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OBM Geriatrics

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Review

The Cellular Mechanism of Aging as Programmed Epigenetic Phenomenon: From Hypothesis to Scientific Evidence

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Academic Editor: Kurt Whittemore

Special Issue: Perspectives on Telomeres and Aging II

OBM Geriatrics
2025, volume 9, issue 3
doi:10.21926/obm.geriatr.2503325

Received: December 04, 2024
Accepted: September 03, 2025
Published: September 10, 2025

Abstract

There is a main difference between theories explaining aging as an adaptive phenomenon that is determined and modulated by genes (i.e., the result of a specific "program") and theories explaining aging as a non-adaptive phenomenon caused by the accumulation of random degenerative events. In fact, for adaptive theories, a genetically determined and modulated program determining aging is indispensable, while for non-adaptive theories, such a program cannot exist. However, there appears to be strong evidence to support the existence of this program as proposed by the subtelomere-telomere (STT) theory with the action of TERRA sequences. The STT theory with TERRA sequences was developed in four successive phases: 1) Aging caused by limitations in cell duplication; 2) Aging caused by progressive telomere shortening; 3) Aging caused by progressive inhibition of particular hypothetical subtelomeric regulatory sequences (r-sequences) determined by progressive telomere shortening; 4) Identification of the r-sequences in the TERRA sequences whose effects are well known and documented. The theory, as proposed in phases 1 and 2, was untenable because the evidence contradicted the predictions. The theory, as proposed in phase 3, was based mainly on the

hypothetical existence of regulatory sequences that required empirical confirmation. The further development of the theory in phase 4 overcame this difficulty. In particular, the identification of hypothetical r-sequences in widely documented sequences such as the TERRA sequences has transformed a hypothesis into a theory confirmed by empirical evidence. The mechanism proposed describes a genetically determined and regulated mechanism of aging as a program causing specific epigenetic modifications. This confirms the thesis that aging is an adaptive phenomenon and invalidates the opposing hypothesis. Among other things, for the validity of the opposite hypothesis of aging as a non-adaptive phenomenon, it would be essential to justify in evolutionary terms: (i) the position of regulatory sequences of great importance for cellular functions in a position where they are inhibited by telomere shortening; (ii) cell senescence which appears to be an oncogenic factor and so cannot be justified as a defense against cancer; and (iii) gradual cell senescence which cannot be hypothesized as an anti-cancer defense. Furthermore, the phenomena referred to in points (ii) and (iii) and TERRA sequences inhibition are entirely reversible with appropriate manipulations, and this is unlikely with their possible interpretation as a consequence of random degenerative phenomena.

Keywords
Aging; epigenetic modifications; TERRA; telomere; telomeric cap; subtelomere; telomere position effect

1. Introduction

Aging may be defined as the phenomenon of age-related increasing mortality observed in populations in the wild [1] or “increasing mortality with age ... actuarial senescence” [2]. Aging is widely documented in the wild for many species and is subject to natural selection as it contributes significantly to reducing the life span in natural conditions [1-3].

There are two opposite types of explanation for aging (discussed in [4, 5]).

- For the first interpretation, aging has no adaptive meaning and is the consequence of multiple random events that cause progressive degeneration of cells, tissues, and organs. So, no program determines and regulates aging. Moreover, this hypothetical program cannot exist because it would be detrimental to the individual in its environment and would undoubtedly be opposed by natural selection [6, 7]. Furthermore, it is maintained, disregarding natural observations, that aging does not exist in the wild and therefore natural selection cannot act on it. Consequently, it is unlikely that a hypothetical mechanism exists that determines aging, and such a mechanism could not have been forged by natural selection [6, 8].
- The second interpretation suggests aging as an adaptive phenomenon. This means that aging (i) must exist in natural conditions; (ii) is regulated and determined by the DNA sequence; and (iii) is favored by natural selection at the supra-individual level [5]. Aging is within the category of phenomena defined as phenoptosis (i.e., “programmed death of an organism” [9-11]) with countless examples in nature, although under other names [11, 12]. For this second

interpretation of aging, the existence of a specific mechanism determining aging is indispensable.

The primary and essential discriminating factor between the two theses is the absence versus the existence of this hypothetical mechanism.

The description of this mechanism and how it consists mainly of epigenetic modifications correlated with the progressive telomere shortening is the subject of this paper.

Other arguments or empirical evidence for or against the two interpretations will not be discussed here, and for them it is helpful to refer to other works (e.g., [4, 5] and the references cited therein).

2. The Four Historical Phases of the Definition of a Cellular Mechanism of Aging

It is possible to demonstrate empirical evidence of a specific mechanism that causes and regulates aging at the cellular level, with consequences for the entire organism. The definition of this mechanism was achieved in four historical phases [5, 13, 14], which will be outlined in the following sections.

2.1 Phase 1: Aging Caused by the Effects of Cell Duplication Limits

Hayflick demonstrated that cells could not reproduce without limits [15, 16], contrary to the previously held belief, which was based on experiments biased by errors [17, 18].

In the following years, it was found that there was a specific correlation between the longevity of a species and the maximum number of possible duplications [19]. Furthermore, it was also observed that, in humans, the number of further possible duplications decreased in relation to age [20]. For example, it was observed “a decrease of 0.20 population doublings per year of donor life in the cultured normal fibroblasts of humans” [21].

As a logical derivation, it was suggested by Hayflick that aging could be a consequence of these limits in cell duplication and that aging could have a cellular origin ([20], p. 163).

However, this hypothesis raised two main objections:

1) Hayflick himself, based on numerous experimental works of various authors, underlined that there are many age-related cellular alterations before the cells lose the ability to divide. Table 4 in [20] summarizes the cellular parameters that increase or decrease before the block of duplication capabilities, alterations that have been further explored in other works (e.g., [22]). As these alterations appear before, not after, the block above, they cannot be a consequence of it.
2) Cell types without turnover (perennial cells, especially most neurons) age similarly to cells with turnover. Any limit in cell duplication did not appear to be a possible cause of the aging of cells that do not duplicate.

Thus, cell duplication limits could not be the primary cause of aging, and indeed depended on another primary cause (or set of causes), without excluding, however, the possibility that such limits in cell reproduction were among the factors contributing to aging.

2.2 Phase 2: Aging as a Consequence of Telomere Shortening (Telomere Theory)

In subsequent years, it was observed that DNA duplication was incomplete in the telomere, and this resulted in a progressive shortening of the telomere [23].

In 1973, it was proposed [24] that telomere shortening was slowed or prevented by a specific enzyme, later identified as telomerase, which was isolated twelve years later [25].

Telomere shortening was shown to be related to cell alterations and the probability of transition to a cellular state, cell senescence, in which duplication was impossible, and there are many cellular alterations [26, 27].

Thus, the progressive shortening of telomeres appears to determine both cell duplication limits and cellular metabolic alterations, as well as tissue aging and organism aging (see [5, 28]).

Regarding the mechanism by which telomere shortening caused such alterations, an interesting intuition was expressed by Fossel twenty years ago based on the experimental evidence then available. He suggested that the telomere and part of the subtelomeric sequence were covered by a hood with a fixed size. With the shortening of the telomere a greater portion of the telomere was covered by the 'hood' and this caused a progressive inhibition of subtelomeric transcription. Moreover, Fossel hypothesized the existence in the subtelomere of regulatory sequences capable of modulating other genes, or groups of genes, even in distant parts of the DNA sequence ([28], p. 50, and [29]).

