Heart-Breaking Telomeres

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Abstract: **Telomeres, the protective ends of linear chromosomes, shorten throughout an individual's lifetime. Accumulation of critically short telomeres is proposed to be a primary molecular cause of aging and age-associated diseases. Mutations in telomere maintenance genes are associated with pathologies referred to as or telomeropathies. The rate of telomere shortening throughout life is determined by endogenous (genetic) and external (nongenetic) factors. Therapeutic strategies based on telomerase activation are being developed to treat and prevent telomere-associated diseases, namely aging-related diseases and telomeropathies. Here, we review the molecular mechanisms underlying telomere driven diseases with particular emphasis on cardiovascular diseases. (*Circ Res*. 2018;123:787-802. DOI: 10.1161/CIRCRESAHA.118.312202.)**

Key Words: cardiovascular diseases ■ mutation ■ telomerase ■ telomeres ■ telomere shortening

Telomeric DNA and Telomeric Proteins

Telomeres are heterochromatic structures at the ends of linear chromosomes that protect them from degradation and DNA repair activities and are, therefore, essential to ensure chromosome stability[1,2]. Mammalian telomeres comprise several kilobases, between 10 to 15 kb in humans and 25 to 50 kb in mice, of tandem TTAGGG DNA repeats.[1] Telomeres exhibit a 30 to 400 nucleotide long 3'-overhang of a G-rich strand, known as the G-strand overhang, which has been proposed to form a loop at the chromosome end. The T-loop structure has been proposed to protect chromosome ends from degradation and DNA repair activities, as well as from telomerase activity[3,4] (Figure 1A and 1B).

Telomere repeats are bound by a specialized complex known as shelterin that plays crucial functions in telomere length regulation, protection of telomeres from the DNA damage response (DDR; through repression of the ATM [ataxia telangiectasia mutated] and ATR [ataxia telangiectasia and Rad3 related] signaling pathways), and in masking the chromosome ends from DNA repair machinery[2] (Figure 1C). The shelterin complex is composed of 6 core proteins: the TRF1 and (TRF2 telomeric repeat binding factor 1 and 2), the TIN2 (TRF1-interacting protein 2), POT1 (protection of telomeres protein 1), TPP1 (TIN2 and POT1 interacting protein), and the RAP1 (repressor/activator protein 1)[5–7]. Homodimers of TRF1 and TRF2 bind double-stranded telomeric DNA and nucleate the assembly of the shelterin complex via TRF homology domains present on both factors, which bind to F/YxLxP motifs present on TIN2.[8] TIN2 binds TRF1 and TRF2 through independent domains and recruits the TPP1-POT1 complex, bridging the different shelterin components.[8–13] TPP1 binds TIN2 and POT1 through its C-terminal and central domains, respectively.[14,15] TPP1 recruits POT1 to telomeres.[16,17] POT1 possesses high specificity for the single-stranded telomeric DNA sequence 5'-TAGGGTTAG-3', thereby binding to the G-strand overhang and probably the displaced G-strand at the D-loop.[18–20] Whereas human cells contain only one *POT1* gene, mouse cells have *Pot1b*.[21,22] POT1a primarily prevents ATR activation, whereas POT1b regulates the 3'-overhang.[22–26] In addition, TPP1 is required for the recruitment of telomerase to chromosome ends in vivo (Figure 1C).[27–29] Finally, RAP1 binds to telomeres via association with TRF2.[30–32] Human RAP1 is required for inhibition of the NHEJ (nonhomologous end joining) pathway in the absence of TRF2, whereas mouse RAP1 is necessary to prevent telomere fragility and inhibits homologous recombination.[33–35] RAP1 also associates to non-telomeric genomic sites where it has been demonstrated to regulate gene expression, in particular of metabolic pathways, protecting from obesity and metabolic syndrome.[33,36–38]

Importantly, owing in part to the so-called end-replication problem, telomeres shorten during each cell duplication cycle because of the inability of replicative DNA polymerases to fully replicate the 3' ends of linear chromosomes.[39,40] In particular, the removal of RNA primers, which provide the required 3'OH group for addition of dNTPs (deoxynucleotide) by DNA polymerases, renders the newly synthesized DNA strand shorter than the parental template. Thus, chromosomes progressively shorten from both ends on repeated cell division, and this telomere shortening underlies the molecular clock proposed by Hayflick to explain the limited lifespan of cells in culture or Hayflick limit[41,42] (Figure 1D). Telomere shortening as a consequence of the removal of the RNA primer of the most distal Okazaki fragment is estimated in ≈5 bases per population doubling. However, the observed sequence loss in primary human cells is considerably larger than that, 100 to 200 bases of TTAGG repeats per cell division, indicating that that replication-associated telomere shortening is caused by a combination of the end-replication problem and the processing of G-strand generation.[43]

Telomerase is a DNA reverse transcriptase polymerase (*TERT* [telomerase reverse transcriptase]) which uses an

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Nonstandard Abbreviations and Acronyms

AAV	adeno-associated vectors
CSC	cardiac stem cells
CVD	cardiovascular disease
DDR	DNA damage response
DMD	Duchenne muscular dystrophy
MI	myocardial infarction
POT	protection of telomeres protein
RAP	repressor/activator protein
ROS	reactive oxygen species
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
TIN	TRF1-interacting protein
TPP	TIN2 and POT1 interacting protein
TRF	telomeric repeat binding factor

RNA template (*TERC* [telomerase RNA component]) for de novo addition of telomeric DNA onto telomeres, thus compensating for the telomere erosion caused by cell divisions (Figure 1C).[44] However, high telomerase expression is normally restricted to early stages of embryonic development, as well as to pluripotent embryonic stem cells and to adult stem cell compartments.[41,42,45–50] Although telomerase is expressed in adult stem cell compartments, this is not sufficient to counteract telomere attrition associated with cell division throughout life and, therefore, telomeres shorten with age in vitro and in vivo.[41,42,45–50]

