as through ISF bulk flow and CSF absorption into the circulatory and lymphatic systems (Tarasoff-Conway et al. 2015). Active transport of Aβ across the BBB is mediated by LDL receptor (LDLR) family members such as LRP1, and ATP-binding cassette transporters (ABC transporters) such as ABCB1 and ABCA1 (Pascale et al. 2011; Fitz et al. 2012; ElAli and Rivest 2013). Clearance of Aβ across the BBB is also mediated by apolipoprotein E (ApoE), α2-macroglobulin (α2M), and LDL-related protein 2 (LRP2) (Tarasoff-Conway et al. 2015). It was reported that perivascular drainage pathways also contribute to the clearance of Aβ from the brain (Preston et al. 2003). The AQP4-dependent glymphatic pathway is another clearance system reported to remove soluble Aβ from the brain interstitium (Tarasoff-Conway et al. 2015). In mice, Aβ clearance is accelerated during sleep, an effect that is mediated by an increase in the volume of the extracellular space and accelerated ISF-to-CSF bulk flow (Xie et al. 2013; McKinley et al. 2013). Interestingly, amyloid accumulation is reduced in elderly patients with narcolepsy type 1 (Gabelle et al. 2019), a condition characterized by excessive daytime sleepiness and cataplexy, and caused by the destruction of orexin neurons (Scammell 2015). These facts might partly explain why sleep impairment increases the risk of AD (Ju et al. 2014). Aβ in circulating CSF can be absorbed either through the arachnoid villi and BCSFB into the circulation or through the perivascular and perineural spaces into the lymphatic system (Tarasoff-Conway et al. 2015). These clearance systems are also impaired by aging (Hawkes et al. 2011; Kress et al. 2014; Pollay 2010). Furthermore, Aβ clearance is decreased in sporadic AD (SAD) patients (Mawuenyega et al. 2010), and perivascular drainage pathways are impaired in cerebral amyloid angiopathy (CAA) and AD (Weller et al. 2008). Age-related alterations in these clearance systems might contribute in part to the accumulation of Aβ in the brain parenchyma and vessel walls.

21.3 Amyloid Pathologies and AD

Aβ accumulates in the extracellular space as "'senile plaques"' or on the leptomeningeal and cortical vessel walls that cause CAA. Senile plaques are one of the most important pathological features of AD. Other major pathological markers of AD include the accumulation of hyper-phosphorylated tau as intracellular neurofibrillary tangles (NFTs), the loss of neuronal cells mainly in the cerebral cortex and hippocampus, and chronic neuroinflammation (Braak and Braak 1991; Hyman et al. 2012). AD is the most prevalent neurodegenerative disease; it is characterized clinically by early memory deficits, followed by a gradual decline in other cognitive functions (Scheltens et al. 2016; Winblad et al. 2016). Aβ pathology commences approximately two decades before cortical tau pathology, neurodegeneration, and the clinical onset of dementia (Bateman et al. 2012; Maruyama et al. 2013). Sporadic late-onset AD (LOAD) accounts for more than 99% of all cases of AD (Campion et al. 1999), with the number of patients continuing to increase dramatically as the world's populations grow older, given that aging is a primary risk factor (Winblad et al. 2016). Early-onset AD (EOAD), which is predominately familial, is caused by mutations in genes that encode amyloid precursor protein (*APP*), presenilin-1 (*PSEN1*), and presenilin-2 (*PSEN2*). Most familial AD (FAD) mutations affect the processivity of γ-secretase, resulting in the release of longer Aβ peptides and shifts in the relative ratios of the different peptides (Welander et al. 2009; Chávez-Gutiérrez et al. 2012). Mutations in the *APP* gene result in the production of longer Aβ peptides or aggregate-prone Aβ species (Rosenberg et al. 2016). Interestingly, an APP missense mutation (A673T) at the second amino acid of Aβ was claimed to reduce the risk of SAD and age-related cognitive decline by decreasing the production of Aβ (Jonsson et al. 2012; Maloney et al. 2014). This mutation also appears to affect the biophysical properties of the Aβ peptide generated (Benilova et al. 2014; Zheng et al. 2015). Mutations that affect Aβ production and influence the risk of AD are cited in many studies and provide hard evidence that Aβ plays a central role in AD pathogenesis (Selkoe and Hardy 2016).

Senile plaques consist of fibrillary amyloid materials and are morphologically diverse (Thal et al. 2006). Amyloid fibrils are composed of aggregated Aβ peptides that are formed from soluble oligomers and display a β-pleated sheet conformation. Senile plaque pathology begins with the first diffuse plaques, followed by the appearance of cored and neuritic plaques (Thal et al. 2006). Classical cored plaques are characterized by a dense central core and a peripheral halo. The core can be stained by amyloid stains such as Congo red and thioflavin S. The cored plaques are associated with dystrophic neurites and microglia. Large diffuse plaques often lack these cores and are weakly positive or negative for amyloid stains. Diffuse plaques are often found in cognitively normal, elderly persons, but the distribution of these plaques is restricted to certain areas of the brain such as the neocortex, basal ganglia, and hypothalamus (Thal et al. 2004). On the other hand, in AD patients, senile plaques are much more widespread and occur in many areas of the brain (Braak and Braak 1991; Thal et al. 2002b). In addition, cored and neuritic plaques are frequently observed in AD brain and occur only in late stages of Aβ deposition. These facts indicate that cored and neuritic plaques represent mature forms developed from

diffuse plaques or emerge in brain regions that are already diseased, whereas diffuse plaques develop in normal areas (Thal et al. 2006). Although the major form of Aβ peptides in senile plaques is $A\beta_{1\text{-}42}$, other forms including longer Aβ species such as $A\beta_{43}$ (Welander et al. 2009; Saito et al. 2011; Sandebring et al. 2013) or N-terminal-truncated forms such as $A\beta_{3\text{-}40/42}$, $A\beta_{11\text{-}40/42}$, and $A\beta_{17\text{-}40/42}$ also have amyloidogenic properties and are known to accumulate in plaques (Harigaya et al. 2000). In addition, other proteins such as ApoE, α-macroglobulin, and interleukin-1α were also shown to be involved (Thal et al. 2006). The progression of amyloid pathology is relatively uniform in AD and was staged by Thal and colleagues (2002b). In the first phase, amyloid deposition is restricted to the neocortex. The plaques extend to allocortical regions in phase 2, and then to the diencephalic nuclei, striatum, and cholinergic nuclei of the basal forebrain in phase 3. In phases 4 and 5, several brainstem nuclei and the cerebellum are involved, respectively. These characteristic progression patterns raised a hypothesis that pathological Aβ species propagate through the brain in a similar fashion to prion proteins in prion disease (Lauwers et al. 2020). Many lines of evidence support this hypothesis in experimental models as well as in human subjects who underwent systemic injections of contaminated growth hormone extracts or dura mater grafts derived from cadavers (Ritchie et al. 2017; Duyckaerts et al. 2018; Kovacs et al. 2016; Hamaguchi et al. 2016; Banerjee et al. 2019).

In CAA, Aβ deposits are observed in leptomeningeal and cortical arteries, veins, arterioles, venules, and capillaries (Greenberg et al. 2020). CAA is often present in AD (Brenowitz et al. 2015) and causes lobar intracerebral hemorrhages and microbleeds. Sporadic CAA is classified into two types depending on the involvement of brain capillaries (Thal et al. 2002a). When Aβ deposition occurs in capillaries, it is classified as CAA-Type I, which tends to be widespread and specifically associated with neuritic plaques and severe AD pathology (Attems and Jellinger 2004), whereas CAA without capillary involvement is classified as CAA type II (Thal et al. 2002a). In contrast to senile plaques, CAA is composed predominantly of $A\beta_{40}$ (Thal et al. 2002a). The distribution of CAA is patchy and segmental, suggesting that vascular Aβ preferentially accumulates at sites of initial Aβ deposition. CAA might contribute to the pathogenesis of AD by affecting the perivascular drainage system. In addition, CAA was linked to inflammatory responses to anti-Aβ immunotherapy in AD clinical trials (Eng et al. 2004; Nicoll et al. 2003). Several mutations in the *APP* gene such as the Dutch (E693Q), Italian (E693K), and L705V mutations were reported to cause a hereditary form of CAA (Levy et al. 1990; Bugiani et al. 2010; Obici et al. 2005). These mutations locate within the Aβ sequence, suggesting that conformational changes of Aβ peptides promote its deposition in vessels in these cases.

The *APOE* gene that encodes the ApoE protein is the strongest genetic risk modifier of LOAD (Belloy et al. 2019; Yamazaki et al. 2019). ApoE is related to lipid metabolism and is produced mainly from astrocytes in the brain under physiological conditions. Compared to the most common *APOE ε3* allele, the *APOE ε4* allele increases and the *APOE ε2* allele decreases the risk of developing LOAD depending on the number of alleles (Farrer et al. 1997; Genin et al. 2011). In AD brains from *APOE ε2* carriers, amyloid plaques were fewer than those from *APOE*

ε3/3 individuals, whereas *APOE ε4* carriers harbor more abundant amyloid pathology (Serrano-Pozo et al. 2015; Polvikoski et al. 1995). Although the mechanism (s) underlying the effects of the *APOE* gene variants remains unclear, several possible roles of ApoE in Aβ metabolism have been suggested (Yamazaki et al. 2019). These include the clearance of soluble Aβ from the brain ISF (Castellano et al. 2011), the removal of Aβ via transport across the BBB (Deane et al. 2008), cellular uptake, and subsequent degradation of Aβ by glial cells (Lin et al. 2018; Jiang et al. 2008), promotion of Aβ fibril formation by accelerating the initial seeding or nucleation of Aβ peptides (Liu et al. 2017), and regulation of APP expression (Huang et al. 2017).

Although tau pathology is more closely related to neurodegeneration and cognitive dysfunction in AD (Bateman et al. 2012), amyloid pathology itself may have detrimental effects on neuronal functions or neural circuits (Zott et al. 2018). Aβ oligomers can cause synaptic dysfunction and structural changes in experimental models (Selkoe and Hardy 2016; Cline et al. 2018). Soluble oligomers of $A\beta_{42}$ extracted from AD patient brains can decrease synaptic function and number and induce cognitive impairment in rats (Shankar et al. 2008). Utilizing a selective enzyme-linked immunosorbent assay (ELISA) against Aβ oligomers, Esparza et al. revealed that concentrations of Aβ oligomers in the brains of AD patients were tightly correlated with the severity of amyloid plaques (Esparza et al. 2013). Interestingly, this relationship was not as apparent in cognitively normal AD patients with comparable amounts of amyloid pathology. These results indicate that the link between plaques and oligomers may be a key pathophysiological event underlying cognitive dysfunctions in early AD.

Neuronal hyperactivity is a potential feature caused by Aβ in early AD. It was reported that neuronal hyperactivity in the hippocampus is observed before the clinical onset of AD (Bookheimer et al. 2000; Quiroz et al. 2010; Mormino et al. 2012). Seizures occur in 10–22% of patients with AD (Vossel et al. 2017), and subclinical epileptiform activity was detected in 42% of patients (Vossel et al. 2016; Lam et al. 2017). In transgenic mouse models of AD, seizures and epileptiform activity can be observed even before Aβ plaque deposition, possibly caused by Aβ oligomers (Busche et al. 2012). Synthetic and AD brain-derived soluble Aβ dimers induced hyperactivity in hippocampal CA1 neurons in mice, which required neuron-specific baseline activity (Zott et al. 2019). Neuronal hyperactivation was induced by accumulation of perisynaptic glutamate, which was caused by an Aβ-dependent suppression of astroglial excitatory amino-acid transporter 2 (EAAT2)-mediated glutamate uptake. Aβ-induced hyperexcitation might drive a vicious cycle of hyperactivation via these components (Zott et al. 2019).

Aβ may cause impairment of the default mode network (DMN) in the subclinical stage of AD (Zott et al. 2018). DMN is a resting-state network and composed of three major subdivisions: the ventral medial prefrontal cortex, the dorsal medial prefrontal cortex, and the posterior cingulate cortex (PCC) and adjacent precuneus plus the lateral parietal cortex (Raichle 2015). Although precise functions of the DMN remain elusive, it may be involved in cognitive function, including the activation of episodic memory (Raichle 2015). Amyloid accumulation first occurs

in the PCC and precuneus, two core regions of the DMN (Palmqvist et al. 2017). Furthermore, functional disruption of the DMN occurs about 20 years before the clinical onset of AD in patients with inherited forms of the disease (Chhatwal et al. 2013). This suggests that amyloid pathology might have detrimental effects on neural circuits.

21.4 Animal Models to Study Amyloid Pathology and Aging

Animal models are a prerequisite for investigating the effects of aging on Aβ metabolism and accumulation in the brain. We recently generated next-generation mouse models that precisely recapitulate amyloid pathology, neuroinflammation, and cognitive impairment, but without the overexpression of APP protein (Saito et al. 2014; Sasaguri et al. 2017) (Fig. 21.3). We humanized a murine Aβ sequence and introduced two FAD mutations ($App^{NL\text{-}F}$ mice, KM670/671NL, Swedish and I716F, Beyreuther/Iberian mutations) or three mutations ($App^{NL\text{-}G\text{-}F}$ mice; E693G: Arctic mutation as a third mutation) into the endogenous mouse *App* gene (Saito et al. 2014). $App^{NL\text{-}F}$ and $App^{NL\text{-}G\text{-}F}$ mice exhibited increased $A\beta_{42}$ production and a

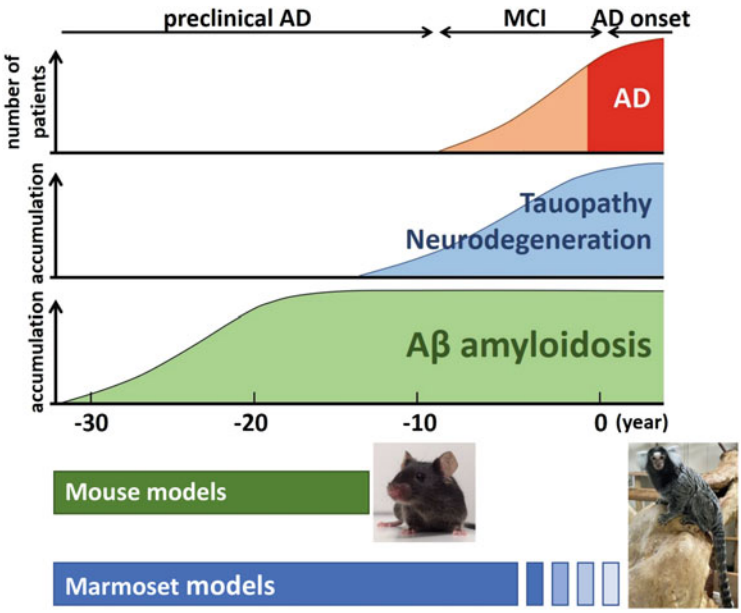

Fig. 21.3 Animal models and cortical pathology in preclinical AD, MCI, and AD. AD mouse models generally recapitulate amyloid pathology, but not tau pathology or neurodegeneration. AD marmoset models are expected to reveal these pathologies because of their biological similarities to humans (The picture of marmoset; Drs. Erika Sasaki & Wakako Kumita's courtesy (Central Institute of Experimental Animals, CIEA, Japan))

high $A\beta_{42}/A\beta_{40}$ ratio without alterations in the expression levels of APP or other fragments. Exceptionally, the *App* knock-in mice produced more CTF-β and thus sAPPβ, fragments produced by BACE1-mediated cleavage, compared with wild-type mice because of the effects of the Swedish mutation to increase total amounts of Aβ by increasing the susceptibility of APP to the β-secretase pathway (Saito et al. 2014). App^{NL} mice that carried only the Swedish mutation and produced similar amounts of CTF-β and sAPPβ to $App^{NL\text{-}F}$ and $App^{NL\text{-}G\text{-}F}$ mice showed no amyloid pathology or behavioral abnormalities, confirming that these levels of CTF-β and sAPPβ exerted no effects on the pathology or cognitive functions of the mice (Saito et al. 2014; Masuda et al. 2016). $App^{NL\text{-}F}$ mice exhibited pathological Aβ deposition in the cerebral cortex and hippocampus, which was accompanied by enhanced neuroinflammation from 6 months of age. Importantly, the amyloid plaques in $App^{NL\text{-}F}$ mice consisted mainly of pathogenic $A\beta_{1/3pE\text{-}42}$ (Saido et al. 1995) in a manner similar to the brains of AD patients. $App^{NL\text{-}F}$ mice also developed memory dysfunction at 18 months of age. The $App^{NL\text{-}G\text{-}F}$ mice exhibited substantially earlier onset of AD pathology and cognitive abnormalities compared with the $App^{NL\text{-}F}$ mice. Masuda et al. analyzed the *App* knock-in mice using an automated behavioral analysis system, IntelliCage, and determined that the $App^{NL\text{-}F}$ and $App^{NL\text{-}G\text{-}F}$ mice exhibited different cognitive dysfunctions depending on their age and pathology (Masuda et al. 2016). Furthermore, by utilizing deep learning, we found that even in earlier stages of amyloid pathology (8−12-month-old animals), these *App* knock-in mice showed deficits in compulsivity control, learning, and attention (Sutoko et al. 2021).

As a consequence of studies on next-generation AD mouse models, several new lines of evidence support the notion that abnormal Aβ metabolism and accumulation cause detrimental effects in vivo. In $App^{NL\text{-}F}$ mice, Zhang et al. demonstrated that hippocampal mushroom spines are lost and that the STIM2 (stromal interaction molecule 2)-nSOC (neuronal store-operated calcium entry) pathway is altered from as early as 3 months of age in a time-dependent manner (Zhang et al. 2015). Extracellular $A\beta_{42}$ levels correlated with the spine loss. The authors concluded that $A\beta_{42}$-induced hyperactivation of metabotropic glutamate receptor 5 (mGluR5) and the subsequent overload of endoplasmic reticulum (ER) Ca^{2+} signaling likely represent the main cause for mushroom spine loss in *App* knock-in mice. Jun et al. analyzed neuronal activity in the hippocampus and medial entorhinal cortex (MEC) during spatial memory tasks in $App^{NL\text{-}G\text{-}F}$ mice (Jun et al. 2020) and found that CA1 neurons including place cells in these mice showed disrupted remapping. In addition, MEC neurons exhibited a severe loss of spatial tuning, while grid cells in the MEC were almost absent in 12−13-month-old $App^{NL\text{-}G\text{-}F}$ mice. Mild disruption of MEC grid cells was observed even in younger *App* knock-in mice (3–5 months of age). These results may partly explain pathomechanisms underlying spatial memory impairment in AD. Recently, novel high-throughput analyses such as single-cell RNA sequencing and spatial transcriptomics have been developed. Chen et al. performed spatial transcriptomics in $App^{NL\text{-}G\text{-}F}$ mice and in situ sequencing in the brains of human AD patients to identify gene networks that were specifically related to amyloid plaques. They demonstrated a multicellular gene co-expression network

of plaque-induced genes (PIGs) involving the complement system, oxidative stress, lysosomes, and inflammation that were prominent in the later phase of the disease, whereas early alterations occurred in a gene co-expression network enriched for myelin and oligodendrocyte genes (OLIGs) (Chen et al. 2020).

