Published in final edited form as:

Pharmacol Res. 2016 September ; 111: 422–433. doi:10.1016/j.phrs.2016.07.003.

Friend or Foe? Telomerase as a Pharmacological target in Cancer and Cardiovascular Disease

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Abstract

Aging, cancer, and chronic disease have remained at the forefront of basic biological research for decades. Within this context, significant attention has been paid to the role of telomerase, the enzyme responsible for lengthening telomeres, the nucleotide sequences located at the end of chromosomes found in the nucleus. Alterations in telomere length and telomerase activity are a common denominator to the underlying pathology of these diseases. While nuclear-specific, telomere-lengthening effects of telomerase impact cellular/organismal aging and cancer development, non-canonical, extra-nuclear, and non-telomere-lengthening contributions of telomerase have only recently been described and their exact physiological implications are ill defined. Although the mechanism remains unclear, recent reports reveal that the catalytic subunit of telomerase, telomerase reverse transcriptase (TERT), regulates levels of mitochondrial-derived reactive oxygen species ($mROS$), independent of its established role in the nucleus. Telomerase inhibition has been the target of chemotherapy (directed or indirectly) for over a decade now, yet no telomerase inhibitor is FDA approved and few are currently in late-stage clinical trials, possibly due to underappreciation of the distinct extra-nuclear functions of telomerase. Moreover, evaluation of telomerase-specific therapies is largely limited to the context of chemotherapy, despite reports of the beneficial effects of telomerase activation in the cardiovascular system in relation to such processes as endothelial dysfunction and myocardial infarction. Thus, there is a need for better understanding of telomerase-focused cell and organism physiology, as well as development of telomerase-specific therapies in relation to cancer and extension of these therapies to cardiovascular pathologies. This review will detail findings related to telomerase and evaluate its potential to serve as a therapeutic target.

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Graphical Abstract

2. Introduction

Repetitive DNA elements at the end of chromosomes, called telomeres, protect against chromosomal erosion and rearrangement in the nucleus. When shortened by natural aging (through successive rounds of cell divisions) or the onset of disease, telomeres become critically short, reaching what is called the Hayflick limit. Once this limit is reached, cells undergo replicative senescence and apoptosis. The major enzyme complex that counteracts telomere shortening is telomerase, which is composed of the catalytic subunit telomerase reverse transcriptase (TERT) and its RNA component, TERC. Telomerase is expressed at a high level in malignant cells, thereby conferring increased replicative capacity. This immortalizing role of telomerase has received much attention within the context of cancer biology. However, even permanently-differentiated cells such as endothelial cells (1) or neurons (2) display higher levels of telomerase activity (TA) compared to other somatic cells. These findings suggest that telomerase may be important in non-malignant processes to preserve homeostasis and viability in these highly specialized cells.

Decreased TA is an established factor in the development of senescence and tissue aging. Telomerase, present primarily in the nucleus, can also accumulate in mitochondria under conditions of oxidative stress (3, 4). Recently, TERT has been described as a regulator of mitochondrial-derived reactive oxygen species ($mtROS$) (4, 5). Pharmacological activation of TERT decreases $mtROS$ (6) while ablation of TERT in cell culture and mouse models leads to elevation of $mtROS$ (3). However, it is not known whether these effects require TERT to be physically located in mitochondria ($mtTERT$) or whether they result from lack of nuclear TERT ($nucTERT$) and the associated telomere shortening. Recently, findings from a cell culture model demonstrate that expression of a mutant TERT that cannot enter mitochondria but retains its telomere-lengthening function leads to mitochondrial dysfunction and increased $mtROS$ production (4), supporting the notion that TERT translocation to the mitochondria is necessary to maintain physiological ROS levels. This novel role of telomerase appears to have relevance for cardiovascular pathologies. Consistent with this view of an extra-nuclear, non-telomere-related function of TERT, increased $mtROS$ and hypertension preceded critical telomere shortening in global TERT knockout mice (7). The broader impact of TA on the vasculature itself is controversial. One source reports that decreased global TA is associated with inflammation and atherosclerosis (8), whereas loss of global telomerase function was shown to be protective in a mouse model of atherosclerosis

(9). A recent study from our laboratory revealed the beneficial effects of global telomerase activation in the human vasculature in subjects with coronary artery disease (CAD) through protection against $mtROS$ production. Further clarification of the potential ROS-detoxifying effects of TERT is warranted, especially in relation to the subcellular localization of TERT, given these conflicting reports and the relative lack of emphasis on this novel role of TERT.

The total number of publications found on PubMed related to telomerase [13,523], TA [7112], and telomere length [5588] is substantial; however, most of this work contributes to the understanding of nuclear telomerase and its telomere-lengthening role. Only about 300 of these publications relate to extra-nuclear telomerase function, and fewer than 30 employ human cells or tissue samples. Interestingly cancer- and aging-related studies contribute to the bulk of the knowledge (~10,000 studies), with fewer than 600 publications (~150 TA) investigating the contribution of these basic biological mechanisms to the development of cardiovascular disease (CVD). These numbers illustrate the relative novelty of the concept that reductions in TA outside the nucleus contributes to susceptibility to disease in general, and more specifically to the development of cardiovascular diseases. As a result, telomerase has attracted interest as a new target for anticancer treatment, but most efforts are directed at the canonical function of TERT with little attention paid to TERC or nuclear independent contributions of TERT to physiological processes. This review will highlight exciting pharmacological tools used to inhibit or increase TA and the physiological implications of both traditional and non-canonical functions of TERT within the context of cancer biology and cardiovascular disease.