Telomere Length as a Determinant for Health Span and Aging

Aging is a multifactorial process that results in a progressive functional decline at cellular, tissue, and organismal levels that leads to decreased fitness an increased risk of death. In humans, apart from apparent signs of aging, that is, hair graying, skin wrinkling, muscle wasting, and altered adiposity, the susceptibility to diseases progressively increases as we age. Aging-related diseases mainly include deficient immune function, cancer, diabetes mellitus, depression, cognitive decline, and cardiovascular diseases (CVDs).[51] Several different molecular pathways have been identified as the molecular causes of aging, including shortened telomeres, cellular senescence, genomic instability, stem cell exhaustion, mitochondrial dysfunction, epigenetic alterations, deregulated nutrient sensing, altered cellular communication, and loss of proteolysis[52] (Figure 2). Short telomeres are considered a primary cause of aging and of the onset of aging-associated diseases because it induces many of the above-mentioned hallmarks of aging.[52] Thus, short telomeres can induce a persistent DDR that leads to a growth arrest or apoptosis. In addition, loss of DNA damage checkpoints can also allow the propagation of cells with short/damaged telomeres leading to chromosome end-to-end fusions and genomic instability triggering age-associated diseases like cancer.[53] Of particular relevance is the fact that short telomeres trigger replicative senescence.[54,55] Replicative senescence has been proposed as a mechanism that evolved to protect from cancer with the drawback of promoting age-associated diseases.[56,57] Senescent cells progressively accumulate during life and acquire a senescence-associated secretory phenotype, which entails a striking increase in the secretion of proinflammatory cytokines, proteases, and of insoluble extracellular matrix components.[56,58] Factors secreted by senescent cells exert a paracrine effect promoting degenerative and hyperproliferative changes on neighboring cells that alter the tissue microenvironment and cause chronic inflammation. Thus, senescent cells have been proposed to contribute to age-related pathologies.[56,58] Shortened telomeres also lead to stem cell dysfunction impairing their capacity to self-renew and mobilization to repair tissue damage, which in turn lead to loss of tissue homeostasis and different disease states.[41,42,45–50,59–61] Telomere dysfunction has been associated with impaired mitochondrial biogenesis and function through activation of p53 which in turn represses PGC1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) and PGC1β (peroxisome proliferator-activated receptor gamma coactivator 1-beta) expression, contributing to organ and metabolic failure and to diminishing organismal fitness in the setting of telomere dysfunction.[62] Short telomeres can trigger epigenetic changes at telomeric and at subtelomeric chromatin.[63] In particular, loss of heterochromatic marks at telomeres has been liked to processes that involve loss of differentiation and cellular identity, such as during tumorigenesis and during the induction of pluripotency.[49,64] Telomere length can also impact on human diseases by regulating gene expression through telomere position effects that spread telomeric heterochromatin to silence nearby genes.[65,66] Finally, short telomeres in the telomerase-deficient mice lead to a reduced size of pancreatic islets that results in defective insulin secretion and consequent glucose intolerance, providing a link between telomeres and type 2 diabetes mellitus.[67] Indeed, telomerase overexpression in old wild-type mice results in increased IGF-1 (insulin-like growth factor) levels and improved fasting insulin levels and glucose uptake, clearly connecting telomere maintenance with nutrient sensing[68] (Figure 2). Thus, accumulation of short telomeres could be placed upstream of many of the known hallmarks of aging, suggesting that intervention at that level may be sufficient to revert other hallmarks of aging, including DNA damage, senescence, loss of stem cell, epigenetic modifications, mitochondrial dysfunction, and improper nutrient sensing (Figure 2). Indeed, at the cellular level, overexpression of telomerase is sufficient to counteract telomere attrition and to indefinitely extend the replicative lifespan of primary cells in culture.[69–71]

The first proof that shortened telomeres cause age-related pathologies and determine longevity came from the study of telomerase-deficient mice, which show premature aging phenotypes both in low proliferating (heart, brain) and high proliferating compartments (bone marrow, gut, skin, and testis).[72–76] Telomere shortening with age has also been observed in several mouse stem cell compartments from different tissues independently of high or low tissue proliferation rates[45] (Figure 3). Telomerase-deficient mice show a worsening of phenotypes with increasing mouse generations owing to inherited progressively shorter telomeres, with the later generations showing severe phenotypes and premature death at prereproductive ages.[74,75,77] These observations indicated for the first time a genetic anticipation

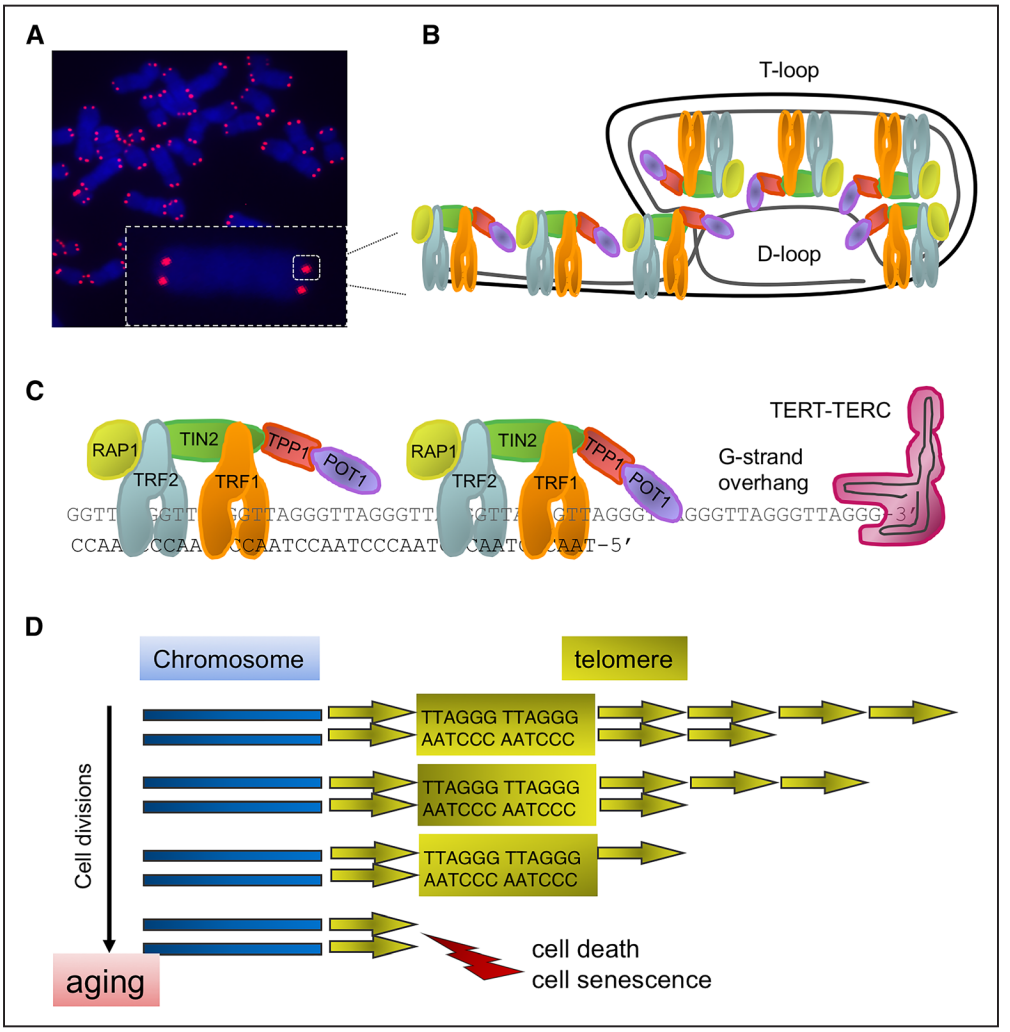

Figure 1. Telomeric DNA, telomeric proteins, and telomere structure. A, Representative image of a metaphasic chromosome stained with DAPI (blue) and the telomeric DNA specific peptide nucleic acid probe (red). **B**, Schematic model of the shelterin complex bound to a telomere in a T-loop configuration. Telomeres contain a double-stranded (ds) region of TTAGGG repeats and a 150 to 200 nucleotide long single-stranded DNA (ss) overhang of a G-rich strand. The G-strand overhang (grey) invades the telomeric ds DNA region to form a protective T-loop, with a displacement D-loop at the invasion site. **C**, Schematic representation of telomere-bound proteins, the shelterin complex, and telomerase. The shelterin complex is composed of the TRF1 and TRF2 (telomeric repeat binding factor 1 and 2), RAP1 (repressor-activator protein 1), the POT1 (protection of telomeres protein 1)-TIN2 (TRF1-interacting nuclear factor 2) organizing protein (TPP1), TIN2, and POT1. TRF1, TRF2, and POT1 bind directly to telomeric DNA repeats, with TRF1 and TRF2 binding to telomeric ds DNA and POT1 to the 3'-ss G-overhang. TIN2 binds TRF1 and TRF2 through independent domains and recruits the TPP1-POT1 complex, constituting the bridge among the different shelterin components. Telomerase is composed of the catalytic subunit (TERT [telomerase reverse transcriptase]) and the RNA template (TERC [telomerase RNA component]) that recognizes the 3'-OH at the end of the G-strand overhang and elongates the telomere. **D**, Telomere shorten with cell division throughout life. This progressive telomere shortening eventually leads to critically short telomeres that can impair the regenerative capacity of tissues and has been proposed as one of the molecular hallmarks of aging. Shortened telomeres induce a DNA damage response that leads to a growth arrest during which cells attempt to repair the damage and if DNA damage is irreparable, triggers replicative senescence or cell death.