In addition to the *App* knock-in mouse models, we have been trying to generate nonhuman primate (NHP) models by introducing FAD-causing mutations in common marmoset (marmosets, *Callithrix jacchus*). Marmosets are small nonhuman primates that belong to the New World Primates (Schiel and Souto 2017). They have been increasingly utilized in neuroscience research because of many advantages over other research animals (Burkart and Finkenwirth 2015; Miller et al. 2016). Marmosets possess genetic backgrounds, physiological functions, brain structures, and complex cognitive and social behaviors similar to those of humans. Their life spans in captivity are 10–15 years, making them suitable for aging research. In association to AD research, the amino acid sequence of Aβ in marmosets is identical to that of humans (unpublished), and Aβ starts to accumulate as plaques in the brains of these animals from 7 years of age (Geula et al. 2002). In addition, Tau hyperphosphorylation was detected in the brains of adolescent marmosets and increased with aging in a more fibrillary form, but not as NFTs (Rodriguez-Callejas et al. 2016). Marmoset gene programs of microglia, including complement, phagocytic, and susceptibility genes to neurodegeneration, such as AD, are closer to those of humans compared to rodents (Geirsdottir et al. 2019). Their metabolic functions resemble those of humans and thus may affect the pathogenic processes related to AD. Marmosets also have sleep phases composed of rapid eye movement (REM) and non-REM cycles similar to those of humans (Crofts et al. 2001). Once AD marmoset models are established, they will almost certainly be important for many types of studies: noninvasive imaging analyses by magnetic resonance imaging (MRI), functional MRI, and positron emission tomography (PET); biomarker identification and development; highly sensitive cognitive analyses such as touch-sensitive technology or for the analysis of sleep disturbances; analyses of neural circuit abnormalities including DMN; and pharmaceutical studies for drug screening prior to clinical trials.

21.5 Conclusions

Recent failures of clinical trials of drugs targeting Aβ raised the possibility that Aβ accumulation represents an epiphenomenon rather than a cause of AD and thus challenged the amyloid cascade hypothesis of AD (Panza et al. 2019). However, because amyloid pathology begins more than 20 years before the clinical onset of AD, and the downstream pathomechanism(s) triggered by Aβ can progress independently of amyloid pathology, it is possible that earlier intervention against Aβ is required to prevent the disease. It is interesting that a small proportion of cases was negative for amyloid plaques even in patients over 90 years of age (Braak et al. 2011). Although the precise mechanism(s) underlying this observation is not clear, it

is presumed that several protective genetic variants such as *APOE* ε2 and *APP* A673T are involved (Snitz et al. 2020; Kero et al. 2013). In addition, this fact may indicate that strategies should be to exist to prevent amyloid pathology from developing in humans. The results of ongoing studies in preclinical or asymptomatic stages of AD and cognitively healthy individuals at risk of AD should provide some answers for the critical question concerning whether Aβ accumulation causes AD or is a byproduct of the AD process. It is also known that the genetic risks for AD and for amyloid deposition are not identical and that conversion from mild cognitive impairment (MCI) to AD is predicted by the polygenic risk score (PRS) (Leonenko et al. 2019). This suggests that different pathomechanisms are in play during the distinct phases of AD. We believe recent advances in technologies based on biochemical analyses and neuroimaging, as well as disease modeling will pave new ways for the pathogenesis of AD to be elucidated and for treatment strategies to be established.

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Chapter 22
Tau Pathology and Neurodegenerative Disorders

Akihiko Takashima, Yoshiyuki Soeda, Riki Koike, and Sumihiro Maeda

Abstract Neurofibrillary tangles (NFTs) are a pathological feature of Alzheimer's disease and the other neurodegenerative disorders. Neuronal loss, followed by neuronal dysfunction, invariably occurs in areas where NFTs are present. The spread of NFTs, from the entorhinal cortex through the limbic system to the neocortex, leads to lower cognitive function. By the time dementia is diagnosed, NFTs are already prominent in areas beyond the limbic system. Introduction of treatment at this stage is futile. Thus, it is worthwhile considering treatment or prevention interventions one stage before, when NFTs begin to form in the entorhinal cortex.

Keywords Neurofibrillary tangles (NFTs) · Tau · Granular tau oligomer · Alzheimer's disease · Entorhinal cortex · Grid cell · Path integration task

22.1 Development of Tau Pathology

Neurofibrillary tangles (NFTs) are intraneuronal inclusions, first described by Gallyas using silver staining, thioflavin S fluorescence, and Congo-red birefringence (Moloney et al. 2021). Under the electron microscopic, NFTs are seen as two differently shaped fibrils: straight filaments (SFs) and paired helical filaments (PHFs) (Hirano et al. 1968; Wiśniewski et al. 1976). The PHF are comprised of two strands twisted around one another; the cyclical helical filaments are spaced at 70−85 nm and have a thickness of 10−20 nm (Miyakawa et al. 1989). In contrast, SFs have a diameter of 10 nm (Cowan and Mudher 2013). While the protofibrils of PHF and SF have a similar structure, they differ in their inter-protofilament packing (Fitzpatrick et al. 2017).

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_22

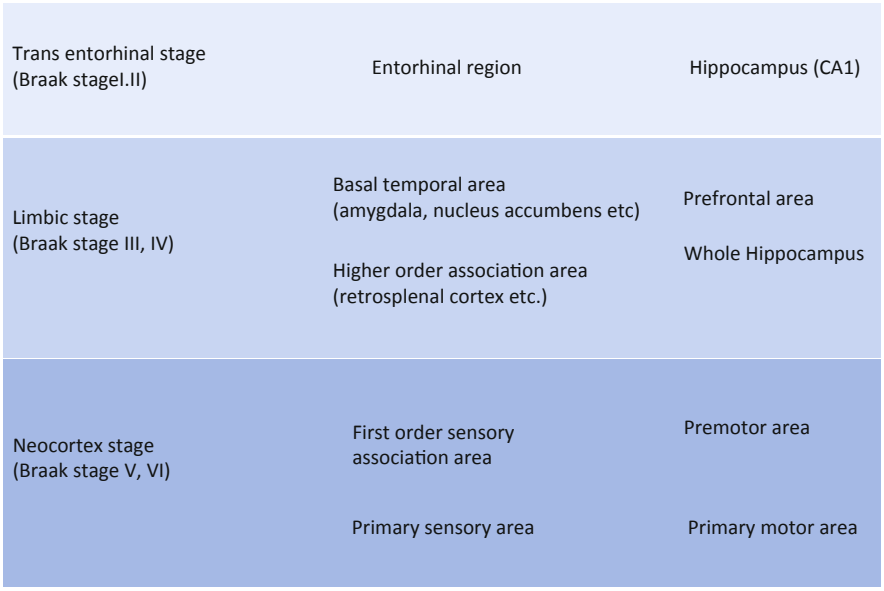

Fig. 22.1 Braak NFT staging in Alzheimer's disease. Braak et al. used postmortem brains of Alzheimer's disease patients and proposed six stages to classify the distribution and extent of tau pathology. In stages I and II, NFT accumulation begins in the trans-entorhinal cortex (stage I) and is confined to the entorhinal cortex and hippocampus CA1 region (stage II); cognitive function is normal at stages I and II. In stages III and IV, NFT pathology extends throughout the hippocampus (stage III) and other limbic areas (stage IV), accompanied by mild cognitive impairment. In V and VI, tau pathology is found in the neocortical and primary sensory areas. The figure is a simplified version of previously published maps (Braak and Braak 1991; Kretzschmar 2009)

Braak et al. defined six stages of tau pathology in Alzheimer's disease on the basis of the distribution of NFTs (Fig. 22.1) (Braak and Braak 1991). At Braak stage I, NFTs first appear in the entorhinal cortex. At Braak stage II, the number of NFT is increased, while the NFT is still confined in entorhinal cortex. At Braak stage III, numerous NFTs as well as ghost tangles are found in layer II of the entorhinal cortex, and a few NFT are present in the hippocampal CA1 region. By stage IV, many ghost tangles are observed in the entorhinal cortex, while the number of NFTs in the hippocampal CA1 increases dramatically and NFTs begin to appear in the CA4 and dentate gyrus. Stages III and IV are referred to as the limbic stage. All regions of the hippocampus as well as the neocortex show a progressive rise in NFT abundance by stages V and VI (also called the isocortical or neocortical stage).

22.2 Mechanism of Tau Aggregation

NFT consists of hyperphosphorylated tau. Tau is initially distributed throughout the neuron but becomes increasingly localized within the axon as the neuron matures (Kosik and Finch 1987). In fact, tau mRNA expression decreases rapidly after neuronal maturation, but tau proteins bind and stabilize axons thereafter. Axonal stabilization results from tau binding to microtubule; therefore, tau is known as a microtubule-associated protein. It has been observed that, in the brains of Alzheimer's disease patients, hyperphosphorylated tau first accumulates in the cell body and dendrites in a state (Wang et al. 2013). Abnormally hyperphosphorylated and distributed tau is thought to be the mechanism responsible for triggering neurodegeneration.

In general, hyperphosphorylated tau binds poorly to microtubules and be able to move to cell body beyond a diffusion barrier in the axon initial segment (Zempel and Mandelkow 2014). Some evidence suggest that the accumulation of hyperphosphorylated tau in cell bodies and dendrites is triggered by β-amyloid (Zempel et al. 2013) and that Fyn-mediated local translation of tau mRNA occurs in cell bodies and dendrites (Li and Götz 2017). On the other hand, we reported that tau mRNA can be normally found in cell bodies and dendrites as ribonucleoprotein (RNP) granules and that glutamate stimulation induces the accumulation of somatodendritic hyperphosphorylated tau through enhanced the translation of tau and the activation of GSK-3β (Kobayashi et al. 2017). While a number of hypotheses have been proposed regarding the mechanism underpinning the accumulation of phosphorylated tau in somatodendrites, the mechanism that initiate NFT formation and, ultimately, neurodegeneration remains obscure.

While the intracellular concentration of tau is about 2 μM (Avila 2010; Drubin et al. 1985; Wegmann et al. 2018), even 10 μM of tau is insufficient to cause tau self-aggregation in vitro, unless polyanions such as heparin sulfate (Goedert et al. 1996; Pérez et al. 1996), arachidonic acid (Wilson and Binder 1997), and RNA (Kampers et al. 1996) are added to enable tau-tau interaction. These polyanions electrostatically interact with tau to induce the MC1 epitope, which reflects a structural change, namely, PHF-like tau (Maeda et al. 2007). In order for soluble monomeric tau to develop into insoluble aggregates with a β-sheet structure, a phase transition—which depends on increases in the local concentration of tau—is necessary. Recently, liquid-liquid phase separation (LLPS) was suggested as a mechanism by which local tau protein concentrations are raised (Alberti et al. 2019). Briefly, LLPS refers to intracellular granules that do not have a cell membrane, e.g., stress granules (Hyman et al. 2014), the core of which is composed of ionized molecules such as RNA and proteins, to which a number of factors such as TIA1 are bound (Protter and Parker 2016). In the LLPS, proteins and RNAs form a highly concentrated mass that retains its liquid properties but is separated from the cytoplasm. Several reports have implicated intracellular binding of ribosomal RNA to tau, and the formation of fibers from highly concentrated droplets of tau with RNA or heparin (Ambadipudi et al. 2017; Apicco et al. 2018; Wegmann et al. 2018) after prolonged incubation,

indicating that tau droplet formation may be the structure that initiates tau aggregation. In summary, neuronal hyperactivation increases tau translation and increases tau concentration in the somatodendritic compartment. Interactions with other (yet undefined) factors cause tau to form droplets that ultimately aggregate (Wegmann et al. 2018).

Analysis of tau aggregation in vitro has shown that tau forms dimers via oxidation-dependent disulfide bonds between cysteine residues (Sahara et al. 2007). Seeding for aggregation occurs in a protracted way in the absence of these disulfide bonds (Sahara et al. 2007; Soeda et al. 2015). First, monomeric tau proteins bind to each other in droplets to form soluble tau oligomers. Once tau oligomers acquire a β-sheet structure, they precipitate as granular tau oligomers. Examination by atomic force microscopy (AFM) revealed that granular tau oligomers vary between 5 and 30 nm in diameter of rigid structures surrounded by flexible protruded N- and C-terminus tau. Laser light scattering showed that granular tau oligomers have an average size of 55 nm average size including the flexible protruded regions and consist of 40 tau molecules, including a flexible region. AFM also showed that 20 nm diameter granular tau oligomers extend in length to form long filaments (Maeda et al. 2007). Recently, cryoelectron microscopic studies revealed the structure of the core region of PHF and SF from Alzheimer's disease patients (Fitzpatrick et al. 2017). In both types of the filaments, the core region comprises tau dimers bound via the microtubule binding domain, and the fibrils are twisted by stacking tau dimers. As mentioned before, tau can form granular oligomers of various sizes; oligomers of a specific size probably have a PHF core and can elongate the fibrils to form PHF. Thus, when tau concentrations rise, tau oligomerizes in different ways, one of which results in elongation and formation tau fibrils. It is conjectured that other tau oligomers, which do not form fibrils, may have neurotoxic properties. Therefore, tau fibrils and neurotoxic tau oligomers can be independent structures but still be formed simultaneously in a cell. This may explain that neuronal loss occurs in brain regions where tau fibrils are found.

22.3 Tau Pathology and Brain Dysfunction

Cognitive tests can prove normal cognition of a subject even though NFTs are present in the entorhinal cortex of the subject. Once NFTs appear in the limbic system, patients may be diagnosed with mild cognitive impairment (MCI) or early dementia (Braak and Braak 1991; Serrano-Pozo et al. 2011). Thus, it appears that the degree of cognitive decline is not merely a function of the amount of NFTs but rather the extent of NFT spread in the brain. Maintenance of normal cognitive function at the trans-entorhinal stage (Braak stage I and II) of NFT formation probably results from compensation by other brain regions for the loss of entorhinal cortex function, thereby maintaining; eventually such compensatory mechanisms may become inadequate to support cognitive functions mediated by the limbic system.

Recently, "grid cells" that facilitate the division of space into hexagons and which become electrically active when the apex of a hexagon is crossed were discovered in the entorhinal cortex (Hafting et al. 2005). These grid cell properties make an important contribution to spatial navigation which is based on self-centered (egocentric) navigation and landmark-dependent allocentric navigation (Maguire et al. 1999). In the path integration task, egocentric navigation prioritizes the use of grid cell functions (Bush et al. 2015; Hafting et al. 2005; Poucet and Save 2009). In contrast, allocentric navigation which is used in tests such as the water-maze mainly depends on the hippocampus (Colombo et al. 2017; O'Keefe 1991; Vorhees and Williams 2014). Interestingly, the ability for allocentric navigation declines in an age-dependent manner (Colombo et al. 2017; Coughlan et al. 2018; Gazova et al. 2013; Rodgers et al. 2012); at the same time, MCI patients perform poorly on path integration tests, their scores correlating negatively with volume of the entorhinal cortex (Howett et al. 2019). This proves that the path integration test mirrors entorhinal cortex function; lower scores in the path integration test may reflect NFT-related dysfunction of the entorhinal cortex; if true, examination of path integration-dependent navigation skills can provide information on events that precede cognitive decline.

22.4 Therapies to Target Tau

Current drug development for Alzheimer's disease is mainly focused on disease-modifying therapies (Cummings et al. 2020); tau is one target. The current approaches for tau targeting drugs are (1) the inhibition of posttranslational modifications that are believed to occur before tau aggregation, (2) the direct inhibition of tau aggregation, (3) the blocking of tau propagation, (4) microtubule stabilization (the main physiological function of tau), and (5) the suppression of tau expression using antisense oligonucleotides (Soeda and Takashima 2020). Here, we will focus on just two of these strategies: the direct inhibition of tau aggregation and the inhibition of tau propagation.

A variety of chemical compounds that inhibit tau aggregation have been screened (Bulic et al. 2009; Crowe et al. 2009; Taniguchi et al. 2005). Among them, the first report of methylene blue as a potential agent is now the subject of an early clinical trial in AD. A Phase II study has shown that, as compared to the placebo group, subjects receiving daily oral administration of 138 mg methylene blue have significantly improved scores on the ADAS-cog, a psychological test assessing cognitive function (Wischik et al. 2014), leading to a Phase III study. However, LMTX, a methylene blue derivative that is well tolerated and absorbed compared to methylene blue, failed to achieve the primary endpoint (improvement in cognitive function) in the Phase III study (Gauthier et al. 2016; Wilcock et al. 2018). Laboratory analysis using recombinant tau proteins has shown that methylene blue inhibits tau fibril formation but increases intermediate aggregates containing granular tau oligomers (Soeda et al. 2019). It was therefore suggested that methylene blue inhibits tau fiber,

but not tau oligomer, formation, providing a possible explanation for the negative Phase III results; this interpretation fits with our previous reports that tau oligomers, rather than fibrils, are involved in tau toxicity (Kimura et al. 2007, 2010; Takashima 2013). These findings point to the need for the development of inhibitors of tau oligomer formation to halt neuronal loss at an early stage and, therefore, to block progression to dementia.

With the aim of finding inhibitors of granular tau oligomer formation, we conducted a screening of tau aggregation inhibitors using a library consisting of natural compounds and their derivatives (Soeda et al. 2015). We identified small-molecule compounds with a 1,2-dihydroxybenzene skeleton that could achieve this by capping the cysteine residues in tau. One of these compounds, DL-isoproterenol, was shown to inhibit tau aggregation and neuronal loss in mice overexpressing P301L mutant tau (Soeda et al. 2015). Recently, we have examined the blood-brain barrier (BBB) permeability of various compounds in a novel in vitro reconstituted system model (marketed by Pharmacocel Corporation) and confirmed that DL-isoproterenol can indeed traverse the BBB (unpublished), increasing the promise of 1,2-dihydroxybenzene-containing compounds to stall tau oligomer formation and aggregation.

