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1. Biological principles of aging and approaches for interventions

Suresh I. S. Rattan
Laboratory of Cellular Ageing, Department of Molecular Biology, Aarhus University
Gustav Wieds Vej 10; DK8000 Aarhus-C; Denmark

Abstract. Aging can be understood at various levels, from evolutionary and biological to psychological and sociological levels. At the biological and molecular levels aging is characterized by the stochastic occurrence and accumulation of molecular damage. Damages in the maintenance and repair pathways comprising homeodynamic space lead to age-related failure of homeodynamics, increased molecular heterogeneity, altered cellular functioning, reduced stress tolerance, increased probability of diseases and ultimate death. Novel approaches for testing and developing effective means of intervention, prevention and modulation of aging incorporate means to minimize the occurrence and accumulation of molecular damage. Mild stress-induced hormesis by physical, biological and nutritional methods, including hormetins, is a promising strategy for achieving healthy aging and for preventing age-related diseases.

Introduction

Two of the pioneers of modern biogerontology, Leonard Hayflick and Robin Holliday, have declared that aging is no longer an unsolved problem in

Correspondence/Reprint request: Dr. Suresh Rattan, Laboratory of Cellular Ageing, Department of Molecular Biology, Aarhus University, Gustav Wieds Vej 10; DK8000 Aarhus-C; Denmark. Email: rattan@mb.au.dk

Table 1. Biological principles of aging and longevity derived from modern biogerontological research.

1.	*Evolutionary life history principle:* Aging is an emergent phenomenon seen primarily in protected environments, which allow survival beyond the natural lifespan of a species, termed 'essential lifespan' (ELS), [3-5].
2.	*Non-genetic principle:* There is no fixed and rigid genetic program, which determines the exact duration of survival of an organism, and there are no real gerontogenes whose sole function is to cause aging and to determine precisely the lifespan of an organism.
3.	*Differential principle:* The progression and rate of aging is different in different species, organisms within a species, organs and tissues within an organism, cell types within a tissue, sub-cellular compartments within a cell type, and macromolecules within a cell.
4.	*Molecular mechanistic principle*: Aging is characterized by a stochastic occurrence, accumulation and heterogeneity of damage in macro-molecules, leading to the shrinkage of the homeodynamic space and the failure of maintenance and repair pathways.

biology [1, 2]. The bold assertion by Hayflick and Holliday underlines the fact that biological basis of aging is well understood and a distinctive framework has been established, based on which general principles of aging and longevity can be formulated, and can be the basis for developing interventions towards achieving a healthy old age. These biological principles of aging and longevity are summarized in Table 1.

Thus, aging is an emergent, epigenetic and a meta-phenomenon, which is not controlled by a single mechanism. Although individually no tissue, organ or system becomes functionally exhausted even in very old organisms, it is their combined interaction and interdependence that determines the survival of the whole. The evidence that genes have a limited (about 25%) influence upon lifespan in human beings has mainly come from the studies performed on centenarians and their siblings, twins and long living families. The value of the genetic determinant of longevity was calculated from studies on Danish twins, and it was shown that the heritability of longevity in men and women was 0.26 and 0.23, respectively [6]. A combination of genes, milieu and chance determine the course and consequences of aging and the duration of survival of an individual [7].

Homeodynamic space and its shrinkage

Survival and longevity are a function of the ability of various maintenance and repair mechanisms to keep up with damage and wear-and-tear. All living

systems have the intrinsic ability to respond, to counteract and to adapt to the external and internal sources of disturbance. The traditional conceptual model to describe this property is homeostasis, which has dominated biology, physiology and medicine since 1930s. However, advances made in our understanding of the processes of biological growth, development, maturation, reproduction, and finally, of aging, senescence and death have led to the realization that homeostasis model as an explanation is seriously incomplete. The main reason for the incompleteness of the homeostasis model is its defining principle of "stability through constancy", which does not take into account the new themes, such as cybernetics, control theory, catastrophe theory, chaos theory, information and interaction networks, which comprise and underline the modern biology of complexity [8]. Since 1990s, the term homeodynamics is being increasingly used to account for the fact that the internal milieu of complex biological systems is not permanently fixed, is not at equilibrium, and is a dynamic regulation and interaction among various levels of organization [9].