associated with telomerase deficiency. However, and in support of critically shortened telomeres being a determinant of aging and longevity, increased TERT expression in the context of cancer resistant transgenic mice was sufficient to delay aging and extend mouse longevity by 40%.[78] More recently, these findings have been translated into a potential therapeutic strategy by using adeno-associated vectors (AAV) to transiently activate telomerase in adult tissues[68,79] (see below). Importantly, by overexpressing telomerase, we could demonstrate that short telomeres are an important determinant of mouse longevity but also of age-associated diseases, as telomerase expression was able to maintain longer telomeres with aging and to delay many age-associated pathologies, including cancer.[68]

In line with mouse studies, many human syndromes were later described to be caused by germline mutations in genes coding for factors involved in telomere maintenance and repair, the so-called telomere syndromes or telomeropathies[80,81] (Figure 3). Human telomeropathies are mainly associated with Hoyeraal-Hreidarsson syndrome, dyskeratosis congenita, aplastic anemia, pulmonary and liver disease and are nowadays considered more as a spectrum disorder than distinct diseases.[80,81] Inherited telomeropathies fall into characteristic patterns that can include ≥1 of the following: loss of immune function through depletion of bone marrow stem cell pool, certain cancers, pulmonary fibrosis, gastrointestinal disorders, liver cirrhosis, and neuropsychiatric conditions.[51] Although these diseases show a wide and complex range of clinical

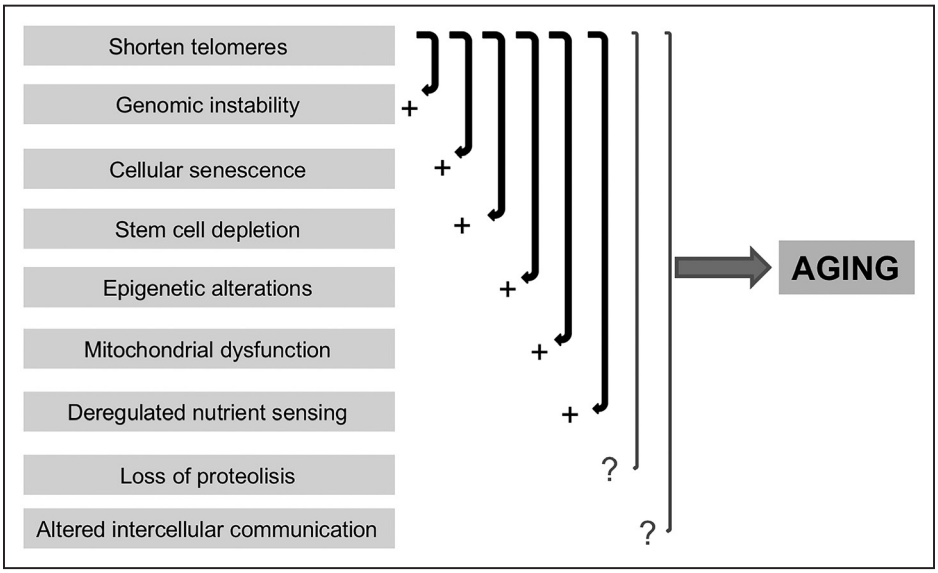

Figure 2. Shortened telomeres as a primary cause of aging: 9 different biological processes have been identified as the molecular and cellular causes of aging and are nowadays considered the hallmarks of aging. These processes cover shortened telomeres, genomic instability, cellular senescence, stem cell exhaustion, epigenetic alterations, mitochondrial dysfunction, deregulated nutrient sensing, altered cellular communication, and loss of proteolysis.[52] Shortened telomeres in its turn induces several of these hallmarks of aging (green arrow). Short unprotected telomeres lead to chromosome end-to-end fusions and genomic instability. Critically short telomeres elicit the induction of replicative senescence and lead to stem cell depletion impairing their tissue and self-renewal capacity. Short telomeres can trigger epigenetic changes at telomeric and at subtelomeric chromatin and have been associated with mitochondrial dysfunction. Short telomeres impair insulin secretion by pancreatic islets and lead to glucose intolerance, providing a link between telomeres and nutrient sensing. No connections between shortened telomeres and either loss of proteolysis or disturbed intercellular communications have been described to date (grey arrows).

symptoms, all of them are characterized by presenting with critically short telomeres. The age of onset and the severity of their clinical manifestations vary among individuals. These syndromes are characterized by premature loss of the regenerative capacity of tissues, affecting tissues with both high and low proliferation rates.[80,82] As in mice, disease anticipation is also found in human families with telomere syndromes, with the mutation generally first manifesting in adults with pulmonary fibrosis and the more severe phenotypes appearing in pediatric populations (immunodeficiency) and young adults (aplastic anemia) from the next generations.[82] Interestingly, patients often present symptoms of accelerated aging, such as hair graying, skin pigmentation, diabetes mellitus, and cardiovascular dysfunction.[51]

Epidemiological studies confirmed that telomeres shorten with aging in humans although the correlation of telomere length and other health markers are still under debate.[83–85] In a study with people over the age of 60 years, it was shown that those presenting shorter telomeres in blood DNA had poorer survival as compared to those with longer telomeres. The higher mortality rate was because of heart and infectious disease, supporting the hypothesis that shortened telomeres in human also contributes to mortality in many age-related diseases.[86] In another work performed with >100 000 individuals, it was shown that telomere length positively correlated with age in subjects older than 75 years, indicative of an association of longer telomeres with more years of survival in those older than 75 years.[87] Other reports, however, do not support the correlation between average telomere length and longevity in the old and oldest.[88,89] In addition to being considered a primary molecular cause of aging, shortened telomeres in total leukocytes or in peripheral blood mononuclear cells has been proposed to be a biomarker of biological aging, with a potential prognostic value for many different age-associated diseases, including infectious diseases, some cancers, diabetes mellitus, and CVDs[90–99] (Figure 3).

In mice, longitudinal studies of telomere length throughout lifespan showed that the rate of increase of short telomeres with time but not average telomere length or the rate of telomere shortening was predictive of individual lifespan.[100] This observation highlights that the presence of few critically short uncapped telomeres in the cell is sufficient to induce replicative senescence or apoptosis and the consequent downstream deleterious effects within tissues.[101] In addition, these findings suggest that it is the ability of different species to maintain telomeres rather than average telomere length per se that may be a determinant of species longevity. This idea is further supported by longitudinal studies in free-living birds in which higher rates of telomere shortening predict mortality.[102,103] Thus, future human epidemiological studies should take into account longitudinal telomere length analysis and the rate of increase of the fraction of short telomeres.

Genetic and Nongenetic Determinants of Telomere Length

Similar to aging and longevity, telomere length is affected by endogenous (genetic) and external factors (nongenetic). Variable longevities across species may be partly explained by differences in the pace of telomere shortening. The average telomere shortening in human blood cells occurs at a rate of 31 to 72 base pairs per year, whereas mouse telomeres shorten around 100× faster than that.[94,100,104] This clearly indicates that in addition to the intrinsic end-replication problem, there are other factors contributing to telomere attrition throughout life.