Seven of the top ten pharmaceutical sales in 2019 were biologics. Since biologics are macromolecules, such as antibodies, that cannot penetrate cells efficiently, their targets are typically extracellular proteins or cell surface protein. Tau is basically an intracellular protein, but when tau pathology propagates, pathological tau is thought to be secreted into the extracellular space (Mudher et al. 2017; Yamada et al. 2011). Immunotherapies in which extracellular tau is targeted are currently under being explored in clinical trials—two of them are based on active immunization (see Table 22.1 for target sites and initial results). The immunotherapies being tested target various regions from the N- to C-terminus of tau (Table 22.1). Basic research indicate that antibodies against the middle region of tau (Antibody D; aa 235-246) are more efficient in blocking tau propagation as compared to antibodies targeting the N-terminal region (Albert et al. 2019). Therapies target tau are expected to delay the progression of disease, by inhibiting neuronal loss in tauopathies including AD.

Table 22.1 Summary of immunotherapeutic drugs for tau

Drugs	Therapy type	Targets	Sponsors	Preclinical study	Clinical trials	
					Subjects	Phase
AADvac-1	Active	Tau a.a. 294–305	Axon Neuroscience SE	AADvac-1 decreased tau hyperphosphorylation and ameliorated the sensorimotor functions of transgenic (Tg) rats (Kontsekova et al. 2014)	AD	Phase 2
					PPA	Phase 1
ACI-35	Active	Phospho-S396/404	AC Immune SA—Janssen	ACI-35 decreased sarkosyl-insoluble phosphorylated tau and ameliorated survival in P301L tau Tg mice (Theunis et al. 2013)	AD	Phase 1b/2a
RG7345	Passive	Phospho-S422	F. Hoffmann-La Roche	RG7345 decreased tau pathology in 3xTg-AD mice (Collin et al. 2014)	AD	Phase 1—discontinued
Gosuranemab	Passive	Secreted N-terminal tau fragments (Tau a.a. 15–24)	Biogen (Bristol-Meyers Squibb; iPerian)	The original mouse antibody, IPN002, decreased the tau secretion in vitro and in vivo (Bright et al. 2015)	AD	Phase 2
Tilavonemab	Passive	Extracellular form of pathological tau (Tau a.a. 25–30)	AbbVie	The original mouse antibody, HJ8.5, decreased tau seeding activity in vitro and in vivo (Yanamandra et al. 2013, 2015)	AD	Phase 2
Bepranemab	Passive	Mid-region of tau (Tau a.a. 235–246)	UCB Biopharma	The original mouse antibody, Antibody D, inhibited tau propagation in vivo and in vitro (Albert et al. 2019; Courade et al. 2018)	PSP	Phase 1
					AD	Phase 1
Zagotenemab	Passive	Same as MC1 antibody (Tau a.a. 7–9, 313–322)	Eli Lilly	Administration of MC1 decreased tau pathology in tau Tg mice (Chai et al. 2011; d'Abramo et al. 2013)	AD	Phase 2
BIIB076	Passive	Monomeric and fibrillar tau	Biogen		AD	Phase 1
JNJ-63733657	Passive	Mid-region of tau	Janssen		AD	Phase 2

(continued)

Table 22.1 (continued)

Drugs	Therapy type	Targets	Sponsors	Preclinical study	Clinical trials	
					Subjects	Phase
Lu AF87908	Passive	Phospho-S396	H. Lundbeck A/S	The original mouse antibody suppressed tau propagation in vitro and in vivo (Rosenqvist et al. 2018)	AD	Phase 1
PNT001	Passive	*cis*-phospho-T231	Pinteon Therapeutics	The original mouse antibody ameliorated traumatic brain injury-related structural and functional impairment in mice (Kondo et al. 2015)	AD	Phase 1
					TBI	Phase 1
Semorinemab	Passive	N-terminal region of tau	AC Immune SA—Genentech—F. Hoffmann-La Roche	RO7105705 decreased brain pathology in P301L tau Tg mice (Lee et al. 2016)	AD	Phase 2
E2185	Passive	HVPGG epitope in the microtubule-binding domain (Tau a.a. 299−303, 362−366)	Eisai	The original mouse antibody, 7G6, inhibited tau aggregation in vitro, and tau seeding and transmission in vivo (Roberts et al. 2020)	AD	Phase 1

Abbreviations: *AD* Alzheimer's disease, *PPA* nonfluent/agrammatic variant primary progressive aphasia, *PSP* progressive supranuclear palsy, *TBI* traumatic brain injury

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Chapter 23
Aging and Parkinson's Disease: Pathological Insight on Model Mice

Shigeto Sato and Nobutaka Hattori

Abstract Excessive loss of dopaminergic neurons leads to Parkinson's disease (PD), the most common neurodegenerative disorder characterized by the loss of nigrostriatal dopamine-producing neurons. Based on the epidemiological studies, aging is the greatest risk factor for developing PD. However, the correlation between age-related cellular mechanisms and the degeneration of dopaminergic neurons remain unclear. In order to elucidate the pathogenesis of PD, we generated genetically modified mice and measured both dopaminergic neuronal loss and motor phenotype over an extended period of time. These PD model mice exhibited age-dependent locomotor impairments, including hindlimb defects and the number of dopaminergic neurons decreased in aged mice, contributing to locomotor dysfunction. We present evidence from our work on dopaminergic neurons of aged mice that demonstrate the markers including impaired proteolysis, mitochondria damage and inflammation, which increase with advancing age. We contend that aging exaggerated dopaminergic neurons vulnerable to degeneration in PD.

Keywords Parkinson's disease · Aging · Dopaminergic neuron · Mitochondria · Aggregate · Mouse model

23.1 Introduction

The mammalian brain contains several dopaminergic systems. The largest such system originates in the midbrain and projects primarily to the basal ganglia. The originating cells for this system are found in the substantia nigra and ventral tegmental area of the midbrain. Parkinson's disease (PD), the most common aging-related motoric neurodegenerative disorder, is characterized by the loss of nigrostriatal dopaminergic neurons and the formation of intracellular Lewy bodies (LBs) (Spillantini et al. 1997). PD typically begins between the ages of 60 and 80. It

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_23

Table 23.1 Parkinson's disease-related genes

Familial Parkinson's disease

Locus	Gene	Inheritance	Protein function/nature
4q21-23	PARK1 α-synuclein	AD	Aggregate
6q25.2-27	PARK2 Parkin	AR	Ubiquitin ligase
4q21-22	PARK4 α-synuclein	AD	Triplication of PARK1
4p14 -15.1	PARK5 UCH-L1	AD	Ubiquitin C-terminal hydrolase
1p35-36	PARK6 PINK1	AR	Protein kinase in mitochondria
1p36	PARK7 DJ-1	AR	Antioxidant
12p11.2-q13.1	PARK8 LRRK2	AD	Protein kinase
1p36	PARK9 ATP13A2	AR	Lysosome
2q36-37	PARK11 GIGYF2	AD	Grb10 interact: signal
2p13	PARK13 Omi/HtrA2	AD	Protease in mitochondria
22q13.1	PARK14 PLA2G6	AR	Phospholipase
22q12-q13	PARK15 FBXO7	AR	F-BOX protein
16q12	PARK17 VPS35	AD	Retromer
7p11.2	PARK22 CHCHD2	AD	Mitochondria

is predicted that PD affects over 1% of the population in the age of 60, but the rate of PD populations is five times increase following 20 years, implicating that the senescence of brain or neurons relates to the onset of PD (de Lau and Breteler 2006; Nussbaum and Ellis 2003; Wood-Kaczmar et al. 2006). Interactions between genetic predisposition and environmental factors are likely to be the main cause of mitochondrial dysfunction and oxidative damage; however, the underlying molecular mechanisms remain poorly understood. For example, aging-related changes in the nigrostriatal systems of advanced PD patients imply that non-pathological aging changes are partially responsible for the restricted onset of PD.

The vast majority of PD cases are sporadic, in contrast to inherited familial forms of PD that account for only 5% of all cases (Dauer and Przedborski 2003) (Table 23.1). The identification of PD-related genes and risk factors including aging has implicated several pathways in PD etiology, with accumulating evidence suggesting a link between dysfunctional intracellular protein catabolism, mitochondrial clearance, and PD age-related pathogenesis (Fig. 23.1). In *PARK1*-linked PD, intrinsically disordered mutant α-synuclein (hereafter referred to as synuclein) initiates the disease process. Given that highly aggregated proteins are deposited in nigral neurons in PD, dysfunctions of proteolytic systems, i.e., the ubiquitin–

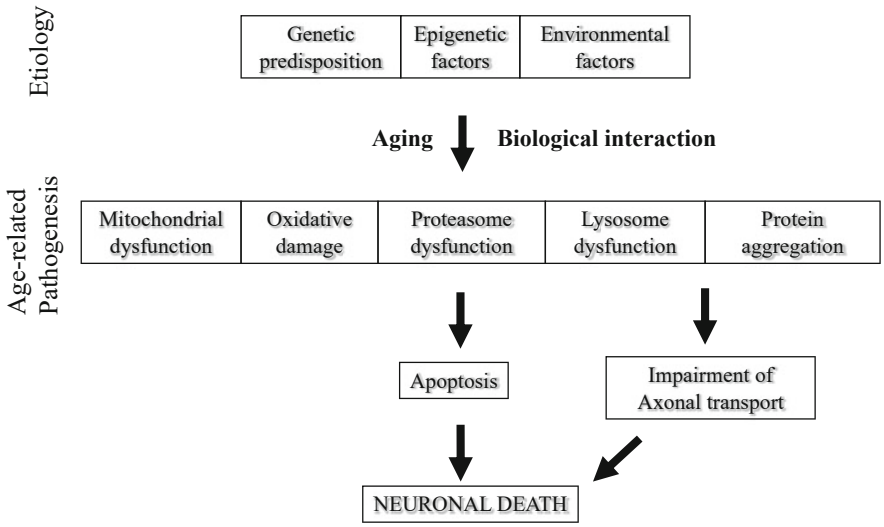

Fig. 23.1 Age-related Parkinson's disease pathogenesis

proteasome system and autophagy–lysosomal pathway, are likely to contribute to the final neurodegenerative process. In particular, in cases of *PARK2*-linked PD, intrinsically disordered mutant Parkin accelerates PD pathogenesis (Kitada et al. 1998). *PARK2*-linked PD is early-onset and is characterized by the massive loss of dopaminergic neurons (Chung et al. 2001; Shimura et al. 2000). Parkin mutations were hypothesized to accelerate the aging of the dopaminergic system in this disease. Since Parkin is associated with mitochondrial degradation in vitro (Narendra et al. 2008), it is thus likely that a defect in mitophagy (i.e., selective mitochondrial autophagy) contributes to the neurodegenerative process. Several lines of evidence indicate that damaged mitochondria are mostly degraded by mitophagy in vitro. In addition, Parkin mutations disrupt the induction of mitophagy in PD and suggest that the accumulation of damaged mitochondria contributes to dopaminergic cell death in aging-related neurodegeneration. In *PARK22*-linked PD, intrinsically disordered mutant CHCHD2 initiates PD pathology (Funayama et al. 2015). Autopsy of an individual with the T61I mutation revealed widespread synuclein accumulation (Ikeda et al. 2019). CHCHD2 localizes to the intermembrane spaces of the mitochondria and plays roles in the regulation of mitochondrial respiration, transcription of complex IV, and mitochondria-associated apoptosis (Baughman et al. 2009; Liu et al. 2015). Loss of CHCHD2 destabilizes cytochrome c and complex IV in cultured cells and causes degeneration of mitochondrial cristae and impairment of oxygen respiration, leading to loss of dopaminergic neurons and motor dysfunction (Meng et al. 2017).

Mitochondrial dysfunction has been considered a major contributor to aging and age-related diseases. Mitochondrial dynamics change in advanced age, e.g., mitochondrial biogenesis decreases, while mitochondrial DNA damage and reactive

oxygen species (ROS) production both increase (Chistiakov et al. 2014; Gonzalez-Freire et al. 2015). The accumulation of ROS and oxidative damage has been linked to multiple pathologies, including neurodegenerative disorders, diabetes, cancer, and aging. Excessive ROS production is considered an important factor that accelerates the aging process (Avantaggiato et al. 2015; López-Lluch et al. 2015). As cellular processes of aging are dynamic and neurodegeneration occurs over a prolonged period of aging stress, it is impossible to determine with any certainty if these aging pathways work independently or converge to a single route to dopaminergic neuronal death. To elucidate the critical role of aging in dopaminergic neurons, we characterized age-related pathologies in PD model mice.

23.2 Change of Dopaminergic Neurons in PD

The relative contribution of genes and environment factor in pathogenesis of PD has been suggested. With median age at onset, age is the most important risk factor for PD (Ascherio and Schwarzschild 2016; Simon et al. 2020). It is recognized in human postmortem studies that PD patients have neuronal loss in the substantia nigra pars compacta. The Braak hypothesis suggests that early pathological changes occur in the medulla oblongata and olfactory bulb before advancing rostrally to substantia nigra and midbrain by which time clinical symptoms and signs are likely to be present. The initial studies showing neurodegeneration in the substantia nigra of patients of PD also reported a neuronal loss in the substantia nigra of the healthy subjects included in the control groups of these studies (McGeer et al. 1977; Stark and Pakkenberg 2004). Healthy aged subjects also showed a decrease in pigmented cells (7−10% decrease per decade) (McGeer et al. 1988; Double et al. 2008). In PD, the striatal decrease of dopamine has also been found in the aged brain where the dopamine level decreases 10−13% per decade of life (Carlsson and Winblad 1976; Kish et al. 1992). Dopaminergic neuron degeneration probably begins in the distal axon and proceeds retrogradely including the inhibition of axonal transport (Galvin et al. 1999; Coleman 2005). Regardless of the underpinning etiology, several key molecular events and hallmarks have been indicated in the postmortem tissue, cell lines, and animal model. These include synuclein misfolding and aggregation, oxidative stress, mitochondria dysfunction, impairment of protein clearance, and neuroinflammation. As these processes occur over the prolonged period of stress, thus, it is crucial for in vivo model studies to evaluate aging effects.

23.3 Age-Related Pathogenesis of PD

23.3.1 Oxidative Stress

Oxidative stress theory that age-related physiological dysfunction is due to the progressive accumulation of oxidative damage is proposed in 1956 (Harman 1956). The mitochondria produce unpaired electrons and facilitate ROS production. The ROS damages essential constituents including proteins, lipids, and nucleic acids and accelerates aging (Oliveira et al. 2010; Barja 2002). Dopaminergic neurons are vulnerable to oxidative stress and aging, because the dopaminergic neurons have specific structure and need high energy. These neurons have a high number of synaptic terminals and a thin unmyelinated axon and consume a high quantity of energy to maintain these activities. And high number of mitochondria produce a number of ROS for dopaminergic neuron life span. Furthermore, dopamine itself produces ROS in the process of metabolization. And in the presence of iron, ROS generation is accelerated by Fenton reactions (Halliwell 1992) (Fig. 23.2). Therefore, dopaminergic neurons have ROS-rich environment and promote aging. The influence of oxidative stress has been suggested in PD to explain the dopaminergic neuronal loss (Olanow and Tatton 1999). There is evidence showing a disruption of mitochondrial electron transport chain complex I in the PD brain (Shapira et al. 2012). As a toxic model in dopaminergic neuron degeneration, complex I inhibitor (MPTP, rotenone, and 6-hydroxydopamine) was administrated to animals to generate PD models. But these models were not completely mimic PD patients.

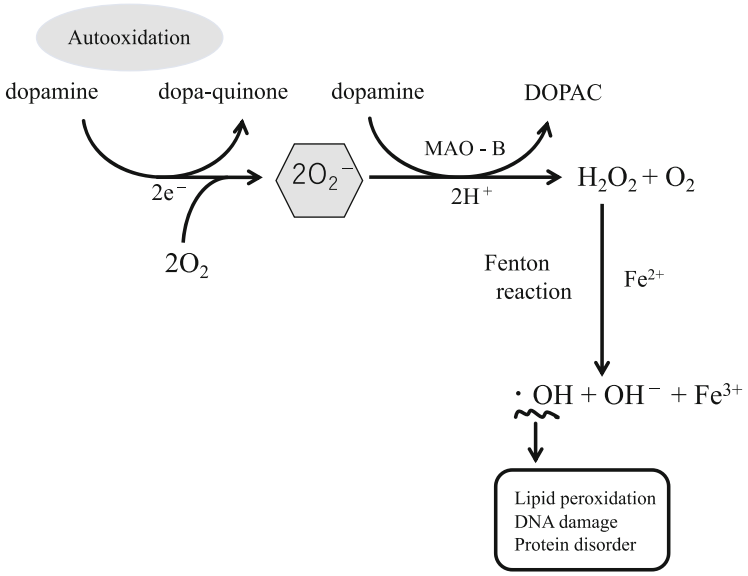

Fig. 23.2 Oxidative stress in dopaminergic neuron

23.3.2 Mitochondria Dysfunction

After the incorporation of the mitochondria damage to oxidative stress hypothesis, the stress-related accumulation of mitochondria DNA (mtDNA) damage began to be considered as a major cause of aging. Neurons were supposed to accumulate a number of mtDNA mutations because of these long life span (Reeve et al. 2013). But mtDNA deletions of PD were a little higher than those observed in healthy aged subjects (Bender et al. 2006). From a different point of view, mitochondria structure is associated with aging (Wilson and Franks 1975; Herbener 1976). In general, damage mitochondria may be repaired by a fusion/fission process. When mitochondria receive great damage, these mitochondria must be eliminated by mitophagy to maintain mitochondrial homeostasis and prevent cell death. Given that Parkin is associated with mitochondrial degradation in vitro (Narendra et al. 2008; Matsuda et al. 2010), it is likely that a dysfunction of mitophagy contributes to the neurodegenerative process. Specifically, experiments in which cultured cells were treated with the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), which causes mitochondrial depolarization, revealed that the PINK1/Parkin pathway is associated with mitochondrial quality control. Insights regarding the functions of PINK1 and Parkin (Pickrell and Youle 2015) have also enhanced our understanding of PD pathogenesis. Great progress has been made toward understanding the pathogenesis of PD, mainly due to the discovery of PINK1/Parkin-mediated mitophagy. However, little is understood about the in vivo functions underlying PD pathogenesis. In the second half of the chapter, we will describe detailed observations of aged-Parkin-deficient mice.

