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Up-Regulating Telomerase and Tumor Suppression: A Two-Step Strategy to Boost Hematopoietic Stem Cell Transplantation

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Abstract

The high levels of morbidity and mortality of chronic non-communicable diseases (most of which highly associated with aging) worldwide indicate the need of studying the underlying mechanisms of physiological aging and aging related-impairments, as well as developing and improving therapeutic approaches such as cell therapy. In this manuscript, two well-established aging mechanisms – telomere shortening and DNA damage accumulation – are briefly reviewed regarding their roles in hematopoietic stem cells function and transplantation. Based on the available literature, up-regulating both telomerase and tumor suppression responses is proposed in a two-step strategy as a promising mechanism to benefit hematopoietic stem cell transplantation qualitatively (by enriching the cell pool for healthy hematopoietic stem cells) and quantitatively (by *in vitro* expansion of healthy hematopoietic stem cells). The applications, limitations and perspectives associated with the proposed strategy are also discussed.

Keywords: Telomere length; Telomerase; DNA damage; Tumor suppression; Hematopoietic stem cells

Abbreviations: ASCs: Adult Stem Cells; HSCs: Hematopoietic Stem Cells; HSCT: Hematopoietic Stem Cell Transplantation

Introduction

The recent trends in the age structure of most populations worldwide has changed the scenario of common causes of morbidity and mortality, with chronic non-communicable diseases (e.g., cardiovascular diseases, cancer and diabetes) featuring as (or among) the most frequent ones [1]. Currently, such diseases are considered a public health concern, indicating the importance of elucidating the mechanisms implicated in physiological aging and aging-related impairments. Such elucidation may contribute to the development and improvement of new therapeutic strategies for age-related diseases, with cell therapy featuring among the most promising approaches [2]. Regarding aging physiology and associated diseases, telomere biology is among the most relevant molecular mechanisms [3]. Briefly, telomeres are guanine-rich (5' TTAGGG 3' in humans) DNA tandem repeats located at the end of eukaryotic chromosomes [4] associated with at least six proteins, (called shelterin) which compose the telomeric structure [5]. The telomeres have several important roles, such as preventing both the recognition of chromosome ends as sites of DNA damage and the occurrence chromosome end fusions [6]. An important aspect of telomere biology is that the telomeres are shortened after a cell division due to the end replication problem (i.e., the incapacity of the replication machinery to replicate the ends of eukaryotic chromosomes) [7], resulting in telomere dysfunction with time. Since telomere dysfunction elicits tumor suppression responses, critical telomere shortening results in loss of cell viability by either senescence or apoptosis [8].

In spite of telomere shortening being a natural consequence of DNA replication, telomerase activity counteracts the end replication problem by promoting reverse transcription-based telomere lengthening. Telomerase is a ribonucleoprotein enzymatic complex composed by two main subunits: the telomerase reverse transcriptase (the catalytic subunit, encoded by *TERT* in humans and *Tert* in mice – GeneEntrezIDs: 7015 and 21752, respectively) and the telomerase RNA component (the RNA template for reverse transcription, encoded by *TERC* in humans and *Terc* in mice – GeneEntrezIDs: 7012 and 21748, respectively) [9,10]. The relevance of telomere biology (especially of telomerase) for human health and disease is such that the 2009 Nobel Prize in Medicine and Physiology was awarded to telomerase discoverers [11]. Telomerase activity is present in primitive cell types, such as embryonic stem cells, germline stem cells and adult stem cells (ASCs) [12-14]. In the last, however, telomerase activity is sufficient only to delay telomere shortening [15,16], thus resulting in ASCs eventually reaching a critical telomere length state after several cell divisions. Given the importance of ASC to maintain homeostasis by allowing tissue self renewal during the lifespan, telomere-related ASC exhaustion is currently among the best established aging mechanisms. In disease, telomerase activity plays a major role in cancer, being present in 85-90% of human cancers [17,18]. This makes telomerase one of the most prevalent cancer makers and indicates that telomerase activation is the most frequent mechanism of replicative immortality. It is important to note, however, that telomerase activity is commonly regarded as a consequence of genetic instability rather than an early event in the carcinogenic process. Interestingly, telomere dysfunction is considered an early cancer event that is highly associated with genetic instability [19], indicating that telomere length/integrity, telomerase activity and tumor suppression responses interact in a complex fashion, which may regulate their association with ASC impairment and cancer [20].

