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Telomerase as a "stemness" enzyme

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Pluripotent or multipotent stem cells are involved in development and tissue homeostasis; they have the ability to self-renew and differentiate into various types of functional cells. To maintain these properties, stem cells must undergo sustained or unlimited proliferation that requires the stabilization of telomeres, which are essential for chromosome end protection. Telomerase, an RNA-dependent DNA polymerase, synthesizes telomeric DNA. Through the lengthening of telomeres the lifespans of cells are extended, or indefinite proliferation is conferred; this is intimately associated with stem cell phenotype. This review highlights our current understanding of telomerase as a "stemness" enzyme and discusses the underlying implications.

stem cells, telomerase, telomere, genomic stability, iPS cells, TERC, TERT

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Eukaryotic chromosomes terminate with tandem arrays of repetitive DNA sequences known as telomeres. This telomeric DNA, together with its binding proteins, forms a protective cap at the ends of chromosomes for maintaining genomic integrity and stability [1,2]. In human somatic cells, telomere sequences are 8−15 kb long TTAGGG repeats that shorten progressively with each round of cell division owing to the end replication problem; this shortening of telomeres commences during fetal development [1,3−5]. When telomeres become too short (dysfunctional) to protect chromosomes, the DNA damage response is activated, triggering permanent growth arrest of cells (replicative senescence) or apoptosis. Progressive telomere erosion is believed to function as a mitotic clock for controlling cellular lifespan and preventing abnormal cell proliferation [2,5]. Certain types of normal human cells exhibit strong replication potential, while malignant cells are capable of proliferating indefinitely. In all these cases, telomere shortening coupled with cell replication must be compensated for to circumvent senescence and apoptosis. Telomerase, an RNA-dependent DNA polymerase, synthesizes or extends telomeric DNA, and is widespread in those cells. It has been established that activation of telomerase is the most common strategy to stabilize telomere length in both normal and malignant cells, thereby maintaining sustained and unlimited proliferation capacity [5−7].

Stem cells are defined as cells with two important properties: they have the capacity for self-renewal; and they are able to differentiate into any somatic cell type [8,9]. In mammals, it is generally considered that there are embryonic, germinal and adult stem cells. All these stem cell types possess a remarkable ability to undergo asymmetric mitotic divisions that produce two daughter cells. Alternatively, they can undergo symmetric division in a stochastic manner to produce more stem cells and/or differentiating cells. One daughter cell possesses the properties of a stem cell, while the other differentiates to replenish specialized cell types. Embryonic stem cells (ESCs) are present in the inner cell

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mass of blastocysts and give rise to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. Germinal stem cells of the embryo undergo differentiation to generate either eggs or sperm, whereas adult stem cells (ASCs) divide and differentiate to replace damaged cells in tissues, providing the body with an internal repair system. In recent years, reprogramming technology has been used to successfully convert differentiated cells into pluripotent stem cells, which have been coined induced pluripotent stem cells (iPSCs) [10–12]. These iPSCs are very much similar to ESCs [12]. In addition to the stem cells described above, many malignancies contain a fraction of cells that have functional similarities to stem cells. These cells are usually responsible for tumor initiation, development, metastasis and recurrence; the so-called cancer stem cells (CSCs) [13,14].

Regardless of stem cell origin, sustained or indefinite proliferation potential is a key characteristic of all stem cells required for self-renewal. To achieve this, stem cells must be able to stabilize their telomere length. Accumulating evidence indicates that telomerase activation is widespread in stem cells, with telomerase levels rate-limiting for the self-renewal potential of stem cells. Therefore, telomerase is believed to act as a "stemness" enzyme. The present review summarizes our current understanding of telomerase as a stemness enzyme, and discusses its potential biological and clinical implications.

