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REVIEW

Is Evidence Supporting the Subtelomere–Telomere Theory of Aging?

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Abstract—The telomere theory tries to explain cellular mechanisms of aging as mainly caused by telomere shortening at each duplication. The subtelomere–telomere theory overcomes various shortcomings of telomere theory by highlighting the essential role of subtelomeric DNA in aging mechanisms. The present work illustrates and deepens the correspondence between assumptions and implications of subtelomere–telomere theory and experimental results. In particular, it is investigated the evidence regarding the relationships between aging and (i) epigenetic modifications; (ii) oxidation and inflammation; (iii) telomere protection; (iv) telomeric heterochromatin hood; (v) gradual cell senescence; (vi) cell senescence; and (vii) organism decline with telomere shortening. The evidence appears broadly in accordance or at least compatible with the description and implications of the subtelomere–telomere theory. In short, phenomena of cellular aging, by which the senescence of the whole organism is determined in various ways, appear substantially dependent on epigenetic modifications regulated by the subtelomere–telomere–telomeric hood–telomerase system. These phenomena appear to be not random, inevitable, and irreversible but rather induced and regulated by genetically determined mechanisms, and modifiable and reversible by appropriate methods. All this supports the thesis that aging is a genetically programmed and regulated phenoptotic phenomenon and is against the opposite thesis of aging as caused by random and inevitable degenerative factors.

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INTRODUCTION

This section is the synthesis of concepts and facts already exposed in recent works [1-3] and, for the sake of brevity, most of the references will be omitted.

There are two opposite paradigms to explain aging. For the first ("non-programmed or non-adaptive aging paradigm"), aging is supposed to be caused by the random accumulation of effects of various degenerative phenomena insufficiently countered by natural selection.

Many theories belong to the first paradigm [3]. A first large group of theories proposes that aging is an inevitable consequence of the accumulation of damage of various types. In this group of theories, many of them, only historically interesting, are of the nineteenth-century and the first half of the twentieth century and attribute aging to cellular "wear and tear", mechanochemical deterioration of cell colloids, inherent changes in specified tissues (nervous, endocrine, vascular, and connective), toxic products of intestinal bacteria, accumulation of "metaplasm" or of metabolites, etc. Then there are various popular modern or contemporary theories of this group that attribute aging to the accumulation of chemi-

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cal damage due to DNA transcription errors, deleterious effects of oxidation, oxidative effects of free radicals on the whole body, mitochondria, or DNA, inflammatory phenomena ("inflamm-aging"), and immunological alterations related to age (some references for these theories are cited in the section "Aging, oxidation, and inflammation").

Other popular non-adaptive aging theories, such as mutation accumulation hypothesis, antagonistic pleiotropy hypothesis, and disposable soma hypothesis, try to take into account the mechanisms of evolution.

Despite the great variety of these theories, one fundamental concept unites them: as aging is certainly harmful to an individual, natural selection can only work to counteract aging; consequently, it is not admissible or conceivable that there are adaptive physiological mechanisms of any kind that could determine aging.

For the second paradigm ("programmed or adaptive aging paradigm"), aging is considered to be determined and modulated by genes favored by natural selection at the supra-individual level, even if they appear certainly harmful at the individual level, and belongs to the category of phenoptotic phenomena (sacrifice of an individual favored by the supra-individual selective mechanisms) [4, 5].

The theories belonging to the second paradigm are few [3] and necessarily require an existence of specific mechanisms that determine aging. Consequently, empirical data showing the existence of such mechanisms strongly support the validity of theories belonging to the second paradigm and are against the admissibility of the first paradigm and of its theories.

For a certain period, mainly in the context of and in support of the second paradigm, it has been believed that the progressive shortening of telomeres at each duplication could be a direct and sufficient explanation of aging (this may be defined as "telomere theory"). However, the implications of the theory appeared to be in contradiction with various facts, e.g.:

– hamsters and mice have longer telomeres than humans, but have much shorter life spans [6]. In comparing various species, there is a lack of correspondence between the longevity and telomere length [7]. In various experimental cases, there was an analogous lack of correspondence in individuals within the same species but with different telomere length in the first germ cells (e.g., between the donor and the cloned animal [8, 9]; for other examples and a broader discussion, see [10], pp. 59-61).

– Elizabeth Blackburn [11] observed that in synchronously dividing cell cultures, cell senescence was not reached after a constant number of duplications, but, from the first duplications, there was a progressively increasing probability of cell senescence activation [12, 13], i.e., cell senescence is not triggered when telomere reaches a critical length, but there is a probabilistic relationship between the activation of cell senescence and reduction of telomere length.

A possible solution for the shortcomings of the telomere theory, which in fact represented an excessively and unacceptably simplified vision, considers the effect of telomere shortening on subtelomere [10], and has been defined as the "subtelomere–telomere theory" [1-3]. It will be briefly explained below, referring to the original works for most of the evidence that supports it:

– telomeres are the terminal parts of chromosomal DNA molecules, and therefore, the number of telomeres is twice the number of such molecules, as each of chromosomes has two ends. In germ cells, the length of each telomere varies and is hereditary [14-16];

– in the first cell of an organism, each telomere must be covered by a heterochromatin hood or cap modeled according to the telomere length (hypothesis of the theory);

– the length of each hood does not change in any subsequent duplication (hypothesis of the theory), even if the telomere at each duplication is shortened due to the incomplete action of DNA polymerase (if the shortening is not fully compensated for by telomerase enzyme, which shows an activity depending on the cell type);

– concerning the telomere shortening, the telomeric cap, which has been assumed to be of a fixed length, slides on the portion of DNA molecule adjacent to the telomere, defined as the "subtelomere R" ("R" means Regulatory) [2], that includes the TERRA sequences coding for primary regulatory RNA sequences (TERRA transcripts) and not proteins. Two types of TERRA sequences are known (TelBam3.4 and TelSau2.0) [17, 18], but it is not excluded that other types of TERRA sequences exist;