Aging, senescence and death are the final manifestations of unsuccessful homeostasis or failure of homeodynamics [10, 11]. A wide range of molecular, cellular and physiological pathways of repair are well known, and these range from multiple pathways of nuclear and mitochondrial DNA repair to free radical counteracting mechanisms, protein turnover and repair, detoxification mechanisms, and other processes including immune- and stress-responses. All these processes involve numerous genes whose products and their interactions give rise to a "homeodynamic space" or the "buffering capacity", which is the ultimate determinant of an individual's chance and ability to survive and maintain a healthy state [10, 11]. A progressive shrinking of the homeodynamic space is the hallmark of aging and the cause of origin of age-related diseases.

In a normal, healthy and young individual, the complex network of maintenance and repair systems (MARS) constitute a functional homeodynamic space. Since no MARS can be 100% perfect 100% of the time, there is a probability of imperfect homeodynamics giving rise to a zone of vulnerability, manifested in age-independent diseases and mortality. However, a progressive accumulation of molecular damage and its effects on the interacting molecular networks leads to the reduction in the functional homeodynamic space, and effectively increases the vulnerability zone, and thus allows for the occurrence and emergence of age-related diseases. Alzheimer's disease, cancer, cataract, diabetes-2, osteoporosis, Parkinson's disease, sarcopenia and other age-related diseases are the result of reduced homeodynamic space of the individual.

Molecular theories of aging

At the molecular level, aging is best characterized by the accumulation of molecular damage. There are three major sources of damages within a cell:

(1) Reactive oxygen species (ROS) and free radicals (FR) formed due to external inducers of damage (for example ultra-violet rays), and as a consequence of cellular metabolism involving oxygen, metals and other metabolites;
(2) Nutritional glucose and its metabolites, and their biochemical interactions with ROS; and
(3) Spontaneous errors in biochemical processes, such as DNA duplication, transcription, post-transcriptional processing, translation, and post-translational modifications.

The so-called mechanistic theories of biological aging have often focused on a single category of inducers of molecular damage as an explanation for possible mechanisms of aging. Of these, two theories, which have been the basis of most of the experimental biogerontology research, are the free radical- and the protein error-theory of aging. Although neither of them can be considered to be the complete theory of biological aging, their contributions in providing a solid scientific footing to experimental aging research and anti-aging interventions cannot be overestimated.

Free radical theory of aging (FRTA)

FRTA, proposed in 1954, arose from a consideration of the aging phenomenon from the premise that a single common biochemical process may be responsible for the aging and death of all living beings (for an update, see [12]). There is abundant evidence to show that a variety of ROS and other FR are indeed involved in the occurrence of molecular damage, which can lead to structural and functional disorders, diseases and death. The chemistry and biochemistry of FR is very well worked out, and the cellular and organismic consequences are well documented [13]. However, the main criticisms raised against this theory are with respect to its lack of incorporation of the essential and beneficial role of FR in the normal functioning and survival of biological systems [14, 15]. Additionally, FRTA presents FR as the universal cause of damage without taking into account the differences in the wide range of FR-counteracting mechanisms in different species. Furthermore, a large body of data showing the contrary and/or lack of predictable and expected beneficial results of anti-oxidant and FR-

scavenging therapies have restricted the FRTA to being only a partial explanation of some of the observed changes during aging [16-18].

Protein error theory of aging (PETA)

The history of PETA, also known as the error catastrophe theory, is full of controversy and premature declaration of its demise [19-21]. Since the spontaneous error frequency in protein translation is generally several orders of magnitude higher than that in DNA replication and RNA transcription, the role of protein errors and their feedback in biochemical pathways has been considered to be a crucial one with respect to aging. Several attempts have been made to determine the accuracy of translation in cell-free extracts, and most of the studies show that there is an age-related increase in the mis-incorporation of nucleotides and amino acids [19-21]. It has also been shown that there is an age-related accumulation of aberrant DNA polymerases and other components of the transcriptional and translational machinery [19-24].