1 Telomerase activity and subunit expression in normal and cancer cells

Telomerase is a large ribonucleoprotein complex containing many known and uncharacterized elements. The core enzyme comprises telomerase reverse transcriptase (TERT), an RNA component (TERC) and dyskerin (Figure 1) [15]. TERT is a catalytic subunit, while TERC serves as the template for the addition of TTAGGG telomere repeats [6,7]. Human TERC RNA is ubiquitously expressed in various normal tissues and cells. Dyskerin, encoded by *DKC1*, is widespread in normal cells and is required for hTERC stability and recruitment [16,17]. In contrast, TERT expression is repressed in most differentiated cells during the later stages of fetal development and after birth [3,6,7]. Generally, TERT expression is associated with acquisition of telomerase activity; the introduction of TERT into telomerase-deficient cells is sufficient to activate telomerase [6,18]. Therefore, it is widely considered that TERT is a crucial rate-limiting component for controlling telomerase activity.

Telomerase activity is detectable in up to 90% of human malignancies. This is in sharp contrast to that seen in normal differentiated cells, and suggests broad re-activation of telomerase during oncogenesis [6,7]. Alterations in the levels of various telomerase components could contribute to its activation to a certain extent, however, the induction of TERT expression is the determining step for acquisition of telomerase activity during cellular transformation [6]. A close association between presence of telomerase activity and TERT expression is seen in both primary cancers and cancer-derived cell lines. Given these observations, numerous efforts have been made to elucidate how the *TERT* gene is activated during oncogenesis. Results from studies involving cancer cells have significantly contributed to the understanding of telomerase/TERT regulation in normal cells as both normal and malignant cells share considerable similarities with respect to regulating telomerase activity and TERT expression [19].

2 Telomerase in normal stem cells

Human stem cells are one of the few normal cell types that exhibit TERT and telomerase activity [20]; however, there is a significant difference in telomerase biology between ESCs and ASCs (Figure 1). *In vitro* cultured ESCs exhibit an immortal phenotype with the ability to proliferate indefinitely [21]. Consistently high levels of telomerase activity, coupled with stabilized or long telomeres, are seen in these cells. When human ESCs undergo differentiation, their telomerase activity and TERT expression is down-regulated. As a result of this down-regulation, telomere shortening can no longer be compensated for. It has been suggested that such high telomerase activity is an essential element for maintenance of the immortal and pluripotent phenotypes of human ESCs. Inhibiting TERT expression and telomerase activity led to reduced ESC proliferation, with an increased number of cells in the G1 phase and a reduced number in the S phase [22]. Suppression of TERT leads to loss of pluripotency and differentiation of human ESCs towards extraembryonic and embryonic lineages [22,23]. In contrast, ectopic TERT expression and increased telomerase activity stimulate the proliferation and colony-forming ability of human ESCs, and increase the number of cells in the S phase of the cell cycle [22].

The regulatory mechanism underlying TERT expression and telomerase activity in ESCs has recently been investigated. Krüppel-Like Transcription Factor 4 (KLF4), a core component of the pluripotency transcriptional network, plays a key role in regulating TERT transcription in human ESCs [24]. KLF4 binds to its motif on the TERT core promoter and activates TERT transcription. When KLF4 was inhibited using RNA interference (RNAi), TERT expression and telomerase activity were significantly reduced in human ESCs. Furthermore, β-catenin, another pluripotency-related transcription factor, stimulates TERT transcription by interacting with KLF4 [25,26]. β-catenin-deficient murine ESCs expressed lower levels of TERT and telomerase activity. Using RNAi to screen regulators for telomerase in murine ESCs, Counssens et al. identified hypoxia inducible factor-1α (HIF-1α) as a potent trans-activator of the *TERT* gene