– TERRA sequences are characteristic of eukaryotic species and are evolutionarily conserved [19];

– TERRA sequences may be defined as "first-level regulatory sequences" and the sequences regulated by them as "second-level regulatory sequences", because TERRA transcripts have three types of concurrent regulatory effects, which include: (a) action (up-regulation) on the portion of DNA molecule adjacent to subtelomere R and defined as "subtelomere A" ("A" means Amplifier) [2] on the same telomere and on other telomeres, containing second-level regulatory sequences that amplify and multiply the effects of TERRA transcripts; (b) action (up-regulation or down-regulation) on other second-level regulatory sequences located in other non-subtelomeric areas both on the same DNA molecule or on other DNA molecules of the cell; (c) regulatory action of telomere protection by the telomeric hood. Experimental evidence for these statements has confirmed that: (i) "TERRA binds chromatin targets throughout the genome. ...TERRA binds both in *cis* at telomeres and in *trans* within or near genes" [20]. (ii) There are "...significant changes in expression of

TERRA targets relative to non-targets after TERRA depletion..., indicating that TERRA target genes were more likely to be affected by TERRA depletion... Interestingly, subtelomeric target genes were consistently downregulated... Internal target genes could either be up- or down-regulated..." [20]. (iii) "TERRA binds to many genomic loci outside telomeres where the noncoding DNA appears to play important regulatory functions related to gene expression [19-21]". (iv) "The vast majority of TERRA-binding sites were found outside of telomeres, mostly in distal intergenic and intronic regions of the genome where TERRA regulates gene expression. Importantly however, TERRA depletion in ES (embryonic stem) cells was also associated with telomere deprotection, suggesting that TERRA is nevertheless important for mouse telomeric integrity..." [19]. (v) The depletion of T-transcripts appears to be associated with reduced protection of telomeres [20, 21].

– It is likely that there are innumerable second-level regulatory sequences ("In the mouse ES [embryonic stem] cell genome, we identified thousands of *cis* and *trans* chromatin binding sites" [20]), which influence the function of innumerable cellular genes and other possible regulatory sequences.

– In relation to the increasing degree of repression of TERRA sequences, there is a gradual alteration of cellular functions defined as "gradual cell senescence" [2] and reduction in telomere protection with an increased risk of triggering cell senescence, a "fundamental cellular program" [22] characterized by stereotyped modifications (maximal alteration of cellular functions, i.e., gradual cell senescence to the highest degree, senescence-associated secretory phenotype, or SASP, which increases oncogenic risk [23], replicative capacity block or replicative senescence [22, 24], resistance to apoptosis [25, 26]). An increasing repression of TERRA sequences determines an increasing number of cells in (i) gradual cell senescence or (ii) cell senescence, i.e., with altered functions, SASP included, to varying degrees. The accumulation of these two types of cells with altered functions and the declining number of proliferating cells determined by cell senescence leads to the increasing alterations in all tissues and organs, defined as "atrophic syndrome", and so to the fitness decline, i.e., aging [3].

– Eventually, Blackburn's observation was solved by the same author, in accordance with the functions later attributed to TERRA sequences (see before), by suggesting that (i) the telomere is loosely covered by a hood and that it can oscillate between the two conditions, "capped" and "uncapped"; (ii) in the uncapped phase it is vulnerable to activate the replication capacity block (i.e., cell senescence); and (iii) the proportion of the uncapped phase increases with the telomere shortening. So, the cells appear to exist "in two states: cycling and exited from the cell cycle. Even from the very beginning of passaging, cells are stochastically dropping out of the cycling population. They do so with ever-increasing frequency until the population as a whole cease doubling ...even at the beginning of the passaging, the telomeres, although relatively long, are predicted to have a finite (although initially low) probability of becoming uncapped... the uncapped telomere signals the cell to exit the cell cycle... stochastic and increasing probability to switching to the uncapped/non-cycling state. It can explain several observations. In human cells in culture, two mitotic sister cells can have vastly different proliferative potentials [13] even though they have telomeres that are similar in length... in telomerase knockout mice, the telomere fusions and phenotypic effects of telomerase deficiency increase steadily in frequency and severity with increasing generations, rather than showing up abruptly only in late generations... In yeasts, cellular senescence is also stochastic and progressive in the cell population... the new model obviates the need to define a 'critical' telomere length" [11].

These concepts are summarized in Fig. 1.

As for perennial cells, their decline is explained by the decline of their satellite cells, a subject discussed elsewhere [27].

In the present work, we tried to verify if other experimental facts contradict or, on the contrary, confirm the subtelomere–telomere theory (or if they are at least compatible with the theory).

AGING AND DNA METHYLATION

DNA epigenetic modifications are highly related to age. In particular, DNA methylation (cytosine-5 methylation within CpG dinucleotides) is the subject of an increasing number of studies, showing, among other things, that they are close to zero for embryonic and induced pluripotent stem cells (iPSCs) and correlate with the cell passage number [28, 29]. DNA epigenetic characteristics vary according to the cell and tissue type [30, 31], and analogous variation is observed for their age-related changes [32, 33].

Age-related DNA methylation in some cases is hypermethylation and, in others, hypomethylation [32, 34-36].

Aside from epigenetic variations related to the cell and tissue type, sex, and disease conditions, there are CpG sequences that exhibit age-dependent DNA methylation [35-40].