23.3.3 Protein Degradation

Given that highly aggregated proteins are deposited, which consist primarily of synuclein and ubiquitin in PD, dysfunctions of proteolytic systems, i.e., the ubiquitin–proteasome system and autophagy–lysosomal pathway, seem to contribute to the final neurodegenerative process. Macroautophagy (hereafter, referred to as autophagy) is a highly conserved bulk protein degradation pathway in eukaryotes. Cytoplasmic proteins and organelles are engulfed within autophagosomes, which fuse with the lysosome, where they are degraded along with their cargo (Levine and Klionsky 2004). Several lines of evidence indicate that synuclein is predominantly degraded by autophagy but also by the proteasome. However, mutant forms of synuclein and oligomers are dependent on the autophagy–lysosome pathway for their clearance (Webb et al. 2003; Spencer et al. 2009; Yu et al. 2009). Although the phenotypes of mice harboring brain-specific deletion of *Atg5* or *Atg7* reveal the critical role of autophagy in the removal of aggregated proteins (Hara et al. 2006; Komatsu et al. 2006), dopaminergic neuron-specific autophagy deficiency leads to the restrictive presynaptic accumulation of synuclein in the dorsal striatum,

suggesting that impaired autophagy plays a role in PD pathogenesis (Friedman et al. 2012). Oxidative stress, gene mutation, and overexpression can influence synuclein conformational change, and some of these species, which exhibit toxicity (Giguère et al. 2018) can seed and spread synuclein pathology cell to cell. In the human brain, synuclein deposition and neuronal degeneration are accentuated in distal regions (Uchihara and Giasson 2016). Because dopaminergic neurons have a unique structural characteristic, long hyper-branched projection axons, that innervate wide areas in the brain (Sulzer and Surmeier 2013), it is likely that this feature increases the chance of developing deposits in peripheral axons and may disturb axonal transport.

23.3.4 REST Localization

REST (the repressor element-1 silencing transcription factor), also known as neuron-restrictive silencer factor (NRSF), is transcriptionally induced in the aging human brain, and the induction is declined in Alzheimer's disease (AD) patients (Lu et al. 2014). They also show that the induced REST is detected in the nucleus and represses the transcription level of proapoptotic and AD-related proteins in normal aging brain, suggesting that the REST induction and nuclear accumulation is neuroprotective. On the other hand, REST has been known as a primary factor in the transcriptional and epigenetic regulatory circuitry that modulates the expression of genes containing NRSE element in their promoter region (Chong et al. 1995; Schoenherr and Anderson 1995). As NRSE is found in the most of neuronal specific genes, REST is believed to act exclusively in nonneuronal cells where it represses the gene expression of NRSF controlled neuronal genes. In PD patients, REST is sequestered in LBs, inhibiting the entry of REST into the nucleus and therefore impairing REST function (Kawamura et al. 2019). Given the lack of LBs formation model, it could be interesting to speculate that lower-grade aggregates, such as oligomers, could also bind REST, inhibiting its DNA binding function and neuroprotective effects. Furthermore, REST is neuroprotective against manganese-induced toxicity in immortalized cell lines (Pajarillo et al. 2020). These observations suggest REST as a key mediator of synuclein, mitochondrial dysfunction, and dopaminergic neuron vulnerability in PD (Ryan et al. 2021).

23.3.5 Change of Dopaminergic Neurons in Aged Human and Mouse

Parkinsonism is very common among people over the age of 65, and its prevalence increases markedly with age (Bennett et al. 1996). Aging-related neuronal loss has been reported in several areas of the brain. The number of nigral neurons decreases in direct proportion to age, with 48% loss by the age of 60 years (McGeer et al.

1977). Other studies support the concept of nigral neuronal loss with aging and progressive dopamine decrease (Mann et al. 1984; Fearnley and Lees 1991; Tooyama et al. 1994; Ma et al. 1999). In humans, striatal dopamine decreases throughout adult life, but little is known about the time course of changes during mice aging. In mice, dopamine levels decrease within striatal regions in 24–30-month-old mice and C57BL/6 mice clearly do not undergo progressive dopamine loss between 3 and 21 months (Osterburg et al. 1981). Compared with humans, mouse dopaminergic neurons may be affected by aging past 100 weeks.

23.3.6 Locomotor Impairment of Aged C57BL/6 Mice

We observed C57BL/6 mice over a longer period of time. These aged-C57BL/6 mice begin to exhibit age-dependent motor behavioral deficits. These physiological abnormalities could be evaluated by both the rotarod and runway tests. In the rotarod test, latency to fall times was diminished at 120 weeks, compared with young mice. Moreover, in the runway test, young mice exhibited well-coordinated movement and almost no slips of either the forepaw or hindpaw from the beam. By contrast, aged mice could hardly move on the beam and slipped frequently. In particular, the hindpaws of mice at 120 weeks of age often slipped off the beam. Gait disturbance progressed with aging.

23.3.7 Tyrosine Hydroxylase Neuron Number Decreases in Aged Mice, Which Contributes to Their Locomotor Dysfunction

To determine how decreasing tyrosine hydroxylase (TH) neuron number contributes to aging, we compared the number of TH neurons between aged and younger mice. As demonstrated by the rotarod test, aged mice suffered from locomotor dysfunction. We sacrificed these mice (70-week-old mice, $n = 5$; 120-week-old mice, $n = 18$) and counted the number of TH neurons in three sections: the ventral tegmental area (VTA), the center area of the substantia nigra pars compacta (SNcc), and the lateral area of the substantia nigra pars compacta (SNcl). Mice at 120 weeks of age had fewer TH neurons than at 70 weeks of age. The reduction in TH cell number was most prominent in the VTA. No neuronal loss was observed in young mice that did not exhibit motor dysfunction. The loss of dopaminergic neurons may contribute to the motor impairment observed in aged mice. In addition, we tested dopamine physiology in 120-week-old aged mice by neurochemical analysis of the dorsal striata. High-performance liquid chromatography revealed a reduction in striatal dopamine levels and metabolites in aged mice relative to control mice.

23.3.8 Accumulation of Fragmented Mitochondria in Aged Mice

To further characterize these mitochondria, we performed ultrastructural analysis in dopaminergic neurons of 120-week-old mice. We observed small, round, and fragmented mitochondria in dopaminergic neurons in aged mice, but not in young mice. Precise quantification revealed that mitochondria area was reduced in dopaminergic cells, but there was no significant difference in aged cerebellar and cerebral cortical neurons. In electron microscopy, these fragmented mitochondria have a normal outer membrane structure and normal internal structure, including the matrix and cristae (Noda et al. 2020a, 2020b).

23.4 PD Model Mice

23.4.1 Dopaminergic Neuron-Specific Atg7-Deficient Mice

23.4.1.1 Characterization of Locomotor Impairments

We generated TH cell-specific *Atg7* conditional knockout mice ($Atg7^{flox/flox}$:TH-Cre) by crossing the previously characterized *Atg7* floxed mice ($Atg7^{flox/flox}$) with TH-Cre mice (TH-Cre) harboring the Cre recombinase coding sequence downstream of a characterized fragment of the TH promoter (Savitt et al. 2005).

$Atg7^{flox/flox}$:TH-Cre mice were viable at birth and indistinguishable in appearance from their littermates, and their survival rate was not markedly diminished. Although $Atg7^{flox/flox}$:TH-Cre mice have not yet been observed over the entire life span, they began to exhibit impairment in motor coordination tasks around 100 weeks and apparent motor behavioral deficits around 110 weeks. These clinical abnormalities were ascertained by the runway and rotarod tests. In contrast to $Atg7^{flox/flox}$ mice, which exhibited well-coordinated movement and almost no slips of the forepaw or hindpaw from the beam, the $Atg7^{flox/flox}$:TH-Cre mice could hardly move on the beam and slipped frequently. In particular, the hindpaws of $Atg7^{flox/flox}$:TH-Cre mice often slipped off the beam. Furthermore, in the accelerating rotarod test, fall latency was reduced in $Atg7^{flox/flox}$:TH-Cre mice. Gait disturbance progressed, and by the terminal stage, the majority of affected mice could hardly move.

23.4.1.2 Age-Related Development of p62 Inclusions in the Dopaminergic Neuron

Previous studies showed that deletion of *Atg5* or *Atg7* in the central nervous system leads to formation of inclusions positive for the autophagy adaptor/receptor protein p62 in multiple neuronal populations (Hara et al. 2006; Komatsu et al. 2006).

However, the mechanisms underlying age-related progression and intracellular localization of these inclusions in dopaminergic neurons remain elusive. In $Atg7^{flox/flox}$:TH-Cre mice, these neurons contained eosinophilic aggregates, which are characteristic of LBs. In addition, inclusions containing ubiquitin and p62 were present in TH-positive neurons. In fact, p62 and ubiquitin colocalization strongly predict LBs. In contrast to $Atg7^{flox/flox}$ mice, in which no aggregates were seen even at the age of 18 months, p62-positive inclusions were present in $Atg7^{flox/flox}$:TH-Cre mice at the age of 2 months. In the same animals at 18 months, the p62 inclusions were larger and mainly localized outside the soma. Specifically, p62 inclusions were present along the TH fibers and were mainly located in the branches of dopaminergic neurons. In ultrastructural analysis of the substantia nigra, fibrous inclusions localized in the soma and neurites in dopaminergic neurons. These inclusions contained autophagosome-like structures.

23.4.2 p62-Positive Inclusions Contain Endogenous Synuclein

Previous in vivo analysis of dopaminergic neurons suggested that synuclein regulation is linked to autophagy (Friedman et al. 2012; Ahmed et al. 2012), but the association between synuclein accumulation and PD pathology remained unclear. To address this issue, we asked whether endogenous synuclein colocalizes with p62-positive inclusions in $Atg7^{flox/flox}$:TH-Cre mice. Around 9 months, we pathologically confirmed endogenous synuclein accumulation in somata and neurites. High-resolution confocal images through the substantia nigra revealed immunofluorescence labeling of TH and synuclein, where $89.30 \pm 1.65\%$ synuclein inclusions localized in TH fibers. Although p62 is a representative substrate of autophagy that is rapidly influenced by dysfunction of the pathway, not only p62 but also endogenous synuclein was regulated to some extent by autophagy. $Atg7^{flox/flox}$ mice exhibited no p62-positive aggregates or endogenous synuclein accumulation. On the other hand, $Atg7^{flox/flox}$:TH-Cre mice exhibited synuclein deposition and inclusions that colocalized with p62 in cell bodies. Interestingly, we observed these inclusions not only within the soma but also outside the soma. Thus, loss of autophagy induces synuclein accumulation and formation of LBs, similar to the pathology observed in dopaminergic neurons.

23.4.2.1 Abundance of Tyrosine Hydroxylase Neurons Is Reduced in Aged $Atg7^{flox/flox}$:TH-Cre Mice

To date, many genetically modified mice have been developed as PD models, but most of them do not exhibit neuronal loss. To assess the influence of aggregate formation, we compared the number of TH neurons between aged $Atg7^{flox/flox}$:TH-

Cre and $Atg7^{flox/flox}$ mice. $Atg7^{flox/flox}$:TH-Cre mice exhibited locomotor dysfunction at ages above 110 weeks. We sacrificed these mice and counted TH neurons in three sections (VTA, ventral tegmental area; SNcc, center area of substantia nigra pars compacta; SNcl, lateral area of substantia nigra pars compacta). $Atg7^{flox/flox}$:TH-Cre mice had fewer TH neurons than $Atg7^{flox/flox}$ mice. The reduction in TH cell number was most prominent in the center area of substantia nigra pars compacta (SNcc). Consistent with our results, in PD, the reduced abundance of TH neurons in PD also occurs primarily in the substantia nigra pars compacta. Dopaminergic neuronal loss may contribute to motor impairment observed at the late stages of disease. Furthermore, we tested dopamine physiology in 120-week-old $Atg7^{flox/flox}$:TH-Cre mice by neurochemical analysis of the dorsal striata. High-performance liquid chromatography revealed a reduction in striatal dopamine levels and metabolites in $Atg7^{flox/flox}$:TH-Cre versus control mice. Thus, dopamine content is affected by dopaminergic neuronal loss (Sato et al. 2018).

23.4.3 Parkin-Deficient Mice

23.4.3.1 Locomotor Impairments of Aged Parkin Knockout Mice

In general, the phenotypes of *Parkin* knockout mice do not accurately reflect the symptoms of PD (Goldberg et al. 2003; Periquet et al. 2005). Previously, we generated mice deficient in exon 2 of *Parkin* and characterized them in the early part of life. When young, the mice exhibited very few phenotypes other than downregulation of dopamine release and upregulation of D1 and D2 dopamine receptors in the striatum. At 48 weeks, the abundance of dopaminergic neurons was not reduced (Sato et al. 2006).

Parkin knockout mice were viable at birth, indistinguishable in appearance from their littermates, and had a normal survival rate. Although these mice have not yet been observed over their entire life span, they began to exhibit motor behavioral deficit at 110 weeks. These clinical abnormalities could be observed by the runway, footprint, and rotarod tests. In the runway test, wild-type mice exhibited well-coordinated movement and almost no slips of the forepaw or hindpaw from the beam; by contrast, Parkin knockout mice could hardly move on the beam and frequently slipped. In particular, the hindpaws of Parkin knockout mice often slipped off the beam. Next, because a short stride is a characteristic of PD, including patients with *PARK2* mutations, we conducted the footprint test. Parkin knockout mice had a shorter foot range than wild-type mice. Furthermore, in the accelerating rotarod test, fall latency was reduced in Parkin knockout mice.

23.4.3.2 Mitochondrial Fragmentation in Dopaminergic Neuron

Accumulating knowledge regarding PINK1 and Parkin, both of which are associated with mitochondria, has increased our understanding of these proteins' cellular functions (Pickrell and Youle 2015). The PINK1/Parkin pathway is associated with mitochondrial quality control, but it remains unclear how Parkin influences mitochondrial function or structure in vivo. To evaluate its in vivo function, we analyzed mitochondria in dopaminergic neurons in 110-week-old Parkin-deficient mice, which exhibit locomotor impairments. To identify dopaminergic neurons, we performed immunofluorescence staining for TH and cytochrome c. In Parkin knockout mice, small mitochondria accumulated in the substantia nigra to a greater extent than in wild-type mice. These mitochondria were observed not only in the cytosol but also outside the cell bodies. We speculated that the phenomenon would also be observed in axons and dendrites. To evaluate mitochondria in dopaminergic neurons, we analyzed mitochondrial area in TH-positive cell bodies. In Parkin knockout dopaminergic neurons, mean mitochondria area was smaller than in wild-type mice. Together, these observations suggest that mitochondrial fragmentation might be facilitated in the dopaminergic neurons of aged mice. We speculated that the damaged mitochondria fragmented, resulting in an increase in the number of mitochondria.

23.4.3.3 Accumulation of Damaged Mitochondria

To further characterize these mitochondria, we performed ultrastructural analysis in dopaminergic neurons of 110-week-old mice. We observed small, round, fragmented mitochondria in Parkin-deficient dopaminergic neurons. Precise quantification revealed that mitochondria area was reduced, and the number of fragmented mitochondria per unit of cytosolic area was elevated, in Parkin-deficient dopaminergic neurons. In order for damaged mitochondria to be degraded by autophagy, they must be segregated by fission (Twig et al. 2008). Observation of mitochondrial microstructure in Parkin knockout mice by electron microscopy revealed that the fragmented mitochondria had normal outer membrane structure, but irregular inner structures, i.e., the normal structures of the matrix and cristae, were broken. This observation indicates that damaged mitochondria accumulate due to abrogation of Parkin-mediated mitophagy.

23.4.3.4 Abundance of Tyrosine Hydroxylase Neurons Is Reduced in Aged Parkin Knockout Mice

Although the molecular components required for mitophagy have been identified through extensive in vitro work, the physiological pathology and context of these pathways remain largely unknown (Rodger et al. 2018). Many genetically modified

mice have been developed as PD models, but most of them do not exhibit neuronal loss. To assess the contribution of the accumulation of damaged mitochondria, we compared the number of TH neurons between aged Parkin knockout and control mice. We sacrificed these mice and counted TH neurons in three sections (VTA, SNcc, and SNcl). Parkin knockout mice had fewer TH neurons than wild-type control mice. The reduction in TH cell number was most prominent in SNcc. We conducted stereological quantification of the area of cell bodies but did not identify any difference between Parkin wild-type and Parkin knockout. Furthermore, we conducted stereological quantification of striatal dopaminergic fibers. Parkin knockout mice exhibited significant loss of dopamine fibers in the striatum. Consistent with our results, a reduction in the abundance of TH neurons also occurs in PD, primarily in the SNcc. Loss of dopaminergic neurons may contribute to the motor impairment observed in the late stages of disease. In addition, we tested dopamine physiology in these 120-week-old Parkin knockout mice by neurochemical analysis of the dorsal striata. High-performance liquid chromatography revealed a reduction in striatal dopamine levels and metabolites in Parkin knockout mice relative to control mice. Thus, dopamine content is affected by loss of dopaminergic neurons (Noda et al. 2020a, 2020b).

In general, Parkin knockout mice show rare phenotype; namely, they do not exhibit dopaminergic neuronal loss or movement dysfunction. Previously, we reported that there is no loss of neurons in mice harboring a deletion of Parkin exon 2 at ages up to 12 months (48 weeks) (Sato et al. 2006), and Perez and Palmiter reported no loss of neurons at up to 22 months (88 weeks) (Perez et al. 2005). Thus, Parkin knockout mice do not recapitulate central signs of early-onset PD. However, our long-term observations revealed dopaminergic neuronal loss at 120 weeks. Previous reports indicated that Parkin-deficient mice exhibit inconsistent phenotypes (Itier et al. 2003; Goldberg et al. 2003; Von Coelln et al. 2004; Palacino et al. 2004). The age-related motor dysfunction and pathology we observed in Parkin-deficient mice suggest that impairment of mitochondrial clearance may underlie PD pathology. Our PD model mice exhibited accumulation of damaged mitochondria and neuronal loss. Furthermore, dopamine levels were affected by dopaminergic neuronal loss. These PD models will provide insight into mitochondria-associated PD pathology (Noda et al. 2020a, 2020b).

23.4.4 CHCHD2-Deficient Mice

23.4.4.1 Locomotor Impairments of Aged CHCHD2 Knockout Mice

To determine the function of CHCHD2 in vivo, we generated *CHCHD2* knockout mice and characterized the locomotor impairments of CHCHD2 knockout mice. Although these mice have not yet been observed over their entire life span, they began to exhibit motor behavioral deficits around 115 weeks. These clinical abnormalities could be observed in the runway test and rotarod tests. In the runway test,

wild-type mice exhibited well-coordinated movement and almost no slips of the forepaw or hindpaw from the beam; by contrast, CHCHD2 knockout mice could hardly move on the beam and frequently slipped. In particular, the hindpaws of CHCHD2 knockout mice often slipped off the beam. Furthermore, in the accelerating rotarod test, fall latency was reduced in CHCHD2 knockout mice.