Telomere Biology in Hematopoietic Stem Cell Physiology and Transplantation

Considering the importance of telomere biology for tissue self-

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renewal by regulating ASC viability, it is intuitive that telomere-associated diseases are most prominently manifested in high turnover tissues, such as the skin and the blood. In the hematopoietic compartment the cell turnover is estimated around 10^9 cells produced per hour [21] and is known to be highly dependent on hematopoietic stem cells (HSCs) [22]. In telomere syndromes (phenotypes – normally a disease-related state – caused by genetic profiles associated with telomere biology [23]) such as dyskeratosis congenita, blood-related manifestations are particularly common, being bone marrow failure the most common cause of death (approximately 60% to 70% of cases) [24]. These manifestations are well-characterized consequences of HSC exhaustion due to premature critical telomere shortening, resulting in dyskeratosis congenita being considered a stem cell disease [25]. Although dyskeratosis congenita is not a particularly frequent condition, it serves as an useful model for understanding telomere biology implications for disease [26]. Regarding stem cell therapy and tissue engineering, the notion that HSCs are especially dependent on telomere integrity due to their very high turnover strongly suggests that HSC transplantation (HSCT) has the potential to be improved by exploring telomere-related strategies.

There is a number of studies that investigated the roles of telomere biology in HSC. As pointed by others [23], there is solid evidence (from investigations in both humans and mice) that quantitative and qualitative defects in HSCs caused by telomere shortening result in HSC exhaustion, and a significant portion of such evidence came from studies on telomere syndromes [27-33]. Regarding the importance of telomere biology for HSCT, one of the earliest studies provided convincing evidence for telomere shortening in HSC *in vivo* by doing serial transplantations in murines [34]. In the following year, two studies added further evidence to this notion: one study showed that neutrophil telomere length was shorter in human HSCT recipients (when compared to their donors) at engraftment and 6 and 12 months after HSCT [35]; the other study evidenced, in mice, that stimulated T cells present telomere lengthening by telomerase activity, suggesting that telomerase in HSCs may be important to extend proliferative capacity after transplantation [36]. By this time, the potentially benefic effects of overexpressing *TERT* to enhance HSC replicative potential during transplantation were already recognized [37] due to the understanding that the limited proliferative potential of HSC associated with short telomeres being a relevant concern regarding *ex vivo* HSC expansion for therapeutic purposes [38]. A subsequent study reinforced the notion that transplanted HSCs suffer replicative stress due to telomere shortening, resulting in accelerated senescence [39]. In addition, two key studies provided direct evidence for the potential of telomerase activation to improve HSCT. By comparing wild type and telomerase-deficient mice donors, it was shown that telomerase allows HSC to be viable after more rounds of serial transplantation and that telomerase counteracts telomere shortening that occurs during transplantation [30]. The second study overexpressed (using retroviral vectors) *TERT* in $CD34^+$ and $AC133^+$ cord blood cells and provided evidence for a role of telomerase in HSC proliferation and differentiation abilities [40].

DNA Damage Implications for Hematopoietic Stem Cells Culture

It is well evidenced that telomeres and telomerase have major roles in HSC senescence and that such roles can be exploited in clinical applications of HSC by approaches based on telomere biology manipulation in order to delay HSC replicative exhaustion [41]. It is important to note, however, that telomere shortening is not the only mechanism proposed for organism aging, which is currently considered a multifactorial trait [42]. Among such mechanisms there is DNA damage accumulation [43,44], which is of special relevance for this manuscript since it has been studied in HSCs. By observing HSC of mice deficient for different genomic maintenance pathways (including nucleotide excision repair, telomere maintenance and non-homologous end-joining), it has been shown that accumulated DNA damage functionally impairs HSC (but does not depletes HSC reserves) with age, culminating with HSC functional exhaustion. Evidence for DNA damage accumulation in wild type HSCs has also been provided by this study, indicating that such event occurs physiologically [33]. These findings are in agreement with another study using a mice model for Ligase IV syndrome, which are deficient for DNA double-strand break repair by non-homologous end-joining. Based on their findings, the authors suggested that HSCs sensitivity to non-homologous end-joining deficiency (a conclusion extensible to other types of DNA damage accumulation) is a key factor for HSCs to withstand culture and transplantation [45]. The findings of these studies, which have been corroborated and complemented by additional investigations [46], provide strong evidence for the notion that genomic stress is a causal factor of HSCs aging by limiting their ability to maintain tissue homeostasis [47]. In addition to the diminished capacity of HSC caused by DNA damage accumulation, it is important to note that such event is among the most well-established cancer mechanisms [48,49]. This reasoning suggests that *in vitro* culturing HSCs naturally induces their decay (which is well established by the literature, as discussed in the present text) and may result in accumulation of potentially dangerous characteristics in the context of cancer.