For our species, indicators that predict age have been proposed using only these types of age-related DNA methylation [28, 40, 41]. The most reliable appears to be the one proposed by Horvath that shows age correlation = 0.96 and error = 3.6 years [28]. Similar epigenetic modifications have been described for mammals in general and in the study on 59 tissue-types from 128 mammalian species of 15 phylogenetic orders (with the maximum life spans from 3.8 to 211 years and adult weights

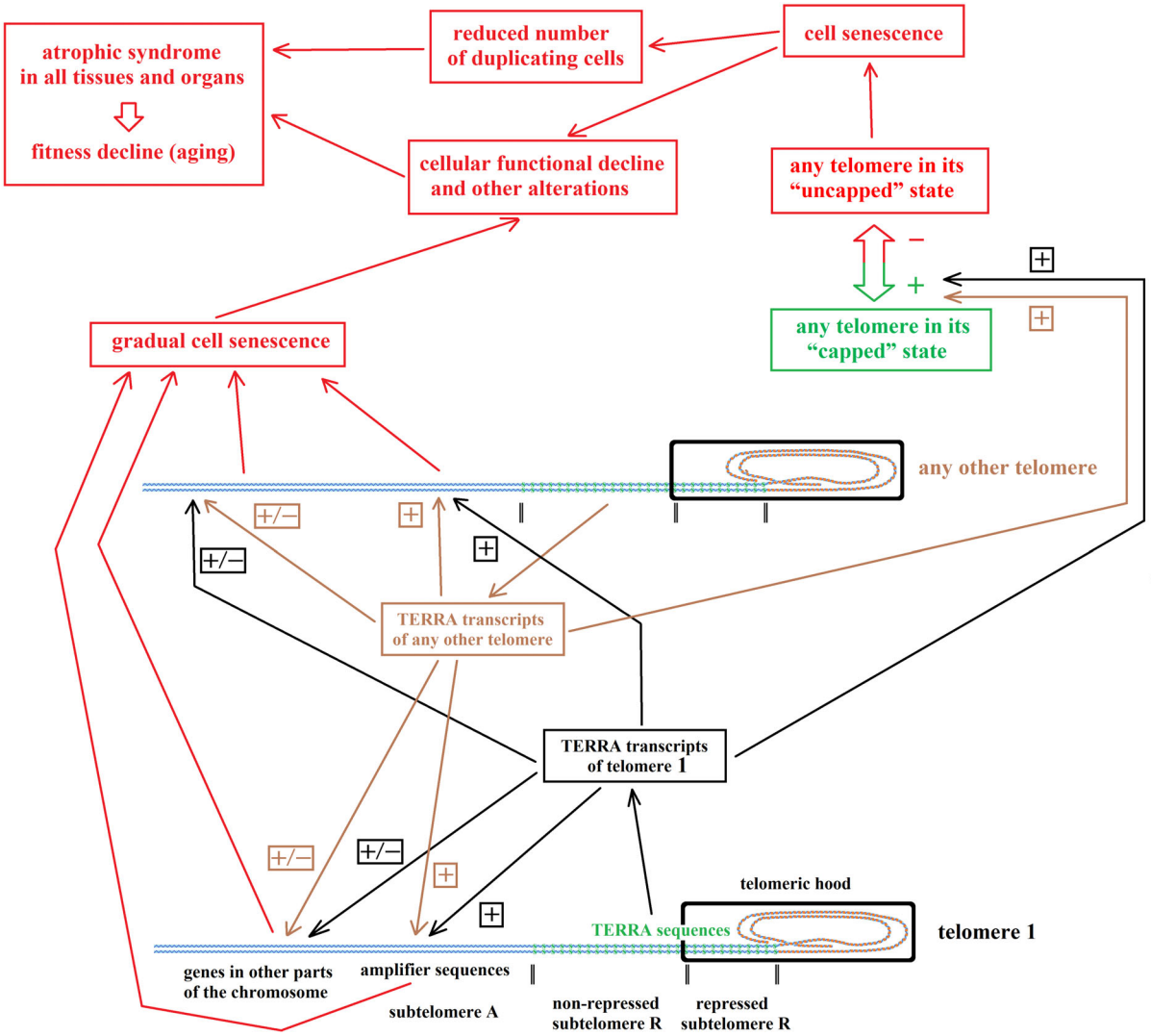

Fig. 1. The transcription of TERRA sequences in subtelomere R produces TERRA transcripts that up-regulate or down-regulate other regulatory sequences in subtelomere A or other chromosome regions both on the same chromosome and on other chromosomes. The sliding of the telomeric heterochromatin hood progressively represses subtelomere R while the telomere shortens, and the decline of the TERRA transcription determines gradual cell senescence and increasing probability of cell senescence. In the tissues and organs, this causes a growing decline of mean cell functionality and duplicating cell number, leading to an overall organism fitness decline, i.e., aging; [+] and [–] indicate up-regulation and down-regulation, respectively, on other sequences or on telomere state; black color indicates the action of TERRA sequences or transcripts on other sequences of the same DNA molecule (defined as telomere 1); brown color indicates the action of TERRA transcripts on the sequences of any other DNA molecule; red color shows the actions on complex phenomena that reduce fitness; non-telomeric DNA, telomeric DNA and the loop of the ending part of telomere are graphically differentiated by design and color without specific meanings.

from 0.004 to 100,000 kg), an epigenetic clock that is "remarkably accurate ($r > 0.96$)" and with "a median relative error of less 3.5 percent" was defined [29].

In addition to DNA methylation, there are other age-related epigenetic changes, which include reduction of heterochromatin, nucleosome remodeling, changes in histone marks [42, 43], histone methylation [44, 45], "reduced bulk levels of the core histones, altered patterns of histone posttranslational modifications..., replacement of canonical histones with histone variants, and altered noncoding RNA expression" [46]. However, no reliable age prediction index is proposed for such epigenetic modifications as the indexes defined for DNA methylation [28, 29].

These data have become the basis for the concept that, at the cellular level, aging is an epigenetic phenomenon (e.g., [47]), with the implication that aging of a whole organism also depends on cellular epigenetic modifications. However, this idea is based on well-documented data but does not necessarily mean that epigenetic

changes are primary and not consequential to other mechanisms.