3. Cancer Biology – Basic Role of Telomerase

Cancer is defined by uncontrolled, clonal proliferation. This proliferative process is not possible without significant cellular changes. In the absence of malignant transformation, terminally differentiated cells have a finite replicative lifespan; because of the nature of discontinuous DNA replication, pieces of the ends of chromosomes, called telomeres, are lost at each division. Approximately 50 divisions occur before gradual replication-related erosion of telomeres triggers replicative senescence (10). These telomeres are continually replaced and extended in dividing cells by enzymes such as telomerase, which repairs the shortening that occurs with each cell division. After the discovery of telomerase in yeast, and subsequently in humans, in the late 1980s (11, 12), a natural next question was whether this enzyme contributes to tumorigenesis, given that tumor cells appear to be unencumbered by the restraints of the Hayflick limit and can undergo repeated and prolonged cell division. This question began to be answered in the early 1990s, when telomerase and TA were detected in embryonic and cancer tissues. Critically, expression of the enzyme appeared to be limited in terminally differentiated cells, with enzymatic activity most apparent only in malignant tumors and embryonic stem cells, highlighting the pro-replicative, “immortalizing” effects of elevated TA (13). Indeed, the vast majority of tumor cells express telomerase or have engaged some alternative mechanism of telomere lengthening (ALT) (reviewed in (14)). This essential characteristic shared by tumor cells across cancer sites has made telomerase an important target in cancer research and therapeutic development.

3.1. Telomerase as a Key Player in Oncogenesis

The role of telomerase and its catalytic subunit TERT in cancer is well-established and has been reviewed numerous times. This review will focus on non-traditional and controversial roles of TERT in cancer development and progression. Given the necessity of immortalization to enable long-term replication, in vitro experiments have evaluated the role of telomerase in the transformation of normal cells to a transformed, cancerous state. Much of this early work was conducted with murine cells, which provided an interesting reference point for future studies. More recent work suggests that there are species differences in the transformability of different cell types, with human cells being much harder to transform and requiring significant additional mutations versus their murine counterparts, even in the presence of telomerase activation (15). Of note, immortalization with telomerase has not been shown to be sufficient to induce transformation of human cells to a cancerous state. Because human cells require a higher mutational load for transformation to occur, telomerase may increase the number of cell divisions that can occur and thereby provide additional opportunities for mutations and malignant transformation to occur, but is not, by itself, sufficient. One group showed that ectopic overexpression of telomerase significantly enhances the ability of human fibroblasts to be oncogenically transformed. Telomerase was identified as an essential element of a transforming “cocktail” of oncogenes in early work (16), although subsequent studies revealed that it was also possible to develop transformed human fibroblasts without the addition of telomerase (17). This indicates that telomerase may not, in all cases, be a required element for oncogenesis, although it is likely to be a permissive factor that helps to catalyze the process of transformation. Interestingly, when tumors derived without telomerase transfection were explanted and grown in culture, they suffered a telomere crisis within 40–50 passages. Ultimately, this resulted in the outgrowth of a population of tumor cells that do express telomerase and are capable of continuing to proliferate. This seminal work suggests that telomerase activation is not a required element for tumor transformation in all cell types and in all contexts; however, the central problem of telomere crisis remains. As a result, as the transformation process continues, and cancerous cells proliferate, a crisis point is eventually reached which necessitates either reactivation or expression of the telomerase enzyme or use of alternative means to lengthen telomeres in order to promote continued proliferation. Alternatively, others have shown that when telomerase is exogenously overexpressed in tumor cells to generate artificially long telomeres, and then excised, tumor cell proliferation can continue for up to a year. However, once the telomeres are exhausted, at least in this model system, the cancer cells lose their replicative capacity. This may indicate that the acquisition of alternative means to lengthen telomeres can be context and tissue type dependent (18).

Open questions remain regarding the importance of telomerase in human disease. Genome wide association studies (GWAS), which are designed to identify genetic polymorphisms associated with pathologies such as cancer, have yielded mixed results regarding the role of telomerase in cancer. In certain cancers, such as urothelial carcinomas, dramatic associations between cancer development and mutations in the telomerase promoter that lead to higher levels of TERT have been identified (19). Methods to elongate telomeres become critical to tumor growth, which can either take the form of telomerase expression as tumors progress (the bulk of cancers), or engagement of ALT. Although much less common than telomerase

reactivation, ALT can involve a number of mechanisms. Generally, most ALT is thought to be an adaptation of the homologous repair pathway which is activated by DNA damage (e.g. by telomere shortening), and can be co-opted by cancerous cells to extend telomeres and enable continued replication (reviewed in (20)). Other studies, however, have failed to reveal a link between telomerase promoter alterations and cancer. For example, although mutations in the TERT-CLPTM1L gene locus have been identified that are associated with lung cancers, the culprit of cancer development may not be TERT but rather increased expression of CLPTM1L (21, 22). A recent study reported that cell passaging of embryonic stem cells can generate hyper-long telomeres in the absence of increases in telomerase activity, suggesting that this cell passaging technique recruits ALT to extend telomeres and may provide an experimental model for studying mechanisms underlying ALT. These data support the central thesis that has emerged from the in vitro work conducted to date, which indicates that telomerase is often associated with transformation and tumor development, as well as tumor progression, but is not a requirement in all instances.

In vivo data also support a critical oncogenic role for telomerase, which is essential for the formation of most tumors, and the need for telomerase re-expression or ALT for continued progression. In one early study, female mice engineered to overexpress telomerase developed significantly more mammary tumors with a more aggressive phenotype as they aged. This result may require additional interpretation, since rodents naturally have longer telomeres than humans, potentially suggesting that telomerase overexpression can predispose to cancer through a mechanism that is telomere independent (23). Since this initial work, numerous other studies using TERC^{-/-} models have consistently revealed that cancer risk is dramatically *reduced* when TA is abrogated in vivo (reviewed in (24)). As with all biological systems however, there is a delicate balance related to telomerase expression, overexpression and inherent cancer risk. While a TERC^{-/-} state where telomerase lacks catalytic activity dramatically decreases cancer risk on a genetically normal rodent background, the story becomes more complex when mutations in common tumor suppressors are introduced. Cells isolated from TERC^{-/-}/p53^{-/-} mice are much more readily transformed by transfection of known oncogenes. Although this result may seem paradoxical, it may be explained by examining the biology that underlies this phenomenon: critically short telomeres serve as a pro-apoptotic signal that depends on p53. In the absence of p53, cells with critically short telomeres are able to survive and undergo chromosomal rearrangements that can cooperate with expression of oncogenes to increase transformation efficiency, at least in the short term (25). Overall, the in vivo data suggest that telomerase is an important mediator in the oncogenic process for the vast majority of tumor types, and that re-activation of the enzyme plays a critical role in tumor progression. However, a myopic view of telomerase as solely an agent of cellular immortalization and tumorigenesis in cancer misses the nuance of telomerase biology as a regulator of both transformation, and in some rare cases, tumor suppression.