23.4.4.2 Age-Related Development of Inclusions Are Associated with Mitochondria Function

In contrast to younger CHCHD2 knockout mice, in which no aggregates were seen even at the age of 12 months, in aging mice, p62 inclusions were present in dopaminergic neuron cell bodies and outside the soma. To determine the location of these inclusions, we conducted double staining with p62 and TH antibodies and showed that p62 inclusions were present along the fibers, mainly in the branch of dopaminergic neurons. Previous in vivo analyses of dopaminergic neurons suggested that synuclein regulation is linked to autophagy (Friedman et al. 2012; Ahmed et al. 2012), but it remained unclear how synuclein degradation is regulated. But, in this model, we pathologically confirmed endogenous synuclein accumulation and colocalization with p62 inclusions. Although p62 is a representative substrate of autophagy that is rapidly influenced by its dysfunction, it seemed that not only p62 but also endogenous synuclein was regulated to some extent by autophagy. Thus, loss of CHCHD2 might be associated with autophagy dysfunction and formation of LBs. Accumulating knowledge regarding CHCHD2, which is associated with mitochondria, has increased our understanding of the protein's cellular functions. In fact, CHCHD2 is associated with mitochondrial respiration (Funayama et al. 2015), cytochrome c oxidase expression (Aras et al. 2013), complex IV activity (Baughman et al. 2009), and inhibition of apoptosis (Liu et al. 2015) in vitro; however, it remains unclear how CHCHD2 influences autophagy, mitochondrial structure, or mitochondrial function in vivo. To further characterize the mitochondria, we performed ultrastructural analysis in dopaminergic neurons of 120-week-old mice and specifically observed small, round, fragmented mitochondria in CHCHD2-deficient dopaminergic neurons. Furthermore, fragmented mitochondria had normal outer membrane structure but exhibited an irregular inner structure, i.e., the normal structures of matrix and cristae were broken. In order for damaged mitochondria to be degraded by autophagy, they must be segregated by fission (Twig et al. 2008). Thus, CHCHD2 knockout mice may have damaged mitochondria. Indeed, the activities of complexes I and III were reduced in the brains of CHCHD2 knockout mice brain.

23.4.4.3 Abundance of Tyrosine Hydroxylase Neurons Is Reduced in Aged CHCHD2 Knockout Mice

To assess the contribution of accumulation of damaged mitochondria, we compared the number of TH neurons between aged CHCHD2 knockout and control mice. As demonstrated in the rotarod test, CHCHD2 knockout mice exhibited locomotor dysfunction at ages above 115 weeks. We sacrificed 120-week-old mice and counted TH neurons in three sections (VTA, ventral tegmental area; SNcc, center area of substantia nigra pars compacta; SNcl, lateral area of substantia nigra pars compacta). CHCHD2 knockout mice had fewer TH neurons than wild-type control mice. The reduction in TH cell number was most prominent in SNcc. We conducted stereological quantification of the area of cell bodies, but we did not observe a difference between CHCHD2 wild-type and CHCHD2 knockout mice. Loss of dopaminergic neurons may contribute to the motor impairment observed in the late stages of disease. In addition, we tested dopamine physiology in these 120-week-old CHCHD2 knockout mice by neurochemical analysis of the dorsal striata. High-performance liquid chromatography revealed a reduction in striatal dopamine levels in CHCHD2 knockout mice relative to control mice (Sato et al. 2021).

23.5 Conclusion

Until now, we have analyzed several kinds of PD model mice and followed aging-dependent motor function and dopaminergic neuronal loss. In the process, we found that focusing on dopaminergic neurons using aged mice is necessary to analyze PD pathogenesis. In this study, we carried out detailed observations of aged PD model mice, which exhibit motor dysfunction and loss of dopaminergic neurons. Specifically, PD model mice began to exhibit impairment in motor coordination tasks over 100 weeks of age along with apparent motor behavioral deficits. In contrast to wild-type mice, PD model mice showed severe phenotype and SNcc dominant dopaminergic neuronal loss with aging (Table 23.2). Mitochondrial dysfunction has been considered a major contributor to aging and aging-related diseases. Changes in mitochondrial volume, shape, and length seem to be a general feature of the human aging process. Besides morphological changes, significant functional alterations were demonstrated in mitochondria during aging. Although we require further examination to verify our findings, mitochondria may play a key role in the pathophysiology of aging and may be useful targets for preventing and treating chronic disease, as well as for promoting healthy aging.

Table 23.2 Age-related phenotype for Parkinson's disease model mice

Age-related phenotype for Parkinson's disease model mice

	Dopaminergic Neuron	Striatal Dopamine	Locomotor Dysfunction	Mitochondria Size	Mitochondria Structure	Aggregate Formation
Wild-type	VTA ↓	↓	↓	↓	-	-
Atg7 F/F: TH-Cre	SNcc ↓↓	↓↓	↓↓	↓↓	+	++
Parkin knockout	SNcc ↓↓	↓↓	↓↓	↓↓↓	++	-
CHCHD2 knockout	SNcc ↓↓	↓↓	↓↓	↓↓	+	+

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Part VII
Anti-aging: Intervention and Epidemiology

Chapter 24
Evaluating the Brain Aging Through Eyes: The Potential Use of Hyperspectral Imaging Cameras to Diagnose Alzheimer's Disease Risk

Nozomu Mori, Hisashi Oki, Airi Sasaki, Mari Mori, and Toru Nakazawa

Abstract The retina of the human eye contains about a million retinal ganglion cells directly connected to the brain, and it has been noted that in dementia, especially in patients with Alzheimer's disease, there is a change in the thickness of the retinal layer structure and atrophy of the optic nerve bundle. The deposition of amyloid-β and phosphorylated tau, typical pathology in Alzheimer's disease brains, has also been observed in the retina as well as in the postmortem brains of patients. The retina of the eye can be viewed as a "window into the brain." Recently, a new technology called hyperspectral imaging (HSI) cameras has been developed to measure amyloid-β levels, a risk factor for Alzheimer's disease, through noninvasive retinal imaging. It could be used to diagnose the risk of developing dementia in the elderly with a simple fundus examination. In this chapter, we summarize conventional optical coherence tomography (OCT) studies of the retina of Alzheimer's disease patients, compare and contrast data from recent studies using the HSI camera, and discuss the prospects and cautions for future use of this method.

Keywords Amyloid beta · Alzheimer's disease · Tau · Mild cognitive impairment (MCI) · Fundus examination · Retina · Optical coherence tomography (OCT) · Hyperspectral imaging (HSI)

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_24

24.1 Introduction

Alzheimer's disease (AD) is preceded by an undiagnosed condition called mild cognitive impairment (MCI) (Petersen 2011). MCI is associated with some levels of forgetfulness and with residual cognitive ability without impairment, and various diagnostic criteria for MCI have been proposed (American Psychiatric Association 2013; World Health Organization 1993; Morris 1993; Albert et al. 2011; Dubois et al. 2014). MCI could be detected at a slightly earlier stage than dementia in terms of the neuropsychological test scores used to examine dementia. However, there are some difficulties in making a definitive diagnosis of AD. Studies have shown that aggregation of amyloid-β (Aβ), a peptide derived from amyloid precursor protein (APP), accumulates to form senile plaques, causing damages to the synapses in the neural networks in the brain. In addition, neurofibrillary tangles (NFTs) are formed from abnormal hyperphosphorylation of the tau protein, a microtubule-associated protein unique to nerve axons. The NFT accumulation impairs axonal flows required for neuronal excitation and metabolism. Evaluating these two characteristic pathologies in the brain is the only accurate way to assess AD progression. However, brain biopsies are not practical until postmortem. Without performing a brain biopsy, the only way to determine cognitive ability is by asking questions. There are no useful biomarkers that can be used to make a diagnosis for MCI and AD. Recently, studies have shown that both amyloid and tau can be detected by positron-emission tomography (PET). However, PET technology is costly and requires radioactive tracers. While PET has been used in patients with AD for research purposes for many years, it was only recently approved by the FDA for clinical application.

The incidence of dementia, including AD, is rapidly increasing in developed countries worldwide, including Japan, Western European countries, and the United States. In aging societies, dementia is not only a problem for the patients themselves but also a societal problem; the increase in the number of patients with dementia causes financial, physical, and emotional burdens on their families, caregivers, and supporters. Therefore, developing a noninvasive and reliable method to assess the risk of developing dementia is urgently needed from a health economics perspective. In addition, there are increasing desires to detect and control the onset of AD and/or dementia at an early stage when the disease is asymptomatic, as in MCI. Most of the current diagnostic criteria for MCI rely on neuropsychological evaluation due to the lack of reliable and objective biomarkers, indices, or images.

Ideal methods to screening for MCI and AD would be a simple blood test, a hearing or vision screening, or other mass screening methods. Such methods could be performed on seniors when they renew their driver's licenses at ages 75–80. From a public health perspective, easy and noninvasive screening tests would be desirable rather than costly and labor-intensive tests.

24.2 The Retina as a "Window into the Brain"

The retina of the eye is the only central nervous tissue that can be observed noninvasively. Fundus examination with optical coherence tomography (OCT), widely available in most ophthalmology clinics, measures the thickness of the cell layers of the retina and the optic nerve fibers, i.e., the axonal bundles of retinal ganglion cells (RGCs) that run through the outermost layers of the eye: retina. Information from photoreceptor cells (rods, cones, retinal ganglion cells) passes through the bipolar cells. All of the axonal bundles of the retinal ganglion cells in the retinal cell layer become thick bundles forming optic nerves that connect the retina to the interior of the brain, i.e., the lateral geniculate body (LGB) located in the posterior thalamus. The thalamus is an inner brain region originating from the developing diencephalon, as does the retina and its related eye structures. Thus, the eye and the brain maintain a direct relationship from infancy to adulthood and even to elderhood. Retinal neurons maintain synaptic connections with the LGB, optic nerves, and optic tracts throughout life, even after age-related diseases develop, such as glaucoma and macular degeneration.

The retinal ganglion cells are prone to senile degeneration similar to that observed in hippocampal and cortical neurons. Age-related macular degeneration is a serious neurodegenerative disease in the elderly similar to AD. The resulting symptom is not dementia but rather a visual impairment. This parallel may enable inference of geriatric changes in the brain by observing the retinal changes as well. The retina can readily be examined noninvasively in most eye clinics. There is a quick and painless way to obtain retinal fundus images.

Thus, the retina is a window to the brain, potentially affording an ideal examination method of the brain by retinal exam (London et al. 2013). The feasibility of such a diagnostic method would depend on the correlation evidence between brain and retina degeneration.

Therefore, the extent to which the retinal changes reflect cerebral changes in elderly and AD patients has been studied over the last 30 years. Specific questions include whether it is possible to observe the accumulation of amyloid plaques and neurofibrillary tangles and/or the levels of Aβ and p-Tau in the retina (Lim et al. 2016). If amyloid and tau could be detected in the retina, it may be used to test for AD risks through the eyes. While dementia including AD is clinically diagnosed based on history, neuroimaging and neuropsychological assessment, a retinal exam may serve as an effective screening for AD-type dementia risk. Its plausibility also depends on the degree of correlation between the brain and the retina.

To what extent does the "window" of the retina reflect the phenomena in the brain? We first need to answer this question.

24.3 Retinal Findings on OCT Fundus Examination in AD and MCI

Much has been studied over the past 30 years or so to determine what lesions occur in the eye of AD patients using the retina as a "window" into AD development. Below, we discuss those articles to date. In addition to the following discussion, readers may also refer more to recent reviews on this topic (Liao et al. 2018; Szegedi et al. 2020; Lee and Apte 2020; Ngolab et al. 2019; Lemmens et al. 2020a, b; Mirzaei et al. 2020; Fereshetian et al. 2021).

Carol Miller at the University of Southern California in the mid-1980s first pointed out the significance of fundus findings in AD patients (Hinton et al. 1986), introducing the critical importance of examining the retina-brain interrelationship. They reported a reduction in the levels of retinal ganglion cell numbers and a shrinking of the nerve fiber thickness in patients with AD compared to the age-matched healthy individuals as controls. They concluded that retinal observation of the eye could predict AD development. This finding was subsequently analyzed in more detail by Blanks et al. (1989, 1996a, b). With the advent of scanning laser ophthalmoscopy (SLO) and optical coherence tomography (OCT) ophthalmoscopy at the turn of the century, it became possible to obtain precise data across the entire retinal cell layer in vivo in real-time imaging, allowing for detailed analysis of fundus findings in patients with AD. Subsequent data from many SLO and OCT studies have shown definite atrophy, i.e., decreased layer thickness, of the nerve fiber layer (Berisha et al. 2007; Kergoat et al. 2001; Danesh-Meyer et al. 2006). They observed a marked trend, particularly a reduction of the supraorbital region. Subsequent large-scale studies of patients with AD and those with MCI in the preclinical stage of AD showed that the optic ganglion cell changes in the macula correlated well with the disease state rather than the atrophy of the nerve fiber layer thickness (Cheung et al. 2015).

In summary, a decrease in the ganglion cell layer and a decrease in the nerve fiber layer were observed in patients with AD and MCI. However, these changes were also noted in glaucoma, a prevalent condition in middle-aged and older patients for unknown pathophysiology. Some data also point to a decrease in synaptic and neural microtubule changes prior to a decrease in neurons themselves, such as retinal ganglion cells. While such changes precede neuronal death and can be considered a "precursor" to neurodegeneration, detecting subtle changes was challenging. In this regard, some studies have shown that polarized light-sensitive OCT (PS-OCT), which can sort out the birefringence properties of fibrous structures, can be used to detect AD-specific signals (Pircher et al. 2011). In addition to conventional OCT scans, recent attempts have been made to derive more sensitive AD indicators by simultaneously analyzing other parameters in combination: angle-resolved low-coherence interferomatory (a/LCI) systems (Song et al. 2020). The LCI study evaluating changes in the thickness of the conventional retinal cell layer and optic nerve bundle was performed on AD mouse models. Further evaluation of AD indicators using LCI on human patients with AD is necessary.

A group from Barcelona, Spain, reported a large-scale study of cognitive function testing (Marquié et al. 2020). They performed brain MRI, FBB (^{18}F-florbetaben) PET, APOE genotyping, and retinal OCT combined with retinal OCT on 200 middle-aged and people over 55 years old, in order to determine if it is possible to detect MCI at a very early stage, even at the level of mere forgetfulness. The mean age was 65 years, and about 15% of them were FBB-PET-positive. Among those in the FBB-PET-positive group, a trivial decrease in retinal layer thickness on OCT scans could be detected 2 years before the onset of AD. Thus it was possible to detect retinal changes through OCT even at the MCI stage.

There were many studies on retinal vascular distribution and blood flow changes in patients with AD. Based on the studies to date, subtle changes specific to AD have not been identified. Atrophy of the choroid in the eyes of patients with AD has been noticed by a deep examination of the retinal layer using OCT at 1060 nm. However, it was not clarified whether the change is specific to AD or nonspecific change due to aging. For further information, please refer to the aforementioned review articles (Liao et al. 2018; Szegedi et al. 2020; Lee and Apte 2020; Ngolab et al. 2019; Lemmens et al. 2020a, b; Mirzaei et al. 2020; Fereshetian et al. 2021).

24.4 Detection of Amyloid Beta and Phosphorylated Tau in the Retina of AD Patients

Detection of the characteristic pathology of AD—plaques and tangles—in the retina is also crucial, i.e., deposition of amyloid-β (Aβ) in the nerve synapse and aggregate deposition of phosphorylated tau (p-Tau) in the neuronal axons. The first report to examine the presence or absence of Aβ in the retina of the elderly was Karin Leffler et al. at the University of Illinois at Chicago (Löffler et al. 1995). They observed a histological accumulation of amyloid precursor protein (APP) and Aβ in the intercellular spaces of the retina. However, it was not evident in this study whether it was caused by AD. Since then, various studies have been conducted by at least seven laboratories in Europe and the United States, but no clear conclusions have been reached with various conclusions on Aβ and p-Tau deposition in the retina, some positive and some negative. Table 24.1 summarizes the key findings of these studies. Many of the early studies were tend to be negative, whereas more recent studies have concluded to be positive.

In particular, as early as a decade ago, Maya Coronyo et al. at the Cedars-Sinai Medical Center in Los Angeles, hypothesizing that the traditional methodology of fragmental histology was limited in detecting Aβ, stained the entire retina first and then sectioned it for continuous comparison. As a result, they concluded that both the 2011 report and the recent 2017 report were indeed positive for Aβ in the retina of AD patients (Koronyo-Hamaoui et al. 2011; Koronyo et al. 2017). In addition to human data, her group also used a transgenic AD mouse model (APP(SWE)/PS1 (ΔE9); with a mutation in the Swedish amyloid precursor protein and a mutation in

Table 24.1 Experimental comparisons of amyloid-β and phosphorylated tau deposition in the retina of elderly and AD patients

References	Institutes	Retinal subjects	Methods	Antibody	Results
Hinton et al. (1986)	University of Southern California	4 (AD)	Histological staining	—	Tangle-free, plaque-free, vascular amyloid-free, but RGC depletion and axonal degeneration detected
Blanks et al. (1989)	University of Southern California	12 (AD)	Histological staining	—	Tangle not detected, vascular amyloid not detected
Löffler et al. (1995)	University of Illinois	24 (elderly)	Immunostaining	Anti-tau	No age-related changes in tau, APP staining in elderly, Aβ deposition near retinal pigment epithelium in elderly
				Anti-APP	
Leger et al. (2011)	University Hospital of Bordeaux	19 (elderly, age 49–87)	Immunostaining	Anti-tau	Tau aggregation in old age, Aβ and p-Tau not detected
				Anti-p-Tau	
				Anti-Aβ	
Koronyo-Hamaoui et al. (2011)	Cedars-Sinai Medical Center	8 (AD), 5 (HC)	Immunostaining	Anti-Aβ	Aβ detected in retinas of AD patients
			Curcumin staining		
Ho et al. (2014)	Johns Hopkins University	6 (AD), 6 (HC)	Immunostaining	Anti-A	Non-detectable Aβ, non-detectable tau, but α-synuclein deposition detected in the retina of AD patient
				Anti-p-Tau	
				Anti-α-synuclein	
Koronyo et al. (2017)	Cedars-Sinai Medical Center	37 (AD, HC)	Immunostaining	Anti-Aβ	Aβ detected in the retina of AD patient
			Curcumin staining		
Williams et al. (2017)	Massachusetts General Hospital	17 (AD), 2 (HC)	Immune staining	Anti-Aβ	Aβ not detected, p-Tau not detected, TDP-43 not detected
				Anti-ubiquitin	
				Anti-α-synuclein	
				Anti-TDP-43	
den Haan et al. (2018)	Amsterdam Free University	6 (AD), 6 (HC)	Immunostaining	Anti-Aβ	Aβ and APP detected in the retina of AD patients, but qualitatively different from those in the brain. p-Tau elevated in the retina of AD patients (inner and outer reticular layers)
				Anti-tau	
				Anti-p-Tau	

Aβ amyloid beta, *AD* Alzheimer disease's, *APP* amyloid precursor protein, *HC* healthy control

the presenilin gene) to compare Aβ accumulation in the brain and the retina and found that Aβ accumulation in the retina was positive. They clearly demonstrated that retinal Aβ detection in patients with AD is possible (Leger et al. 2011). Furthermore, they demonstrated that Aβ deposition could be detected in vivo 2 days after administration of curcumin, a major component of turmeric that has a high affinity with Aβ, using a conventional OCT camera.