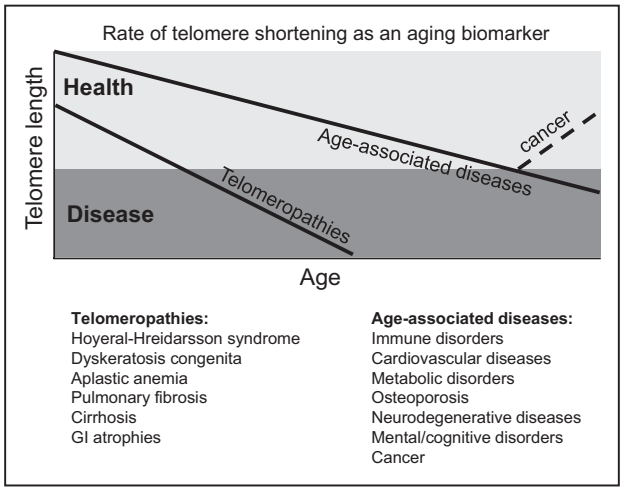

Figure 3. Impact of telomere shortening on age-associated diseases and telomeropathies. A, Telomeres progressively shorten with increased cell divisions throughout individuals' lifetime. Shortened, unprotected telomeres elicit a DNA damage response that induces replicative senescence. Senescence impacts the regenerative capacity of tissues giving rise to a whole range of age-associated diseases. Spontaneous mutations that activate telomerase expression or the alternative lengthening of telomere pathway allow genetically unstable checkpoint-deficient cells to divide unlimitedly and eventually to become a cancer cell. Age-associated diseases include immune disorders, cardiovascular diseases, metabolic disorders, osteoporosis, neurodegenerative diseases, mental/cognitive disorders, and cancer. Germline mutations in genes coding for factors involved in telomere maintenance cause a premature telomere shortening and trigger the development of telomeropathies. Genetic anticipation is associated with telomere shortening across generations, whereby diseases show a progressively earlier age of onset and aggravation of symptoms. Telomeropathies include Hoyeraal-Hreidarsson syndrome, dyskeratosis congenita, aplastic anemia, pulmonary fibrosis, liver cirrhosis, and gastrointestinal (GI) atrophies. The rate of telomere shortening has been proposed as an aging biomarker.

In fact, telomere shortening can occur as a consequence of oxidative stress and inflammation that cause DNA damage and breaks with potential loss of telomeric sequence.[105] Because of its high guanine content, telomeric DNA is particularly susceptible to DNA breaks originated by reactive oxygen species (ROS)[106] (Figure 4).

Telomere length is strongly determined by the genetic make-up. Twin and family studies demonstrate that telomere length is highly heritable, with heritability estimates ranging from 34% to 82%.[104,107–109] Although a strong maternal inheritance and paternal age effect on telomere length has been reported in a meta-analysis comprising nearly 20 000 subjects,[109] there is still some debate about whether the inheritance of telomere length correlates more strongly with paternal or maternal inheritance pattern.[110] Meta-analyses addressing the association between sex and telomere length have shown that females have longer telomeres than males.[94,111] Race/ethnicity has also been associated with telomere length, with Hispanics and blacks being those as having longer telomeres.[112] Genome-wide association studies identified several genomic regions containing variants associated with telomere length in blood cells that can account for the genetic determination of telomere length.[113–115] Thus, age, sex, race, parental age at birth, and genetic mutations/variants are nonmodifiable factors that contribute to telomere length[116] (Figure 4).

In the recent years, several studies have shown that nongenetic factors, such as psychosocial, environmental, and behavioral, influence on telomere length homeostasis and consequently have an impact on health span and disease causes.[51,116] Thus, smoking, unhealthy diet (eg, high cholesterol, alcohol intake), or obesity might lead to telomere shortening by provoking tissue inflammation and oxidative stress.[117–126] Chronic and acute psychological stress has also been shown to accelerate telomere shortening in leukocytes.[93,127–129] Indeed, shorter telomere length has been shown to associate to major depressive disorder, a severe illness which shows signs of premature aging.[130–133] Shorter telomeres are also associated with cognitive impairment in the elderly.[94] On the contrary, some nutritional habits, meditation, good quality sleep, and physical exercise have been shown to reduce telomere erosion and slow down the pace of aging[116,134–139] (Figure 4).

The interplay among the genetic and nongenetic determinants of telomere maintenance would ultimately dictate the individual's telomere length dynamics and, therefore, their life expectancy and susceptibility to develop age-related diseases.

Telomere-Based Therapeutic Interventions

The knowledge of the implications of telomeres and telomere proteins in human disease has considerably improved in recent years. The current research challenge focuses on how our knowledge of telomere biology and its connection with human disease can be translated into the clinic to improve human health. Given the proven effects of shortened telomeres on aging and age-related diseases, there is a growing interest in the development of telomere-based therapeutic interventions to treat age-related diseases, as well as the so-called telomeropathies associated with mutations in telomere maintenance genes. The use of telomerase activators in the treatment of aging-associated conditions is widely studied. The telomerase chemical activator TA-65 (astragalosid IV), a small molecule derived from *Astragalus membranaceus* extracts, has been shown to lead to a moderate telomere lengthening and to improvement of some aging-related parameters in mouse and humans, including immune remodeling, metabolic, bone, skin, and cardiovascular health markers, although no effect on longevity has been observed[140,141] (Figure 4). Interestingly, androgen therapy has been applied as a treatment for aplastic anemia for many years without knowing its mechanism of action[142,143] (Figure 4). It was subsequently reported that sex hormones activate TERT transcription.[144] Indeed, testosterone therapy in mice having aplastic anemia associated with short telomeres was shown to upregulate telomerase expression, rescue shortened telomeres, and to extend the lifespan of these mice[145] (Figure 4). In humans, administration of a synthetic androgen, Danazol, to patients with telomeropathies resulted in telomere elongation in circulating leukocytes in association with improvement of hematologic parameters.[146] More recently, therapeutic interventions based on telomerase-based gene therapy are currently being investigated in mouse models for their potential to improve health and extend lifespan, and as a treatment for short telomere syndromes.[147]

In particular, we have developed a therapeutic strategy by using AAV to activate telomerase in adult tissues[68,79]

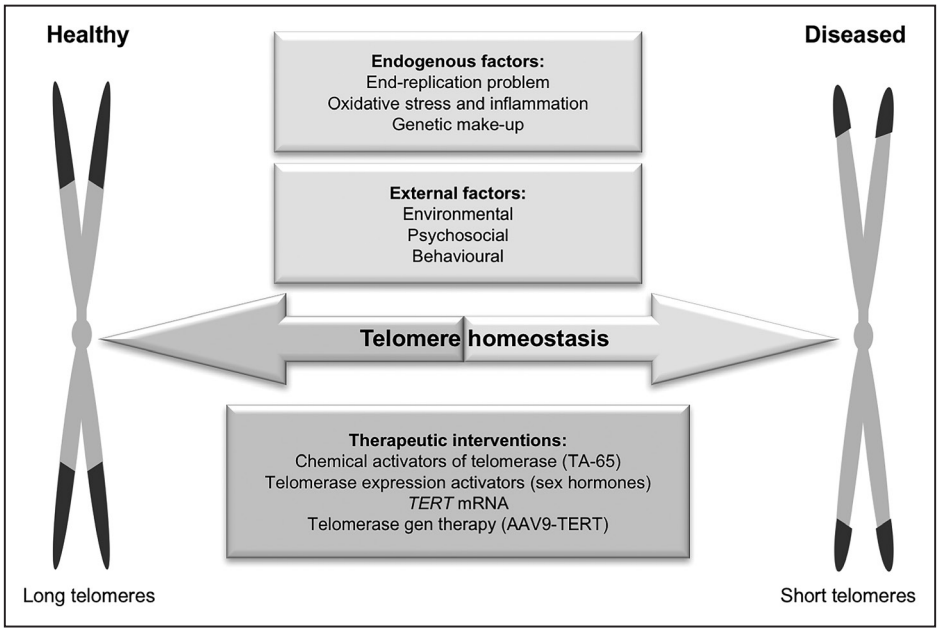

Figure 4. Endogenous and external factors, as well as therapeutic interventions affecting telomere-mediated diseases. The pace of telomere attrition during life is determined by endogenous and external factors that may either accelerate or slow it down. Endogenous factors include telomere shortening associated with the end-replication problem, oxidative stress, inflammation, and the individual's genetic make-up. External or nongenetic factors, such as environmental, psychosocial, and behavioral do also impact on telomere length homeostasis. Several therapeutic interventions are being assessed to counteract shortened telomeres, among others, chemical activators of telomerase (TA-65 [astragalosid IV]), activators of the telomerase reverse transcriptase (*TERT*) transcription (sex hormones), intracellular administration of *TERT* mRNA, and telomerase gene therapy (adeno-associated vector 9 [AAV9]-TERT).