3.2. Telomerase: More than an Immortalizer?

New evidence suggests that telomerase biology has broader effects in cancer cells that transcend chromosomal lengthening and cellular proliferation. Overexpression of the enzyme is associated with the acquisition of a distinct and discernible “telomerase gene

expression pattern”, which includes many genes involved in cell cycle regulation (26). As cancers in most cases are proliferating and consist of immortalized cells it is not surprising that elevated TERT levels are associated with cancer. It is currently believed that telomerase stabilizes the genome of an initiated cancer to permit progression and metastasis (27), but *increased telomerase alone has not been linked to tumorigenesis* (28) While this direct connection to cell cycle regulation does not come as a surprise, some of the genes included in this signature are surprising. For instance, upregulated integrin beta 1 (ITGB1) is a feature of telomerase overexpression. ITGB1 is known to play an essential role in tumor cell metastasis – expression of ITGB1 is significantly elevated in metastatic cells, and plays an important role in extravasation, while also upregulating expression of matrix metalloproteinases that break down extracellular matrix (29). Telomerase was also recently found to regulate metabolism and glycolysis in melanoma cells, directly impacting their ability to metastasize (30). These non-canonical tumor-promoting functions of the telomerase enzyme have also been validated in other models of cancer in vivo. A murine model of conditional telomerase reactivation proved illustrative of this point. Mice deficient in telomerase still generate prostate tumors, but these tumors were smaller. Interestingly, when telomerase was reactivated, tumors showed increased signs of DNA repair, acquisition of many other mutations and new phenotypic properties, including increased size and growth rate, and the development of bone metastasis, not simply increased proliferative capacity (31). This may suggest that telomerase may not always be necessary for transformation and replication itself, but that the acquisition of many of the pro-replicative features of cancer require or are enhanced by telomerase reactivation. In a number of human cancers, high TA is associated with a negative prognosis. The reasons for this negative prognostic role are not entirely clear, although a recent study showed that a dominant negative hTERT splice variant decreased the tumorigenicity of neuroblastoma cell lines, despite having little effect on telomere length, supporting a non-telomeric role for telomerase in cancer (32). Telomerase may also play a role in allowing tumor cells to overcome transforming growth factor beta (TGFB) induced growth arrest, enabling tumor cells to overcome the circuitry designed to shunt pre-cancerous cells toward a quiescent state. Overexpression of telomerase in human mammary epithelial cells bathed in TGFB allows these cells to continue to proliferate. Interestingly, proliferative ability is directly correlated to telomerase activity, but not to telomere length itself (33).

Recently, variants of telomerase that are catalytically inactive have been identified as well. These variants are particularly common across many cancer types, yet their role in the disease process remains poorly understood. The fact that a transcript which is catalytically inactive would accumulate in aggressive cancers suggests that these variants are evolutionarily favored in cancer, although their significance to biology has not yet been elucidated. Provocative early work suggests that overexpression of a variant of telomerase which is catalytically inactive is capable of protecting tumor cells from apoptosis induced by cytotoxic agents such as cisplatin (34). Alternative splicing variants are not limited to this single, catalytically inactive form; there are currently 22 distinct telomerase splice variants that have been observed in humans. With the exception of full-length telomerase however, none of these appears to have telomere-extending capability (reviewed in (35)). Intriguingly, although many of these isoforms have the capability to inhibit telomerase activity, several

also appear to have unique functions that add to our understanding of the complexity of telomerase biology in cancer. For instance, a human telomerase isoform that contains a deletion of exons 4 and 13 and is expressed in some cancers cannot extend telomeres, but does stimulate cell proliferation through activation of the Wnt signaling pathway (36).

3.3. Inhibiting Telomerase in Cancer

Because of the central role of telomerase in cellular immortalization through telomere-dependent and independent mechanisms, and the perceived key role of the enzyme in tumorigenesis, significant effort has been expended to develop pharmacologic means of inhibiting the enzyme (Table 1). In general, these approaches fall into one of four categories: 1) approaches that directly target the hTERT catalytic component of the holoenzyme; 2) approaches that target the TERC component of the holoenzyme; 3) approaches that use immunotherapy to target telomerase and 4) unintended off-target effects of chemotherapy drugs that either inhibit or activate telomerase.

Despite the prominence of telomerase as an anti-cancer target, there has been limited success to date in developing pharmacologic inhibitors of the enzyme. The major developmental challenge has been synthesizing small molecules and inhibitors that specifically inhibit telomerase at an effective dose that is not toxic to the host.

3.4. Targeting hTERT Catalytic Activity

Because telomerase functions as a reverse transcriptase, a number of anti-retroviral therapies that are currently used may also have off target effects on this enzyme and utility as the basis for telomerase inhibitors. A growing body of clinical evidence suggests that many HIV/AIDS patients treated with non-nucleoside reverse transcriptase inhibitors (NNRTIs) also have decreased TA (Table 1) (37, 38). Efforts to more specifically inhibit telomerase with greater potency have also been explored, leading to the creation of several lead candidates that more specifically inhibit telomerase. Chief among these is BIBR1532, which is mechanistically similar to many of the existing NNRTIs, although it is a non-competitive inhibitor that inhibits enzyme processivity (39). We have recently shown that BIBR1532 can specifically inhibit TA to induce a vascular phenotype in healthy vessels that is similar to that observed in subjects with coronary artery disease, adding to the growing body of evidence suggesting that this class of telomerase inhibitors may have significant off-tumor side effects (Table 1) (40).