First of all, it should be noted that epigenetic alterations:

– are limited to specific DNA parts. DNA methylation concerns DNA stretches where CpG nucleotides are frequent (about 1 per 10 bp; these stretches are defined as CpG islands or CGIs) but these sections constitute only 2% of the entire DNA [31]. The CGIs often have the same position of transcription start sites of genes [48]. CGIs methylation is correlated with the silencing of any promoter present in them [49], while demethylation restores expression of the promoter [50];

– in some points, they are so evolutionarily conserved that they have allowed the definition of a reliable index that is valid for mammals in general [29];

– are reversible through the transformation of adult somatic cells into iPSCs. Evaluating age on the basis of DNA methylation (DNAm age), these values are close to zero for embryonic stem cells and iPSCs [28].

– the number of previous cell duplications correlates with the DNA methylation values [28].

These characteristics are not compatible with the possible hypothesis of random alterations and indicate that precise, genetically defined and regulated, and evolutionarily conserved mechanisms determining such modifications exist. Consequently, age-related epigenetic modifications do not appear as the primary determinant of aging, while, on the contrary, they could be a fundamental part of a more general mechanism that determines and regulates aging. This concept has been explicitly stated: "Aging is often perceived as a degenerative process caused by random accrual of cellular damage over time. In spite of this, age can be accurately estimated by epigenetic clocks based on DNA methylation profiles from almost any tissue of the body. Since such pan-tissue epigenetic clocks have been successfully developed for several different species, it is difficult to ignore the likelihood that a defined and shared mechanism instead, underlies the aging process" [29].

Let us now see if epigenetic modifications are compatible with the mechanisms proposed by the subtelomere–telomere theory:

– "CpG dinucleotide-rich DNA islands, shared among multiple human chromosome ends, promote transcription of TERRA molecules" [18].

– "Subtelomeric DNA methylation is ...decreased in conjunction with telomere shortening in $Terc^{-/-}$ mice" [51].

– "Both healthy controls and sarcoidosis patients showed that long telomeres (>9.4 kb) decrease and short telomeres (<4.4 kb) increase with aging, accompanying relative increases of long telomeres with subtelomeric hypermethylation and short telomeres with subtelomeric hypomethylation. This suggested that the aging-related telomere shortening is associated with the surrounding subtelomeric hypomethylation" [52].

– In mice, there is a relationship between telomere shortening and methylation of subtelomeric DNA [53]. "Furthermore, the abrogation of master epigenetic regulators, such as histone methyltransferases and DNA methyltransferases, correlates with loss of telomere-length control, and telomere shortening to a critical length affects the epigenetic status of telomeres and subtelomeres" [53].

– In human leukocytes, "...shorter telomeres are associated with decreased methylation levels of multiple cytosine sites located within 4 Mb of telomeres... significant enrichment of positively associated methylated CpG sites in subtelomeric loci (within 4 Mb of the telomere) ($p < 0.01$)" [54]. Shorter telomeres modify gene expression and increase the risk of age-related diseases [54].

These data appear to indicate that first-level regulatory sequences (TERRA sequences, whose promoters are CpG sequences) and second-level regulatory sequences (near telomeres and in other parts of DNA) are up- or downregulated by epigenetic mechanisms, such as methylation and demethylation. The same data do not indicate how the up/downregulation of possible third or higher level regulatory sequences occurs, even if it is plausible that similar epigenetic mechanisms act on them. In addition to these gaps that must necessarily be filled by appropriate studies, no specific study shows that the sequences controlled by TERRA via multi-level regulatory mechanisms coincide with the sequences discussed above whose epigenetic status varies with age.

However, an important element could be given by the study of senescent cells with an expected correspondence to the condition of maximum repression of the TERRA sequences.

One work has shown that, for mesenchymal stem cells (MSCs), the condition of cell senescence is associated with DNA methylation at specific CpG sites and histone marks of aging such as trimethylation of specific targets [55] and that "expansion of MSC has a very consistent impact on DNA-methylation profiles"; "517 CpG sites were consistently differentially methylated in early *versus* late passages"; "The DNA-methylation pattern has been shown to be linked to histone modifications – especially methylation of histone H3" [55].

In aged MSCs, both hypomethylation and hypermethylation have been reported: "Almost one third of the CpG sites reveal age-associated changes on DNA methylation, of which 60% become hypomethylated and 40% hypermethylated upon aging" [56].

This set of data, although with gaps that need to be filled, appears to indicate that DNA methylation and, in general, age-related epigenetic modifications, are closely correlated with repressive mechanisms of TERRA sequences, which are dependent on a system consisting of subtelomere, telomere, telomeric hood, telomerase, other regulatory sequences, and their relative regulatory proteins. Therefore, the experimental evidence presented

above about DNA methylation and other epigenetic age-related changes appears to confirm or at least to be compatible with the subtelomere–telomere theory. Consequently, it would seem legitimate to integrate the statement that cellular aging is an epigenetic phenomenon with the addition that the phenomenon appears to be regulated by the subtelomere–telomere system.

AGING, OXIDATION, AND INFLAMMATION

Numerous papers highlight the correlations between aging and an increasing accumulation of substances that cause oxidation (free radicals [57], reactive oxygen or nitrogen species [58, 59]) or molecules oxidized/damaged by such substances (oxidized proteins [60], damaged or shortened telomeres [61]), or a chronic state of inflammation and immunological alterations defined as inflammaging [62]).

These correlations have given rise to various theories in which the aforementioned substances or their effects have been considered the fundamental cause of aging (e.g., oxidative effects of free radicals and ROS on the whole body [63, 64], mitochondria [65, 66], or DNA [67, 68]; inflammaging and immunological alterations related to age [69, 70]).