The fact that telomerase is associated with cancer is a significant concern for the use of telomerase activation in cell culture with clinical purposes. As discussed elsewhere [50], there are alternatives to reduce this risk, such as using moderate and transient *TERT* up-regulation by chemical activators instead of lentivirus-based systems and co-treatment with differentiation agents (which would not apply to HSCT). However, it is important to not underestimate the cancer risk associated with telomerase, especially when considering the emerging evidence for non-canonical (i.e., telomere-independent) roles of telomerase in processes such as apoptosis resistance [51,52], DNA damage repair [53] and in cancer-related signaling pathways [54]. Interestingly, *TERT* up-regulation has also been shown to reduce intracellular reactive oxygen species production [55,56], indicating that telomerase activity stimulation by *TERT* up-regulation may be accompanied by antioxidant defenses, which has potentially beneficial applications for HSC *in vitro* culture. Such application is to be cautiously considered, however, since reducing oxidative stress could potentially lead to maintenance of certain injuries/damages that would normally be eliminated from the HSC pool by triggering intracellular reactive oxygen species production that results in cell cycle arrest or death.

A Two-Step Strategy for HSC *In Vitro* Expansion: A Hypothesis to Boost HSCT

Tumor suppression responses are known (mostly from cancer research) to be key factors to prevent or reduce DNA damage accumulation along cell divisions [49]. Tumorigenesis is characterized by increased mutability rates, which is significantly favored by compromising the tumor suppression machinery that monitor genomic integrity and detect DNA damage, triggering either senescence or apoptosis [57-59]; and *in vivo* experiments confirm the implications of such pathways for cancer [60]. Interestingly, tumor

suppression responses have been evidenced to prevent (or at least reduce) accumulation of cells with DNA damage within a given ASC pool, since damaged ASCs undergo either senescence or apoptosis [61]. All this evidence supports the notion of up-regulating both telomerase activity and tumor suppression responses in order to increase cell and organism life-span without increasing cancer risk.

Such hypothesis has been addressed in an elegant experiment using mice that overexpressed either *Tert* (in the epithelia, regulated by *Krt5 – GeneEntrezID: 110308 – promoter* or tumor suppression genes, and a group that overexpressed both (called the SUPER-M mice) [62], resulting in mice with less aging but no increase in cancer rates [63]. The median survival values (both overall and cancer-free) for each mice group are shown in figure 1. These results, in light of the biology already elucidated, allowed the proposal of new stem cell-based models for aging, focusing on the roles of telomerase and tumor suppression [64]. In fact, the median survival values shown in figure 1 indicate that telomeres and tumor suppression are effect modifiers of each other, since the median survival increase of the SUPER-M mice compared to the control group is greater than the simple addition of the median survival increase due to overexpression of *Tert* and tumor suppressors individually, also compared to the control group (which can be easily identified by comparing groups 1-3 and 2-4, from top to bottom). This evidence for effect modification strongly suggests that telomerase and tumor suppression interact in a complex fashion [20], and such interaction can be exploited in cell culture with clinical application purposes.