(Figure 4). Treatment with *TERT* gene therapy using nonintegrative replication incompetent AAV9 vectors of adult mice was able to delay aging and increase longevity by decreasing age-related pathologies, such as osteoporosis, glucose intolerance, as well as neuromuscular and cognitive decline. Furthermore, the onset of cancer was also delayed in the TERT treated mice.[68] *TERT* gene therapy could constitute an attractive clinical treatment of human telomere syndromes associated with telomerase mutations, as well as with short telomeres. Indeed, by using the mouse preclinical model of aplastic anemia provoked by short telomeres in the bone marrow, we demonstrated that AAV9-*Tert* rescued aplastic anemia and mouse survival by inducing telomere lengthening in peripheral blood and in bone marrow cells.[148] Similarly, AAV9-*Tert* therapy was also recently shown to be effective in the treatment of pulmonary fibrosis induced by short telomeres by rescuing short telomeres in alveolar type II cells and enhancing their proliferative capacity.[149,150] An alternative nonintegrative method to transiently express TERT based on *TERT* mRNA delivery into human cells in culture has been developed, but it has not yet been tested in vivo[151] (Figure 4).

Telomerase activation emerges as a potential strategy to prevent or treat age-related diseases.[68,147–149,152] A recent report from our group demonstrated that increased telomere length in the absence of telomerase activation by using epigenetic means was also able to delay various age-related molecular markers in mice.[153] Chimeric mice generated with embryonic stem cells with longer telomeres than those of the natural species accumulated fewer short telomeres, less DNA damage burden, and lower levels of p53 with age.[153] Importantly, they did not show any disadvantageous effects or enhanced predisposition for cancer development compared with control chimeric mice with normal telomere length, indicating that long telomeres do not cause negative consequences to the organism.[153] Thus, the up-to-date experimental data in preclinical settings support the viability of telomerase activation strategies to counteract the accumulation of critically short telomere and its associated consequences. In addition, the use of *TERT* gene therapy constitutes a promising candidate in the prevention and treatment of human telomeropathies mediated by TERT mutations that deserve further research efforts for clinical implementation.

Telomeres and CVDs

The term CVD encompasses all heart-related disorders, including myocardial infarction (MI), atherosclerosis, hypertension, and heart failure. CVDs constitute the major cause of chronic disability and the leading cause of death worldwide.[154] The prevalence of CVDs progressively increases with age. In addition to aging, psychological stress, smoking, obesity, sedentary lifestyle, inflammation, and ROS constitute risk factors for CVDs. Interestingly, all of these factors have also been associated with telomere shortening (see above).[51,116]

Observational epidemiological studies have shown an association of short telomeres in leukocytes with CVDs and cardiovascular mortality.[139,155–158] It has been estimated that each shortened kilobase pair of telomere in blood cells results in a 3-fold higher risk of MI and stroke.[155] In a systematic review and meta-analysis, it was shown that shortened leukocyte telomere length significantly associated not only with stroke and MI but also with type 2 diabetes mellitus.[157] Similarly, population studies have also demonstrated association between telomere length and atherosclerotic vascular disease.[159,160] Benetos et al[160] showed that the rate of telomere length shortening in

atherosclerotic patients and controls was similar during adulthood suggesting that increase telomere attrition in early life is more likely the explanation of the shorter telomeres observed in atherosclerotic patients. However, average telomere length and short telomere load in circulating leukocyte were shown not to be independent determinants of subclinical atherosclerosis.[161]

The effect of aging on shortened telomeres specifically in cardiac tissue remains, however, controversial. Some studies showed that myocardial telomere length is significantly reduced at a rate of 20 bp per year,[162] whereas others did not observe telomere length changes with age in healthy human hearts.[163] However, telomere length analyses in biopsied diseased heart tissues and in cardiomyocytes have demonstrated unequivocal association of shortened telomeres in the adult heart with cardiac diseases and heart failure.[162–164] Indeed, a recent study aimed to elucidate the cell-specific changes in telomere length of diseased hearts confirmed that patients with heart failure because of either hypertrophic cardiomyopathy or to ischemic cardiomyopathy exhibit shorter telomeres compared with nonfailing cohorts and demonstrated that telomere shortening is specific of cardiomyocytes and not to cardiac smooth muscle cells of the same heart.[163] The authors also showed that cardiomyocyte telomere shortening in patients with hypertrophic cardiomyopathy is associated with extensive DNA damage in the same cell type.[163] These observations highlight the central role of cardiomyocytes in heart pathology and raise the question whether shortened telomeres is the consequence of injury-induced cardiomyocyte proliferation or whether short telomeres are causative of heart damage. Both scenarios are indeed conceivable.

Studies performed in human vasculature have also shown that telomeres of coronary endothelial cells are shorter in patients with atherosclerosis compared with age-matched healthy controls, suggesting that telomere shortening of endothelial cells might play a role in atherogenesis.[165] However, analysis in endothelial cells of the human abdominal aorta as a function of age and atherosclerosis revealed that telomere length does not significantly correlate with atherosclerotic grade but rather with the cellular turnover.[166] Thus, those areas of the vasculature that undergo greater wall stress have higher cellular turnover rates and consequently shorter telomeres.[166–168]

Genetic evidence of telomeres as causative factors of CVDs has been provided. In particular, several cardiovascular pathologies, including cardiac fibrosis, dilated cardiomyopathy, MI, and pulmonary arteriovenous malformations are nowadays considered additional phenotypes of dyskeratosis congenita, a telomere syndrome caused by germline mutations in telomere biology genes.[169–171] Mendelian randomization approaches have also provided genetic evidence on the association of several genetic variants in telomere maintenance genes (*TERT*, *TERC*, *NAF1*, *STN1*, and *RTEL1*) with a higher risk for heart diseases.[113,172] Some works have shown that the effect of telomere length genetic determinants on CVDs is partially mediated by fasting insulin, suggesting that targeting fasting levels of insulin might constitute a potential therapeutic intervention to counteract the detrimental effects of shortened telomeres on CVDs risk.[173] However, longitudinal cohort studies have suggested that individuals with short telomeres are more likely to develop insulin resistance and other metabolic disorders, but the presence of insulin resistance does not accelerate telomere attrition.[174,175] Thus, further large-scale and longitudinal studies are needed to ultimately validate the casual relationship between shortened telomeres, fasting insulin, and CVDs.