3.5. TERC/TR Competitors

Other pharmacologic approaches have included the use of antagonists of hTR (TERC). GRN163L (imtelstat) is a compound which is currently in phase II/III trials in both cancer and pulmonary hypertension therapy (Table 1). Imtelstat is a nucleic acid drug, with modified thiol residues designed to resist degradation by nucleases, as well as a palmitoyl lipid moiety that significantly improves cellular uptake, enabling delivery without a lipid carrier. GRN163L is an antagonist of TR or TERC the RNA subunit of telomerase, competing with TR for binding to TERT (Table 1). GRN163-hTERT complexes do not have enzymatic activity and are not able to bind telomeric DNA. Preclinical work reveals that GRN163L effectively inhibits telomerase activity, resulting in morphological changes in

tumor cells – although the development of a senescent phenotype requires significant cell passaging. This may imply that the canonical nuclear actions, not the cytosolic/mitochondrial actions, are involved as the TERC dependent mechanism does not operate in the cytosol/mitochondria. Regardless, this finding highlights the fact that TA inhibitors such as GRN163L may best be used in combination with other drugs, due to the relatively long time frame required for anti-proliferative effects to become apparent (41). GRN163L has advanced to clinical trials, with mixed results. An early study of GRN163L in breast cancer patients was terminated due to lack of efficacy (reviewed in (42)). However, subsequent studies in hematologic malignancy have been promising. One recently completed small study in essential thrombocythemia was particularly promising, with 100% of patients responding to therapy (18/18) and 89% (16/18) exhibiting complete responses. Decreases in TA were detected in all treated patients. Side effects in this study included neutropenia and anemia (43). Other ongoing studies aim to evaluate the use of GRN163L in combination with other chemotherapeutic agents. Because GRN163L serves as a competitor to TERC, it may logically follow that GRN163L more specifically targets the telomere-extending functions of telomerase which are dependent on TERC, rather than targeting non-telomeric or extra-nuclear activity of the enzyme which may not be dependent on TERC.

3.6. Immunologic Approaches Targeting Telomerase-Expressing Cells

Because telomerase expression was thought to be largely restricted to rapidly proliferating cells and to tumor cells, efforts to develop vaccines against telomerase (hTERT) were pursued, leading to several vaccine candidates in advanced stage clinical trials. GV1001, a peptide corresponding to 16 amino acid residues within the catalytic domain of hTERT has been used in several Phase III trials (Table 1). Interestingly, vaccination against telomerase was shown to generate an active T cell-mediated response in a subset of patients. For a narrower subset of these patients, responses correlated with long-term survival in a Phase II trial of Stage III/IV non-small cell lung cancer patients. Despite some evidence of efficacy, few patients have benefited, and benefit has been largely constrained to the relatively small group of immune responders (44). This may be largely due to the difficulty in priming an immune response to a native self-protein, which has been the strategy with telomerase. Unfortunately, a recent trial Phase III trial of GV1001, given with chemotherapy to pancreatic cancer patients revealed no improvement in overall survival (45). Following the use of GV1001 in clinical trials, novel immune responses against new regions of the telomerase protein were identified in patients with sustained remissions and durable responses after GV1001 vaccination. This phenomenon, termed epitope spreading, is believed to be a result of the immune response evolving over time to identify more potent regions of the protein in terms of antigen presentation, which ultimately correlates with vaccine efficacy. Using these newly identified epitopes commonly found in responders to GV1001 vaccination, next generation peptide vaccines are currently under development and beginning Phase I/II trials. It is hoped that these epitopes will prove to be more effective than the GV1001 epitope in a broader cohort of patients. (UltimoVacs) Other vaccines have included shorter peptides (GV1001 is a relatively long 16 amino acid vaccine) that have also been trialed clinically in an effort to identify the best possible epitopes to target telomerase. Other immunologic recent efforts have included the development of dendritic cell based vaccines, which are a cellular therapy product derived from each individual patient. Isolated

dendritic cells are pulsed with telomerase peptides, or loaded with DNA encoding specific epitopes of the enzyme. Coupled with GM-CSF in some cases, the dendritic cells are re-infused into patients to present telomerase antigen and drive an anti-telomerase response (reviewed in (46)).

3.7. Off Target Effects of Targeted and Conventional Chemotherapeutics: Modulators of Telomerase Activity?

Beyond targeted approaches to inhibit telomerase, there has also been an increasing realization that many anti-cancer drugs have secondary, tertiary or direct effects on telomerase itself. Because telomerase must be shuttled into and out of the nucleus, therapeutics that impact the ability of the enzyme to traffic may indirectly impact overall telomerase activity. For nuclear import to occur, telomerase must be phosphorylated by Akt; specific inhibitors of Akt phosphorylation may, as a result, have the secondary effect of blocking telomerase translocation to the nucleus (47). Because of the complicated interplay of cell signaling pathways, it is also conceivable that many tyrosine kinase inhibitors (TKI) may have secondary effects on telomerase. One of the most striking examples is the BCR-ABL inhibitor imatinib. BCR-ABL TKIs, including imatinib, represent the current standard of care in subjects with chronic myelogenous leukemia (CML), and is used indefinitely to suppress expression of the BCR-ABL1 that causes most cases of CML. Recent work has shown that imatinib treatment at clinically-relevant doses is capable of downregulating TA by 50% or more. The downregulation of TA may occur through a combination of secondary events or potentially through a more direct interaction between the tyrosine kinase inhibitor and telomerase itself. Intriguingly, treatment with imatinib directly suppresses transcription of telomerase, potentially indicating additional direct and indirect effects of this drug on telomerase biology (48). Other less targeted agents in hematologic malignancies have also been shown to directly regulate telomerase activity. Thalidomide (and its derivatives) are used in the treatment of multiple myeloma. Although not originally postulated to be the primary mechanism of thalidomide's efficacy, detailed molecular analysis revealed that thalidomide preferentially downregulates genes with GC-rich promoter regions, producing an especially dramatic reduction in hTERT levels in treated multiple myeloma cells (49).