The evidence discussed so far strongly suggests that telomerase activation and tumor suppression have significant implications (either individually and combined, with evidence for interaction between the two mechanisms) for *in vitro* culturing of HSCs, which is required for many of the potential clinical applications regarding cell therapy. In a two-step strategy to boost HSCT by selecting and expanding healthier (i.e., cells with reduced DNA damage – and other markers of stress – accumulation and with relatively long telomeres) HSCs based on up-regulation of both telomerase activity and tumor suppression responses (Figure 2). According to this strategy, the isolated HSCs are basically a pool of cells with different degrees of damage accumulation (according to, for example, donor's age and lifestyle). By up-regulating tumor suppression responses, the probability of detecting such damage and, consequently, of eliminating damaged cells from the HSC pool by senescence or apoptosis, increases. Importantly, there is no telomerase

Mice strain	Median survival	
	Overall	Cancer-free
Wildtype →+Telomerase	9.0	18.0
Wildtype →+Tumor suppressors	11.3	8.7
+Tumor suppresors→ SUPER-M	26.0	38.0
+Telomerase → SUPER-M	28.6	27.1
Wild-type→ SUPER-M	40.2	50.0

Figure 1: Wildtype: Sp53 mice. +Tumor suppressors: Sp53/Sp16/SArf mice. +Telomerase: Sp53/ TgTert. SUPER-M: Sp53/Sp16/SArf/TgTert. The values in each cell represent the median increase in survival, comparing the two strains (reference strain → group of interest). The values for the "Wildtype →**+**Tumor suppressors" and "**+**Telomerase → SUPER-M" comparisons were calculated based on the values for the other comparisons. Blue boxes: overall median survival. Red boxes: cancer-free median survival.

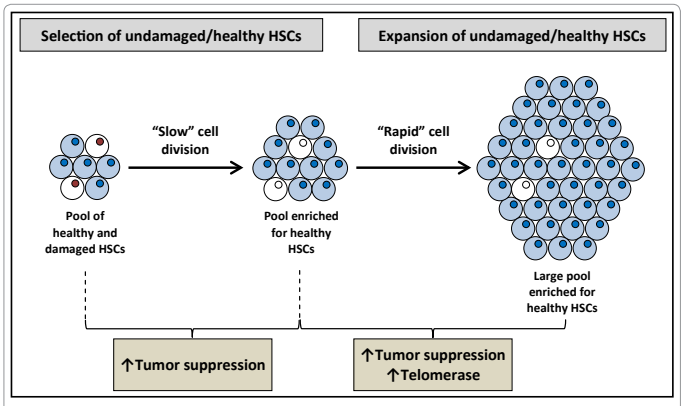

Figure 2: The figure is a simplified illustration of the two-step strategy to boost HSCT by culturing HSCs under up-regulation of telomerase activity and tumor suppression responses. Blue cells: healthy cells. Red cells: damaged cells. Colorless cells: damaged cells that have been sensed and cleared from the HSC pool by the tumor suppression machinery.

up-regulation in this step, which reduces the chances that telomerase activity confers apoptosis resistance to some stress markers present in the HSC pool (since damaged HSCs are likely to be sensed and eliminated by the tumor suppression machinery). This process results in a HSC pool enriched for healthy cells, which can be expanded by serial passages under up-regulation of telomerase activity and tumor suppression responses, in order to avoid undesirable HSC telomere shortening (or even promote HSC telomere lengthening) and protect the cell pool from accumulating stress markers (given the evidence for protective roles of both telomerase activity and tumor suppression responses).

Conclusions: Applications, Limitations and Perspectives

The relevance of the proposed strategy lies on the importance of HSCT and to the plausible generalizability of the benefits of this approach to culture other types of cells (as well as to other applications using HSCs). HSCT is the first form of stem cell therapy used in human medicine [65], and its current applications are not limited to myeloma or leukemia patients [66,67], with therapeutic uses for auto-immune and cardiovascular disease [68,69]. HSCT requires isolation of HSCs, but does not select for healthy cells [70]. The proposed strategy allows expanding isolated HSCs in a selective condition that enriches for healthy cells, thus benefiting HSCT both quantitatively and qualitatively. Moreover, the strategy may also enlarge the range of potential donors. For instance, HSCT using HSCs from old donors are known to increase the risk of graft-versus-host disease and decrease probability of survival [71,72]. Considering that DNA damage accumulation and telomere shortening are aging hallmarks (as already discussed here), it is plausible to hypothesize that applying the two-step strategy would alleviate HSCT difficulties associated with old donors. In this regard, telomere shortening has been associated with other HSCT issues, including graft failure [73]. This is an important consideration given the advantages of receiving HSCs from a close relative (such as a sibling or a parent) regarding graft-versus-host disease risk [74], since it might well be the case that the closest relatives are in the risk age group regarding HSCs donation. Moreover, a recent study suggests that long-term immune reconstitution after haploidentical HSCT (which is a good alternative for patients lacking a human leukocyte antigen-matched donor [75]) largely depends on *de novo* T cell production, with implications for telomere length since naive-enriched $CD4^+$ T cell populations of HSCT recipients presented shorter telomeres than age-matched controls,

