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Telomerase activation in epidermal ASCs: a step towards the Fountain of Youth

Cláudia Cavaco 50150[1], David Ferreira 52565[1], Sofia Ribeiro 57628[1] and Soraia Santos 57877[1]

[1] Master's Degree in Molecular Biology and Genetics, Science Faculty of the University of Lisbon (FCUL), Lisbon, Portugal

Professor Andreia Figueiredo

Introduction

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Aging is often defined as the progressive loss of physiological integrity, which results in compromised functions and a higher vulnerability for diseases and death[1]. In the process of aging, the consequent body dysfunction is believed to be due to the accumulation of senescent cells that lost their proliferative potential and suffered considerable changes in cell morphology, gene expression, metabolism and epigenetics[2,3]. In fact, most somatic cells undergo cellular senescence and aging due to their limited replication capacity resulting from telomere shortening in each cell division[2–4].

Telomeres are non-coding DNA sequences at the end of chromosomal DNA that act as buffer zones, thereby protecting chromosomes from degradation and supporting overall organization and stabilization[5–7]. Human telomeres consist of tandem repeats of TTAGGG sequences where the telomeric protein complex Shelterin and other proteins involved in chromatin remodeling bind to fold into a telomere-protective structure known as T-loop[6–8]. This structure protects the coding DNA sequences upstream the telomeres from exonucleases or DNA damaging agents[7,8]. However, telomeres length shortens by 50–200 bp at each somatic cell division due to the incapacity of DNA polymerase to completely replicate the 3' end of the DNA during lagging strand replication[6,8]. Consequently, telomeres reach a critical length at which they signal cell proliferation arrest, preventing cellular and DNA damage[7].

The preservation of telomeres is mainly regulated by the telomerase complex and the human telomerase reverse transcriptase gene (*hTERT*), which encodes the catalytic subunit and rate-limiting determinant of the enzymatic activity of telomerase identified as telomerase reverse transcriptase protein (TERT)[6]. This enzyme is expressed during embryogenesis and adds tandem short-sequence repeats at the end of chromosomes to maintain the length of telomeres[7,9]. Nonetheless, telomerase is inactivated postnatally in most cells, limiting its proliferation potential to 50–70 cell doublings. Germ cells and specific subsets of stem and progenitor cells are exceptions that retain some levels of telomerase, however in progenitor cells and adult stem cells (ASCs) these levels are insufficient to prevent telomere shortening on the long term[8,9].

A healthy and young-looking appearance influences social and reproductive status[10,11]. As a result, the skincare market has been growing in the latest years[12]. Recent studies have been focused on epigenetic mechanisms that regulate skin homeostasis and regeneration[13]. So far, promising results have been achieved with significant rejuvenation of both methylome and transcriptome of dermal fibroblasts from middle-aged donors by around 30 years[14]. However, a previous study using different generations of telomerase-deficient mice, ranging from slightly reduced in length to critically short, showed that telomere length and TERT are crucial in the mobilization and proliferation of epidermal stem cells[15]. This suggests that ectopic expression of telomerase could be a potential therapeutic approach to counteract aging[3,8].

Virtually all studies analyzing telomerase's role in the aging of epithelial stem cells were done in transgenic mice that either never expressed telomerase during development or overexpressed it in all tissues, generating questions about the effect of telomere increase in natural aging of stem cells[16,17]. Mice stem cells are also very different from human stem cells, with different differentiation stages and crucial molecules. The presented study addresses these issues by selectively activating telomerase in human ASCs that have aged normally *in vivo.* Previous studies have also failed to characterize telomerase's activation effect in stem cells on

a cellular level, focusing instead on histological related changes[17]. Thus, the proposed study will provide an in-depth look at TERTs effect on human-aged epithelial ASCs, to assess the possibility of future procedures to rejuvenate the skin by specific telomerase overexpression on pre-existent stem cells.

Experimental Design

To date, telomerase has only been studied in transgenic mice with an overexpression of telomerase or with no expression at all. Although age is associated with several pathways, such as oxidative stress, telomere damage/shortening, DNA damage and epigenetic dysregulation[18], the impact of increased telomerase in human ASCs aging has not been yet analyzed in a detailed whole-system approach. Therefore, the following experimental design (Figure 1) aims to understand the effect of telomerase activation in aged human ASCs obtained from middle-aged donors.

Cell isolation and sampling

The skin cells will be collected from fresh human skin scalp from facelift procedures and punch biopsy from individuals over 50 years old as described in[19]. Then, the ASCs will be isolated using fluorescence-activated cell sorting (FACS). These cells will be transfected with a *hTERT* gene with a cumate-inducible promoter. To avoid further variation, the ASCs clones from the same medium will be analyzed before and after the *hTERT* activation. The cultures of ASCs will be performed as described in[19].