This intuition was not explored further by Fossel, but will constitute an essential stimulus for the subsequent Phase 3 (see below).

Regarding the aging of perennial cells, which do not replicate and therefore cannot exhibit telomere shortening and suffer its consequences, a logical solution has been proposed for eye photoreceptor cells [30] and for other neurons not subject to turnover [30, 31]. This proposal was reiterated and deepened in subsequent works [5, 32].

In particular, eye photoreceptors, specialized neurons without turnover, are dependent for their trophism and survival on retinal pigmented cells (RPCs), a specific type of glial cells that show turnover. Each RPC serves approximately 50 photoreceptors and phagocytizes roughly 10% of the internal membranes of these photoreceptors daily, on which photopsin is located. The decline in the turnover of RPCs determines a progressive decay of photoreceptor functions and vitality. So, there is the accumulation of various substances (in particular, lipofuscin, A2E, and β-amyloid protein) and the formation of holes in the retinal pigmented epithelium with the consequent death of the photoreceptors. This degeneration is more marked and causes more serious problems starting from the macula, determining the age-related macular degeneration.

A similar mechanism was suggested for other neurons of the central nervous system and their satellite cells (microglia cells, astrocytes, oligodendrocytes, all cells that are specialized gliocytes). When the turnover of these cells declines, there is a progressive accumulation of various substances (in particular, β-amyloid protein and α-synuclein), and then neuronal suffering and death. This causes Parkinson's disease and Alzheimer's disease. A detailed exposition of the hypothesis that the functional decline of non-dividing cells results from impaired support by satellite cells is reported, in particular, in [5, 32], and I refer to these works for the conspicuous evidence in this regard.

In all the phenomena outlined above, the levels of activity of the telomerase enzyme were critical, as proven in various works. In normal human cells, a series of experiments showed that telomerase induction or reactivation not only restored telomere length and the ability to divide but also

reversed some cellular biomarkers of aging [33-36], and the treated cells appeared in a "phenotypically youthful state" [33].

In vitro, telomerase activation was induced in human aged fibroblasts with significant alterations in gene expression. Then the fibroblasts were used to reconstitute the skin, which turned out to be equal to that obtained using young fibroblasts [37].

In mice, artificially induced telomerase activity delayed aging manifestations, resulted in significant improvements in balance and neuromuscular coordination tests, and increased longevity, but not cancer risk [38].

In old mice with artificially blocked telomerase and a long series of age-related alterations in cells and organs, telomerase reactivation resulted in significant improvements of all these alterations, particularly reversing the degeneration of nervous tissues by restoring cell populations of oligodendrocytes, neural progenitors, and newborn neurons [39].

In the study of some "animals with negligible senescence", that is, with mortality rates not increasing in a detectable way in relation to age, the same levels of telomerase activity were found in old and young individuals of the same species (e.g., lobster and rainbow trout [40, 41], and rockfish species [42]). In particular, for rockfish, unlike other species that show age-related increasing mortality (i.e., that age), Black, a science writer, reports that, comparing old and young fish, it was observed in older individuals no increase in oxidative damage and undegraded proteins, and no reduction of telomerase activity [42].

However, the telomere theory suggested that, when comparing species, a relationship exists between longevity and telomere length. A species with longer telomeres was expected to age more slowly and live longer, whereas the opposite was likely to occur for species with shorter telomeres. The evidence clearly contradicted this. e.g.:

- In comparison with humans, mice and hamsters have relatively longer telomeres but shorter longevities [43];
- in rodent species, telomere length and longevity are not related [44, 45];
- two *Mus* strains that have telomeres with quite different lengths (10 and 20 kb), show equal life spans and rhythms of aging [28], p. 60;
- in mice strains characterized by inactivated telomerase (mTR-/- strains), after one or two generations, the telomeres are shortened, but no marked alterations in fertility and viability are evident in laboratory conditions, which are, however, observed after 4-6 generations when the telomeres are significantly shortened [46, 47];
- Cloned animals are obtained from somatic cells with shorter telomeres than the germinal cells of the donors. Despite the different telomere lengths, no differences in longevity and aging rates were observed [48, 49].

Therefore, the theory that aging was dependent on initial telomere length was untenable. However, the evidence did not rule out the idea that telomere shortening contributed to cellular changes that cause aging.

2.3 Phase 3: Aging as a Result of Subtelomere Repression Caused by the Shortening of Telomeres (Subtelomere-telomere Theory)

However, some facts had to be taken into consideration:

1) Regarding telomere length, apart from the differences between species (for mammals reported in [50]), in a species the length of telomeres in the germ cells varies (i) between two chromosomes of the same individual; (ii) between one arm and another of the same chromosome; and (iii) between the same chromosome arms in different individuals [51], while the length of each telomere is heritable [52, 53]. Therefore, any mechanism hypothesizing a repression of subtelomeric DNA as a function of telomere shortening must take into account this significant variability in telomere length.
2) A gene artificially inserted in a subtelomeric position is repressed [54], and this phenomenon, defined as telomere position effect, is correlated with telomere shortening [55];
3) Yeast, a unicellular species, divides into two cells in each duplication. One of these cells, defined as a "daughter" cell, retains unchanged cellular functions, while the other cell, described as a "mother" cell, exhibits cellular alterations that increase with each duplication [56]. In the wild strains of this species, where the telomerase enzyme is always active, there is no shortening of the telomeres at each cell division. However, the individuals of the "mother" lineage exhibit, in relation to the number of previous duplications, a progressive accumulation of extrachromosomal ribosomal DNA circles (ERCs) on the subtelomere [57], which is associated with progressive cellular alterations [58]. These alterations are also associated with an increasing probability of triggering a mechanism analogous to cell senescence, which in yeast activates cell apoptosis [58], whereas in multicellular species, it is characterized by resistance to apoptosis [22]. In a yeast mutant strain with defective telomerase (*tlc1Δ* mutants), for the individuals of "daughter" lineage, no accumulation of ERCs is observed. However, telomeres are shortened by each duplication, and there are the same metabolic alterations shown by yeast cells of the "mother" lineage with the same number of previous duplications [58]. These alterations are likely caused by subtelomere inhibition. In yeast *tlc1Δ* mutants, to justify subtelomere inhibition in "daughter" lineage, this could be explained if there is a telomeric cap of fixed size, i.e., not shortening in relation to telomere shortening. This cap, when the telomere shortens, progressively covers and represses a larger portion of the subtelomere.

These facts and the aforementioned intuition of Fossel ([28], p. 50) led to the proposal of the subtelomere-telomere theory of aging [59-61], which can be summarized in the following key points:

(i) In the first cell of any organism, for each telomere there is the formation of a cap with a size that is proportional to the length of the telomere;
(ii) The size of this cap (or hood) remains unchanged in the following cell divisions, even though the telomere shortens;
(iii) In relation to telomere shortening, the cap covers a greater portion of the subtelomere (we could say that it slides on the subtelomere) and increasingly inhibits particular hypothetical regulatory sequences ("r-sequences") existing in the subtelomere;
(iv) The repression of these r-sequences alters the functioning of other regulatory sequences, and so there are increasing alterations in cellular metabolism;
(v) One of these alterations is an increasing instability of the telomere-telomeric cap complex and therefore an increasing probability of the transition to the state of cell senescence.

Therefore, the proposed theory hypothesizes a situation analogous to that of the cells in the daughter lineage of a yeast mutant strain with defective telomerase for human somatic cells.