3.8. Risk and benefit of targeting telomerase in cancer

Given the fundamental role of telomerase in cancer biology, which has been well established through both in vitro and in vivo studies (described above), telomerase is a tantalizing target in cancer that requires further exploration. Current therapies targeting the enzyme have shown some promise, but as detailed in Table 1, they are not without side effects, potentially due to lack of recognition of differential non-nuclear roles that TERT may play in other healthy, somatic tissues. It should also be noted that many of these investigational therapies have been trialed in cancer patients with highly advanced disease, potentially masking side effects of therapy that may be attributed to disease progression. As clinical studies progress and advance further, additional toxicities associated with treatment duration may be revealed. Given the increasingly appreciated non-canonical roles of TA discussed above, as well as newly identified roles for telomerase in vascular biology (detailed in subsequent sections), new therapies that aim to target TA in new ways that maximize therapeutic benefit while minimizing side effects are needed. This may become especially important if long-

term administration of anti-telomerase agents is required. Imatinib and other small molecule inhibitors used in the treatment of chronic leukemias are prototypic examples of agents that are used for years at a time to treat patients, which may also have an off-target impact on telomerase. The long-term cardiovascular side effects including heart failure associated with some anti-retroviral therapies given to manage HIV/AIDS could potentially be a result of off-target telomerase inhibition. Still, since direct, telomerase-inhibiting drugs remain in clinical trials, there has been little opportunity to assess the long-term impacts that these more specific inhibitors might have. Given the increasingly appreciated side effects associated with these drugs, there is added impetus to think carefully about the long-term effects of treatment with anti-telomerase agents. The recent work by DePinho *et al* revealing that inhibition of telomerase can lead to the evolution of ALT (50), and potentially to more highly resistant cancers, after a period of tumor regression should also give the field some pause.

One of the major challenges in oncology is the highly toxic nature of chemotherapeutic drugs. A number of cytotoxic therapies that remain broadly used to treat a variety of malignancies, including breast cancers are limited by the dosages that can be given to patients due to toxicity. One of these drugs, doxorubicin, causes congestive heart failure in up to 8% of treated patients. A recent study evaluating susceptibility factors to doxorubicin-induced cardiac toxicity identified lower basal mitochondrial metabolism as the key risk factor in doxorubicin-induced congestive heart failure (51). Given the known role of mitochondrially-localized telomerase in enhancing and preserving mitochondrial function, particularly under conditions of oxidative stress (52), such as those generated by chemotherapies like doxorubicin, targeting of TA could also potentially increase the side effects of many mainline chemotherapeutic drugs, such as doxorubicin. The design and development of next generation anti-telomerase agents will need to include thorough consideration of side effects and a rational strategy to effectively target cancers while minimizing opportunities for tumor evolution toward a more aggressive state after therapy failure. Next generation agents that attempt to separate the pro-tumorigenic effects of nuclear telomerase from the non-canonical, non-nuclear, and protective roles that telomerase appears to play in normal vascular biology may be particularly valuable.

4. Role of Telomerase in of Cardiovascular Disease

While the goal of therapy in oncological settings is telomerase inhibition, recent work in the cardiovascular realm (3) and neurodegenerative disease (6, 53) show beneficial effects of telomerase activation. These differential effects of TA in different disease settings (cardiovascular vs oncological) may be explained in part by the distinct nuclear and mitochondrial roles of telomerase. Age-related alterations in cellular homeostatic mechanisms make the aged cardiovascular system more susceptible to the damaging effects of risk factors such as obesity and smoking. Amongst a range of proposed mechanisms, telomerase dysfunction is taking place as a potential pathophysiological mechanism for cardiovascular diseases (54–57).

4.1. Differential regulation of TERT in vascular cells

Recent data suggest that preserved telomerase function is essential for the maintenance of cardiovascular homeostasis and prevention of cardiovascular aging and disease. For example, cellular senescence of aged endothelial cells (EC) and vascular smooth muscle cells (VSMC) plays an important role in the early stages of a developing vascular lesion that ultimately leads to an atherosclerotic plaque (94–96). Preclinical studies suggest that differential regulation of telomerase can delay or even reverse the senescent phenotype of aged vascular cells and restore the physiological function relevant to these cells (63, 97, 98). This section will expand upon this emerging paradigm and highlight the involvement of telomerase in promoting physiological cardiovascular health by describing the differential roles of TERT in SMC and vascular ECs; the dysregulation of telomerase and associated cardiovascular pathologies; and pharmacological tools to activate telomerase to combat cardiovascular aging and disease (Figure 1).

4.2. TERT in endothelial cells

Telomerase contributes to preservation of endothelial homeostasis via repression of inflammation, oxidative stress, and senescence. Many studies of gain- or loss- of activity have described the important role that telomerase plays in maintaining the proliferative potential and viability of endothelial cells (EC)(58–62). After ischemia-reperfusion injury, vascular regrowth via angiogenesis and collateral formation are key factors in reestablishing blood flow into the injured area (63). Pallini et al. (64) have described a direct correlation between hTERT mRNA expression in ECs of newly formed vessels and the histological grade of human tumors, thus supporting a role of telomerase in angiogenesis. In addition, overexpression of TERT in human dermal microvascular EC improves the formation of durable microvascular structures in immunodeficiency mice (65) and the endothelial progenitor cell's proliferative and migratory capacities and survival (60).