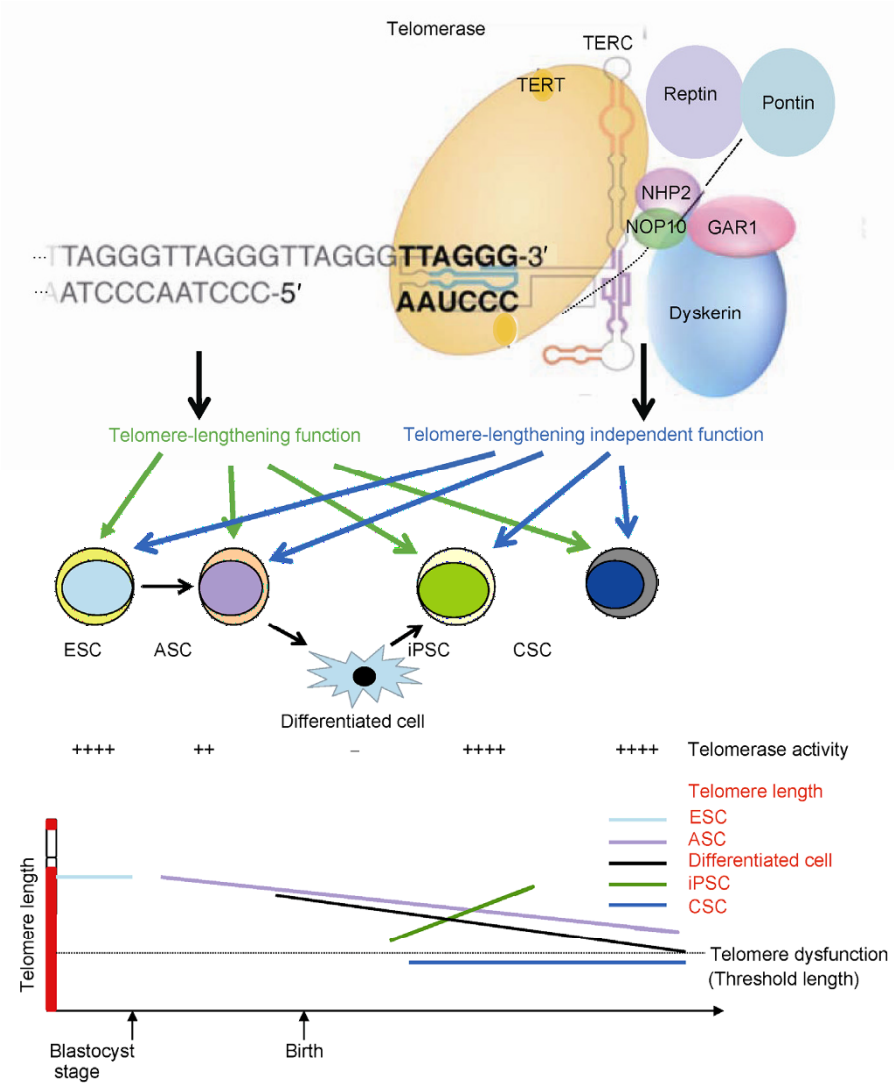

Figure 1 Telomerase in human stem cells. The telomerase complex comprises TERT (telomerase reverse transcriptase), TERC (telomerase RNA template), Dsykerin, NOP10, Reptin, Pontin and other factors that are yet to be identified. Telomere lengthening is the canonical function of telomerase. Embryonic stem cells (ESCs) express high levels of telomerase activity that are required to maintain telomere length. Adult stem cells (ASC) also express telomerase, but the levels of expression are lower than those in ESCs. ASCs exhibit gradual telomere shortening with increasing age and become senescent (telomere dysfunction). Induced pluripotent stem cells (iPSCs) acquire telomerase, and their telomeres are restored to lengths similar to those in ESCs. In general, cancer stem cells (CSCs) have shorter telomeres than those in normal cells, however high levels of telomerase activity stabilize their telomeres. Telomerase has multiple activities, independent of its telomere lengthening action. These functions might also significantly affect the phenotype and other characteristics of ESCs, ASCs, iPSCs and CSCs.

[27]. Taken together, various stemness factors and signaling pathways cooperate to maintain high levels of TERT expression and telomerase activity in ESCs.