Further evidence in support of PETA comes from experiments which showed that an induction and increase in protein errors can accelerate aging in human cells and bacteria [19-21, 25, 26]. Similarly, an increase in the accuracy of protein synthesis can slow aging and increase the lifespan in fungi [27-29]. Therefore, it is not ruled out that several kinds of errors in various components of protein synthetic machinery, including tRNA charging, and in mitochondria do have long-term effects on cellular stability and survival [30-33]. However, almost all these methods have relied on indirect *in vitro* assays, and so far direct, realistic and accurate estimates of age-related changes in errors in cytoplasmic and mitochondrial proteins, and their biological relevance, have not been made. Similarly, applying methods such as two-dimensional gel electrophoresis, which can resolve only some kinds of mis-incorporations, have so far remained insensitive and inconclusive [19-21]. It will be necessary to combine several methods, such as electrophoresis, mass-spectrometry, protein-protein interactions and antibody-based detection of molecular heterogeneity to find out the extent of protein errors and their biological role in aging.

From FRTA and PETA to higher order theories

Both the FRTA and PETA provide molecular mechanisms for the occurrence of molecular damage. Additionally, nutritional components, specially the sugars and metal-based micronutrients can induce, enhance and amplify the molecular damage either independently or in combination with other inducers of damage. The biological consequences of increased levels of

molecular damage are wide ranging and include altered gene expression, genomic instability, mutations, molecular heterogeneity, loss of cell division potential, cell death, impaired intercellular communication, tissue disorganization, organ dysfunctions, and increased vulnerability to stress and other sources of disturbance. Historically, each of these biological consequences has been used as the basis of putting forward other theories of aging, such as replicative senescence theory, neuroendocrine theory, pineal gland theory, immunological theory and many more [5, 15, 34].

Genetics, post-genetics and epigenetics of aging

Since all molecular processes in a living system are based in and regulated by genes, an attractive research strategy has been to discover genes for aging, termed gerontogenes [35-37]. However, the evolutionary explanation for the origin of aging, and limited lifespan discussed above, have generally ruled out the notion of any specific genetic program involving specific gerontogenes. But a lack of specific gerontogenes with the sole purpose of causing aging and terminating the lifespan of an individual does not imply that genes do not or cannot influence survival, longevity and the rate of aging.

There is ample evidence from studies performed on yeast and other fungi, nematodes, insects, rodents and humans that mutations in various genes can either prolong or shorten the lifespan, and some of these are also the cause of premature aging syndromes in human beings [38-40]. Additionally, genetic linkage studies for longevity in mice have identified major histocompatibility complex regions and quantitative trait loci on several chromosomes as putative genes for aging. In gene association studies with human centenarians, certain alleles of HLA locus on chromosome 6, regions of chromosome 4, different alleles of APO-E and APO-B, and DD genotype of angiotensin converting enzyme (ACE) have been linked to exceptional longevity. Similarly, several other studies have reported an association between human longevity and single nucleotide polymorphisms (SNP) in a variety of genes in various biological pathways, including heat shock response, mitochondrial functions, immune response, cholesterol metabolism and others [40-46].

An analysis of the various functions of the genes associated with aging and longevity shows that these genes cover a wide range of biochemical pathways, such as energy metabolism, kinases, kinase receptors, transcription factors, DNA helicases, membrane glucosidases, GTP-binding protein coupled receptors, chaperones, and cell cycle check point pathways [40, 46].

What is clear from the identification of the genes influencing aging and longevity is that whatever their normal function and mechanism of action may be, these gerontogenes did not evolve to cause and accumulate molecular damage, to cause functional disorders, and to terminate the life of the organism.

Most of these genes have well defined roles in normal metabolism, in intra- and inter-cellular signaling, and in maintenance and repair functions including stress response. It is the damage-induced changes in the regulation, structure and/or activity of their gene products, which result in their altered biological role with age. Therefore, such genes have been termed "virtual gerontogenes" [36, 47]. Furthermore, a lack of evolutionary selection of virtual gerontogenes has given rise to the notion of post-genetics or "post-reproductive genetics" as an explanation for different biological roles played at different ages by the same genetic variants [48].