The situation of the yeast cells in the three previously exposed cases is illustrated in Figure 1. For humans, a comparison with yeast cells is shown in Figure 2, where the daughter lineage of *tlc1Δ* mutant strains with inactive telomerase is depicted.

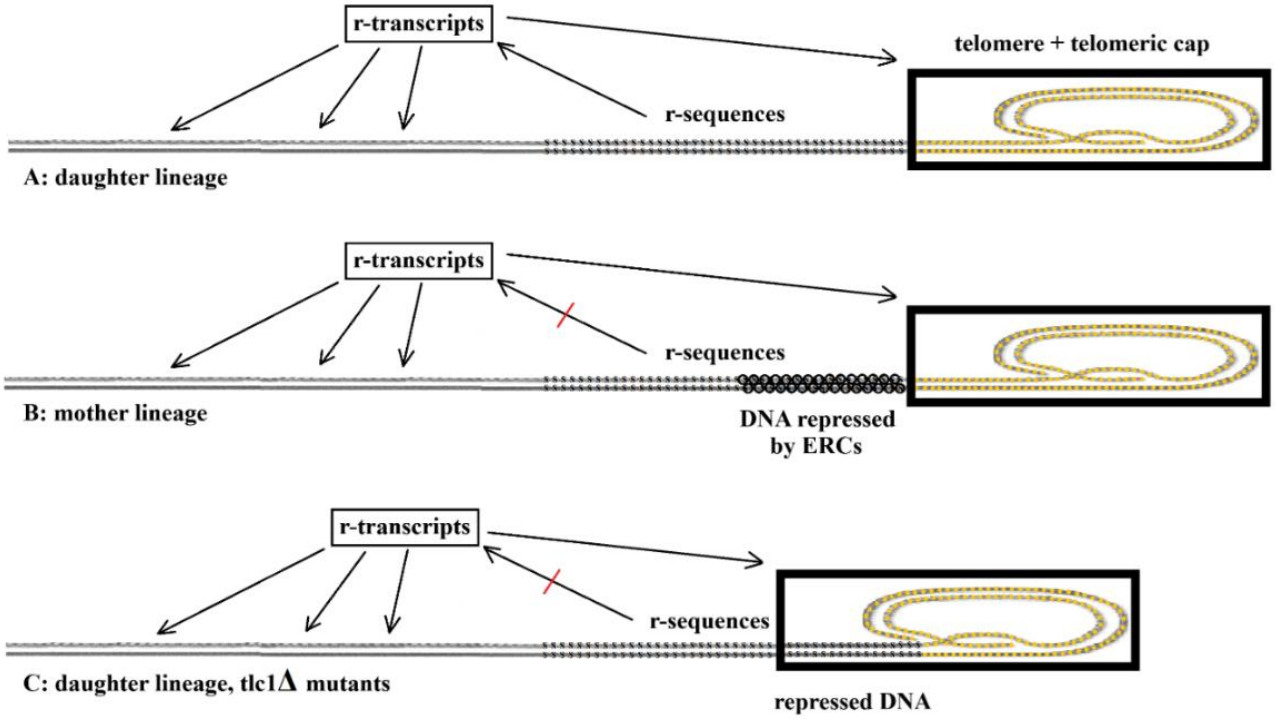

Figure 1 Yeast cells after some duplications. A: daughter lineage (no telomere shortening, no ERC accumulation, and no subtelomeric repression); B: mother lineage (subtelomeric repression due to accumulation of ERCs); C: daughter lineage of *tlc1Δ* mutants (subtelomeric repression by sliding on the subtelomere of the telomeric cap). Some hypothetical subtelomeric regulatory sequences ("r-sequences") are transcribed into "r-transcripts", which regulate many cellular functions, including the stability of the telomere-telomere cap complex that influences the probability of triggering cell senescence. In cases B and C, it is suggested that r-sequences repression hinders the transcription of these sequences.

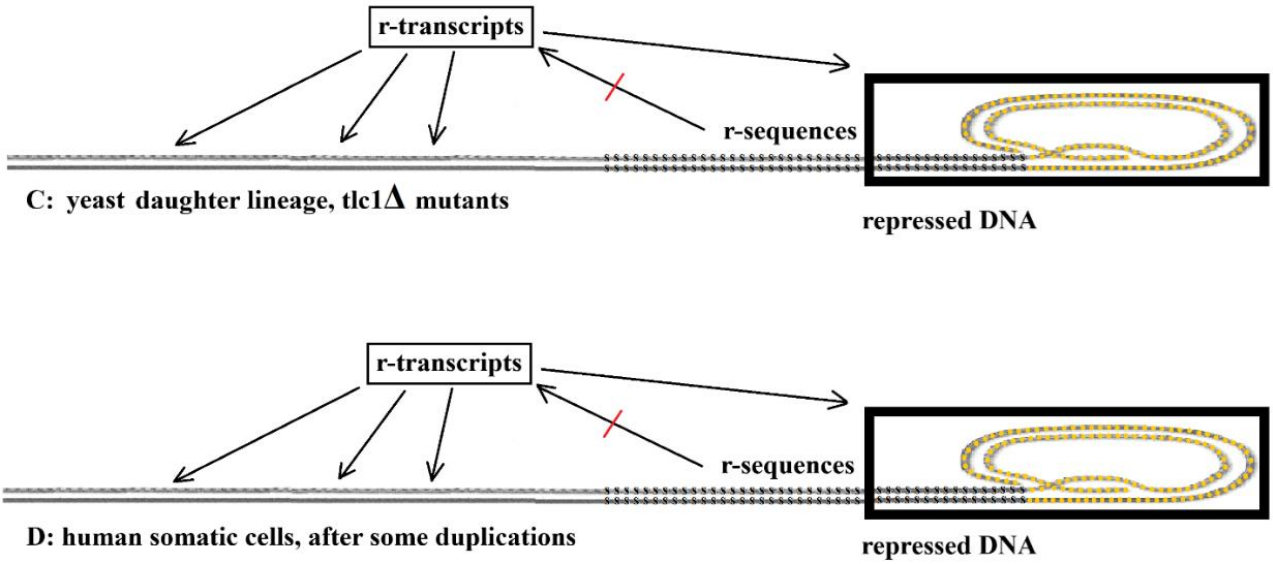

Figure 2 The subtelomere-telomere theory suggests that the condition in human somatic cells where telomerase is inactive is analogous to that in yeast cells, daughter lineage, of *tlc1Δ* mutant strains with telomerase inactive.

It is helpful to note that cell senescence in yeast determines the elimination of the individual through apoptosis. On the contrary, in human somatic cells, cell senescence determines resistance to apoptosis. However, functional alterations in senescent cells reduce cellular efficiency and that of the tissues and organs to which the cells belong, thus decreasing the organism's fitness and causing an increase in mortality.

A scheme of the theory is shown in Figure 3. The gradual repression of the subtelomere causes the phenomena discussed in the following paragraphs 2.4.7 Cell senescence and gradual cell senescence and 2.4.8 Decline of cell duplication capacity.