Unlike human ESCs, ASCs are not immortal, and exhibit signs such as decreased self-renewal, reduced clonal stability, reduced homing and engraftment, and biased lineage commitment [2,8,9,28]. Telomerase activity and TERT expression, coupled with relatively longer telomeres, are widely observed in stem cell compartments in many tissues and organs [20,29]. Among human ASCs, hematopoietic stem cells (HSCs) are the most widely studied. It has been noticed that HSCs undergo telomere erosion with increasing age, but at a slower rate compared with differentiated progenies or lymphocytes; this would suggest inadequate telomere maintenance [30,31]. Telomerase activity is detectable in HSCs, but at levels lower than those observed in human ESCs. Despite this reduction, moderate levels of telomerase activity are required for HSCs to support rapid turnover of differentiated blood cells [32].

The critical role of telomerase in ASCs, especially in human and mouse HSCs, has been shown through studies on individuals with telomerase gene alterations and using telomerase knockout mice. Dyskeratosis congenital (DC) is a genetic disease, with bone marrow failure occurring in 80% of DC patients [33]. Mutations in *DKC1*, which encodes dyskerin, are etiological for X-linked DC. Autosomal

forms (recessive or dominant) of DC are due to mutations in *TERT*, *TERC* or other telomerase components. These mutations result in defective telomerase activity, leading to accelerated telomere shortening [16,17,33–35]. Another consequence of this is the severe impairment of HSC self-renewal and proliferation, which eventually results in bone marrow failure. The stem cells of DC patients in other organs and tissues are affected by shortened telomeres, clinically manifesting as nail dystrophy, oral leukoplakia, abnormal skin pigmentation and increased cancer risks [17]. Additionally, *TERT* and *TERC* mutations have been observed in other human diseases, including aplastic anemia and myelodysplastic syndrome, where HSCs are damaged by accelerated telomere erosion because of insufficient telomerase activity [35–37]. Many of these telomerase disease-related manifestations are observed in telomerase knockout mice [38–40]. In addition to the direct effects of telomerase deficiency on ASCs, mouse experiments have also shown that environmental alterations mediated by telomere dysfunction due to lack of telomerase significantly impair HSC function and engraftment [41].

Recent studies have revealed multiple biological activities of telomerase or TERT, in addition to its telomere lengthening function (Figure 1) [42]. *In vitro* murine ESCs that overexpressed TERT were much more resistant to apoptosis and oxidative stress [43]. *In vivo* studies showed that TERT was a critical determinant for the mobilization and proliferation of quiescent epidermal stem cells. TERT overexpression promoted stem cell mobilization and hair growth, while an absence of TERT resulted in inhibited mobilization and proliferation of stem cells out of their niche, and impaired hair growth [44,45]. Mechanistically, TERT functions as a transcriptional modulator of the Wnt/β-catenin signaling pathway [46]. TERT directly regulates Wnt/β-catenin signaling by serving as a cofactor in a β-catenin transcriptional complex. In cultured cells and *in vivo*, TERT activates Wnt-dependent reporters by interacting with BRG1, a SWI/SNF-related chromatin remodeling protein [46]. More recently, a global decline in genomic CpG methylation levels coupled with the down-regulation of DNA methyltransferase 3b expression was observed in ESCs derived from TERT knockout mice, triggering unstable differentiation of ESCs. Those findings revealed an unexpected role for TERT and/or telomere stability in the genome-wide epigenetic regulation of cell differentiation [39]. Additionally, TERT knockout-mediated telomere dysfunction was shown to be associated with impaired mitochondrial biogenesis and function, decreased gluconeogenesis, cardiomyopathy, and increased levels of reactive oxygen species in mice [47]. It is likely that these TERT-related mitochondrial defects can affect the fates of stem cells [48]. Based on the above findings, the independent telomere lengthening function of telomerase is important for the phenotype of stem cells.