Cardiac Regeneration

The notion of the heart as a terminally differentiated organ unable to undergo mitosis and without self-renewal potential has been challenged in the last years. Recent data indicate that mammalian cardiogenesis occurs during adult life.[176] Different cardiac cell types possess variable turnover rates; endothelial cells having a high turnover (>15% per year), mesenchymal cells an intermediate renewal (<4% per year), and cardiomyocytes a low turnover that gradually decreases throughout life (<1% per year).[177] In addition, adult cardiac stem cells/progenitor cells (CSC) that are self-renewing, clonogenic, and multipotent, exhibiting biochemical differentiation into cardiomyocytes, smooth muscle cell, or to endothelial cell lineage have been identified in rats, mice, and humans.[178–180] When injected into an ischemic heart, these CSCs are indeed able to differentiate into cardiomyocytes and to regenerate large amounts of functional myocardium.[178,180] The number of CSCs increased in response to ischemic injury in human hearts.[181] However, the extent to which CSCs contribute to cardiomyocyte renewal remains controversial.[182] Interestingly, human chronological age and chronic heart failure show a progressive decrease in competent CSC that present shortened telomeres, reduced telomerase activity, telomere-induced focis, as well as increased expression of p21 and p16 senescence markers.[183,184] The identification of a subset of hCSCs presenting telomere-induced focis that progressively increases with chronological age and with the concomitant presence of heart failure gives rise to the hypothesis that telomere-induced foci incidence in hCSC might represent a potential quantitative marker of physiological and pathological aging. Although future work analyzing age-matched healthy and failing hearts is needed to confirm this hypothesis, this study showed for the first time that dysfunctional telomeres in hCSCs are biomarkers of aging and heart failure. In addition, it shows that loss of telomere integrity is at least one of the factor involved in the decline of CSC function in the aged and failing heart, similar as for other adult stem cell populations.[45,183]

Despite the recognition of the regenerative potential of the adult heart and of CSCs, the fact is that adult mammals compensate for disease in injured hearts mainly through hypertrophy and extensive scarring instead of regeneration. This is in marked contrast to what is known about cardiac regeneration during embryonic development and soon after birth in mammals (Figure 5). Other vertebrates can regenerate myocardium throughout life.[182] The mechanisms of cardiac regeneration in model organisms and in mammal neonates are similar and consist of a cascade of events, including inflammation, cardiomyocyte proliferation, CSC mobilization, matrix deposition, and remodeling (Figure 5).[182] Understanding the molecular reasons underlying the lack of regeneration in the adult mammalian heart is, therefore, crucial for the design of effective therapies for the treatment of CVDs. In this

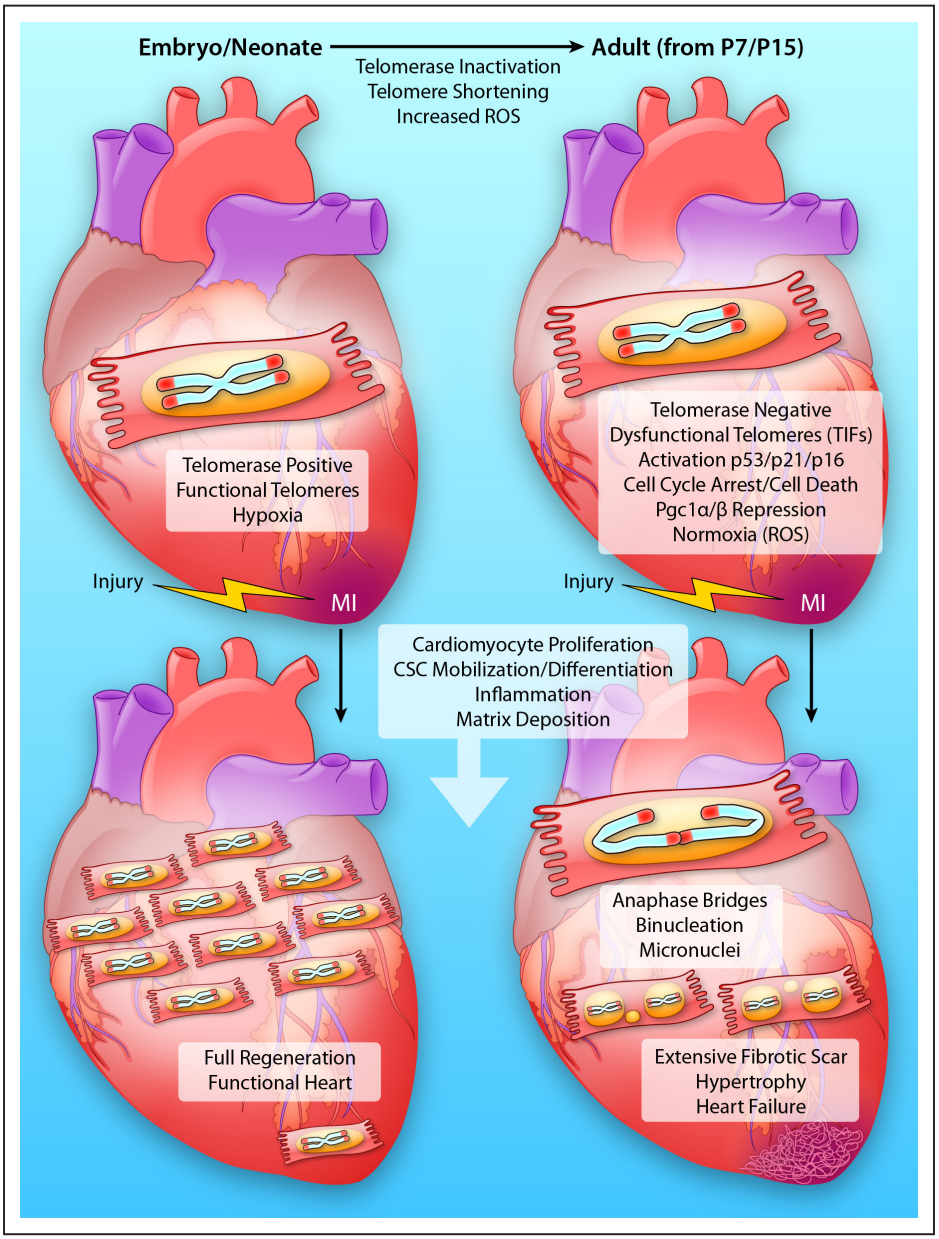

Figure 5. Role of telomeres and telomerase during embryonic/neonatal and adult cardiac regeneration. In response to myocardial infarction (MI), mammal neonates activate a cascade of events, including inflammation, cardiomyocyte proliferation, cardiac stem cells (CSC) mobilization, matrix deposition, and remodeling that result in full regeneration of a functional heart. During embryonic development and the first 7 d after birth, cardiomyocytes express telomerase and present long functional telomeres allowing them to proliferate and regenerate the heart. During the second week after birth, most of the cardiomyocytes inactivate telomerase and shortened telomeres. Telomere shortening is further enhanced by an increase in reactive oxygen species (ROS) because of the higher oxygen concentration in neonate as compared to embryo hearts and to a metabolic rewiring from anaerobic glycolysis to mitochondrial oxidative phosphorylation as main energy sources. This sudden telomere shortening leads to dysfunctional damaged telomeres and chromosome fusions which give rise to anaphase bridges, micronuclei, and binucleation of cardiomyocytes. Damaged telomeres elicit a DNA damage response characterized by the activation of p53/p21/p16 cell cycle inhibitors. Activation of p53 represses PGC1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) and PGC1β (peroxisome proliferator-activated receptor gamma coactivator 1-beta) expression, affecting mitochondrial function and ROS production. Upregulated p53/p21 triggers cell cycle arrest, senescence, and apoptosis and impairs the proliferative potential of cardiomyocyte and cardiac regeneration on injury (MI). Because of the reduced proliferative potential of cardiomyocytes and of CSCs, adult mammals compensate for disease in injured hearts through hypertrophy and extensive scarring instead of regeneration, eventually leading to heart failure. (Illustration Credit: Ben Smith.) TIF indicates telomere-induced foci.