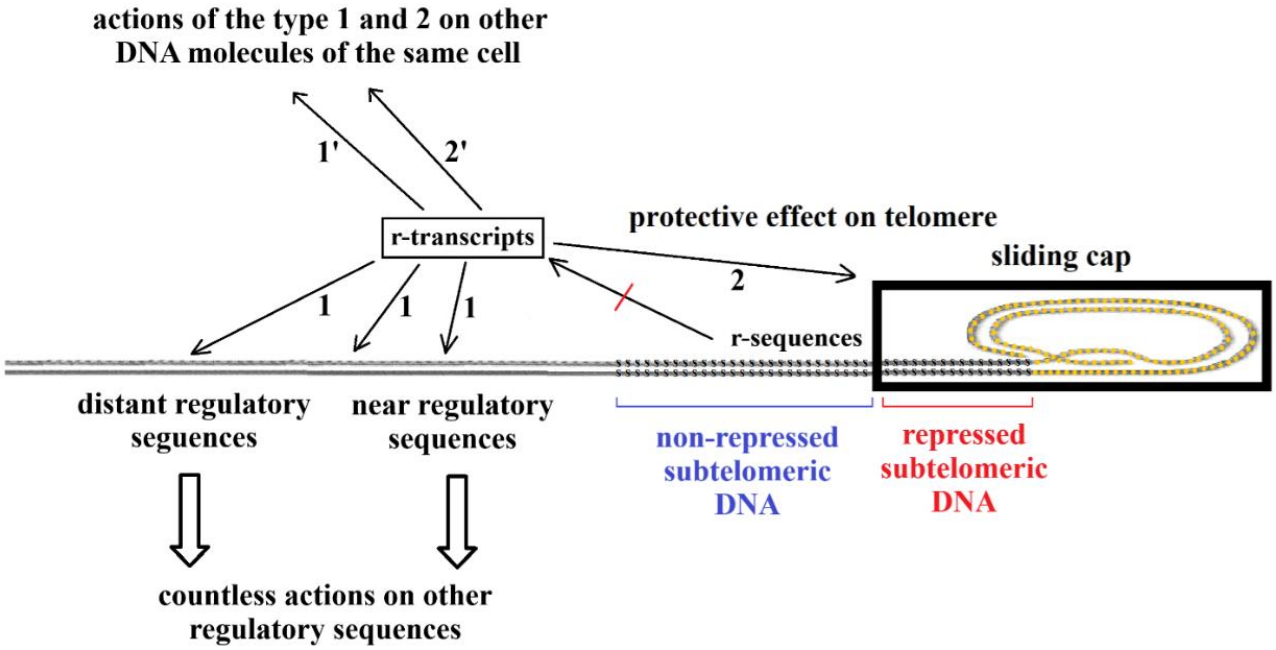

Figure 3 Scheme of the subtelomere-telomere theory.

The subtelomere-telomere theory explained the empirical data well, but the hypothetical r-sequences needed experimental confirmation of their existence and of how they exerted the supposed regulatory activities. Furthermore, there was no evidence that the telomere cap maintained a fixed size despite telomere shortening.

2.4 Phase 4: Subtelomere-telomere Theory with T-sequences

Sometimes there is no direct communication between different researchers, and this can be a significant obstacle to understanding a phenomenon. Sequences with functional characteristics that corresponded exactly to those proposed for r-sequences by the subtelomere-telomere theory were already known under another name. Moreover, for some years, they have been the subject of authoritative studies without there being awareness of their importance as possible key elements of cellular aging mechanisms.

Over thirty years ago, two non-protein-coding subtelomeric sequences (TelBam3.4 and TelSau2.0) were identified [62]. Later, it was demonstrated that there were two evolutionarily conserved regions with distinct structures, which were precisely characterized. Both sequences have a section that is approximately 1.4 kb long for TelBam3.4 and 1.3 kb long for TelSau2.0, and with three different repeating subsections. The intricate details of these two structures are reported in [63].

TelBam3.4 and TelSau2.0 were described as TElomeric Repeat-containing RNA (TERRA [64]; here "T-sequences" for brevity).

T-sequences:

- are non-protein-coding, but are transcribed with the production of RNA sequences, which here are called "T-transcripts" for brevity [65];
- appear to be pivotal elements in many critical cellular processes and constitute a general characteristic of eukaryotic cells [66]. They have been described in our species [64, 67], yeast [68-70], plants [71], Zebrafish and mice [67];
- in vertebrates and many other species are evolutionarily conserved as documented for vertebrates in general [72] and for yeast, plants, mammals, birds, and fish [73].

2.4.1 Transcription of T-sequences

In mammals, T-sequences transcription begins from subtelomeric DNA, is operated by the enzyme RNA polymerase II, proceeds toward the telomere, and includes some of the repeated telomeric motif [64, 67, 74]. The transcription begins from specific subtelomeric promoters that are observed on no less than two-thirds of the ends of chromosomes [63, 75, 76].

In humans, the subtelomeric promoters of T-sequence transcription are composed of DNA islands rich in CpG dinucleotides, characterized by a peculiar structure with repeated sequences, and located approximately 1 kb from the telomeric sequence [66].

The T-sequences are present in the subtelomeric parts of almost all chromosomes, with greater evidence for the sex chromosomes and chromosomes 2, 9, 13, and 18 [77].

2.4.2 Targets and Actions of T-transcripts

T-transcripts bind DNA targets (i) in every part of the genome; (ii) also at sites far from the telomere; (iii) also within genes, where introns are favored; (iv) in cis at telomeres; and (v) in trans near or within genes [77].

Most of the binding sites of T-transcripts are in distal intergenic and intronic sections of chromosomes, and T-transcripts regulate the expression of genes [66].

The expression of T-transcript targets changes significantly when there is a decrease in T-transcript levels. Target genes near the subtelomere are downregulated considerably, while among the internal target genes, some are upregulated and others are downregulated. Thousands of binding sites of both cis and trans types have been identified in mouse embryonic stem cells [77].

In general, the targets of T-transcripts appear to be non-protein-coding DNA sequences with critical regulatory functions on gene expression [77, 78].

T-transcripts are also crucial in regulating telomerase activity [79].

2.4.3 Relations between T-sequences Transcription and Telomere Shortening

The blockage of certain central epigenetic regulators, which enable DNA methylation, correlates with a loss of control over telomere length. Telomere shortening to a critical level alters the epigenetic condition of both subtelomere and telomere [80].

In mice, telomere shortening appears related to decreased subtelomere methylation [80].

In Terc (-/-) mice, the methylation of subtelomeric DNA decreases in conjunction with telomere shortening [29].

In human leukocytes, a relationship has been observed between shorter telomeres and significant methylation of CpG sites in the region within 4 Mb of the telomere [81].

Modified gene expression is related to the shortening of telomeres and increased risk and severity of various age-related diseases [81].

In healthy subjects, the mean length of telomeres decreases with aging. Moreover, longer telomeres are associated with hypermethylation of subtelomeric sequence, while hypomethylation is associated with shorter telomeres. The same was observed for sarcoidosis patients [82].

These data indicate that longer telomeres correlate with greater levels of methylation of T-sequences, while shorter telomeres correlate with smaller levels of methylation. This appears to signify less repression of T-sequences with longer telomeres and greater repression with shorter telomeres.

2.4.4 Relations between T-sequences and Telomere Protection

In mice, in embryonic stem cells, reduced levels of T-transcripts are associated with lower telomere protection [77, 78].

The transcription of T-sequences activates the mechanisms for the telomeric DNA damage response [83]. This is in relation to alterations in the capping function and the consequent loss of telomeric integrity [77].

Considering the protein ATRX, studied for alpha thalassemia mental retardation X-related syndrome, T-transcripts and ATRX are functionally antagonistic for hundreds of target genes they

share. In general, T-transcripts activate a target gene while ATRX represses it. About binding to telomeric DNA, T-transcripts compete with ATRX and contribute to telomere stability [77].