Telomerase's ability to promote endothelial homeostasis is not limited to its angiogenic effects. Aging induces phenotypic changes within ECs to an activated inflammatory state by expressing markers such as VCAM-1 and ICAM-1 and cytokines (99). These changes are accelerated at sites of disturbed flow such as the iliac artery bifurcation, where the telomeres of human EC are demonstrably shorter (100) and number of senescent EC is increased, suggesting an association between reductions in telomerase activity and corresponding vascular aging in the endothelium (101, 102). This accelerated aging at vascular bifurcations may be due in part to the hemodynamic activation of inflammation in ECs by low and oscillating shear stress (103, 104), which contributes to the formation of atherosclerotic plaques at these sites (105). Studies suggest that this pathologic cycle could potentially be reversed by telomerase activation, which leads to reductions in vascular inflammation (97, 106). Our lab has further demonstrated that the activation of telomerase plays an important role in protection against mitochondrial ROS production in endothelial cells and therefore increases the bioavailability of NO in the human microcirculation (Figure 1) (3). In addition, aged human aortic EC manifest many attributes of a senescent vasculature, including reduced ability to proliferate and respond normally to shear stress, to generate NO, and to resist adhesion of leukocytes. Interestingly, overexpression of telomerase is able to reverse ECs' senescent characteristics and retrieve their primary phenotype as demonstrated by the

ability to generate NO (62) and maintenance of endothelial proliferative, migratory and survival capacities (53). These findings suggest that activation of telomerase may serve as a means to counteract the elevated ROS production and inflammation seen in the vascular endothelium in diseases like atherosclerosis.

ii. TERT in vascular smooth muscle cells—Although telomerase activation is desirable in the vascular endothelium due to its antioxidant, anti-inflammatory, and pro-survival effects, this same strategy may not be as useful in the vascular smooth muscle layer. Aged VSMC undergo a switch from a differentiated to a proliferative secretory phenotype that leads to medial thickening, loss of elasticity, and sclerosis of the media (107) and contributes to the pathogenesis of many cardiovascular diseases. Excess VSMC proliferation pathways remain relatively understudied and infrequently targeted for therapies. TA has been shown to be involved in VSMC proliferation. Indeed, it has been demonstrated that increased TA and telomere length play a causal role in the abnormally increased proliferation of VSMC in genetically hypertensive rats (108). Relevant to its role in vascular disease, TERT is activated by mitogens, which are upregulated in diseases characterized by VSMC proliferation (56) (Figure 1). In spontaneously hypertensive rats (SHR), both telomerase protein expression and activity are induced in the aorta but not in other tissues before the onset of hypertension, and this correlates with a maintained telomere length and increased medial SMC proliferation (66). The inhibition of TERT using antisense RNA caused apoptosis in cultured SMCs through a p53-dependent mechanism. It was concluded that TERT activation in aortic SMC affects the balance between the cell proliferation and apoptosis resulting in the vascular remodeling observed in genetic hypertension. This evidence suggests that activating TERT in SMC allows for pathologic remodeling associated with the development of hypertension.

Therefore, global telomerase activation, though useful in the endothelium, may pose a problem in VSMCs. We believe that a possible solution to this problem is to exploit the distinct mitochondrial and nuclear roles of telomerase. The pro-proliferative function of telomerase in VSMCs is likely the result of its nuclear, telomere-lengthening activity. We propose that a targeted approach in which telomerase's antioxidant *mitochondrial* function alone is enhanced – without elevation in nuclear telomerase activity – will decrease oxidative stress without increasing pathological VSMC proliferation. Exploring this more targeted, compartmentalized approach is a necessary next step.

4.3. TERT and vascular ROS

Turning away from the canonical, pro-replicative effects of TERT in VSMCs, recent reports highlight the extranuclear, mitochondrial ROS-detoxifying effects of TERT in the vasculature. Reactive oxygen species (ROS) are formed as a byproduct of the normal metabolism of oxygen in the mitochondria, and ROS production increases in response to cellular stress. In physiology, ROS have important roles in cell signaling and homeostasis (67). However, during times of environmental stress, ROS levels can increase dramatically and result in oxidative stress, leading in the short term to a variety of cellular stressors including mitochondrial dysfunction and in the long term to a pathological state represented by aging, senescence and cardiovascular diseases (68, 69).

Clinical and basic science studies have highlighted the importance of short telomeres and impaired mitochondrial function in driving the age-related functional decline in the cardiovascular system (70–72). Telomerase appears to be critically involved in these pathologic changes associated with aging. The TERT knockout model develops systemic mitochondrial defects (73) supporting a direct role for TERT in mitochondrial function or regulation. Moreover, the ability of the catalytic subunit of TERT to translocate from the nucleus to the mitochondria following drug treatments or increase of oxidative stress (69, 74–76) opens a new window of putative pathways for cellular compartmentalization of the effects of TERT and suggests a distinct mitochondrial function of telomerase.

The role of TERT in the mitochondria is particularly focused on protection against oxidative stress. In addition to its role in *mt*DNA repair (77), it was observed that cells overexpressing TERT display improved respiratory chain function by enhancing complex I activity (75) and complex IV activity (cytochrome c oxidase) (68) and reduced ROS production during oxidative stress. It was also confirmed that these cells overexpressing TERT show a higher resistance to H₂O₂-induced apoptosis (75) (68). Therefore, regulating the mitochondrial-specific role of TERT will be a promising target for achieving breakthroughs in the development of therapeutic telomerase treatments in human aging and related diseases without producing telomere-lengthening, tumorigenic effects.

4.4. TERT and cardiovascular disease

Telomerase is essential to overall cardiovascular health, and loss of telomerase is implicated in several disease processes. Decreased TA is intimately linked to the senescent character of EC and SMC in the atherosclerotic plaque (8, 78) and coronary artery disease (8). Of note, the Atherosclerosis Risk in Communities (ARIC) study has shown that TERT polymorphisms are associated with increased risk of incident coronary heart disease, incident ischemic stroke, and mortality in African-Americans participants (79). Stress reduction and lifestyle modification in this same high risk population increase telomerase gene expression and produce an associated reduction in blood pressure (BP), suggesting that enhanced telomerase gene expression may either be a biomarker for reduced BP or a mechanism by which stress reduction and lifestyle modification reduces BP (80). Moreover, telomerase deletion increases endothelin ET-1 levels in the plasma suggesting that loss of telomerase may contribute to increased vasoconstriction and hypertension (81). Despite this overarching link between reduced telomerase function and cardiovascular disease, the tissue-specific contributions (SMC vs. EC; and others) of telomerase have yet to be determined on a physiological/organism level.