These non-programmed aging theories are based on the assumption that aging is a consequence of the cumulative effect of these substances and implicitly exclude the idea that aging originates from other mechanisms and that the accumulation of these substances and their effects are secondary.

On the contrary, the subtelomere–telomere theory implies the exact opposite, namely that mechanisms centered on the telomere–subtelomere–telomerase system are the primary origin of all the alterations above.

Apart from other arguments and data of empirical evidence against non-programmed aging theories, which have been discussed in another paper [71] and are not the subject of this review, about the idea that such alterations are secondary and not primary, we have some important evidence:

– The transfer of a nucleus from a differentiated cell into an enucleated unfertilized oocyte produces an undifferentiated egg cell from which it is possible to get a new individual that is a clone of the donor of the differentiated cell [72];

– The fusion of human embryonic stem cells with somatic cells (human fibroblasts) generates hybrid tetraploid cells with the characteristics of the embryonic stem cells: "Analysis of genome-wide transcriptional activity, reporter gene activation, allele-specific gene expression, and DNA methylation showed that the somatic genome was reprogrammed to an embryonic state" [73];

– The transformation (reprogramming) of adult fibroblasts into iPSCs by the introduction of four factors (Oct3/4, c-Myc, Sox2, and Klf4) restores the growth properties and cell marker genes of embryonic stem cells [74].

These phenomena indicate that the alterations above are not an inevitable effect of possible causes intrinsic to biochemical mechanisms or of random, inevitable, and irreversible accumulation of various kinds of damages, emphasizing the ones related to the action of reactive oxygen species. On the contrary, they appear to be the effect of precise, genetically determined and modulated mechanisms, which may be reversed by appropriate genetic manipulations. Moreover, the balance between the oxidative and reducing potential within cells and tissues appears thoroughly regulated, and, in turn, the emission of oxidative agents is a part of a signaling system, acting as an effector from the cellular to the organismal level and responding to internal or external regulatory stimuli [75, 76]. All these statements are perfectly compatible with the thesis of the subtelomere–telomere theory.

TELOMERE PROTECTION

The subtelomere–telomere theory maintains that telomere protection, i.e., the limitation of the risk that an unprotected telomere can activate cell senescence, depends on the subtelomere–telomere system, in particular, on the activity of TERRA sequences:

– in mouse embryonic stem cells, depletion of TERRA sequence transcripts correlates with the reduced telomere protection [20, 21]. TERRA depletion is associated with "defects in the capping function. With telomere-specific probes, DNA FISH analysis of metaphase spreads revealed loss of telomeric integrity after 24 h TERRA knockdown..." [20];

– TERRA transcripts antagonize ATRX (a protein related to alpha thalassemia mental retardation X-related syndrome, ATR-Xs) and protect telomeres. "TERRA and ATRX share hundreds of target genes and are functionally antagonistic at these loci: whereas TERRA activates, ATRX represses gene expression. At telomeres, TERRA competes with telomeric DNA for ATRX binding, suppresses ATRX localization, and ensures telomeric stability" [20];

– downregulation of TERRA transcription achieved by various methods determines the activation of the DNA damage responses at telomeres [77];

– deletion of the 20q locus causes a dramatic decrease in the TERRA levels and induces massive DNA damage response. This fact is interpreted as a "demonstration in any organism of the essential role of TERRA in the maintenance of telomeres" [78].

So, various papers correlate the activity of TERRA sequences with telomere protection, that is, with the limitation of the probability that the cell senescence program is activated. The details of how TERRA transcripts

ensure such protection are only partially known. However, the assumption of the subtelomere–telomere theory about a relationship between telomere shortening, downregulation of TERRA transcription, and telomere protection appears in its general lines confirmed by the evidence.

TELOMERIC HETEROCHROMATIN HOOD

The subtelomere–telomere theory requires that, in a cellular period that could be defined as a "reset phase" [79], in the first cell of an organism (or even in the case of transformation of somatic cells into iPSCs), each telomere must be covered by a heterochromatin hood or cap with a length/size proportional to the initial telomere length. This length varies from species to species, from chromosome to chromosome, and even between the two arms of a DNA molecule ("...telomere lengths within the same cell are heterogeneous and certain chromosome arms typically have either short or long telomeres" [14]). In the numberless subsequent duplications of the germ cell and its derived cells, while the telomere shortens to an extent related to telomerase repression, the hood does not adapt to the new length of the telomere but remains constant in length and represses an increasing subtelomeric portion.

For the theory, it is therefore required the telomeric hood with a specific molecular composition and the existence of specific molecular mechanisms:

– in the reset phase, to adapt the length of the cap to the telomere length;

– in each cell duplication, to duplicate the hood reproducing the exact length before duplication and without mirroring any reduction in the telomere length;

– in telomere shortening at each duplication, to indicate to the telomerase complex, in proportion to its degree of activation, to what extent it must lengthen the telomere.

In large part, these questions, which are of fundamental importance for the subtelomere–telomere theory, have no answer or do not have a sure answer. However, some essential elements are known:

– The telomere is covered by several copies of the shelterin protein complex, well defined in its main components (proteins TRF1, TRF2, RAP1, TIN2, TPP1, and POT1 [80, 81]) and in the arrangement of these components [80].

– The heterochromatin hood could be composed of a chain of shelterin complexes linked together by some biochemical link between the POT1 protein of one complex and the TRF1 and TIN2 proteins of the next complex, as shown in Fig. 2.