A substantial decrease in T-transcript levels is a manifestation of the loss of the 20q locus. This causes a massive response to telomeric DNA damage and should be considered evidence of the key role of T-transcripts in the defense and preservation of telomeres [84].

How T-transcripts protect telomeres are the subject of active research. For example, it has been suggested that telomere protection involves the formation of "TERRA R-loops at chromosome ends" [85].

2.4.5 Relations between Aging and Epigenetic Changes

Epigenetic modifications of DNA are age-related and depend on cell types and tissues [86, 87]. The most studied form of epigenetic modification is cytosine-5 methylation within CpG dinucleotides (DNA methylation) [88, 89].

The effects of DNA methylation are correlated with the number of previous cell divisions, while they are practically absent in embryonic cells and also in induced pluripotent stem cells (iPSCs) [88, 89]. The reversibility of DNA methylation is shown by the possible transformation of adult somatic cells into iPSCs, where DNA methylation is practically irrelevant as in embryonic cells [88].

Age-related DNA methylation is not a phenomenon that randomly affects any CpG sequence. There are specific parts of DNA showing this phenomenon [90-93]: in particular, the CpG islands, or CGIs, which are characterized by the abundance of CpG nucleotides (about 1 per 10 bp), constitute only 2% of the DNA [86], and often are the transcription start site of a gene [94]. For these sites, methylation appears to silence the promoters present in them [95], while the expression of these promoters is restored by demethylation [96].

In relation to age, there is hypomethylation for some CGIs, while for others, hypermethylation [92, 93, 97, 98]. However, the measurement of age-related DNA methylation has been proposed as a valuable and reliable biological indicator to evaluate the age of individuals of our species [88]. This index (Horvath's index) allows for assessing the age with an error of 3.6 years and a correlation index equal to 0.96.

The Horvath index is not the only epigenetic clock available, and it is by no means the best in any case. For example, PhenoAge and, in particular, GrimAge are superior epigenetic clocks to the Horvath index in assessing human aging [99]. However, in our discussion, the crucial point is not which epigenetic clock is more reliable, but that (i) epigenetic modifications are observed in precise parts of the DNA molecule's sequence and not in random points; and that (ii) such modifications are similar in many species, allowing for comparative measurements.

The age-related effects of DNA methylation are observed for mammals in general. An indicator similar to Horvath's index was proposed for mammals in general after an extensive study on 128 mammalian species (with a significant variety of adult body weight and maximum longevity ranging from 3.8 to 211 years). This index was found to have an error of less than 3.5% and a correlation greater than 0.96 [89].

The definition of an index valid for mammals in general is possible, in part, because the CGIs subject to DNA methylation appear to be quite evolutionarily conserved [89].

In the senescence of mesenchymal stem cells (MSCs), it was observed DNA methylation in specific CGIs and histone trimethylation at specific targets [100]. The repeated duplication, or expansion, of

MSCs significantly modifies DNA methylation profiles, and many CpG sites exhibit different methylation patterns in relation to the increasing number of duplications [100]. One study found that, regarding age, approximately one-third of CpG sites show different methylation states, with 60% of sites being hypomethylated and 40% hypermethylated [101].

Physical exercise appears to be related to an increase in TERRA levels (i.e., T-transcripts) in myocytes from healthy young subjects [76], an observation compatible with the idea that physical activity is a protective factor against aging.

Epigenetic changes are not limited to DNA methylation. A complete evaluation of epigenetic changes would require considering, among other phenomena, histone methylation, changes in histone marks, nucleosome remodeling, and altered noncoding RNA expression [102-104]. However, in relation to age-related changes, no reliable markers such as those mentioned above to assess age in our species or in mammals in general have been proposed.

The works mentioned above document strong and complex relationships of T-sequences and their T-transcripts with: (i) epigenetic modifications of numerous DNA targets and the consequent actions; (ii) telomere shortening; (iii) telomere protection; and (iv) aging changes.

All these relationships demonstrate that T-sequences and their transcripts exhibit the characteristics predicted by the subtelomere-telomere theory for r-sequences and r-transcripts, respectively. Therefore, the scheme proposed in Figure 3 can be redrawn by simply replacing the hypothetical r-sequences and r-transcripts with the documented T-sequences and T-transcripts (Figure 4). This transforms the subtelomere-telomere theory of aging from a theory requiring empirical evidence into a theory confirmed by scientific evidence.

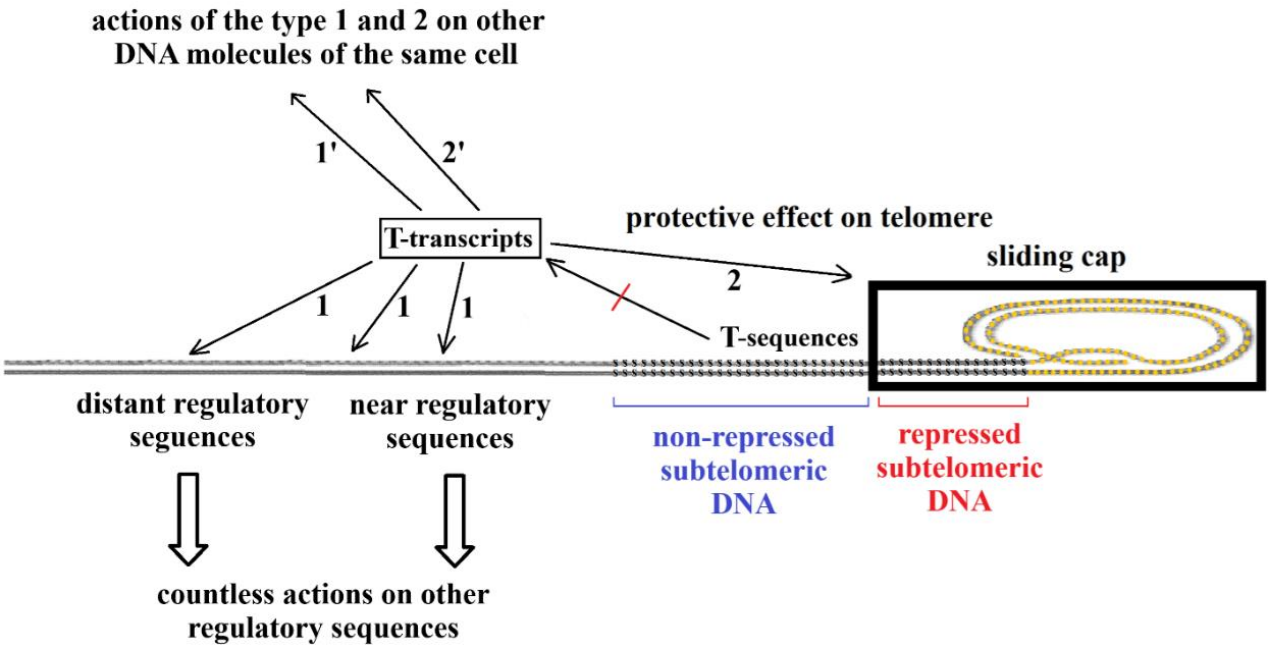

Figure 4 Scheme of the subtelomere-telomere theory with T-sequences: substitution of the hypothetical r-sequences and r-transcripts with the documented T-sequences and T-transcripts, respectively.

2.4.6 The Fixed Size of Telomere Cap

The subtelomere-telomere theory of aging also requires that, for each telomere, the size of the telomeric cap, after its formation in the first cell of an organism, remains constant in subsequent divisions even if the telomere shortens.