3 Telomerase in iPSCs

Reprogramming differentiated somatic cells into pluripotent stem cells has emerged as a way of producing patient-specific stem cells as these cells could be a possible source for autologous pluripotent cell transplantation [49]. Initially, a single cell *via* somatic cell nuclear transfer technology was used for reprogramming somatic cells, and the cloning of animals such as Dolly the sheep [49,50]. Yamanaka et al. [10,11] successfully reprogrammed differentiated fibroblasts into iPSCs using four key transcription factors (Oct4, Sox2, c-Myc, and Klf4), which ushered in a new era in stem cell and regenerative medicine research. To date, iPSCs have been generated using different types of cells of various origin with different combinations of reprograming factors [12].

Terminally differentiated normal human cells have a limited lifespan due to telomere shortening. Telomere erosion must be prevented during reprogramming for iPSCs to acquire sustained proliferation potential. Yamanaka et al. [10,11] found that telomerase was activated in iPSCs; this was confirmed in later studies by other researchers [51–53]. Mathew et al. [54] analyzed the regulation of TERT and telomerase activation in reprogramed human fibroblasts; *TERT* was transcriptionally activated in reprogramed cells, however, levels of TERT expression and telomerase activity differed depending on the extent of reprograming [54]. Fully reprogramed human fibroblasts exhibited the highest levels of TERT expression and telomerase activity [54]. However, TERT transcription and telomerase was also activated in incompletely reprogramed fibroblasts, but their telomere length was not restored. Only iPSC clones with the highest TERT expression/telomerase activities that expressed all pluripotency markers exhibited robust replication potential and formed teratomas [54]. These findings strongly suggest that TERT/telomerase expression levels and telomere length are important parameters for determining whether an iPSC line is fully reprogramed.

The essential role of telomerase in iPSCs is further demonstrated in studies of telomerase-defective or deficient murine cells [52]. Somatic cells derived from third generation TERC-knockout/telomerase null mice failed to generate iPSCs, while the reintroduction of telomerase enabled the cells to be efficiently reprogrammed [29]. This indicates the importance of telomerase in iPSC generation and functionality. When reprogramming fibroblasts derived from individuals with loss-of-function mutations in the *TERT* or *TERC* genes, Winkler et al. [55] observed lower rates for telomere elongation and defective hematopoiesis in mutant telomerase-derived iPSCs. In another study, reduced telomerase activity and progressive telomere erosion was associated with loss of self-renewal potential and early senescence of iPSCs derived from patients with mutated *TERT*, *TERC* or *dyskerin* genes [56]. In contrast, Agarwal et al.

[53] derived iPSCs from two patients with mutations in *DKC1*, and from one patient with a heterozygous null mutation in *TERC*. They observed telomere elongation in iPSCs, and *TERT* and *TERC* upregulation comparable with those in wild-type iPSCs. The implications of these seemingly contradictory results remain to be resolved. Clonal variation, secondary genetic alterations, and differing culture conditions might contribute to the discrepancies observed, however, further studies are required to elucidate these issues.

It is evident from the above data that telomerase is highly activated during somatic cell reprogramming, and that telomerase-mediated telomere homeostasis is essential during iPSC generation, self-renewal and differentiation.

4 Telomerase in CSCs

Many human malignancies are hierarchical with a unique self-renewing population of cells at the top of the hierarchy; this has given rise to the CSC hypothesis [14]. CSCs have the ability to initiate tumor formation, to self-renew in serial transplantation assays, and to differentiate into non-tumorigenic progenies. Although CSCs share functional similarities to normal stem cells, they are not necessarily derived from normal stem cells. CSCs are known to actively proliferate, which is in contrast to normal stem cells that generally cycle at a slow rate [57]. To maintain these characteristics and functionality, the telomere length of CSCs must be stabilized by some mechanisms. Results from a number of studies have shown the presence of telomerase activity and/or TERT expression in CSCs from different malignancies [20]. Castelo-Branco et al. [58] compared telomerase expression between CSCs and non-CSC cells derived from patients with glioblastoma and neuroblastoma. They found that telomerase was activated in CSCs but not in non-CSC cells [58]. When telomerase activity was inhibited, and thereby telomere maintenance was disrupted, in those neural CSCs, their self-renewal capacities were significantly impaired or even lost. Those findings clearly indicate a critical role for telomerase and/or stabilized telomere length in CSCs. However, analysis of glioma-derived telomerase-proficient GOS-3 cells in a different study revealed a $CD133^+$ CSC subpopulation that expressed TERT mRNA at levels 100-fold lower than those in the $CD133^-$ fraction. Serum starvation of GOS-3 cells led to increased expression of the stemness marker CD133 and down-regulation of TERT expression [59]. Because the authors did not evaluate other CSC properties in GOS-3 cells, it is unclear whether those $CD133^+$ GOS-3 cells were true CSCs. Studies on breast, prostate, and pancreatic cancers demonstrated that telomerase was highly activated in CSCs and indispensable for the maintenance of the CSC phenotype and characteristics [20,60,61].