Cell transfection under an inducible promoter

Stem cell transfection will be performed using lentivirus carrying cumate-inducing clonal vectors expressing *TERT* with inducible GFP markers to access telomerase relative gene expression (Figure 2).

hTERT transcription remains off without cumate present in the cell medium since cumate quantity dictates the transcription levels of telomerase. The amount of TERT can then be detected with parallel levels of GFP signaling. The vectors shall be ordered from https://www.abmgood.com/.

Successfully transfected cells will be selected in puromycin rich medium to which they have acquired resistance. Transgenic stem cells will then go through a series of proposed analyses in the absence of cumate (no telomerase) and with cumate in the medium (functional telomerase).

Telomerase expression and telomere length

The telomerase expression will be observed using confocal microscopy, since the GFP levels will correspond to the telomerase quantity in the cells. It will also be performed a single

telomere length analysis (STELA), which combines PCR based methods and Southern blotting[20], to evaluate the telomere length of the ASCs.

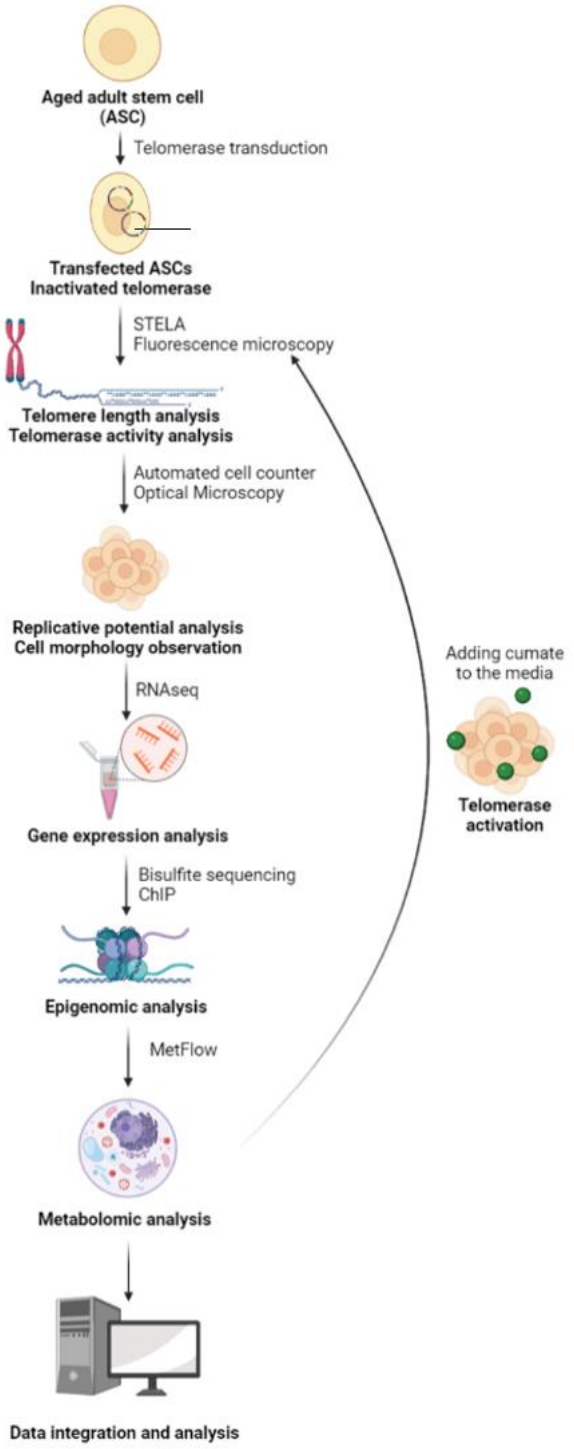

Figure 1 | Experimental design. The ASCs will be transfected with the *hTERT* gene with a cumate-inducible promoter. Before the telomerase activation, these cells will be analyzed for age-related parameters to represent the control group. Afterwards, the ASCs will be induced to express the new *hTERT* gene and the rejuvenated cells will be analyzed for the same age-related parameters as before. Finally, the data will be integrated and analyzed as a whole system. Created in biorender.com