– If the hood comprises a chain of shelterin complexes and has a fixed size that does not vary at each duplication even if the telomere shortens, the total amount of shelterin proteins should not change, passing from the germline cells to the cells with shortened telomeres. On the contrary, if the size of the hood is proportional to the telomere length, the total amount of shelterin proteins should decrease with telomere shortening. A work has studied the amount of shelterin proteins in cells with different telomere lengths with an interesting result: "We used quantitative immunoblotting to determine the abundance and stoichiometry of the shelterin proteins in the chromatin-bound protein fraction of human cells. The abundance of shelterin components was similar in primary and transformed cells and was not correlated with telomere length" [82]. Later, this result has been considered as reliable: "Quantification of the protein levels of shelterin show that the abundance of this complex does not change in relation to telomere length" [82, 83]". Moreover, the same work shows that a particular protein (TZAP) removes excess portions of the telomere when there is "a low concentration of shelterin complex", i.e., a low ratio shelterin complexes/telomere length, and, as underlined by the authors, this implies a necessarily constant amount of shelterin complexes [83]. This evidence would allow the limitation of telomere length as a regulation subordinated to the fixed size of the hood.

As far as the telomeric hood is concerned, these data appear to support the subtelomere–telomere theory but should be considered lacunose and insufficient. However, it is good to consider that any alternative theory should be regarded as much less supported by evidence and with

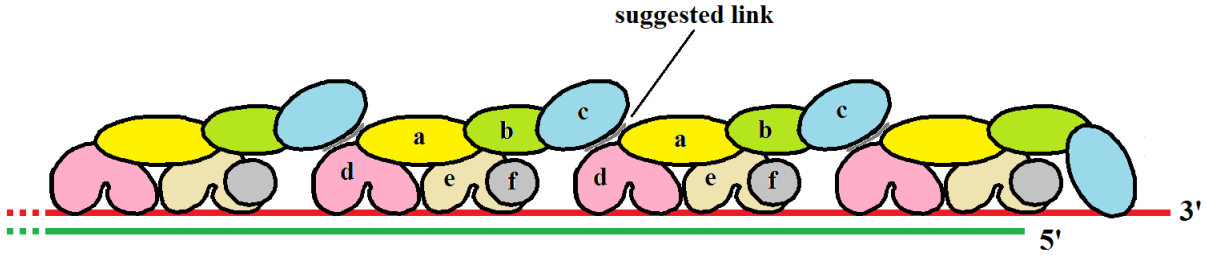

Fig. 2. Possible structure of the chain of shelterin complexes. a-f) TIN2, TPP1, POT1, TRF1, TRF2, and RAP1, respectively.

questions that need to be answered. In fact, if we exclude the hypothesis of a telomeric cap of a fixed length, it would be necessary to explain by some alternative mechanism how the difference in length of the telomeres is conserved after each division [15] and the inheritance of telomere length from parents (e.g., as demonstrated by its high similarity in monozygotic twins and the absence of such similarity in dizygotic twins [15, 16]).

To explain these phenomena without the suggestion of the subtelomere–telomere theory, a hypothetical register with the records of all telomere lengths would be necessary for germline cells. Then:

i) in cells with active telomerase, for each telomere, to compensate for telomere shortening at each replication, a particular mechanism should use the information contained in the hypothetical register to determine how far the telomere needs to be elongated to restore its original length. Furthermore, using the same information, in the case of excessive telomere elongation by telomerase action, another mechanism should eliminate the excess added part;

ii) in cells with partially active telomerase, an analogous mechanism using the information of the initial telomere length contained in the hypothetical register should partially restore the initial length in proportion to the degree of telomerase activity;

iii) in proportion to the telomeric shortening with respect to the initial length, information obtained by different mechanisms that would compare the initial length with that reported in the hypothetical register, phenomena of telomeric position effect, and the probability of triggering the cell senescence would be determined.

However, such a register appears very cumbersome and complex, and there is no evidence of its existence. The concept of this register can be replaced with that of telomeric caps determined in their size in the first cell of the organism and then remaining fixed in their sizes in all subsequent cellular duplications. Thus, telomeric caps may act as "registers" that are specific for each telomere and useful for what is indicated in (i), (ii), and (iii) (i.e., the proposal of the subtelomere–telomere theory), and so we have something that appears realistic, although with various points yet to be defined by experimental proofs.

In short, the hypothesis proposed by the subtelomere–telomere theory of a telomeric hood of length that does not vary with cell duplications, plus all the phenomena connected and dependent on this hypothesis, does not appear to have an alternative hypothesis that is plausible and supported by empirical evidence.

For example, as alternative or partially alternative hypothesis, the shift of balance between the quantity of shelterin complex proteins and the available telomeres or subtelomeric regions, for instance, owing to an excessive telomere shortening, might affect directly the regulation of distal genome regions, including the ones placed far away from the chromosome ends [84], without an increased TERRA repression. Such processes would be associated with the binding by shelterin complex components, otherwise located at telomeres due to a higher affinity, to telomeric repeats in non-terminal regions of chromosomes [85]. The latter may include interstitial telomeric repeats and other genome regulatory elements, also containing telomeric repeats and thus available for binding, though with a lower affinity than the telomeres [86]. Such interactions all together might link the subtelomere–telomere system with other global genome regulators, prompting them to act concordantly throughout complex developmental events, and especially in the regulation of aging. However, this suggestion does not explain the similarity of shelterin abundance in primary and transformed cells "not correlated with telomere length" [82].

GRADUAL CELL SENESCENCE

The subtelomere–telomere theory maintains that, with telomere shortening, subtelomeres are progressively repressed, i.e., progressive repression of TERRA sequences occurs with consequent progressive alteration of cellular functions. This progressive alteration, defined as "gradual cell senescence" [71], must be distinguished from the activation of cell senescence, a "fundamental cellular program" [22], which causes a general stereotypical alteration of cellular functions [87], or a sort of gradual cell senescence to the highest degree, associated to replicative senescence and resistance to apoptosis.