Telomerase is activated and responsible for telomere elongation in the vast majority of malignancies. However, an alternative telomere lengthening (ALT) mechanism operates during telomere stabilization in 10%–15% of telomerase-deficient cancers [62,63]. ALT is an unconserved, telomerase-independent telomere lengthening pathway involving the transfer of telomere tandem repeats between sister chromatids; it is frequently activated in malignancies of mesenchymal origin [63]. Approximately 33% of gliomas acquire ALT activity, and Silvestre et al. [64] characterized CSCs derived from patients with glioblastoma lacking telomerase activity. They found that those CSCs exhibited a typical ALT telomere length profile that was long and heterogeneous; this was observed in all other ALT^+ tumor cells. ALT^+ CSCs expressed neural stem cell markers and formed intracranial tumors in immunocompromised mice. The ALT pathway can maintain the phenotype and function of CSCs as efficiently as telomerase. It has been noted that ALT^+ CSCs appear to be more resistant to radiotherapy than $telomerase^+$ CSCs in glioblastoma patients [64].

Multiple biological activities of telomerase/TERT, beyond its telomere lengthening function, have been demonstrated [42]. It was shown that the effect of TERT on CSCs could be independent of its catalytic activity. In gastric cancer cells, both wild-type TERT and its dominant negative variant were observed to induce the expression of CD44, a CSC marker, to enhance the formation of spheroid colonies, to enhance self-renewal capacities, and to enhance *in vivo* metastasis [65]. Additionally, cancer cells possess strong phenotypic plasticity, and dynamic phenotypic switching can occur between CSCs and non-CSC cells in response to changing intrinsic and microenvironmental conditions. A number of reports have shown that cancer cells, when undergoing epithelial-mesenchymal transition (EMT), frequently acquire CSC properties [13,66]. Similarly, TERT could stimulate the stemness of cancer cells via EMT [65]. These findings suggest that TERT increases the phenotypic plasticity of cancer cells and promotes the conversion of cells from the non-CSC to the CSC form, thereby amplifying the CSC pool.

Collectively, telomerase/TERT contributes to the CSC phenotype via telomere maintenance and independent telomere lengthening mechanisms. In addition, the ALT pathway is capable of maintaining CSC phenotypes and activities in telomerase-deficient malignancies. All these findings provide important rationales for designing telomerase-based strategies that can target CSCs.

5 Conclusion and perspectives

We have discussed recent studies involving telomerase regulation and its role in various stem cells. Current findings suggest that telomerase plays critical roles, in stem cell biology and function. Telomerase would appear to be a stemness enzyme and is rate-limiting for the proliferation, self-renewal and differentiation of stem cells including

ESCs, ASCs, iPSCs and CSCs (Figure 1). In both normal and malignant stem cells, telomerase plays its parts by elongating telomeres and via an independent telomere lengthening function. Aberrant or impaired telomerase activity leads to defective stem cell function, thereby causing diseases. However, we have only touched the tip of the iceberg thus far and many outstanding questions remain to be defined. Nevertheless, the collected data will be very useful for designing telomerase-based strategies in regenerative medicine, the intervention of aging, and cancer therapy. Hopefully, we will be able to specifically manipulate the stemness enzyme in stem cells in time, place and quality for therapeutic purposes in the near future.

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