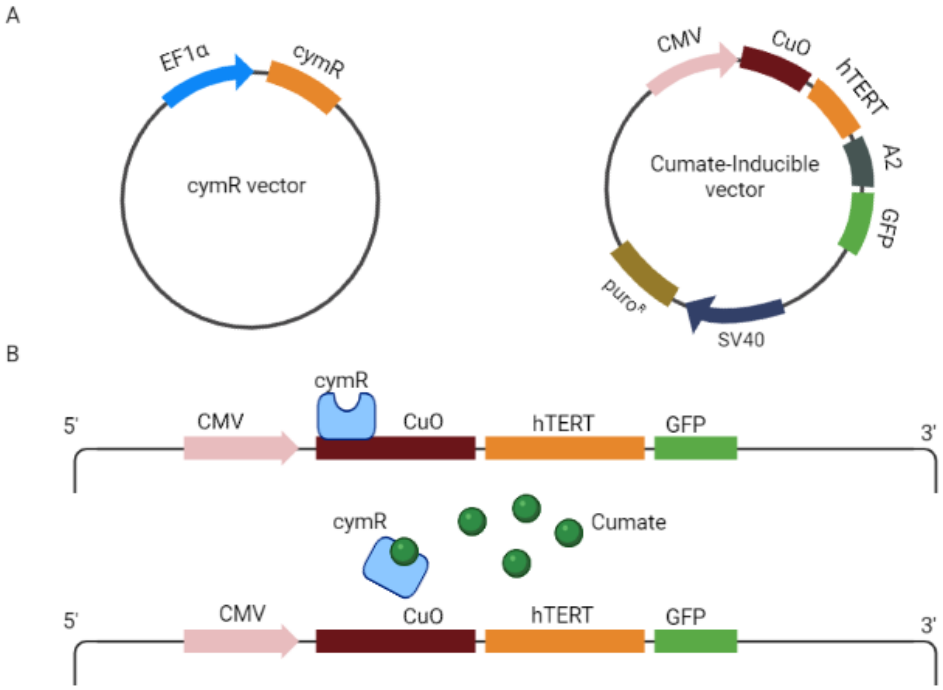

Figure 2 | Cell transduction. A) Lentiviral vector composition. **B)** Cumate mechanism in regulation of *TERT* expression. EF1a: Human EF1a promoter; cymR: CymR repressor; SV40: Simian virus vector promoter; GFP: Green fluorescent protein; A2: Self cleaving peptides; puro^{R}: Puromycin resistance gene; CMV: Cumate operator sequence; TERT: Telomerase reverse transcriptase. Created in biorender.com

Replicative potential and Cell morphology

The replicative potential of the cells will be estimated using an automated cell counter and the ASCs morphology will be observed under optical microscopy. It will be considered that young ASCs are usually rounded and bright while aged ASCs may present a flattened morphology[21].

Gene expression analysis

The gene expression patterns of both (in)activated telomerase ASCs will be evaluated through RNA-Seq transcriptome analysis to identify biomarkers of aging, resorting to outsourcing.

Epigenetic analysis

There are age-related changes in DNA methylation patterns that can be measured by the epigenetic clock[22]. Thus, to understand if telomerase activation can influence the DNA methylation status, a whole-genome bisulfite sequencing will be performed to identify and

quantify the methylation of individual cytosines after DNA treatment with bisulfite in both (in)activated telomerase cells.

Metabolomic analysis

Most cells undergo cellular senescence to prevent cellular and DNA damage. In addition, aging is associated with a decrease in metabolism and antioxidant compound levels. However, senescent cells are functionally and metabolically active, for instance, high concentrations of reactive oxygen species (ROS) lead to oxidative stress, which is one of the mechanisms that promotes the aging process[18]. Therefore, a metabolome analysis will be performed using mass spectrometry (MS), to provide a greater understanding of the ASCs metabolic state in both (in)activated telomerase cells.

Data integration and analysis

The data must be integrated to be analyzed as a whole system biology. These data shall provide information about the pathways related with the telomerase activation and potentially related with aging.

Expected Results and Future Perspectives

Since the correlation between telomere length and aging has been reported, it is expected that ASCs with an activated telomerase can rescue to some extent a younger phenotype, with significant increase in telomere length and in cellular replicative potential. Moreover, it is estimated a decrease in genomic instability and significant differences in gene expression and cell metabolites between ASCs before and after the telomerase activation. If the expected results are confirmed, the next step would be to understand if the transgenic ASCs could originate a phenotypically young skin tissue *in vitro*. Afterwards, the main goal would be to develop a technique able to deliver the extra *hTERT* gene to pre-existing ASCs inside the aged skin tissues *in vivo* to rejuvenate them. It would also be interesting in future studies to establish a parallel between our transgenic aged stem cells and germ-line stem cells that maintain high proliferative potential as well as high levels of telomerase throughout their lives.

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