Epigenetics of aging

Although genes are the foundation of life, genes in themselves are non functional entities. It is the wide variety of gene products, including coding and non-coding RNAs, proteins and other macromolecules, which constitute the biochemical and biophysical milieu for life to exist. "Epigenetics" is the most commonly used term to account for and to explain the consequences of the intracellular and extracellular milieu, which establish and influence the structural and functional stability of genes. These epigenetic effects and alterations generally remain uninherited from one generation to the next, but have strong deterministic effects on the health, survival and aging of the individual.

So far, there is only scanty information available about the involvement in aging of various intracellular epigenetic markers such as methylated cytosines, oxidatively modified nucleotides, alternatively spliced RNAs, and post-translationally modified proteins, including protein folding [49]. The full spectrum of epigenetics of aging is yet to be unraveled and at present is one of the most attractive and challenging areas of research in biogerontology [50, 51]. A major reason for apparent difficulties in fully understanding the epigenetics of aging is the existence of several orders higher complexity and diversity of the constituting components, such as physical, chemical, biological and environmental factors, including psychological factors in human beings. Furthermore, in order to understand how various conditions influence, regulate and modulate the actions, interactions and networks of gene products during aging will require new intellectual and technical tools,

such as systems analysis, bioinformatics, and functional genomics involving simultaneous multiple analyses.

From understanding to intervention

As a biomedical issue, the biological process of ageing underlies all major human diseases. Although the optimal treatment of each and every disease, irrespective of age, is a social and moral necessity, preventing the onset of age-related diseases by intervening in the basic process of ageing is the best solution for improving the quality of human life in old age. According to the three principles of ageing and longevity described above, having the bodies that we have developed after millions of years of evolution, occurrence of ageing in the period beyond ELS, and the onset of one or more diseases before eventual death, appear to be the normal sequence of events. This viewpoint makes modulation of ageing by prevention very much different from the treatment of a specific disease.

Scientific and rational anti-ageing strategies aim to slow down ageing, to prevent or delay the physiological decline, and to regain lost functional abilities. In order to modulate ageing for achieving healthy old age and for extending lifespan, three main conditions need to be fulfilled, as represented by the equation $E = GMC^2$, where genes and milieu are the critical factors [7].

Gene therapy for aging

One of the earliest experimental studies which demonstrated that an induced mutation in a single gene can increase the lifespan of an organism was the discovery of the so-called *age-1* mutant in the nematode *Caenorhabditis elegans* [52, 53]. Since then hundreds of putative gerontogenes or longevity genes have been reported in *C. elegans*, *Drosophila* and rodents, which when mutated result in the extension of average and maximum lifespan of the organism. The methods used for the identification of such genes include induction of mutations and deletions by irradiation and chemical mutagens, alterations in gene expression by knockout, homologus recombination, or by gene addition, and reduction in gene expression by RNAi-induced abrogation of translation (for the latest information on such genes, refer to various online databases, such as: http://genomics.senescence.info/genes/longevity.html, http://wormbase.org/db/misc/site_map?format=searches, http://sageke.sciencemag.org/index.dtl

It is important to realize that in almost all such cases longevity extension had occurred when one or multiple interventions resulted in the reduction or

total inhibition of the activity of one or more genes [54]. Some of the main pathways whose "loss of function" is shown to associate with extended period of survival are: (i) energy generation and utilization in mitochondrial respiratory chain; (ii) nutrition and hormonal sensing and signalling including insulin/insulin-like growth factor-1 and its target forkhead transcription factor FOXO, transcriptional silencing by sirtuin-mediated histone deacetylase; and (iii) translational interference through target of rapamycin (TOR). Similarly, there are other examples which show that several mutant mice strains with defects in growth hormone (GH) pathways including deficiencies of GH levels and GH receptor have extended lifespans. Application of RNAi technology will further identify numerous genes whose normal levels of activities are lifespan restricting [54].