regard, few studies have suggested that telomere dysfunction in adult CSCs and cardiomyocyte might be one of the underlying molecular causes for the inefficient cardiac regeneration in the adult mammalian heart.[79,183–185]

Defective regeneration of the damaged myocardium after MI is thought to be because of the reduced proliferative potential of adult cardiomyocytes.[177,186] In contrast, during embryonic development and during the first few days after birth, mammalian cardiomyocytes actively proliferate. Indeed, damaged heart after ischemic MI of neonatal mice are able to completely regenerate cardiac tissue through proliferation of preexisting cardiomyocytes resulting in full functional heart recovery (Figure 5).[187,188] This regenerative cardiac capacity is lost by 7 days of age coincident with the binucleation

and irreversible cell cycle arrest of cardiomyocytes.[189] From 2 weeks onwards after birth, in the absence of meaningful regeneration, the injured mammalian heart undergoes a series of pathological cardiac remodeling events, including replacement of necrotic tissue with fibrotic scar and hypertrophy of the remaining viable myocardium that ultimately leads to organ failure (Figure 5).[187] The miR-15 family of microRNAs has been implicated in the regulation of neonatal heart regeneration through inhibition of postnatal cardiomyocyte proliferation.[187] Given that members of the miR-15 family engage an ample collection of targets mRNA involved in cell cycle regulation, the molecular mechanisms that drive mammalian cardiomyocytes out of the cell cycle are still elusive. The proliferative state of cardiomyocyte has also been shown to be regulated by epigenetic control through several mechanisms, including DNA methylation, chromatin remodeling, and histone modifications.[182] Adult mice deficient for telomerase with critically short telomeres develop cardiomyopathy characterized by impaired cell division, enhanced cardiomyocyte death, and cellular hypertrophy, which are concomitant with ventricular dilatation, thinning of the wall, and cardiac dysfunction.[76] This observation has led to the hypothesis that short telomeres could be one of the upstream signals that causes permanent cell cycle arrest. Interestingly, it was recently shown that telomerase activity and cardiomyocyte telomere length decreases shortly after birth, resulting in cardiomyocytes with dysfunctional telomeres and anaphase bridges that express the cell cycle regulator p21.[185] Cardiomyocytes with short telomeres from telomerase-deficient mice show accelerated postnatal p21 upregulation, increased telomeric damage, and higher incidence of anaphase bridges (Figure 5).[185] Cardiomyocytes deficient for p21 show a robust proliferation response in 7-day old hearts when wild-type cardiomyocytes have lost their capacity to proliferate after injury. These observations suggest a role for p21 in ceasing cardiomyocyte cell cycle and the regeneration response in postnatal mouse hearts. Indeed, the newborn cardiac regenerative potential after heart injury is lost in telomerase-deficient mice but rescued in the *Terc*$^{-/-}$ *p21*$^{-/-}$ compound mice.[185] These results strongly underscore telomere dysfunction as a trigger for p21 induction and consequent cardiomyocyte cell cycle arrest after birth. However, the dramatic and sudden telomere shortening observed in wild-type mice during the first 15 days after birth, 15% decreased in mean telomere length, and 25% increase in the abundance of short telomeres could hardly be explained solely by the end-replication problem and telomerase inhibition.[185] Mitochondrial ROS–mediated activation of the DDR has also been proposed as an important upstream event that mediates cell cycle arrest of postnatal cardiomyocytes.[190] Transition from embryonic to postnatal states entails a switch from a hypoxic to a normoxic environment, as well as a metabolic rewiring from anaerobic glycolysis to mitochondrial oxidative phosphorylation as main energy sources.[190] As a consequence, the level of ROS significantly increases after birth presumably causing DNA damage and the consequent DDR. In support of this, it has been shown that postnatal hypoxemia, ROS scavenging, or inhibition of the DDR prolong the postnatal proliferative window of cardiomyocytes, whereas hyperoxemia and ROS generator shorten it.[190] It is, therefore, conceivable that the higher level of ROS after birth could result in telomere losses that could also explain the sharp decrease in telomere length observed during the first days of mouse life.[185,191] Future work is needed to elucidate whether the ROS-dependent changes in proliferative potential of neonate cardiomyocytes is mediated through effects on telomere length homeostasis.

Telomere dysfunction and the associated DDR not only induces cellular phenotypes of proliferative arrest, apoptosis, and senescence but also compromises cardiomyocyte function through p53-mediated repression of *PGC1*α and *PGC1*β (Figure 5). The combined PGC1α and PGC1β downregulation results in impaired mitochondrial biogenesis and function, affecting ATP levels, β-oxidation, and fatty acid synthesis in the heart.[62] Thus, telomere dysfunction triggers metabolic changes that may lead to higher level of ROS that in its turn amplify telomere dysfunction.

Evidence supporting telomere length homeostasis and oxidative stress as key determinants in the development of cardio pathologies in the adult organism has been provided by work performed in a mouse model of Duchenne muscular dystrophy (DMD). DMD is a lethal X-linked disease characterized by severe progressive muscle wasting and cardiorespiratory failure. DMD is caused by mutations in DMD (dystrophin), a protein that plays a critical role in plasma membrane integrity in both skeletal and cardiac muscles, being heart failure the primary cause of death of DMD patients.[192] However, mice lacking dystrophin, the *mdx* mouse model, do not develop severe muscle weakness, cardiomyopathy, and reduced lifespan and, therefore, do not recapitulate the hallmarks of human DMD.[193,194] Interestingly, mice lacking dystrophin and telomerase, the compound *Mdx/Terc* knock-out mouse, develop dystrophic skeletal muscle and severe cardiac pathologies, including ventricular dilatation, contractile and conductance dysfunction, and accelerated mortality, faithfully phenocopying the pathophysiology of human DMD.[192,195] In the *Mdx/Terc* mouse, cardiac pathologies were accompanied by telomere shortening, mitochondrial fragmentation, and increased oxidative stress. Treatment with antioxidants delayed the onset of cardiac dysfunction and consequently the death of *Mdx/Terc* mice, suggesting that the oxidative stress generated in the dystrophic heart plays a casual role in cardiac dysfunction.[192] These observations suggest that oxidative stress enhances telomere shortening in *Mdx/Terc* cardiac tissues contributing, therefore, to heart failure. It was not addressed whether antioxidant treatment leads to an improved telomere maintenance in *Mdx/Terc* heart as compared to untreated mice. In support of shortened telomeres as an underlying cause of cardiomyopathy, DMD patients present almost a 2-fold decrease in their cardiomyocyte telomere length as compared to age- and sex-matched controls.[192]

Therapeutic Strategies for the Treatment of CVDs

In spite of recent advance on the knowledge of cardiac biology, adult tissue regeneration and functional recovery of adult heart after MI remain without efficient treatment, underscoring the need of developing new therapeutic strategies. Given the importance of proper telomere maintenance in

cardiac regeneration and the link between CVDs and shortened telomeres, therapeutic strategies aimed at lengthening telomeres and restoring the proliferative potential of adult mammalian cardiomyocytes have been explored as potential treatments for cardiac regeneration after MI. Constitutive *Tert* transgenic expression in the mouse heart from embryonic development onwards improved cardiomyocyte proliferation but was shown to lead to heart hypertrophy.[196] To avoid the undesired effects of constitutive telomerase expression, we tested our previously developed AAV9-*Tert* gene therapy shown to be efficient to delay age-related pathologies and extend mouse longevity.[68] Exogenous *Tert* expression in the adult mouse heart by AAV9-*Tert* gene therapy improved cardiac functional and morphological parameters and reduced mortality associated with heart failure after MI.[79] These beneficial effects were coincidental with an increase in cardiomyocyte telomere length and a lower abundance of cells with short telomeres as compared to control hearts, suggesting that telomere length recovery in the myocardium decreases the associated risk of heart failure. AAV9-*Tert* delivery after infarct led to lower fibrotic scarring of the heart and increased cardiomyocyte proliferation concomitant with transcriptional changes suggestive of a regenerative signature.[79] In fact, the gene expression changes associated with *Tert* treatment in the adult hearts were enriched in the regenerative gene expression signature described in neonatal mice, indicating that reactivation of *Tert* modulates transcriptional programs similar to those in neonatal mice[79] (Figure 6). These findings provide rationale for future clinical testing of telomerase activation for the treatment of human CVDs.