although telomere length was similar in differentiated $CD4^+$ and $CD8^+$ T cells [76].

In spite of the proposed strategy being potentially beneficial for HSCT and other cell therapy applications, it is important to note some of its limitations. First, there are limitations of HSCT that are unlikely to be alleviated by enriching the HSC pool for healthy cells. For instance, although aplastic anemia (a condition associated with short telomeres) can be treated by allogeneic stem cell transplant in humans [23], a recent study on the effectiveness of allogeneic HSCT in 34 dyskeratosis congenita patients reported a 10-year probability of survival of 30%, with ten deaths occurring until 4 months after HSCT due to graft failure (in 6 cases) or other transplant-related complications [77]. Although 9 of these patients had mismatched related or unrelated donors, an additional possibility comes from studies that showed that telomere dysfunction in telomerase-deficient mice induces alterations of the hematopoietic environment, resulting in HSC impairments (including HSC function, engraftment, and B and T lymphopoiesis) independently of the telomere length of HSCs themselves [78-80]. These studies suggest that, for patients with particularly short telomeres (e.g., very old patients or cases of telomere syndromes), the engraftment might be complicated due to the recipient's hematopoietic environment, thus reducing the importance of the quality of the HSCs used for transplantation. Another important consideration is related to DNA damage accumulation of HSCs. There are components other than surveillance mechanisms that compose the DNA maintenance machinery, such as direct DNA damage repair and inactivation or interception of mutagenic molecules before they cause DNA damage [49] and, as discussed here, the former has been strongly evidenced to occur in HSCs as a causal factor of aging. Nevertheless, these two limitations are unlikely to undermine the usefulness of the proposed strategy, since using healthier HSC for HSCT would be beneficial for several applications and the results of the SUPER-M mice study indicate that up-regulating telomerase and tumor suppression (without specifically targeting other pathways associated with DNA maintenance) are sufficient to reduce/delay the occurrence of aging phenotypes at no (significant) increase in cancer risk.

Although the proposed model follows logically from the current knowledge regarding telomere biology and DNA damage accumulation in HSCs, it has not been experimentally tested to date. An important perspective would be to simulate (in mice, for example) different conditions of common application of HSCT in order to verify whether or not (and to what extend) the two-step strategy actually provides better results than standard HSCT. One of the most interesting situations would be to compare the effect of the proposed strategy on HSCT having donors that are likely to have less healthy HSCs, such as late-generation telomerase-deficient mice that had telomerase restored (by using a Cre-LoxP recombination system, for example) or mice subjected to different types of unhealthy factors (e.g., high-fat diet). Another critical perspective concerns the methods for up-regulating telomerase and tumor suppression responses, since temporary modulators (such as molecules added to the culture medium) would be preferred over approaches such as lentivirus-mediated transfection. In this regard, chemical modulators of telomerase activity [81-83] and of p53 (a major tumor suppressor protein [84], encoded by *TP53* in humans and *Tp53* in mice – GeneEntrezIDs: 7157 and 22059, respectively) activity [85] have been identified and studied, showing *in vitro* and *in vivo* activity. It is important to consider, however, the of using genetically engineering HSCs in order to greatly up-regulate telomerase and tumor suppression responses for HSCT procedures of more extreme cases, although the risk of such procedure is yet to be investigated. In conclusion, the proposed two-step strategy is a plausible method to enrich the HSC pool for healthy cells, which may have important beneficial impacts – quantitatively and qualitatively – for HSCT.

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