In contrast to the above, studies have also been performed in which the effects of adding one or multiple copies of various genes leading to the increased expression of their gene products has resulted in the extension of lifespan. Some such transgenic manipulations in model systems include the addition of gene(s) for one of the protein elongation factors, antioxidant genes superoxide dismutase and catalase, sirtuin, forkhead trascription factor FOXO, heat shock proteins (Hsp), heat shock factor (HSF), protein repair methyltransferase and klotho, which is an inhibitor of insulin and IGF1 signalling [54].

Although theses studies have demonstrated longevity-extending effects of various genes in controlled laboratory conditions, there is very little information available on the basic process of ageing in terms of the rate and extent of occurrence and accumulation of macromolecular damage and its physiological consequences in these animals. There is also almost no information available as to what is the physiological price paid for inactivating such genes whose normal function is a part of the general metabolism and signaling. There is some evidence that laboratory-protected longevity mutants in *C. elegans* have reduced Darwinian fitness when competing with the wild type worms under nutritionally challenging conditions [55-58]. Similarly, klotho-induced insulin resistance and the paradox of the insulin/IGF-1 signalling pathways in longevity extension seriously question the practicality of such gene manipulations in humans [58-60].

Another system in which genetic interventions have been tried as potential anti-ageing therapies is the Hayflick system of limited proliferative lifespan of normal diploid differentiated cells in culture [61]. Almost all the genetic interventions by transient or permanent transfection and ectopic expression of various genes on this model system have focused on extending the replicative lifespan of cells by bypassing the cell cycle check-points [62, 63]. One of the most widely used genetic interventions in extending

indefinitely the replicative lifespan of normal cells has been the ectopic expression of telomerase in a wide variety of cells [64, 65]. However, continuous proliferation by such genetically modified non-ageing cells often leads to their genomic instability, transformation and cancer-forming activity [66, 67]. In the case of animals, whereas telomerase negative mice show reduced lifespan and some other abnormalities after six-generations, [68] overexpression of telomerase in the skin increases myc-induced hyperplasia [69] without any extension of lifespan.

Considering that the molecular cause of ageing is the progressive accumulation of macromolecular damage and increased molecular heterogeneity [70], there are at least three major targets for anti-ageing genetic interventions: (1) increasing the repair of damaged macromolecules, for example DNA repair pathways; (2) increasing the removal of damaged macromolecules, for example proteasomal and lysosomal pathways; and (3) decreasing the source of damaging agents, for example reactive oxygen species, other free radicals, and reactive sugar metabolites. Whereas the first two targets basically imply achieving genetic enhancement or genetic improvement, the third target requires resetting the metabolic pathways.

All of the above targets for anti-ageing interventions involve hundreds of genes and gene products, whose expression and action are evolutionarily highly regulated in a cell-type-specific manner. Although there are several approaches in development for gene-based enhancement of physical strength, endurance, appearance and memory, there are serious technical limitations and ethical and safety concerns that remain to be resolved. Preventing or treating one or more age-related diseases by gene therapy, including stem cells, are at best the piecemeal treatments which often are temporary or become unsuccessful since these are overshadowed by the systemic ageing of the whole body. Ideally, gene therapy for the process of ageing requires a significant and "intelligent" redesigning already at the level of the zygote for better maintenance and survival of the body without having to trade-off with growth, development and reproduction. Chances of such an "intelligently redesigned" and directed evolution to succeed in competition with the Darwinian natural selection from much larger random variations and combinations are practically none.

Manipulating the milieu

The second parameter M in the equation $E = GMC^2$ represents milieu – the environment in which living systems operate and survive. The milieu in which genes and gene products function ranges from the intracellular

molecular and ionic milieu to all other levels of organization including cellular, physiological, psychological, and societal. Almost all the ongoing work on aging modulation and intervention at present is aimed at modifying the milieu by either replenishing those enzymes, hormones and other molecules, such as antioxidants and micronutrients, whose levels are reported to decrease during aging. Although some of these approaches have been shown to have some clinical benefits in the treatment of some diseases in the elderly, none of these really modulate the aging process itself.