It is known that the telomere is covered by a cap constituted by copies of the shelterin protein complex, which is known in its protein components (RAP1, TIN2, TRF1, TRF2, POT1, and TPP1) [105, 106] and for their possible arrangement [106].

Suppose the telomeric cap has a fixed size established in germline cells, meaning that this size remains unchanged at each duplication, even if the telomere shortens. In that case, the total amount of proteins that make up the cap should stay constant and not decrease in the transition from germline cells to cells with shortened telomeres.

A vital experiment observed that the abundance of shelterin complex proteins remained unchanged between primary and transformed cells and was not related to telomere length [107]. This indicates that the size of the telomeric cap does not vary with telomere shortening.

2.4.7 Cell Senescence and Gradual Cell Senescence

Cell senescence is not another way to describe the features of an "old cell" but the definition of a precise condition determined by a "fundamental cellular program" [27] with peculiar characteristics:

(i) specific modifications of cellular functions [22, 108, 109], including the alterations of cellular secretions described as senescence-associated secretory phenotype (SASP) [110, 111], related to significant transcriptional modifications [112];
(ii) block of cell replication capacities (replicative senescence) [109, 113];
(iii) resistance to apoptosis [22, 114], which is obtained by selectively blocking the mechanisms that cause apoptosis in malfunctioning cells. Indeed, cell senescence triggers or up-regulates various senescent cell anti-apoptotic pathways, which block apoptotic mechanisms and so are targets for existing or possible senolytic drugs aimed at the selective elimination of senescent cells [22].

The triggering of the cell senescence program is related to telomere shortening, but, as proposed by Blackburn [26], there is no critical telomere length below which the program is triggered. A cap unstably protects the telomere, and, in relation to telomere shortening, telomere protection is reduced, and the probability of activation of the program increases. The evidence supporting the relationships between telomere shortening, increased subtelomere inhibition, and decreased telomere protection from activation of the cell senescence program has been discussed in the previous subsection, "2.4.4 Relations between T-sequences and telomere protection".

The number of senescent cells increases with age, both in absolute numbers and in their fraction of the total number of cells [115, 116], and is related to age-related manifestations and disorders [117, 118].

Senescent cells are harmful for the organism, and their selective elimination reduces the manifestations of aging [118, 119], so that their elimination by senolytic drugs is an essential current therapeutic goal [119, 120].

The fact that senescent cells are undoubtedly harmful to the fitness of the organism and that at the same time are resistant to apoptosis, thus causing persistent damage, is well explainable

assuming that the phenomenon is a general part of aging mechanisms explained as an adaptive phenomenon. Conversely, for the non-adaptive hypotheses of aging, cell senescence requires a specific explanation. In this regard, the only proposed explanation is that the phenomenon is a general defence against the possible proliferation of cancerous cells [121, 122]. So, the damage caused by senescent cells has been justified as an evolutionary trade-off between the damage and the benefits they cause [123]. The unsustainability of this thesis has been discussed in previous works [5, 124] based on various experimental works, including the following findings:

- It is part of the SASP, the secretion of many factors that are associated with inflammation and malignancy [110];
- In humans, a relationship between short telomeres and cancer risk has been shown [125, 126];
- In mice, the selective elimination of senescent cells determined both fewer age-related alterations and a delay in cancer progression [116].

A phenomenon similar but distinct from cell senescence is the gradual decline of cellular functions (gradual cell senescence [4]), caused by the progressive subtelomeric inhibition in cases where the cell senescence program has not yet been activated. The effects of gradual cell senescence can be confused with those of cell senescence because, in a culture, there is overlap between the manifestations of some cells in gradual cell senescence and others in cell senescence. However, there are conditions in which the distinction is specific and clear. In yeast cells of the mother lineage in relation to the number of previous duplications, a progressive inhibition of the subtelomere is observed due to the accumulation of ERCs and progressive alterations of cellular functions [56, 127]. In yeast, there is no possibility of confusion with the phenomenon similar to cell senescence of multicellular eukaryotes because in this species, the phenomenon triggers apoptosis [58].

In vitro, mesenchymal stem cells (MSCs), in correlation with the number of previous duplications, show: (i) gradual changes in the methylation of specific points (hypomethylation or hypermethylation) and the extent of these changes allows to estimate the number of previous duplications [128-130]; and (ii) it possible to observe in the overall gene expression a pattern of significant alterations that are not limited to the latest duplications but which progressively increase in relation to the number of duplications [131].

Moreover, in a study on the effects of telomere shortening, it was observed that the expression of various subtelomeric genes is in relation to the length of telomeres and that significant changes in gene expression (up-regulation or down-regulation) are dependent on telomere shortening long before the reduction of telomere length triggers cell senescence [132].

It is unlikely that this explanation can account for the manifestations of gradual cell senescence, as determined by the accumulation of random alterations. In fact: (i) reprogramming of MSCs to induced pluripotent stem cells (iPSCs) erases the functional alterations that have increased with cell duplications [133]; and these iPCs show the profile of a young cell regardless of cell origin and donor age [133]; (ii) for induced MSCs (iMSCs) the age-related levels of DNA methylation were entirely erased while, in the subsequent culture *in vitro*, iMSCs reacquired the age-related degree of methylation [101]; and (iii) MSCs showing fewer epigenetic changes and better cell functions can be obtained from iPSCs [134].

Cells in gradual cell senescence cannot have any hypothetical significance as a restraint on the reproduction of cancer cells because, for them, there is no replicative senescence. On the contrary,

according to the subtelomere-telomere theory, gradual cell senescence constitutes part of the aging program because it contributes to reducing fitness.

2.4.8 Decline of Cell Duplication Capacity

A simplistic explanation for the decline of cell turnover might be that it is due to the shortening of telomeres to critical levels. However, evidence shows a different situation that requires a brief exposition:

(i) In humans, telomere lengths are similar in different fetal tissues and organs [135];
(ii) telomere lengths in stem cells of tissues with high turnover (e.g., hematopoietic stem cells) are shorter than those of stem cells of low turnover tissues [135];
(iii) in cells derived from the stem cells, excluding perennial cells where there is no turnover, in four types of cells that have quite different turnover rates (leukocytes, and cells from subcutaneous fat, skeletal muscle, and skin), the rhythms of telomere shortening were similar [136];
(iv) in general, the reduction rates of telomere lengths were in most cases within 20-60 bp/year. Only some cell types showed a shortening rate that could be critical (e.g., for liver cells, it was 120 bp/year, and the mean length of telomeres was reduced from 13.7 ± 2.5 kbp in neonates to 8.7 ± 1.4 kbp in centenarians) [137].

These results were interpreted as follows. Stem cells of each cell type show a series of duplications, i.e., an expansion, which is proportional to subsequent turnover rates and determines a proportional telomere shortening. Afterwards, telomeres shorten at constant rates but without reaching critical sizes with a highly probable activation of the cell senescence program [137]. However, following the thesis proposed by Blackburn [26], according to which even with telomeres not shortened, there is always a small probability of triggering cell senescence, stem cells would gradually go into this condition, progressively weakening the capacity for cell turnover.

2.4.9 Aging of Any Tissue or Organ and of the Whole Organism

Cellular aging as described above has effects that extend to every part of the organism and progressively compromise its fitness. In fact, the combined impact of cell senescence, gradual cell senescence, and a reduction in turnover capacity determines an “atrophic syndrome” in every part of the organism, resulting in the manifestations of aging in any tissue and organ, as well as in the whole organism [5].