Hoozemans and his colleagues from the AD Research Center at the Free University of Amsterdam in the Netherlands recently examined in detail the retinal deposition of Aβ and p-Tau in patients with AD (den Haan et al. 2018). They compared the histological accumulation of protein aggregation in the postmortem brain and retina in six patients with AD to six healthy age-matched controls. While no retinal plaques were observed even in patients with cerebral plaques, retinal APP and Aβ were present. However, the retinal histologies were not as specific to patients with AD as those seen in the brains of patients with AD. Furthermore, the apparent presence of both APP and Aβ in elderly controls suggested that the plaques were not AD-specific. On the other hand, the researchers concluded that retinal tau could be a more appropriate biomarker for estimating AD risk because p-Tau was observed in the retina as well as in the brains of patients with AD but not in controls. However, the sample size was small, and thus further studies are awaited.

24.5 Noninvasive Imaging of the Retina Using Hyperspectral Imaging Cameras

Most of the above studies were based on histological examinations of the postmortem brain and eye stock from patients with AD, as AD biomarker evaluation used to be only possible postmortem. Recent imaging technology advancements enabled in vivo assessment of Aβ and other biomarkers.

The hyperspectral imaging (HSI), designed to capture tissue pathology and biomarker changes, can be applied to the retina to demonstrate minute-by-minute change by reconstructing the spectroscopic data taken with a 5 nm increment of the wavelength. Integration of the HSI technology to the fundus camera has received much attention in the ophthalmology field (Kashani et al. 2014; Gao and Smith 2015). This technique allows real-time quantitative observations of retinal Aβ accumulation. Most notable are the works done by Robert Vince at the University of Minnesota, Minneapolis, USA, and Peter van Wijngaarden at the University of Melbourne, Australia. Their works using the human subjects and animal models are summarized below (Table 24.2).

Robert Vince of the University of Minnesota, custom-designed an HSI microscope, and using the technology, in collaboration with Jim Beach, the technology division head of the Alabama company CytoViva, showed in 2014 that Aβ can be detected in the mouse retina (More and Vince 2015). They presented the data on brain and retina analysis in the AD model mouse (APP/PS1) as well as on the

Table 24.2 Comparison of studies on detection of retinal Aβ by fundus examination using HSI camera (2015–2019)

References	Types of the camera	Subjects of the study	Summary of the results
More and Vince (2015)	HSIC/CytoViva	AD model mice (APP/PS1) Human (AD vs. HC)	An HSI camera-equipped microscope system was constructed. Changes in HSI signals in the human brain and retina were detected in the range of 400–1000 nm; Tg mice showed Aβ deposition in the brain at 7 months of age, while HS imaging in the retina showed signals at 6 months of age. In addition, when GSH, a candidate anti-AD drug was administered to these Tg mice, the effect of the drug was detected as a signal in retinal HSI after 2 months
More et al. (2016)	HSIC/CytoViva	AD model mice (APP/PS1)	HS imaging of the mouse retina revealed different vascular images at different wave lengths (510–705 nm), and changes in HSI signal (480–700 nm) in the retina of AD model mice and humans were observed including AD patients. Retinal HSI signal was compared in Tg mice from 3 to 8 months of age. Retinal HSI signals in Tg mice from 3 to 8 months of age were compared, and it was found that Aβ-derived Rayleigh scattering signals (480 nm) were detectable as early as 5 months, earlier than the detection of amyloidopathy at 6 months of age
More et al. (2019)	HSIC/CytoViva	Mice and human, AD patients AD ($n = 19$), non-AD ($n = 16$)	The HSI camera-equipped microscope has been modified into a device suitable for human use in ophthalmology. Cognitive testing and retinal HS imaging were compared in AD patients and non-AD healthy subjects. HSI signals were detected more sensitively at various retinal locations in the MCI group rather than in AD patients with advanced dementia. No significant difference was detected in HSI signals between AD and non-AD patients around the age of 75 years, suggesting that HS imaging can be used to identify MQ selectively
Hadoux et al. (2019)	MHRC/Optina	Human: AD ($n = 15$) vs. HC ($n = 20$) AD model mice (5 × FAD)	Retinal HS imaging in AD and non-AD was compared using Optina's HS camera (MHRC). Data in 450–900 nm range were acquired. With appropriate correction, a significant difference between AD and non-AD was detected at around 550 nm. The signal sensitivity was most obvious at the macula, although a significant difference could be detectable in the whole retina. In comparing with Aβ-PET examination for AD patients, Aβ-PET positivity and HS score were almost proportional. Histological accumulation of Aβ in the retina correlated well with the HS score in AD model mice (5 × FAD)

AD Alzheimer's disease, *HC* healthy control, *HS* hyperspectral, *HIS* hyperspectral imaging, *MCI* mild cognitive impairment, *Tg* transgenic

SH-SY5Y cell line expressing Aβ. While Aβ had been known to aggregate in the brain of AD mouse models at 7 months of age previously, they detected differences in HS signaling in the retina of WT and AD mice as early as 6 months of age. In a small number of cases, they also compared HSI spectra in the human brain and retinal samples and detected significant differences in signals between AD patients and healthy control subjects. The following year, in collaboration with Jim Beach, the Minnesota group developed a small topical fundus imaging and used it to show detailed spectroscopic data on the retina of a mouse model of AD (More et al. 2016). In this AD mouse model, changes in retinal blood vessels, presumably caused by Aβ, were detected after 6 months of age in the form of increased hemoglobin light absorption, especially around 550 nm of wavelength. Light scattering with a peak around 480 nm could be detected as early as 4–5 months of age. According to the authors, this is because Rayleigh scattering (scattering by particles smaller than the wavelength of light) caused by Aβ in the retina is most pronounced at this wavelength. Although the number of cases is small, they also demonstrated that differences in HSI signals could be detected in the retina of humans with AD compared to healthy controls.

Based on this finding, in the fall of 2019, they showed that retinal HSI could be used for preclinical diagnosis of AD in humans using HSI and Aβ-PET data from patients with AD (More et al. 2019). The data showed that the age effect is minimal and the signals are not affected by other ocular diseases such as cataracts and glaucoma. What is distinctive about the data is that they stratified the patients according to the progression of AD (cognitive levels), to show that the difference in signals between AD and controls was greater in the patient group with MCI compared to the patient group with AD with lower cognitive ability, when measured by retinal HSI (rHSI) index. The reason for this, as highlighted in an earlier paper (More et al. 2016), is that rHSI does not directly capture the large aggregation of Aβ (plaque) but instead takes advantage of the "Rayleigh scattering" to capture lowlevel amyloid oligomers present at a preclinical stage.

At about the same time, Peter van Wijngarden's group at the University of Melbourne, in collaboration with the McGill University group in Canada, reported that it was possible to measure Aβ noninvasively with an HSI camera in patients with AD (Hadoux et al. 2019). This group used an HSI camera manufactured by Optina of Montreal, Canada, called the Metabolic Hyperspectral Retinal Camera (MHRC), which builds 91 image cubes in the spectra ranging from 900 to 450 nm by 5 nm increment, in 0.01 s per cube or merely 1 s in total. They also showed the correlation between Aβ detection in the brain and retina in human patients with AD as well as in transgenic mouse model (5 × FAD) and classified cognitively impaired and unimpaired subjects into positive (AD) and negative (control) groups based upon the brain PET imaging study of Aβ. The retinal Aβ signal was detected in all the regions with statistical significance in all patients with AD with relatively higher sensitivity in the supraorbital region (S1) and the peripapillary region (F1).

Thus, with the advent of the HSI camera, it is now possible to visualize retinal Aβ noninvasively with live imaging without any staining. While Maya Koronyo in Los Angeles demonstrated that OCT imaging study with administration of curcumin

2 days prior to the imaging can detect retinal Aβ, the HSI camera is still highly advantageous for its convenience. If further improvements could be made to detect p-Tau levels, the HSI camera may be used as a standard instrument for the preclinical diagnosis of dementia and screen for the AD risk. Even today, based on the paper by Robert Vince, the rHSI index in the retina is greater in patients with MCI than in patients with advanced AD, making it an ideal screening tool for the dementia risk. In contrast to the findings by the Minnesota group, the Wijngaarden laboratory in Melbourne found that the HSI signal detection in the retina was roughly proportional to the levels of Aβ deposition in the brain. The latter group (Melbourne) suggested that Aβ can be detected in the retina in proportion to the detection of Aβ in the brain, whereas the former group (Minnesota) suggested that the rHSI disappears at the time of Aβ deposition but instead selectively detects the early Aβ oligomer formation, thus allowing earlier detection during the progression of dementia. Herein, along with the difference in data between the two groups, some critical differences exist in the interpretation of the HSI technical details.

24.6 Significance of Retinal Amyloid β Detection: Expectations and Cautions for Dementia Risk Assessment

Thus, the introduction of the HSI camera has increased the promise of preclinical diagnosis of AD by noninvasive fundus examination of the eyes. It is indeed possible to detect Aβ on the retina. However, the use of detection of Aβ on the retina alone still needs to be carefully evaluated before it can be considered direct evidence of the risk of AD. Below, we discuss some of the points that should be considered for future research.

First, the detection of retinal Aβ may not be specific to AD but could also be detected in patients with other age-related ocular diseases such as glaucoma. Glaucoma is a progressive neurodegenerative disease that occurs predominantly in middle-aged people and is thought to be caused essentially by elevated intraocular pressure and oxidative stress. Axonal degeneration of retinal ganglion cells (RGCs) eventually leads to cell body dropout (cell death). In this sense, glaucoma can be considered a neurodegenerative disease. The accumulation of Aβ seen in glaucoma is often confined to the RGC layer. In comparison, in AD, Aβ is often spread throughout the entire retinal layers. In addition, phosphorylated tau (p-Tau), which is the cause of tangles in AD brains, is also detected in the inner granular cell layer, the inner reticular layer, and the ganglion cell layer of the retina in patients with AD. In patients with glaucoma, p-Tau is rarely detected, and even when it is detected, it appears to be limited to the inner granular cell layer. HSI cameras may differentiate cases with AD from those with glaucoma based on differences in the distribution pattern of Aβ and p-Tau.

Vince and colleagues at the University of Minnesota concluded that Aβ could be detected in the retina in a mouse model of AD before the onset of memory impairment, and studies in the brains of patients with AD also showed that Aβ begins to accumulate several years earlier than the onset of dementia symptoms. The formation of tangles can then be detected several years later. The onset of cognitive symptoms roughly coincides with the time of detection of tangles. In the brain, there is clearly a time difference between the findings of Aβ and p-Tau. At present, it is not clear whether such a time difference exists in the retinal data; future comparative longitudinal studies are required on the timing of retinal Aβ and p-Tau signal detection in combination with brain PET imaging on patients with AD, as well as on new mouse models with advanced AD with enhanced tau aggregation.

Many cases of Aβ accumulation on the retina were detected around neurons, such as retinal ganglion cells and bipolar cells, while others were detected around blood vessels in the retina. In particular, Koronyo et al. (Los Angeles) showed that OCT after the administration of curcumin can preferentially detect vascular amyloid. Although AD is dementia that originates in neurons, in another type of dementia, e.g., vascular dementia, Aβ may be predominantly deposited in the blood vessels. It may be worthwhile to examine this possibility in detail using mouse models of vascular dementia.

Finally, it is important to note again that although the HSI camera has enabled us to analyze retinal Aβ, it only suggests the presence of Aβ as a "signal" and does not detect the accumulation of Aβ itself in the retinal tissue image in a region-specific manner. In addition to obtaining data from patients with AD and MCI and many control subjects with advanced age without cognitive impairment and performing statistical analysis, more detailed comparative studies of the retina and brain using appropriate AD mouse models are needed at this time. In addition to the current technologies used to detect retinal Aβ signals, as already mentioned, the development of new technologies that can detect p-Tau signals is anticipated. To this end, the P301L mouse model of tauopathies may be useful in comparing the correlation between the retina and the brain tauopathies.

24.7 Perspectives

There are high expectations for a simple fundus examination as a preliminary test for cognitive decline and dementia for the elderly. There are very promising technologies available to screen people for dementia risk in the aging society.

In this article, we discussed the possible application of HSI camera for detecting risk for MCI and AD. However, not all dementias are predicated on hippocampal learning memory. There are many other types of cognitive impairments, such as an inability to perform the activity of daily living, and attention disorders, such as frequent inattention. In short, it depends on which region of the aging brain is responsible for the functional decline. Not all of them may be related to retinal or cerebral Aβ.

Since dementia is a slow and progressive neurodegenerative disease that may not become evident until later in life, early screening is desired for early intervention. In order to live well into old age, it is important to pay attention to brain health after the age of 75. A time may come when HSI cameras are used in the geriatric community to determine whether the citizens have MCI or healthy without dementia risk.

With the advent of the HSI camera, the concept of using the eye as a window into the brain to screen people with dementia may become a reality. News reports of accidents involving elderly drivers driving in the wrong direction on the road and incidents of demented patients entering railway tracks suggest that screening for people at risk for dementia is an issue of serious social concern. There is no doubt that early intervention is essential for society as a whole, as well as it is for the individual. Although it is highly impractical to require all elderly people to undergo amyloid PET examinations, it would not be too much of a public health/medical economics burden to require all elderly people to undergo an HSI camera fundus examination to screen for dementia risk. Such a test could be applied in place of the cognitive function test upon a driver's license renewal over the ages of 75–80. The screening would help protect the aging society from the increasing dangers and save the lives of young and old people.

Acknowledgments We are grateful to David Lapointe and Marie-Claude Marchand at Optina diagnostics for sharing information and stimulating discussion. This study was supported in part by JSPS KAKENHI Grant No. 17K07111 to NM.

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Chapter 25
Healthy Aging in Japan

Hiroshi Shimokata and Rei Otsuka

Abstract The average life expectancy and healthy life expectancy of Japanese people are among the highest in the world. In recent years, the physical and intellectual aging process of Japanese people has slowed down by about 10 years compared to the past. The definition of older adults being 65 years old or older no longer fits the current situation in Japan. The joint committee of the Japan Geriatrics Society and the Japanese Society of Gerontology has proposed that people over 75 years old should be considered older adults. Genetic predisposition may be a factor in the healthy longevity of the Japanese, but the effects of social factors and lifestyle are far more significant. The most important social factor in Japan is a medical system that allows people to visit a medical institution at any time, freely and without worrying about the cost. As for lifestyle, diet is important. A higher intake of vegetables, fruits, grains, and fish, a lower intake of meat and dairy products, less obesity, and less alcohol consumption are thought to contribute to a longer healthy life span.

Keywords Life expectancy · Dietary pattern · Japanese diet · Genotype · Longevity

25.1 Introduction

The average life expectancy in the world varies from country to country by more than 30 years. The longevity of Japanese people is one of the highest in the world. More than 70,000 people lived to be over 100 years old in 2019. We describe the changes in the life expectancy of the Japanese people, review the definition of the

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3_25

older adults, and introduce the factors that determine the longevity of the Japanese people.

25.2 Extension of Life Span

25.2.1 Life Span

Average life expectancy is the expected value of how many more years a person of a given age can expect to live, which is estimated from an age-specific population and mortality data. The average life expectancy at age 0 is the average life span (Our World in Data 2021). Around 1920, the average life expectancy of Japanese people was about 42 years for men and 43 years for women. Thereafter, with the development of medical care and improvements in nutritional conditions, the average life expectancy began to increase rapidly, exceeding 50 years for both men and women in 1947 after World War II, and 60 years in 1951. Since then, the rate of increase has slowed down a bit, and there was a temporary decrease due to the Great East Japan Earthquake, but the average life expectancy will continue to increase in the future. In 2019, the average life expectancy was 81.41 years for men and 87.45 years for women (Ministry of Health, Labour and Welfare Government of Japan 2019). According to data from the Ministry of Health, Labour and Welfare, the average life expectancy of Japanese people was the second highest in the world for men, after Switzerland as a country, and the highest for women. It can be said that Japanese people, both men and women, have one of the highest life expectancies in the developed world.

25.2.2 HALE

The World Health Organization (WHO) defined healthy life expectancy (HALE) as the average number of years that a person can expect to live in "full health" by taking into account years lived in less than full health due to disease and/or injury (WHO 2021).

The Global Burden of Disease Study (GBD) is a good source of internationally comparable HALE data. The GBD is a comprehensive regional and global burden of disease research program on mortality from disease, trauma, and risk factors, as well as the burden of disease from disability, run as an international collaboration of 127 countries led by the Institute for Health Metrics and Evaluation (IHME) at the University of Washington. The GBD determines the disability weights for 291 diseases and 1160 sequelae, calculates years of life lived with disability, and estimates HALE from average life span. The GBD uses this objective method to estimate HALE for countries around the world from 1990 (Kassebaum et al. 2016; Wang et al. 2016).