A critical component of the homeodynamic property of living systems is their capacity to respond to stress. In this context, the term "stress" is defined as a signal generated by any physical, chemical or biological factor (stressor), which in a living system initiates a series of events in order to counteract, adapt and survive. A successful and over-compensatory response to low doses of stressors improves the overall homeodynamics of cells and organisms. This approach for the strengthening of homeodynmaics through mild stress is known as hormesis [71]. Hormesis in ageing is defined as the life supporting beneficial effects resulting from the cellular responses to single or multiple rounds of mild stress. Various mild stresses that have been reported to delay ageing and prolong longevity in cells and animals include temperature shock, irradiation, heavy metals, pro-oxidants, acetaldehyde, alcohols, hypergravity, exercise and food restriction [72]. All such compounds which bring about biologically beneficial effects by causing mild stress and thus stimulating defense pathways are termed as hormetins [71]. Components of various medicinal plants, such as Aswagandha, used frequently in the Indian Ayurvedic system of medicine for potential anti-aging effects, appear to be potential hormetins.

Aging modulatory effects of hormesis have been reported for various human cell types *in vitro*. For example, using a regimen of repeated mild heat shock at 41°C, for 1 hr twice a week, given to cultured normal human skin fibroblasts, keratinocytes, endothelial cells, and telomerase-immortalised bone marrow mesenchymal stem cells, a variety of hormetic effects have been reported. These effects include slowing down of cellular aging, some extension of replicative lifespan, maintenance of youthful morphology, reduction in the levels of oxidatively- and glycoxidatively-damgaed proteins, stimulation of proteasomal activities, increased levels of chaperones, enhancement of stress tolerance, and improvement in differentiation, wound healing and angiogenesis [73]. Other hormetic conditions, which have been shown to have some anti-aging effects in human cells, are irradiation, mechanical stretching and electromagnetic field shock [74-76]. Thus, the proof of the principle regarding the applicability of hormesis as a modulator of aging in human cells is well demonstrated. However, further short term

and long term studies, using a wide variety of human cell types, and a combination of stressors, are required in order to establish the universality of the phenomenological and mechanistic aspects of this issue.

However, not all pathways of stress response respond to every stressor, and although there may be some overlap, generally these pathways are quite specific. The specificity of the response is mostly determined by the nature of the damage induced by the stressor and the variety of downstream effectors involved. Yet, the major pathways of stress response can be used as the screening platform for discovering, testing and monitoring the effects of novel hormetins. Such hormetins may be categorized as: (1) physical hormetins, such as exercise, heat and radiation; (2) biological and nutritional hormetins, such as infections, micronutrients, spices and other sources; and (3) psychological hormetins, such as mental challenge and focused attention or meditation. Hormesis may also be an explanation for the health beneficial effects of numerous other foods and food components, such as garlic, Gingko, and other fruits and vegetables [77-81]. Understanding the hormetic and interactive mode of action of natural and processed foods is a challenging field of research, and has great potential in developing nutritional and other life style modifications for aging intervention and therapies. For example, it may be possible to develop multi-hormetin formulations as anti-aging drugs and nutriceuticals whose mode of action is through hormetic pathways by mild stress-induced stimulation of homeodynamic processes.

Finally, while the G and M components of the $E = GMC^2$ formula for eternal life are being taken care of by various experimental approaches, the third factor C represents chance, which is the probability of stochastic events leading to a cascade of error-catastrophe in complex interacting systems. Recent developments in our understanding of complex networks at all levels of organisation from molecular to societal and global networks have highlighted the vulnerability of all strong and weak links, and has reasserted the significance of chance events which are not amenable to regulation and manipulation. In the context of modulating aging, repeated mild stress-induced hormesis increases the boundaries of the homeodynamic space thus giving cells and organisms wider margins for metabolic fluctuation and adaptation. Slowing down the shrinkage of the homeodynamic space hormetically will reduce the probability of occurrence and emergence of various diseases in old age, and thus extend the health-span.

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