This syndrome consists of a reduction in the number of the primary functional cells of an organ or tissue (i.e., atrophy in the number of cells), with hypertrophy in the volume and activity of the remaining functional cells and replacement of the missing cells with non-functional cells. Furthermore, considering also the cellular alterations determined by cell senescence and gradual cell senescence, including modifications in cellular secretions (SASP), this set of alterations progressively compromises the functionality of the tissue or organ and the fitness of the organism. Finally, telomere shortening is associated with an increased vulnerability to cancer [138].

In the particular case of tissues or organs where the primary functional cells do not show turnover (for example, the neurons of the central nervous system), the alterations above affect their trophic cells, progressively compromising the activity and survival of the primary cells and the overall functionality of the tissue or organ [5, 30-32].

So, the aging of a tissue or organ occurs through the direct decline of its primary functional cells ("direct aging") (Figure 5) or through the decay of the trophic cells of functional cells ("indirect aging") (Figure 6) [5, 32, 60].

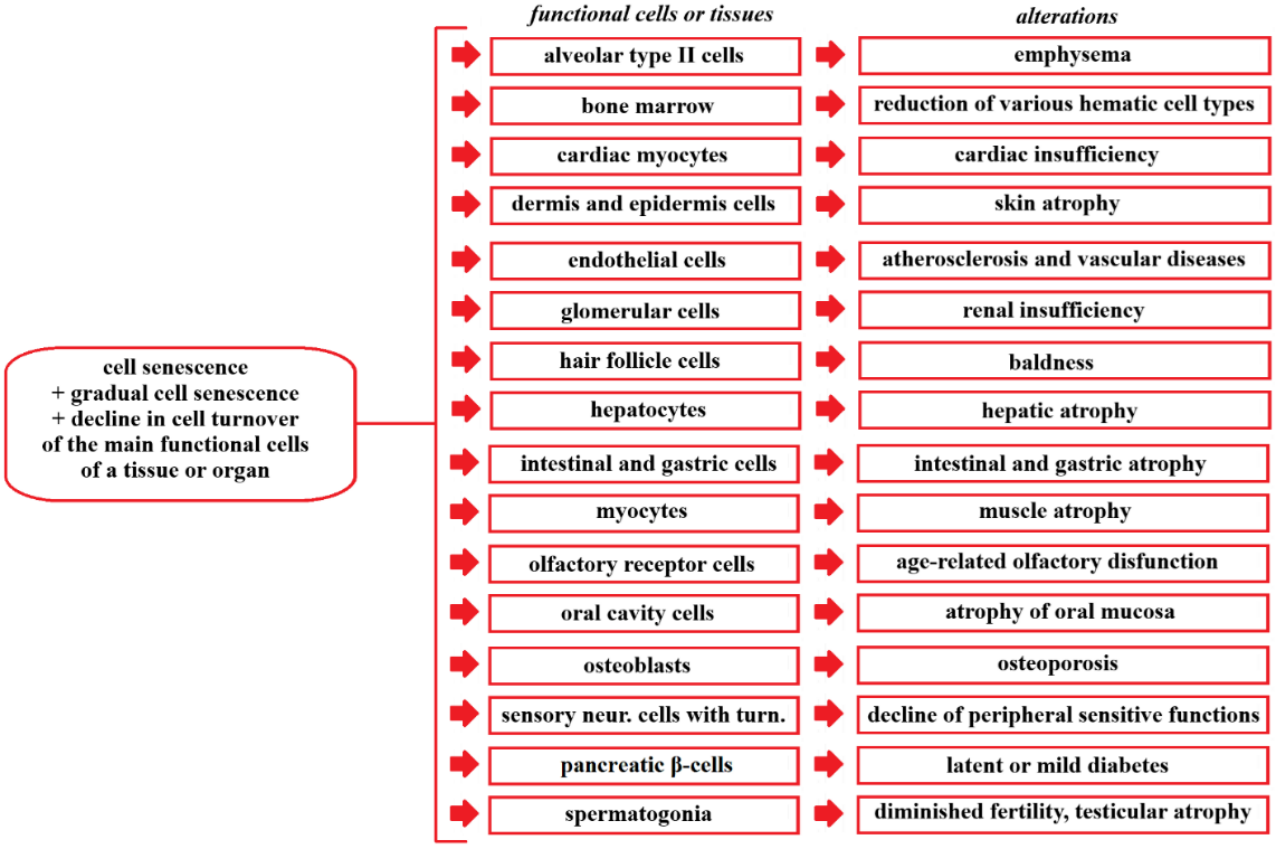

Figure 5 Examples of "direct aging" - Decline of the cells with turnover constituting the primary functional cells of a tissue or organ.

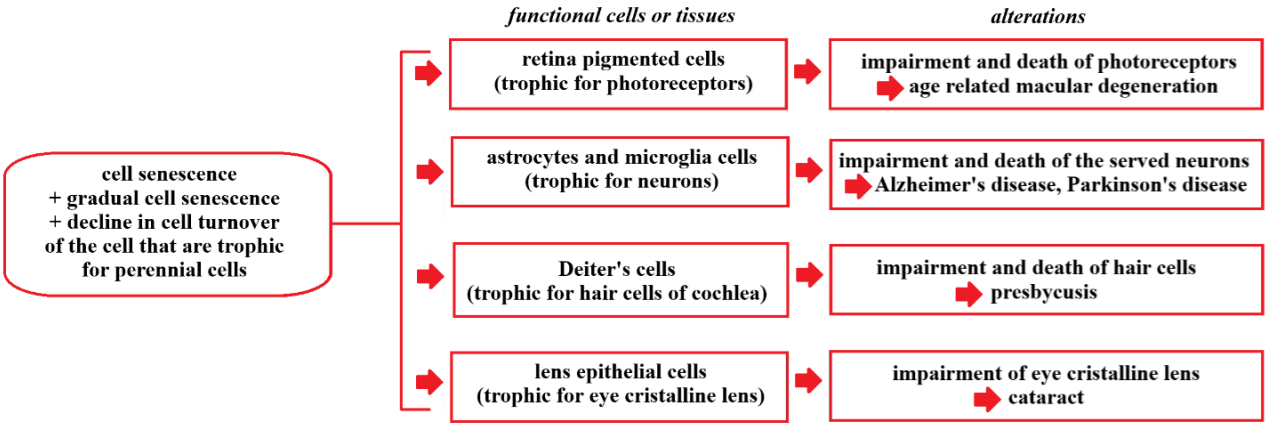

Figure 6 Examples of "indirect aging" - Decay of the cells with turnover that are trophic for the primary functional cells without turnover of a tissue or organ.

2.4.10 Aging as Function Determined by Epigenetic Modifications

There is a growing consensus regarding the association of aging with epigenetic modifications and the description of aging as an epigenetic phenomenon [139-145].

However, two ideas should be avoided.

The first is that the epigenetic modifications associated with aging are random and without functional significance, as they are determined and modulated by natural selection. The above demonstrates that such modifications: (i) occur at specific sites; (ii) are influenced by well-defined subtelomeric sequences, which in turn are progressively inhibited by telomere shortening; (iii) are generally reversible with appropriate manipulations; (iv) are parts of a mechanism that determines aging and is shaped by natural selection. Therefore, the aforementioned epigenetic modifications together with the mechanisms determining them should be defined as a function and not considered as random phenomena.

The second idea to avoid is that aging, understood as a function based on epigenetic modifications, is an anomaly or an exception in the overall organization of the organism.