4.5. TERT-activating Therapeutics

The previously described dichotomy detailing the protective role of telomerase in ECs and pathological role in VSMCs, as well as the differential nuclear and mitochondrial functions of telomerase, may help to explain the seemingly conflicting reports about the benefit of telomerase activation versus inhibition in the cardiovascular system. Some lines of evidence suggest that inhibiting telomerase *in vivo* contributes to the development of vascular maladies, but the exact mechanism is controversial. One source reports that decreased TA is associated with inflammation and atherosclerosis (8, 70, 82). On the other hand, others show

that loss of telomerase function appears to be protective in a mouse model of atherosclerosis (9, 83). Perhaps these conflicting reports relate to an inability to differentiate between nuclear and mitochondrial roles of TERT or localization of effects to the intimal or medial vessel layer. We propose that activation of TERT in the vasculature is desirable due to the large number of studies reporting a protective effect of TERT activation, realizing, however, that the most effect therapeutic approach will likely involve development of novel agents that can more specifically target telomerase function in the nucleus versus the mitochondria. Fortunately, several strategies exist to achieve such global TERT activation in the vasculature (Table 2).

In this context, a very important clinical study conducted over a 5-year period involving an estimated 7000 person population showed interesting results about the effect of TA-65[®], a natural product-derived telomerase activator, on metabolic markers and cardiovascular health. In addition to apparent positive immune remodeling in these patients, TA-65[®] treatment has shown an improvement of metabolic markers with a decrease in the fasting glucose, insulin levels, total cholesterol and low-density lipoprotein cholesterol. In parallel, the systolic and diastolic blood pressures of these patients were significantly ameliorated after treatment. These results suggest that telomerase activation is a rejuvenation strategy for age-associated diseases such as cardiovascular diseases and might prove a therapeutic adjunct or alternative in this setting (84).

The use of thiazolinediones (TZD) may hold real promise for a solution to the differential role of telomerase in the intimal and medial layers through activation of telomerase in ECs and inhibition of telomerase in VSMCs. In the past 15 years, Peroxisome proliferator-activated receptor gamma (PPAR γ), a member of the nuclear receptor superfamily, has emerged as an important player in vascular protection. PPAR γ is expressed in both vascular endothelial and smooth muscle cells, and shown to be critically involved in the development of vascular complications and inflammation and hypertension (85–87). In fact the anti-proliferative, anti-atherosclerosis properties of PPAR γ have been shown to suppress VSMC proliferation, which could be at least in part mediated by its effects on suppression of telomerase activity (pro-proliferation). This is supported by the findings that PPAR γ activation suppresses telomerase in cultured VSMC (88). As PPAR γ is well established to reduce ROS production it is further intriguing to think that these effects in the EC are mediated by TERT upregulation. In cultured endothelial cells, stimulation of PPAR γ with pioglitazone (TZD) improve endothelial stress resistance and reduces susceptibility to senescence stimuli by activating telomerase and reducing senescence marker expression (Table 2) (89). Recent data by Durand et al (90) (supplemental material) further support this notion, as Rosiglitazone restored normal NO mediated, endothelium-dependent dilation in vessels from subjects with CAD (normally mediated by H₂O₂) in a telomerase-dependent manner. The underlying mechanisms determining how PPAR γ differentially regulates TERT, dependent on cell type, has yet to be determined.

An alternative strategy to target telomerase in the vasculature is through endogenous regulators of TERT. One such promising protein is PGC-1 α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha), which, in addition to inducing mitochondrial biogenesis and antioxidant gene expression, is an upstream regulator of TERT with known

beneficial cardiovascular effects (91). Of note, upstream regulation of TERT by PGC-1 α affects both the nuclear and mitochondrial programs of TERT – maintaining telomere length and combatting oxidative stress, respectively. In contrast, downregulation of PGC-1 α exacerbates vascular aging via increased telomere shortening as a result of TERT downregulation. The simultaneous impact on telomeres in the nucleus and mitochondrial oxidative stress pathways underscores the link between these two cellular compartments and reinforces the extra-nuclear role of telomerase. Further justification for targeting TERT *via* manipulation of PGC-1 α , instead of TERT itself, arises from the realization that upregulation of PGC-1 α provides the opportunity to influence additional pathways, such as inflammation (92) and the PPAR- γ pathway outlined above to boost therapeutic potential. Several tools already exist to upregulate PGC-1 α levels, including exercise (93, 94), calorie restriction (95–97), lipoic acid (98), and ZLN005 (a novel transcriptional activator) (99). There is some evidence that increasing PGC-1 α leads to increased tumorigenesis (100), possibly through effects on the nuclear-specific immortalizing effects of telomerase activation, although a recent report describes an anti-tumorigenic role for PGC-1 α (101). Therefore, caution must be used when overexpressing PGC-1 α , and investigators should attempt to discover if there is a critical level of PGC-1 α below which TERT upregulation can be produced to produce ROS detoxification without concomitant tumor-promoting effects. It may also be possible to upregulate PGC-1 α while simultaneously inhibiting the nuclear-specific, telomerase-lengthening actions of TERT to provide cardiovascular protection while avoiding increased susceptibility to cancer development. Additionally, this protective PGC-1 α -TERT pathway must be confirmed in human subjects.

Other reports showed that resveratrol, a type of natural phenol present in some fruits, activates the catalytic subunit of telomerase in human aortic SMC and pulmonary microvascular endothelial cells (Table 2). Similar observations were obtained in resveratrol treated C57BL/6J mouse heart and liver tissues (102). Resveratrol has been shown to produce changes associated with longer lifespan, including increased insulin sensitivity, PGC-1 α activity and mitochondrial number (103). Interestingly, elderly mice fed with resveratrol showed a marked reduction in signs of aging with decreased inflammation and vascular endothelium senescence, and increased aortic elasticity (104). Despite the fact that the exact mechanism by which resveratrol induces TA remains unknown, these findings suggest a strong link with its beneficial effect in anti-aging processes in cardiovascular cells affected by disease.