In support of this theoretical prediction, although it is always necessary to consider the possibility that, in the study of a culture of cells, cell senescence of part of them can simulate a condition of partial senescence of all cells, there is some evidence of gradual senescence as a condition distinct from cell senescence:

– in yeast, a unicellular organism in which telomerase is always perfectly active and so telomeres have constant lengths [88], a cell divides into two cells, the first defined as "mother", in which specific extrachromosomal ribosomal DNA circles (ERCs) are added on the subtelomere, while in the second, defined as "daughter", the ERCs are not added. Regarding the consequences of ERC accumulation, the cells of mother lineage show, with the number of duplications, increasing functional alterations and susceptibility to cell senescence, which in yeast causes apoptosis [89, 90]. Furthermore, in mutant strains in which telomerase is inactive (*tlc1Δ* mutants), the telomeres shorten at each duplication, and in the cells of the daughter line, where there is no accumulation of ERCs, there is a transcriptome similar to the cells of the mother line with an equal number of duplications [91]. This phenomenon in mutants could be a consequence of the sliding of the telomeric cap on the subtelomere and its con-

sequent repression, while in individuals of the mother line of normal strains, the repression of the subtelomere is caused by the accumulation of ERCs [10, 92]. For yeast, as cell senescence causes apoptosis and so the death of an individual, the decline of cellular functions (gradual senescence) cannot be confused with cell senescence.

– "As the telomere shortens, the hood slides further down the chromosome (the heterochromatin hood remains invariant in size and simply moves with the shortening terminus)... the result is an alteration of transcription from portions of the chromosome immediately adjacent to the telomeric complex, usually causing transcriptional silencing... These silenced genes may in turn modulate other, more distant genes (or sets of genes). There is some direct evidence for such modulation in the subtelomere... but overall, while experimental data imply the existence of some undefined... but direct and causal linkage between telomere shortening and changes in gene expression, the mechanisms of the linkage remain unclear and arguable..." [10].

– Mesenchymal stem cells (MSCs) show continuous changes in DNA methylation (hypomethylation or hypermethylation) over subsequent passages, which may be used as reliable biomarkers for the number of passages of *in vitro* culture [93-95]; "mRNA expression profiling revealed a consistent pattern of alterations in the global gene expression signature of MSC at different passages. These changes are not restricted to later passages, but are continuously acquired with increasing passages" [96].

– MSCs show drawbacks related to the number of previous duplications that can be circumvented by their reprogramming to iPSCs [97]. From iPSCs, it is possible to get functional MSCs, named induced MSCs (iMSCs), with minor epigenetic changes and improved cell vitality [98]. "Compared with adult MSCs and irrespective of donor age or cell source, iMSCs show a rejuvenated profile [97]. Nonetheless, DNA methylation, related to age, was completely erased, and iMSCs reacquired senescence-associated DNA methylation during culture *in vitro*" [56]. These works indicate that, by appropriate manipulations, it is possible to switch between various cellular states with different degrees of senescence. This confirms that cellular aging: (i) is not a consequence of inevitable and irreversible damages, but a reversible and modifiable epigenetic condition; and (ii) is gradual, at least until an irreversible condition defined as cell senescence is triggered.

– In the study of the phenomenon defined as telomere position effect over long distance (TPE-OLD), it is reported that: "Our results demonstrate that the expression of a subset of subtelomeric genes is dependent on the length of telomeres and that widespread changes in gene expression are induced by telomere shortening long before telomeres become rate-limiting for division or before short telomeres initiate DNA damage signaling. These changes include up-regulation and down-regulation of gene expression levels" [99], although the authors interpret these results as caused by the lack of formation of particular telomere loops over relatively distant portions of the chromosome when the telomere shortens.

TELOMERE SHORTENING AND ORGANISM DECLINE

For subtelomere–telomere theory (as for telomere theory), possible implications could be that:

– actively duplicating cells of the organism should show a homogeneous reduction in telomere length related to the rhythms of duplication, and aged organism should show critically short telomeres;

– tissue cells with higher duplication rates should show critically shortened telomeres and greater signs of aging, while for cell types with low duplication rates, telomere shortening and signs of cellular aging should be much more limited;

– for perennial cells, telomere shortening would be null, so aging could not be explained by telomere shortening.

The evidence is somehow different and needs some discussion.

In a review regarding the reduction in telomere length over the years, from neonates to centenarians, an annual reduction in telomere lengths was highlighted for many cell types, excluding those with minimal or no cell turnover. However, only for some cell types telomere length reduction appeared critical (e.g., liver cells: yearly reduction rate 120 bp/year; telomere length 13.7 ± 2.5 kb in neonates and 8.7 ± 1.4 in centenarians) [100]. Furthermore, apart from cellular tissues with no noticeable reduction in telomere length (cerebral cortex and cardiac muscle), the reduction rates per year were generally lower, mostly within 20-60 bp per year [100]) and not such as to explain the apparent – at least in approximate terms – homogeneity of aging of the whole organism. Moreover, an explanation for the aging of perennial cells was necessary.

In the study of four types of cells/tissues with very different cell turnover rates (leukocytes, skeletal muscle, subcutaneous fat, and skin), the telomere shortening rates were found to be similar [101].