Autologous cell therapy using stem or progenitor cells has been clinically tested as a treatment for improving the functional recovery after ischemia and restoring heart function in patients with heart failure. The clinical trials up-to-date have implied unfractionated bone marrow–derived mononuclear cell preparation that contains hematopoietic, mesenchymal, and endothelial stem/progenitor cells.[197] Although the results from these small clinical trials have consistently concluded that autologous cell therapy has an overall beneficial effect on cardiac function in patients with acute and chronic ischemia, large-scale blinded clinical trials are warranted to ultimately examine the potential effects of progenitor cell administration on morbidity and mortality.[197] The recognition that the human heart possesses a pool of resident CSCs opens novel therapeutic strategies based on the harvesting of these cells which are primed to acquire a cardiac phenotype and, thereby, be more suited for specific heart repair.[181,198] Aging and risk factors for CVDs are important issues to be taken into account for the use of autologous cell therapy because these factors also affect the functional activity of the endogenous stem/progenitor cell pools and the environment in which the stem cells must be homed. Therefore, age and disease constitute limitations to the therapeutic potential of autologous cell therapy.[198] The use of heterologous cells might be an option to overcome cell impairment during aging and disease. However, this strategy is likely to activate the immune response resulting in cell rejection and serious risk for the treated patients. The pretreatment

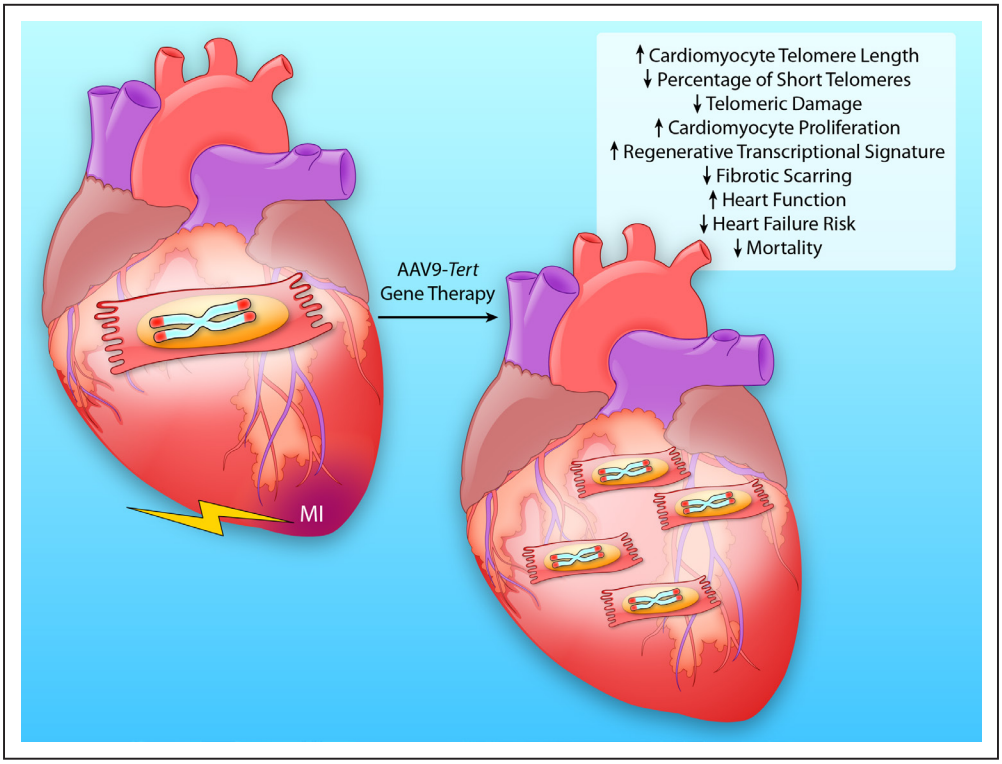

Figure 6. Telomerase gene therapy improves adult heart regeneration after myocardial infarction. Exogenous *Tert* expression in the adult mouse heart by adeno-associated vector 9 (AAV9)-*Tert* gene therapy results in higher telomere length, a decreased abundance of cells with short telomeres and less telomeric damage. AAV9-*Tert* treatment after infarct leads to lower fibrotic scarring of the heart and increased cardiomyocyte proliferation concomitant with transcriptional changes suggestive of a regenerative signature. As a consequence, AAV9-*Tert* treatment improves heart function and reduces mortality associated with heart failure after myocardial infarction (MI). (Illustration Credit: Ben Smith.)

of the patients own cells to restore their proper functions or to direct their differentiation might improve the efficacy of cell therapy in aged or ill individuals. Treatment of the target tissue to favor a better engraftment of the transplanted cells has also been proposed to improve cell therapy.[198] Therapeutic applications based on in vitro telomerase gene transfer into endothelial progenitor cells to compensate for age-associated telomere shortening was assayed in mouse models of acute MI. Overexpression of telomerase enhanced endothelial progenitor cell regenerative activity and improved the functional recovery of mouse heart after acute MI.[199] Telomerase overexpression in endothelial progenitor cells contributed to their angiogenic properties, mitogenic, and migratory activities, as well as cell survival.[199]

Reduction of mitochondrial-dependent oxidative stress has also been proposed as a potential proliferation-based therapeutic approach.[190] The therapeutic role of hypoxemia in adult heart regeneration was recently demonstrated in infarcted mouse heart. Hypoxia induces metabolic reprogramming of adult cardiomyocytes, resulting in cell cycle re-entry and myocardial regeneration. Exposure to hypoxemia 1 week after MI induces a strong regenerative response with reduced myocardial fibrosis and improvement of cardiac function.[200]

Manipulating the epigenetic state of cardiomyocytes during the critical postinjury period has also been proposed as a regenerative therapy. The use of histone deacetylase inhibitors reduces stress-induced cardiomyocyte death, hypertrophy, and fibrosis, thereby inhibiting the pathological cardiac remodeling on injury.[201]

Conclusions and Perspectives

Research during past years has undoubtedly involved telomere biology in the development of age-associated CVDs, including atherosclerosis, hypertension, cardiac remodeling after MI, and heart failure. Dysfunctional telomeres– and mitochondrial ROS–mediated activation of the DDR are placed as critical upstream signals that induce permanent cardiomyocyte cell cycle arrest. The reduced proliferative potential of CSCs and cardiomyocytes underlies the limited regenerative capacity of aged and damaged mammalian heart that ultimately leads to heart failure. Therefore, therapeutic strategies aimed at restoring the proliferative potential of adult mammalian heart constitute promising alternatives for the prevention and treatment of CVDs. In this regards, given the proven beneficial effects of telomerase reactivation in heart functional recovery after MI in mouse models, telomerase gene therapy provides an attractive approach for cardiac regenerative medicine that deserves future studies for clinical implementation.

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Disclosures

None.

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