Another chemical compound, AGS-499, has emerged in the last five years showing neuroprotective effects in the amyotrophic lateral sclerosis (ALS) disease animal model via increased TA. *In vivo* treatment with AGS-499 has increased significantly TA in these animals and improved their life-span (Table 2) (53). Furthermore, AGS-499 treatment, without altering their functionality, protected stem cells from apoptosis and DNA damage produced by long-term exposure to oxidative stress (6). We have also demonstrated that acute activation of telomerase using AGS-499 restored NO bioavailability and limited ROS production in micro-vessels from subjects with coronary artery disease (105).

5. Summary

Currently, the role of telomerase in cancer and aging is heavily investigated. Existing dogma states that telomerase contributes to cancer progression and proliferation but also defends against cellular and organism aging. Increasing awareness of the non-nuclear contributions of telomerase may provide a solution for this therapeutic paradox. Activating mitochondrial-targeted telomerase (protective with against oxidative stress and aging-related phenotypes including cardiovascular disease) and inhibiting nuclear telomerase (protective against excessive cell proliferation) may allow clinicians to circumvent the off-target cardiovascular effects of current chemotherapeutic telomerase inhibitors while providing cardiovascular protection. Such an approach may also function as a new therapy for cardiovascular disease. It remains to be determined whether this novel paradigm can be translated directly to conclude that increased mitochondrial TERT improves quality of aging without unwanted side effects of nuclear telomerase activation in humans. Novel pharmacological tools and animal models are needed to address this pressing question as to date there are only limited means to modulate TA on a subcellular levels using either genetic or pharmacological approaches. Generation of subcellular modulators of TERT capable of specifically targeting nuclear versus mitochondrial TERT appears to be a promising next step with the potential to improve cancer therapy and extend such therapies into new contexts to combat chronic diseases.

Acknowledgments

The authors appreciate the critical review provided by Elisabeth Jacobs, MD and David D. Gutterman, MD.

7. Sources of Funding

This work was supported by the National Institutes of Health Grant R21-OD-018306 to AMB and American Heart Association Grant 16POST26430075 K.A.-A.

Non standard abbreviations

ALS	amyotrophic lateral sclerosis
ALT	alternative telomere lengthening mechanisms
ARIC	Atherosclerosis Risk in Communities
BP	blood pressure
CAD	coronary artery disease
CML	chronic myelogenous leukemia
CVD	Cardiovascular disease
EC	endothelial cells
ET-1	Endothelin 1
GWAS	Genome wide association studies

H₂O₂	Hydrogen peroxide
ITGB1	integrin beta 1
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NO	Nitric Oxide
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
ROS	reactive oxygen species
SHR	spontaneously hypertensive rats
TA	Decreased telomerase activity
TERT	telomerase reverse transcriptase
TGFB	transforming growth factor beta
TZDs	Thiazolidinedione
VSMC	Vascular smooth muscle cells

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Figure 1. Differential regulation of telomerase within vascular tissue

Within the Endothelial cells (EC), increased telomerase activity mediates increase of nitric oxide (NO) bioavailability, antioxidant levels and anti-senescent phenotype and therefore prevents the progression of atherosclerosis. Unlike the EC, telomerase activated in smooth muscle cells (SMC) increases proliferation-induced hypoxia and promotes atherosclerosis progression.

Figure 1. Telomerase regulation under cardiovascular diseases vs Cancer

By its mitochondrial targeted ($mtTERT$) function, telomerase activity induces beneficial and protective effects during cardiovascular disease. While in cancer, its nuclear targeted ($nucTERT$) inhibition is necessary to reverse the immortal, proliferative and chemo-resistance phenotype.

Table 1

Selected Examples of Telomerase Inhibitors

Compound	Study	Mechanism	Effect on CV	Reference
NRTIs/NNRTIs	Human	Nuclear localization	<ul style="list-style-type: none"> Increases LDL and triglycerides Increased risk of cardiomyopathy 	(106)
BIBR1532	Human tissues/cells	Post-translational	<ul style="list-style-type: none"> Increases oxidative stress Decreases NO bioavailability in favor of H₂O₂ 	(3)
GRN163L (imtelstat)	Human/Mice	Post-translational	<ul style="list-style-type: none"> Hematologic responses Elevates liver enzymes Reduces Hypoxia-induced pulmonary hypertension in SMC 	(107, 108)
GV1001 & Other anti-telomerase vaccines	Human	Vaccine	<ul style="list-style-type: none"> None reported 	
Selected Tyrosine Kinase Inhibitors	Human	Off Target Effect related to telomerase inhibition (potentially)	<ul style="list-style-type: none"> Imatinib, dasatinib, nilotinib, sunitib, sorafenib, lapatinib Congestive heart failure ECG changes Decrease LV ejection fraction 	(109)

Table 2

Different means of telomerase activation in the cardiovascular system

Compound	Study	Pathway	Effect on CV	Reference
TA-65®	Human subjects	Transcriptional	<ul style="list-style-type: none"> Improves immune remodeling Decreases fasting glucose, insulin levels, total cholesterol and LDL. Ameliorates blood pressure 	(84, 110)
TZDs	Human samples/Mice	Transcriptional	<ul style="list-style-type: none"> Increases resistance to Oxidative stress Improve EC resistance to senescence Reduces ROS production and inflammation Enhance NO production 	(89, 111–114)
Resveratrol	Human cells/Mice	Post-translational	<ul style="list-style-type: none"> Delays aging-related deterioration Increases the mitochondrial number Increases the insulin sensitivity Decreases inflammation, and apoptosis in the vascular endothelium, Increases aortic elasticity 	(102–104)
AGS-499	Human tissues/Mice	Transcriptional	<ul style="list-style-type: none"> Neuroprotective effect Protects from apoptosis and DNA damage Normalizes the FMD and ROS production 	(3, 6, 53)