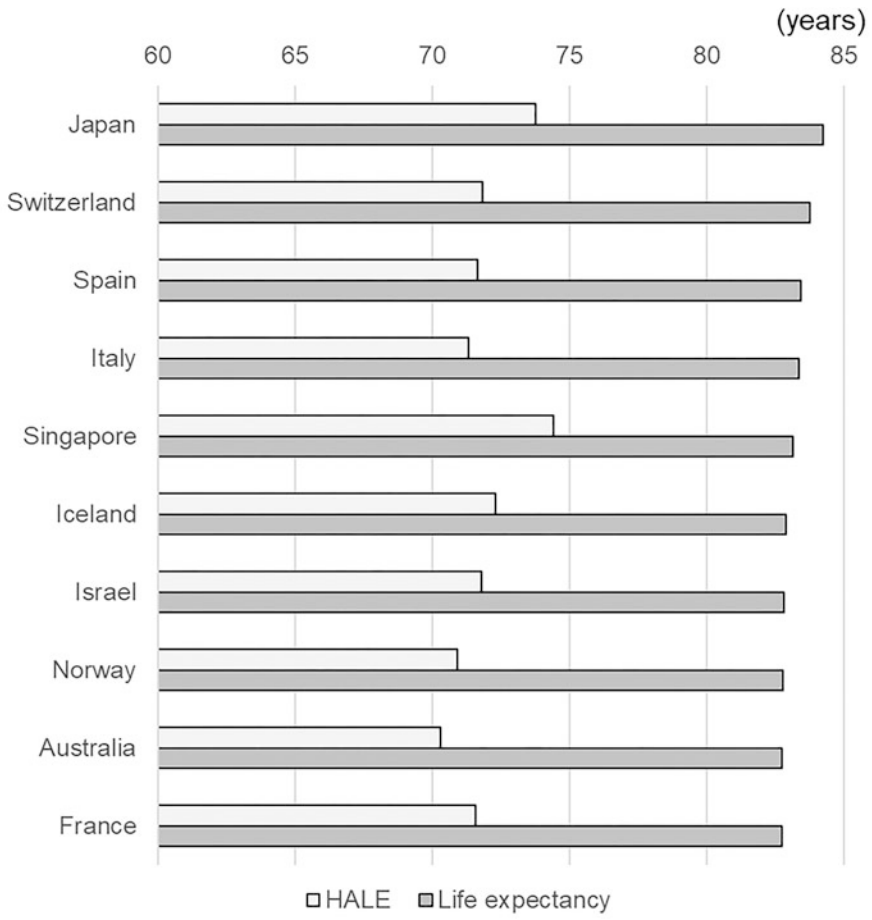

Fig. 25.1 Life expectancy and healthy life expectancy (HALE) in the top ten countries with populations of 1 million or more (World Bank 2020; GBD 2021)

Figure 25.1 shows the top ten countries for male and female average life expectancy and HALE in 2018 according to GBD and World Bank statistics (GBD 2021; World Bank 2020), in order of life expectancy. Japan has the highest average life expectancy in the world, with 84.21 years for men and women combined, followed by Switzerland, Spain, and Italy. The difference between Japan and second-place Switzerland is 0.46 years. Singapore ranks first in terms of HALE, while Japan ranks second. The difference between Singapore and Japan in the case of HALE is 0.67 years, and the difference between Japan and Iceland, which is in third place, is 1.45 years, which is a large gap (World Bank 2020). Countries with longer life expectancy generally have longer HALE, but the relationship between average life expectancy and HALE is not necessarily constant. As shown in Fig. 25.2, Singapore

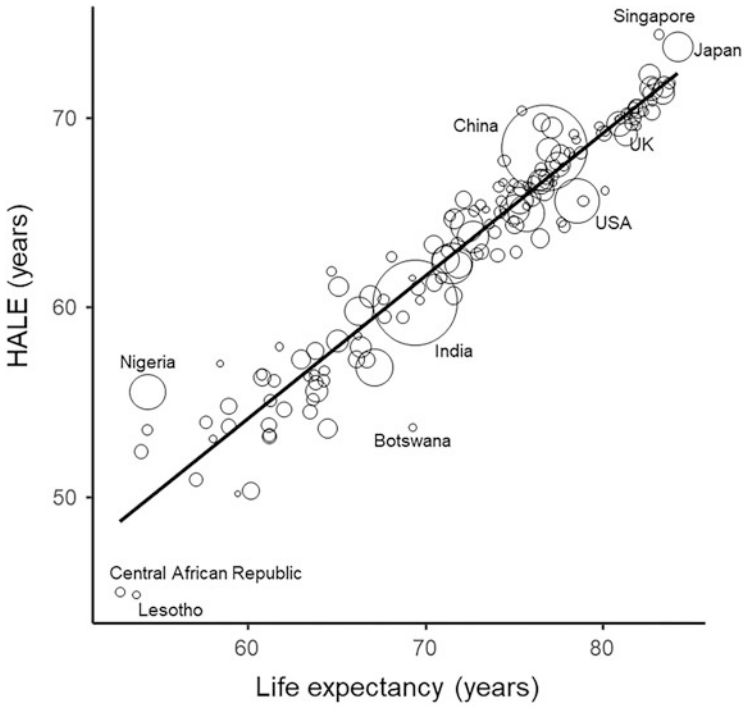

Fig. 25.2 Relationship between life expectancy and healthy life expectancy (HALE) in 2018 in countries with populations of 1 million or more based on Global Burden of Disease (GBD) and World Bank statistics (GBD 2021; World Bank 2020). The size of the bubble indicates population

and Japan have relatively high HALE compared to average life expectancy, while the United States has relatively low HALE.

25.3 Redefining the Older Adults in Japan

The definition of the older adults as those with a calendar age of 65 years or older has been adopted by many countries around the world. However, there is no medical or biological basis for this definition. In Japan, the progression of physical and mental aging is slowing down, although there are individual differences, and many people over 65 are becoming more youthful and active, and it has come to be considered that it is inappropriate to use this as the definition of older adults (Ouchi et al. 2017). This section outlines the redefinition of the older adults in Japan by clarifying the recent trends in age-related changes in physical function and intelligence in the Japanese population.

25.3.1 Changes in Physical Functions with Age

In order to clarify the pattern of aging change and the transition of physical and mental functions in Japanese, grip strength and walking speed were used as physical functions, and intelligence as a psychological function. We used a generalized mixed additive model to clarify the changes in these functions with average aging over time. Subjects of the study were participants of the National Institute for Longevity Sciences-Longitudinal Study of Aging (NILS-LSA) (age 40−79 years at the start of the study) who were randomly selected from the local population stratified by age and sex. The NILS-LSA has been conducting detailed medical, psychological, exercise, body composition, nutritional, social background, and lifestyle surveys on seven participants each day since 1997, with follow-up every 2 years (Shimokata et al. 2000). The cohort is a dynamic cohort with the same number of participants as dropouts during follow-up. Seven surveys were completed by 2012, with a total of 3983 participants and 16,338 measurements.

Grip strength was measured using a hand dynamometer, starting with the right hand, twice on each side. The maximum value for both right and left hands was adopted, and the average value for both sides was used as the measurement value. Walking speed was calculated as the speed in minutes when walking at "normal walking speed" on a 10-m walking path.

Age-related changes in grip strength and gait speed were examined for each gender using a generalized mixed additive model with a smoothed spline curve applied to the interaction term between age and time of measurement. In the generalized mixed additive model examining age-related changes in grip strength, the interaction term between age and time of the survey was significant for both men and women ($p < 0.001$), and the adjusted R-square was 0.404 for men and 0.209 for women. In terms of walking speed, the interaction term between age and time of survey was significant for both men and women ($p < 0.001$), and adjusted R-square was 0.171 for men and 0.234 for women. Figure 25.3 shows the changes in grip strength and walking speed with age based on the survey. Seven surveys have been conducted almost every 2 years.

The rate of decline in grip strength due to aging was relatively slow in both men and women in their 40s but declined more quickly and almost linearly after their 50s. The grip strength of both men and women in their 40s decreased from the first survey to the seventh survey, but for those in their 50s and above, there was little effect of the survey period. The walking speed of both men and women declined sharply from their mid-60s. However, the walking speed increased from the first to the seventh survey. Comparing the first and seventh surveys, the decline in walking speed was slower by 5–15 years.

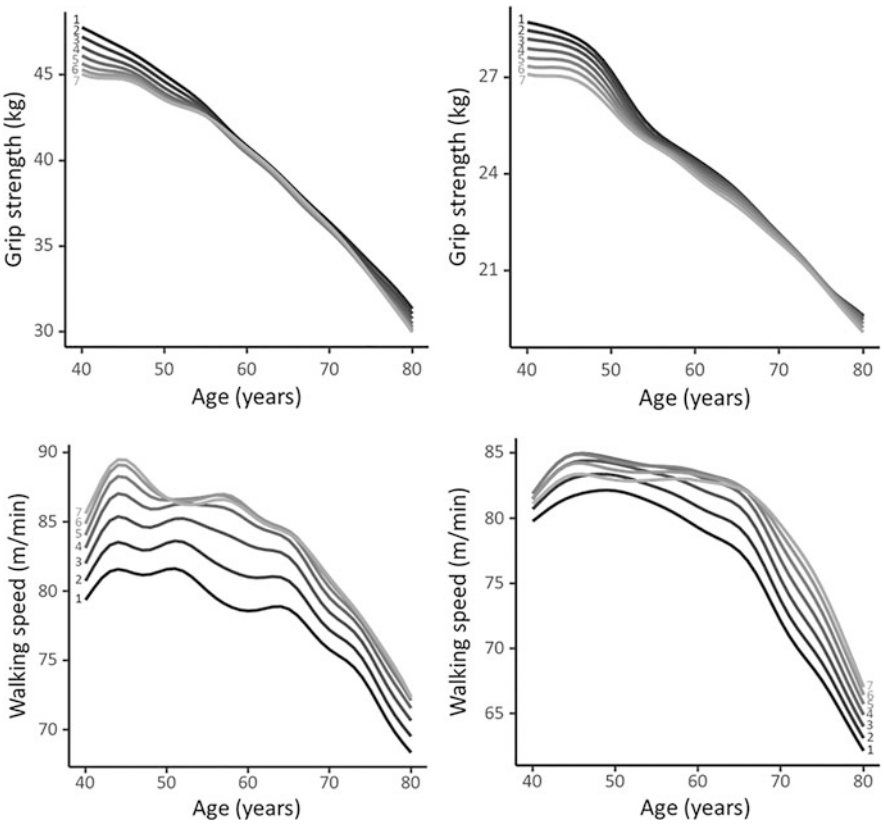

Fig. 25.3 Age-related changes in grip strength and walking speed by gender. A generalized mixed additive model with a smoothed spline curve applied to the interaction term between age and survey period was used to show aging change and secular variation over the 12-year period from the first (1997–2000) through seventh (2010–2012) surveys of the National Institute for Longevity Sciences-Longitudinal Study of Aging (NILS-LSA)

25.3.2 Age-Related Changes in Intelligence

As with physical functioning, age-related changes in intelligence were also examined in the NILS-LSA participants. Intelligence was assessed by the Wechsler Adult Intelligence Scale—Revised—Short Form (WAIS-R-SF), which used Z-transformed scores of four subtests for analysis: Information (amount of general knowledge), Similarity (logical and abstract thinking), Picture Completion (recall and matching of visual long-term memory), and Digit Symbol (speed of information processing). Age-related changes in intelligence were examined using a generalized mixed additive model with a smoothed spline curve applied to the interaction term between measurement time and age for the four subtests of Information, Similarity,

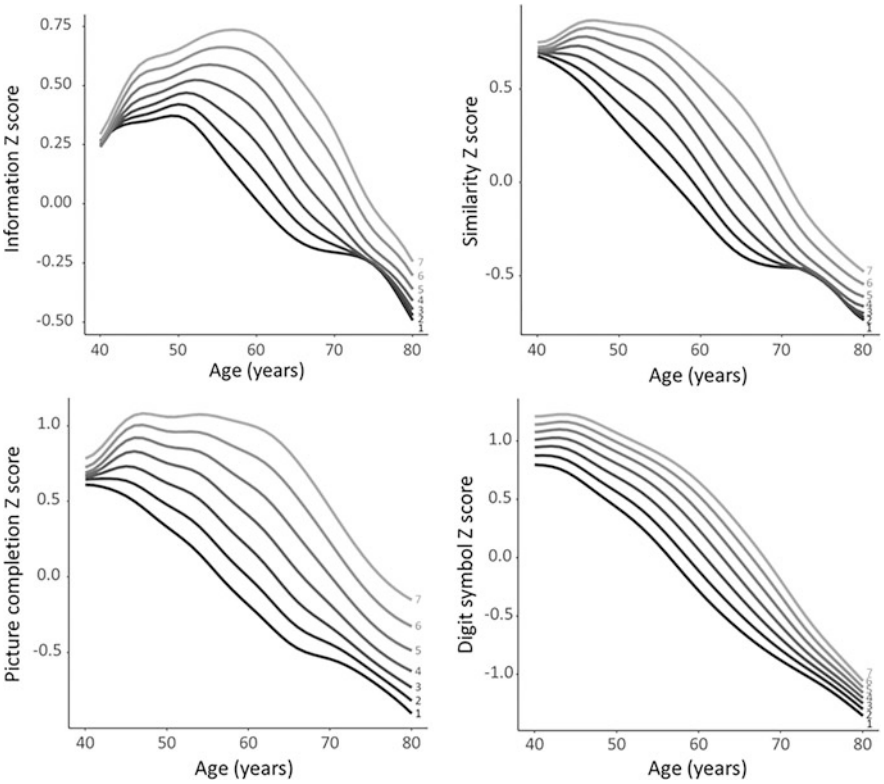

Fig. 25.4 Age-related changes in WAIS-R-SF subtests. Changes in aging and secular variation over the 12-year period from the first to the seventh survey are shown by a generalized mixed additive model with a smoothed spline curve applied to the interaction term between age and survey period, adjusting for gender and the initial effects of examination. Labels 1–7 on the aging curves indicate the first (1997–2000) through seventh (2010–2012) surveys of the National Institute for Longevity Sciences-Longitudinal Study of Aging (NILS-LSA)

Picture Completion, and Digit Symbol, controlling for gender and the initial effect of testing.

In the model, the interaction term between age and time of survey was significant for all subtests ($p < 0.001$). The adjusted R-square was 0.140 for Information, 0.258 for Similarity, 0.310 for Picture Completion, and 0.608 for Digit Symbol.

Figure 25.4 shows the age-related changes in subtests of intelligence by WAIS-R-SF for each survey. After age 60, all scores of subtests decreased with age, but the scores at the same age became higher over the course of the surveys. The peak age of the Z-score for information moved from around 50 to 60 years old as the first to seventh surveys were conducted. The Z-scores for Similarity and Picture Completion began to decline at age 40 in the first survey, but in the seventh survey, the Similarity score did not decline until the late 50s and the Picture Completion did not decline until the 60s. For Digit Symbol, the decline in scores began in the 40s, and

although the movement of the peak age was not clear, the values of the scores at each age became higher over the course of the surveys.

25.3.3 New Definition of the Older Adults

For grip strength, the secular effect on age-related changes was not clear, but for walking speed and intelligence, age-related changes were, on average, about 10 years slower. Analysis of various data on the physical and mental health of the older adults has revealed a "delayed aging phenomenon," in which the onset of age-related changes in physical and mental functions, such as walking speed and intelligence, is delayed by 5–10 years in today's older adults compared to 10–20 years ago (Suzuki 2018). Even among those aged 65 and over, who were previously considered older adults, especially those aged 65−74, both physical and mental health are maintained, and the majority of them are able to participate in active social activities. For these reasons, the joint committee of the Japan Geriatrics Society and the Japanese Society of Gerontology recommended in 2017 that 75 years and older be defined as old, 65–74 years as pre-old, and 90 years and older as oldest-old or super-old (Ouchi et al. 2017).

25.4 Why the Japanese Live Longer

Why do Japanese people have one of the highest average life expectancies and HALE in the world? Genetic factors, medical systems, socioeconomic factors, and lifestyle habits may be the factors that contribute to longevity. In this section, we will explore these factors.

25.4.1 Genetic Factors

25.4.1.1 Longevity-Related Genes in Japanese

Life span is a complex trait that is controlled by multiple genetic and environmental factors. Twin studies have shown that the heritability of longevity is 20−30% (Skytthe et al. 2003). Furthermore, a greater genetic than environmental influence on longevity has been observed in families with exceptional longevity than in the normal population, suggesting the existence of genetic variants associated with extreme longevity (Schoenmaker et al. 2006; Sebastiani and Perls 2012). Many longevity-related genes have been reported so far. We have also reported the longevity-related genes in Japanese people (Tanisawa et al. 2017). The epigenome-wide association study on two independent Japanese cohorts consisting

of 530 nonagenarians/centenarians demonstrated that the G allele of CLEC3B missense variant p.S106G was associated with extreme longevity at the exome-wide level of significance ($p = 2.33 \times 10^{-7}$, odds ratio [OR] = 1.50). The CLEC3B gene encodes tetranectin, a protein implicated in the mineralization process in osteogenesis, as well as in the prognosis and metastasis of cancer. Such genetic polymorphisms may be associated with extreme longevity, which may be exceptional, but not necessarily with average life expectancy or HALE in the general population.

25.4.1.2 Apolipoprotein E (APOE) Genotype

The APOE ε4 allele, a well-known Alzheimer's disease-associated gene polymorphism, shortens life span, while ε2 has been pointed out as a possible life span enhancer (Sebastiani et al. 2019). The ε4 allele of the APOE gene is associated with increased cholesterol levels and heart disease. The APOE ε2 allele could be identified as a "thrifty" allele (Corbo and Scacchi 1999). There are large regional differences in the global distribution of APOE ε2 allele. In high-latitude cold environments and low-latitude hot environments, metabolic rate is elevated, which could require higher cholesterol levels (Singh et al. 2006). In a time when humanity was starving, having the APOE ε4 allele was effective in retaining cholesterol, but in modern society where large amounts of saturated fatty acids are consumed, it causes heart disease, Alzheimer's disease, and shortened life expectancy.

25.4.1.3 Gene-Environment Interaction

Growth hormone (GH)/insulin-like growth factor-1 (IGF-1)/insulin signaling is one of the most plausible biological pathways regulating growth, aging, and longevity. In our examination of genes associated with height, we have reported that accumulation of polymorphisms in genes that result in short stature is a factor in longevity (Tanisawa et al. 2018). Height is associated with nutritional status in childhood, and the relationship between environmental factors and genes is also important. In the general population, polymorphisms in genes related to lifestyle-related diseases such as cancer, obesity, hypertension, diabetes, and lipid disorders seem to be more important for life expectancy than longevity genes (Shimokata and Ando 2012). The interaction between these disease genes and lifestyle factors may be the most important factor in determining life expectancy and HALE.