The same study showed that while telomere lengths are similar in different organs of the human fetus [102], telomere length in hematopoietic stem cells was shorter than that of the cells of somatic tissues with low turnover. From this, the authors deduced that the stem progenitor cells of various cell types in the growth period undergo proliferation proportional to the subsequent rhythms of cell turnover (e.g., massive expansion for high turnover cells such as hematopoietic cells and modest expansion for cells with slow turnover as those of skeletal muscle). In this expansive phase, the length of the telomeres was

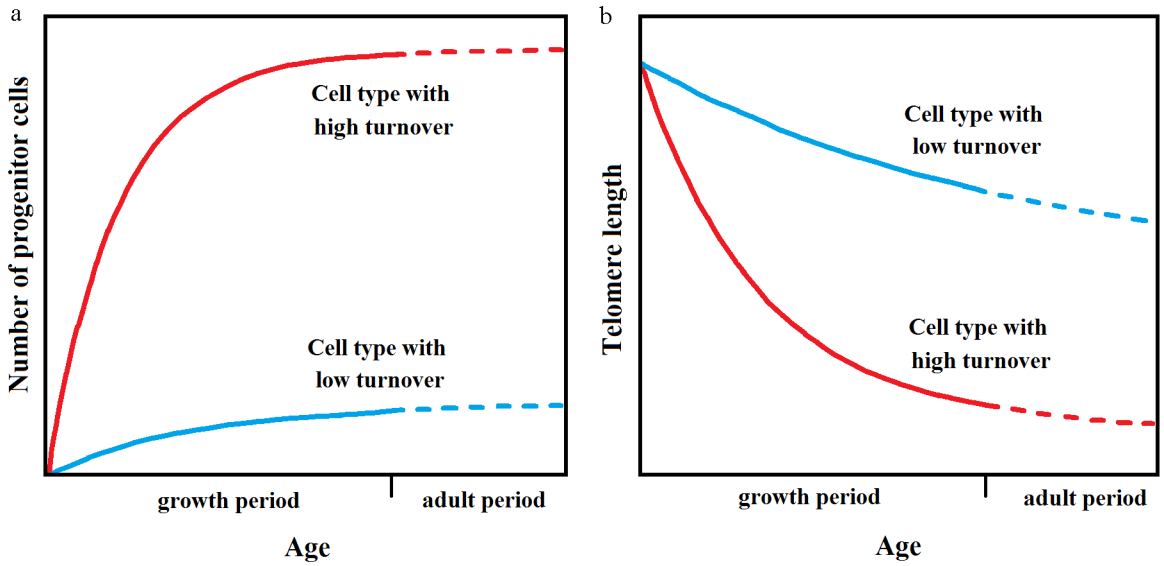

Fig. 3. a) Progenitor cells of a cell type with a high turnover (e.g., hematopoietic cells) in the growth period undergo massive expansion while progenitor cells with a low turnover (e.g., skeletal muscle cells) undergo modest expansion. b) In the growth period, telomere shortening is proportional to the expansion, while in the subsequent periods of life, the rate of telomere shortening is equal for both types of cells (inspired by Fig. 4 in [101]).

reduced as a function of the degree of expansion, while later, the telomeres were reduced with constant rhythms [101] (Fig. 3).

Let's now see how these data can explain the aging of the organism in the context of the subtelomere–telomere theory:

(i) Telomere shortening reduces overall cell function (gradual cell senescence). In particular, for MSCs, before it has been pointed out that their expansion, in relation to the number of previous duplications, besides limiting the number of possible subsequent duplications, causes consistent epigenetic changes [55];

(ii) As telomerase is partially active or inactive in stem progenitor cells, in stem cells, and somatic cells, there is always the possibility of passing to the condition of cell senescence, i.e., the blocking of replicative capacities [11]. This process gradually impoverishes cell turnover capabilities, in particular by reducing stem progenitor cells.

(iii) In the tissues/organs, the increasing number of cells under cell senescence conditions and their abnormal secretions (SASP) reduce the overall function. Indeed, the selective elimination of senescent cells is a reasonable and realistic goal to reduce some of the manifestations of aging [1].

(iv) With regard to perennial or non-renewing cells or structures (most of the neurons of the central nervous system, retina photoreceptor cells included, and eye lens fibers), the effects of the phenomena reported in the previous points on indispensable satellite or trophic cells with the turnover (gliocytes of various types, retina pigmented cells included, and lens epithelium cells) determines their aging [27].

These phenomena lead to a condition, defined as "atrophic syndrome", that is completely compatible with the subtelomere–telomere theory and is a substrate of organism aging [3, 103].

CONCLUSIONS

"The Human Genome and ENCODE Projects have shown that the protein-coding potential of the mammalian genome is extremely limited... Although only 2% of the genome is coding, >90% is transcribed. This transcriptional activity largely produces long noncoding RNAs (lncRNA), the functions of which have remained mostly unknown" [20].

The number of protein-coding genes is similar in very different species. "The scientific community was astonished that the number of human genes is equal to that of a rather unsophisticated nematode" [104], a situation which implies that the enormous differences between species mainly depend on non-protein-coding sequences. The transcription of the large part of the genome that does not encode proteins largely generates regulatory elements for other parts of the genome, both on other segments with regulatory functions and protein-encoding parts, and these actions largely appear to take place through epigenetic modifications [104].

It is, therefore, possible to consider as a general rule that cellular functions, both for a single cell of any uni-

cellular or multicellular organism and, for multicellular organisms, concerning the development and organization of the organism to which the cell belongs, are epigenetic phenomena.

From this, it follows that, if specific mechanisms determine aging, it should be an epigenetic phenomenon, which coincides with what is shown by the evidence and with the description of aging by the subtelomere–telomere theory.

Apart from this general consideration, what is described by the subtelomere–telomere theory appears to find considerable confirmation in the evidence, and in any case, there are no facts that may be considered incompatible with this theory.

It is important to underline that for any theory within the programmed or adaptive aging paradigm, the existence of genetically determined and regulated mechanisms that define various characteristics of aging is indispensable. The mechanisms proposed by the subtelomere–telomere theory appear to satisfy this necessity and find correspondence in the evidence.

On the contrary, for any theory belonging to the opposite paradigm of the non-programmed or non-adaptive aging, the existence of such mechanisms irremediably falsifies the theory unless alternative evolutionary explanations are found.

Ethics declarations. Authors declare that they have no conflicts of interest. This article does not contain any studies involving human participants or animals performed by any of the authors.

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