Until a few decades ago, the discovery of protein-coding by specific DNA sequences (defined as “genes”) and of countless and disparate capacities of proteins led to the belief that all the functions of the organism, both those internal to the cells and those related to the development and organization of tissues, organs and the entire organism, depended exclusively on the protein-coding DNA sections. However, it was observed that:

- A limited part of DNA encodes protein sequences. The hasty and erroneous deduction was that the remaining parts of the DNA had no function, so much so that they deserved the definition of “junk” DNA [146];
- If the enormous differences between the various species in functions, morphological development and organization depended only on genes, a very complex organism like the human one should have a much greater number of genes than much simpler species. However, it was disconcerting to find that our species and a simple nematode had an equal number of genes [147];
- The ENCODE and the Human Genome Projects have highlighted that in mammals, only 2% of the DNA is protein-coding, while >90% is transcribed and produces long noncoding RNA sequences with functions mostly unknown [77].

The logical deduction is that the central part of a species' DNA program is not located in the protein-coding sections (genes), but rather in a network of non-protein-coding sequences that regulate, directly or indirectly, the expression of genes. Plausibly, this regulation occurs through a myriad of epigenetic modifications.

A magnificent example of this theoretical argument was offered by a recent work that demonstrated the differences in epigenetic markers among the many cell types of our species [148]. These differences are precise to the point that it is possible to identify the developmental affinities between the various cell types. Furthermore, it was observed that the different DNA methylation patterns of the various cell types allow for the reconstruction of the ontogenesis of the key types of cells [148].

Consequently, it is possible to suggest that any complex function of the organism is determined and governed by epigenetic modifications regulated by non-coding sequences. This means that

aging, described as a function defined by epigenetic changes, is not an exception but the rule for any complex function.

3. Topics Requiring Further Exploration

In any new theory, there are certainly topics requiring further exploration and clarification, and the theory discussed in this paper is no exception. A detailed examination of these topics is beyond the subject of this article, and it is appropriate to limit ourselves to mentioning some of them:

1) The disastrous effects of a drastic decrease in T-transcript levels [84] or of a defective activity of the ATRX protein, which antagonizes the effects of T-transcripts [77], have already been mentioned in the previous section 2.4.4. However, it would be helpful to evaluate the impact of a partial artificial repression of T-sequences *in vivo*, in experimental animals, and *in vitro*, in suitable cell cultures.
2) A detailed evaluation of all the regulations depending on T-sequences also seems necessary;
3) Regarding the telomere cap and its relationship to the telomere, many aspects need to be investigated in detail, including: (i) how, in the first cell of an individual, this cap is shaped in relation to telomere length; (ii) how the cap is reformed with each subsequent cell duplication, respecting the initial size of the cap and not the possibly reduced length of the telomere; (iii) when telomere length is restored by telomerase action, what is the role of the telomere cap and how telomerase restores the previous length of the telomere without further lengthening it;
4) How does the protective action of T-sequences on the telomere-telomeric cap complex affect the probability of activating cell senescence?

4. Possible Methods for Controlling the Aging Mechanism

Suppose aging is determined by a specific mechanism and is not the consequence of the accumulation of random alterations. In that case, this suggests that this mechanism can be modified, slowed, or even blocked by appropriate methods.

A detailed discussion of this topic is beyond the scope of this paper; therefore, it is helpful to refer to other works (e.g., [149]). Here, based on the above, only the mention of some methods will be given:

1) Activation of the telomerase enzyme. This activation restores telomere length and should reverse the effects of gradual cell senescence and limit the likelihood of cell senescence activation;
2) Elimination of cells in the state of cell senescence, particularly through the action of senolytic drugs;
3) Restoration of original stem cell levels through reprogramming of differentiated cells. This topic appears to be rich in studies and perspectives [150, 151].

Each of these methods does not have the effects of the other two, and all should be used in parallel for better aging control.

Conversely, the idea of targeting the myriad cellular effects of T-sequence repression (that is, interventions downstream of the actions of the T-sequences) does not seem rational, given the multiplicity and variety of these effects.

5. Conclusion

The subtelomere-telomere (STT) theory with T-sequences, which provides clear support for the thesis that aging is a genetically regulated and determined phenomenon, was elaborated in four subsequent phases, summarized in Table 1.

Table 1 Phases in the Elaboration of the Subtelomere-Telomere Theory with T-sequences.

Phase/Theory	Main features	Objections
Phase 1: Aging caused by the effects of cell duplication limits	Cell duplication limits were hypothesized as the cause of aging.	The theory did not explain: (i) the multiple cellular alterations preceding the blocking of cell duplication capacity; and (ii) the aging of perennial cells, i.e. cells without turnover.
Phase 2: Aging as a consequence of telomere shortening (telomere theory)	The progressive shortening of telomeres was proposed as the cause of both the block of cell duplication and the cellular changes preceding this block. The aging of cells without turnover was explained by the decline of satellite cells subject to turnover.	According to the theory, a correlation between telomere length and longevity was expected, but this was contradicted by the evidence. Furthermore, telomere length varies from telomere to telomere within the same individual, and it was difficult to reconcile this variability with the proposed mechanism of aging.
Phase 3: Aging as a result of subtelomere repression caused by the shortening of telomeres (subtelomere-telomere theory)	It was proposed that (i) in the first cell of an individual, a telomeric cap is formed with a size proportional to telomere length; (ii) in subsequent duplications, the size of the cap remains fixed even if the telomere shortens; and (iii) so, the cap progressively inhibits hypothetical subtelomeric general regulatory sequences.	The hypothetical subtelomeric general regulatory sequences required empirical confirmation. Furthermore, the hypothesis of invariance of telomere cap size even with telomere shortening needed to be demonstrated.
Phase 4: Subtelomere-telomere theory with T-sequences	The hypothetical regulatory sequences were identified in particular sequences (TERRA, T-sequences) that, with ample evidence, show characteristics corresponding to those predicted for the hypothetical regulatory sequences.	None.

In support of the STT theory with T-sequences, the evidence shows the existence of a specific mechanism regulating cellular functions and progressively causing cellular aging and hence that of the entire organism. This mechanism is based on key subtelomeric sequences that are general regulators of cellular functioning. They act through countless direct or mediated epigenetic modifications and are progressively repressed in relation to telomere shortening.

Anyone who still wants to support the opposing thesis of aging determined by random and non-adaptive alterations should, among other things, falsify the evidence supporting the existence of this mechanism and the details of its functioning. Moreover, it would be necessary to justify in evolutionary terms:

- why T-sequences, which have a pivotal importance for cellular functions, are in a position highly vulnerable to repression by telomere position effect [54]);
- the existence of cell senescence (for which the hypothesis of an anti-cancer defense appears to be untenable [5, 14]);
- the existence of gradual cell senescence, which cannot be proposed as an anti-cancer defense [5, 14].

So, the hypothesis of aging as an adaptive and programmed phenomenon, determined by precise epigenetic modifications, appears as a theory confirmed by evidence, while the contrary is true for the opposite theory.

Abbreviations

T-sequences	TERRA sequences or simply TERRA
T-transcripts	TERRA transcripts
RPCs	retinal pigmented cells
ERCs	extrachromosomal ribosomal DNA circles
SASP	senescence-associated secretory phenotype
ATRX	protein related to alpha thalassemia mental retardation X-related syndrome

Author Contributions

The author did all the research work for this study.

Competing Interests

The authors have declared that no competing interests exist.

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