25.4.2 Obesity and Dietary Habits

Our body is made up of the food we eat. Dietary habits are probably one of the most important factors that affect health and life expectancy. The characteristics of the

Japanese are less obese, having a unique diet based on fish, vegetables, and rice, and a genetically determined lower capacity to metabolize alcohol, relative to the other populations. These factors may be related to the longevity of the Japanese.

25.4.2.1 Obesity

The WHO defines obesity as body mass index (BMI) of 30 or more, and overweight as BMI between 25 and 30. In 2015, 107.7 million children and 603.7 million adults worldwide were estimated to be obese, representing 5.0% of children and 12.0% of adults. Between 1975 and 2014, the prevalence increased from 3.2 to 10.8% in males and from 6.4 to 14.9% in females and is expected to reach 18% in males and 21% in females by 2025 (Non-Communicable Diseases Risk Factor Collaboration 2016). Even though childhood obesity is less common than adult obesity, the rate of childhood obesity is increasing faster than adult obesity.

In the past few decades, obesity has increased as societies have become more affluent. Even today, the percentage of obese people is not as high as it is in poorer countries. Until the GDP per capita reaches about $10,000, the number of obese people increases rapidly as the country becomes richer, but after that, the percentage of obese people hits a plateau and does not increase (Fig. 25.5). This is most likely due to lifestyle, social systems, and disparities. With health education and lifestyle support for obesity, there are many cases where obesity is rather high in poor families, as in the United States. Japan, South Korea, and Singapore are relatively affluent countries, yet percentages of obese people are exceptionally low in these countries. In Japan, the energy intake of both men and women has been decreasing every year since 1997, and the percentage of people with a BMI of 25 or higher has been increasing only among adult men, which is different from the global trend (Nishi 2015). This low rate of obesity probably contributes to the fact that Japan is one of the top countries in the world in both average life expectancy and HALE.

Obesity is responsible for 4 million deaths worldwide, two-thirds of which are due to cardiovascular diseases such as heart disease and stroke (Afshin et al. 2017). Heart failure, which accounts for the majority of heart disease, can cause difficulty walking, respiratory failure, and edema. Strokes can also shorten HALE by leaving behind aftereffects such as paralysis and loss of speech. A meta-analysis of the combined results of 20 follow-up studies in the United States reported that severe obesity shortens life expectancy by 6.5–13.7 years (Kitahara et al. 2014). Obesity is making people unhealthy and shortening their life span.

25.4.2.2 Traditional Japanese Diet

One of the characteristics of Japan's dietary culture is the variety of foods. The rich nature of Japan, with its four seasons, has nurtured many foods (Tsugane 2020). There are no religious contraindications, and the food culture of other countries has been flexibly adopted. Japanese food also provides excellent nutritional balance. In a

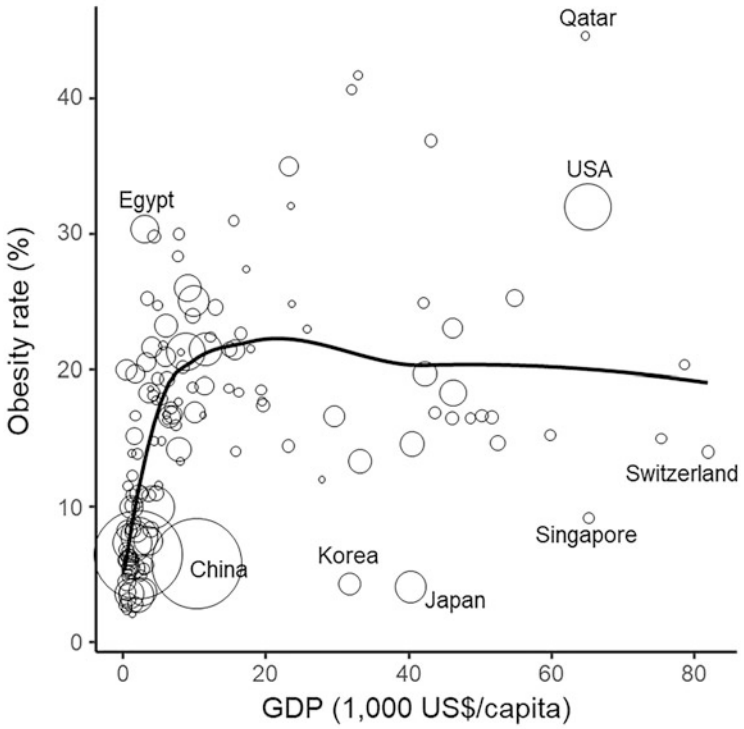

Fig. 25.5 Gross Domestic Product (GDP) per capita and obesity rate in 2018 based on Global Burden of Disease (GBD) statistics (GBD 2021)

typical Japanese meal, foods are served as a staple food, a main dish, a side dish, and a soup. The staple food is rice or other cereal, the main dish is meat or fish, the side dishes are vegetables, and soup such as miso (fermented bean paste) soup. Energy can be obtained from the grains in the main dishes, protein from the main dishes, vitamins, minerals, and fiber from the side dishes, and not only water from the soup but also antioxidants and minerals from seaweed and miso in the soup. It is possible for each individual to get a good balance of nutrients and energy by adjusting the amount of each staple food, main dish, and side dishes by increasing or decreasing the amount of each. Energy is obtained from carbohydrates such as grains, not from sugary snacks and beverages.

Eating too much or too little carbohydrates is not good for health. Both high and low percentages of carbohydrate diets were associated with increased mortality, with minimal risk observed at 50–55% carbohydrate intake in the Atherosclerosis Risk in Communities study with 15,428 adults aged 45–64 years followed up for 25 years (Seidelmann et al. 2018). The percentage of carbohydrates in the Japanese diet is within this range. As mentioned above, Japanese food is nutritionally well-balanced, but one of the disadvantages of Japanese food is that it tends to be overly salty, and the other is that it tends to be deficient in calcium due to the lack of dairy products.

Meta-analyses on calcium intake, bone mineral density, and fractures have reported no association with the incidence of proximal femur fractures (Bischoff-Ferrari et al. 2007). In addition, the effectiveness of calcium alone as a dietary treatment for osteoporosis is low, and the evidence is not clear enough to recommend that such treatment be done. Calcium supplements and calcium medications are often used when calcium intake is inadequate. However, it has been reported that the use of calcium medication increases the risk of cardiovascular diseases such as myocardial infarction (Li et al. 2012; Xiao et al. 2013). It has also been reported that the use of calcium supplements in women with cerebrovascular disease may increase the risk of dementia (Kern et al. 2016). The Mediterranean diet, like the Japanese diet, is characterized by a lack of dairy products. In order to prevent lifestyle-related diseases, it may be important to consume the appropriate amount of calcium without too much.

As for salt intake, the American Heart Association recommends no more than 1500 mg sodium (3.8 g salt) per day for most adults (Whelton et al. 2018), and a salt intake of less than 5 g (approximately 2000 mg sodium) per person per day is recommended by the WHO for the prevention of cardiovascular diseases (WHO 2012). The annual National Health and Nutrition Examination Survey in Japanese people is conducted using the dietary record method. In 2018, dietary salt intake was 10.1 g for men and women overall, 11.0 g for men, and 9.3 g for women. In general, nutrition surveys based on dietary records assess intakes to be lower. There are few national surveys using urinary sodium measurements, but actual salt intake may be much higher.

A study of urinary sodium excretion and the risk of death or cardiovascular disease in 133,118 people found that the lowest risk was associated with a salt intake of about 12 g per day (Fig. 25.6) (Mente et al. 2016). This result was the same in an analysis of 63,559 people with hypertension. In an analysis of 69,559 people without high blood pressure, salt intake of less than 12 g increased the risk, but increasing salt intake to more than 12 g did not increase the risk. In a study of salt intake and HALE by country, the countries with a salt intake of about 12 g per day, such as Japan, had the longest HALE (Fig. 25.7) (Messerli et al. 2018). Countries with high salt intake often consume foods including a variety of fruits and vegetables that contain minerals, which may explain the longer life expectancy in countries with relatively high salt intake.

We have created a system to calculate the Traditional Japanese Dietary Score (TJDS) as an indicator to determine the traditional Japanese food pattern and analyzed the relationship between obesity, ischemic heart disease, and HALE. The higher the TJDS, the lower the prevalence of obesity and ischemic heart disease, and the longer the HALE (Imai et al. 2019). The traditional Japanese diet has the potential to reduce obesity and ischemic heart disease and, as a result, increase life expectancy and HALE.

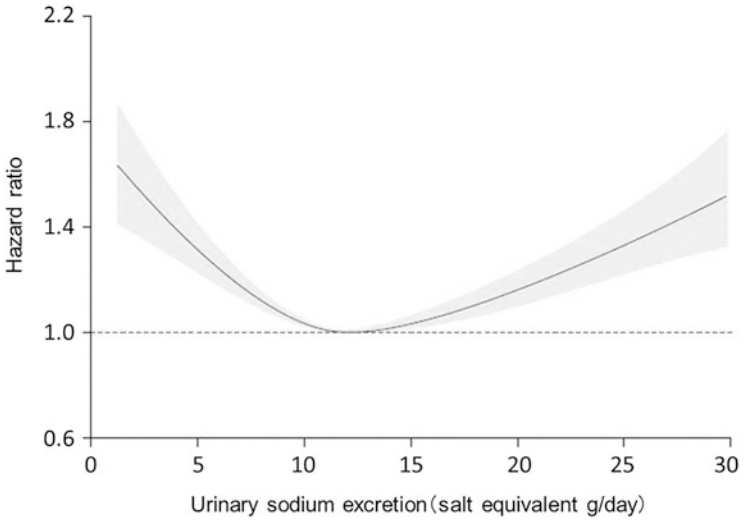

Fig. 25.6 Hazard ratio and 95% confidence interval of death and major cardiovascular events by urinary sodium excretion in 133,118 individuals (median age of 55 years) from 49 countries in four large prospective studies (Mente et al. 2016)

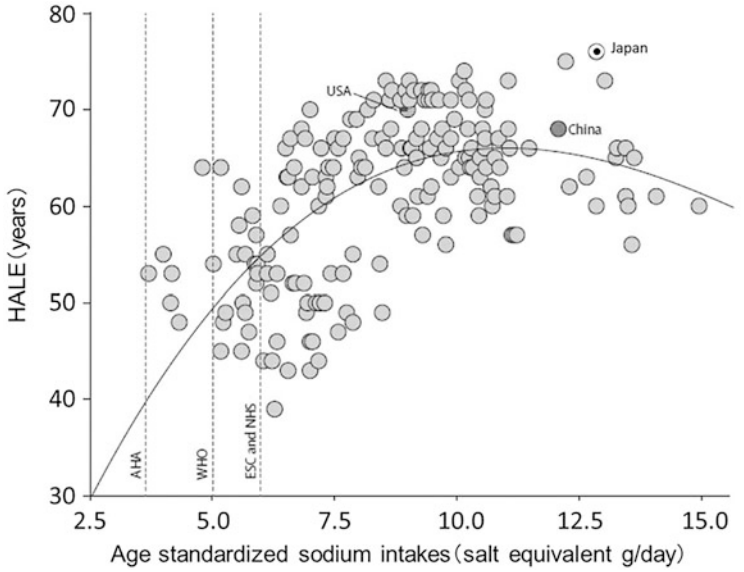

Fig. 25.7 Age-standardized estimated sodium intake and healthy life expectancy (HALE) at birth in 182 countries (Messerli et al. 2018). *AHA* American Heart Association, *ESC* European Society of Cardiology, *NHS* United Kingdom National Health Service. *Dotted lines* show recommended daily intake thresholds

25.4.2.3 Alcohol and Longevity

What about the effects of alcohol consumption on life expectancy? Nearly half of Japanese people have a genotype to metabolize alcohol more slowly than other people (Eng et al. 2007; Wall et al. 2016). There are few people with this genetic abnormality except for the Japanese. Among Organisation for Economic Co-operation and Development countries, Japan has the lowest alcohol consumption, along with Turkey and Israel. It is believed that moderate amounts of alcohol consumption may be good for the prevention of lifestyle-related diseases and longevity. This is true for diabetes and ischemic heart disease (Howard et al. 2004; Ronksley et al. 2011), but not for cancer, liver disease, and dementia. In a report that examined the relationship between alcohol consumption and the risk for all diseases weighted by the disability-adjusted life-years in 195 countries with 26 years of follow-up, the lowest amount of alcohol consumption associated with disease risk was 0 g (Fig. 25.8) (Griswold et al. 2018). Similarly, in 83 studies of current drinkers, with a total of 599,912 people followed, the healthiest amount of drinking was 0 g (Wood et al. 2018). The relatively low consumption of alcohol may be one of the factors contributing to the longevity of the Japanese.

25.4.3 Social Factors

Social systems are also important for longevity. Affluence, stability, a good health care system, and low inequality are all necessary to increase average life expectancy and HALE. In fact, social factors are the biggest determinants of average life expectancy and HALE. In general, the richer the country, the longer the average life expectancy. However, when the GDP per capita exceeds $10,000 per year, the relationship between life expectancy and wealth becomes weaker (Fig. 25.9). The medical system, social system, and lifestyle become more important.

The life expectancy of Japanese people may be longer due to the medical system and social factors such as the relatively small gap between the rich and the poor, the well-developed medical insurance system that allows people to visit medical institutions without worrying about the cost (Sasaki et al. 2015), and the high level of medical care with low infant and neonatal mortality rates, in addition to the characteristic lifestyle of Japanese people.

25.5 Conclusion

Life expectancy is increasing in countries around the world, and the number of people over the age of 100 is also increasing (Robine and Cubaynes 2017). The average life expectancy and HALE of Japanese people are among the highest in the

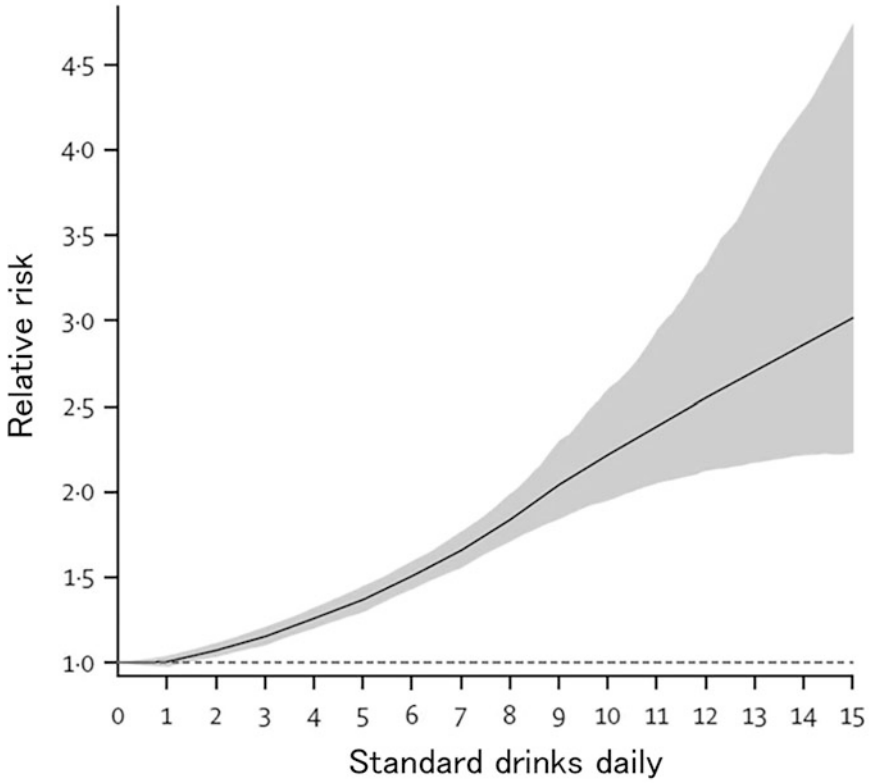

Fig. 25.8 Weighted relative risk of alcohol for all attributable causes, by standard drinks consumed per day (Griswold et al. 2018). Age-standardized weights determined by the disability-adjusted life-year rate in 2016, for both sexes. The *dotted line* is a reference line for a relative risk of 1. One standard drink = 8 g ethanol

world. In contemporary society, obesity has become a particular problem. Obesity is the cause of many diseases and death from heart disease, stroke, and cancer. Improving lifestyle habits, especially a healthy diet, will prevent diseases and lead to healthy aging. The prevention of aging and rejuvenation is probably one of the biggest themes in medical research. Although there is no miracle to achieve immortality, the Japanese have proven that it is possible to delay aging and maintain good health by improving social systems and lifestyles.

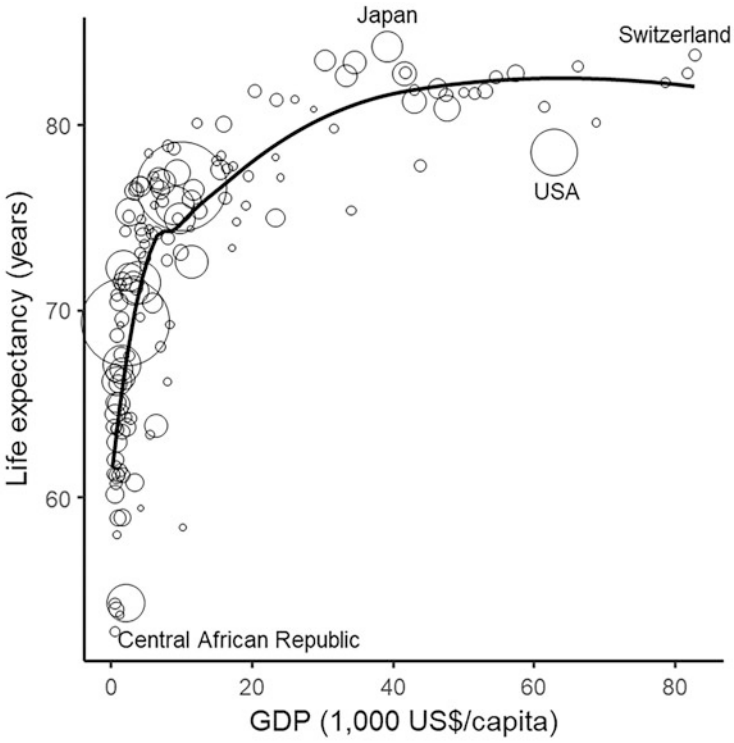

Fig. 25.9 Association between Gross Domestic Product (GDP) per capita and life expectancy by countries with more than 1 million population in 2018 (Compiled from Global Burden of Disease (GBD) and World Bank statistics (World Bank 2020; GBD 2021))

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3

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N. Mori (ed.), *Aging Mechanisms II*, https://doi.org/10.1007/978-981-16-7977-3