Measuring Telomerase Activity In Senescent Human T Cells Upon Genetic Modification

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Summary

Telomerase, a RNA-dependent DNA polymerase that adds telomeric DNA at the 3' ends of eukaryotic chromosomes, is essential for the lifelong preservation of the proliferative potential of antigen specific T lymphocytes. However, senescent T cells that have low telomerase activity, short telomeres and lack of replicative capacity accumulate in old humans, patients with chronic viral infections and cancer. The mechanisms inhibiting telomerase in these cells are poorly understood. Here I describe a strategy that was successfully applied to identify pathways causing telomerase dysfunction in primary human senescent T lymphocytes. Such strategy couples lentiviral vector-based gene manipulations to functional and signaling readouts directly *ex vivo*, in humans.

Key words: T cells, Telomerase, Senescence, Intra-Sensory Signaling & AMP-responsive-protein kinase. **Running title:** Molecular Tuning Of Telomerase Activity in Senescent Human T Cells

1. Introduction

The ends of the eukaryotic chromosomes are characterized by repeated hexameric sequences of GT-rich nucleotides known as telomeres that provide genomic protection and stability (Blackburn, 2000). Functional telomeres require minimal telomere length (above 4-kilo bases (kb)), an intact G-rich 3' DNA overhang and the interaction with specific telomere-binding proteins, the shelterin (Blasco, 2005). When telomeres become critically short after repeated cell divisions, senescent cells spontaneously activate DNA damage response (DDR) cascades and the replicative capacity ceases (Campisi and d'Adda di Fagagna, 2007). This can be delayed by the up-regulation of the enzyme telomerase, a RNA-dependent DNA polymerase firstly identified in 1985 in the protozoa *Tetrahymena Thermophila* (Greider and Blackburn, 1985). The holo-enzyme, which consists of a catalytic core (hTERT) and a RNA template (hTR or hTERC), acts by physically binding to and extending the G-rich 3' DNA overhang of the telomere (**Figure 1**), to replenish the loss of genetic material that is due to the semiconservative mechanism of DNA replication (Verdun and Karlseder, 2007). Hayflick was the first to identify the existence of a limit, imposed by mechanisms of telomere erosion, to the extent at which mammalian cells can proliferate (Hayflick limit) (HAYFLICK and MOORHEAD, 1961). This dictates that adult somatic cells in which telomerase activity is generally very low or undetectable, reach replicative senescence after about 50 cell passages *in vitro* (HAYFLICK and MOORHEAD, 1961). Unlike most adult somatic cells, human T lymphocytes are able to re-activate telomerase upon antigenic challenges and this is

essential for the life-long preservation of their proliferative potential *in vivo* (Hodes et al., 2002). However after repeated episodes of activation, T cells progressively differentiate and lose the capacity to up-regulate the enzyme telomerase, which leads to telomere erosion, loss of proliferative capacity and ultimately telomere-dependent senescence. Conversely, elevated telomerase activity in cancer cells confers virtually unlimited proliferative potential (Cong et al., 2002). Fine-molecular tuning of telomerase is therefore needed to control mammalian cell proliferation and ensure a balance between the delaying of cellular senescence and the risk of developing malignancy. Below, I briefly discuss the current understanding of the main mechanisms controlling telomerase expression, nuclear import and activation.

The biological importance of telomerase is highlighted by the complexity of both transcriptional and post-translational mechanisms tuning the expression of its catalytic sub-unit, hTERT (Tesmer et al., 1999). The hTERT promoter is well characterized and comprises binding sites for both inhibitory and activatory transcription factors (Kyo et al., 2008). For instance, the transcription factor c-MYC binds to the hTERT promoter at regulatory sites known as 'E-boxes', hence inducing hTERT transcription (DePinho et al., 1991). This has been well documented in studies where elevated hTERT expression was directly induced by increased c-MYC activity, in cancer cells (Kyo et al., 2008). Between the E-boxes, the hTERT promoter possesses at least five distinct GC-rich DNA sequences (CpG islands) that are important for Sp1-mediated hTERT up-regulation. Similarly, the p65 sub-unit of the transcription factor NF-KB has been reported to up-regulate hTERT

expression (Akiyama et al., 2003), however the precise binding regulatory site has not been identified. Conversely, E2F1 is an inhibitor of hTERT transcription, and four related binding sites have been identified, in which its action is coordinated by the histone deacetylase HDAC (Crowe, 2001). It is also well recognized that the tumour suppressor p53 inhibits hTERT expression, possibly preventing the binding of Sp1 to the hTERT promoter (Xu et al., 2000).

Although the transcriptional regulation of hTERT has been initially considered the sole regulatory mechanism, it has recently arisen that post-translational mechanisms are also involved in telomerase regulation. In accordance with this, Liu and colleagues firstly reported the existence of notable differences between the levels of hTERT transcripts and telomerase activity in human lymphocytes (Liu et al., 2001). In an extension of this work, the kinase AKT has been shown to phosphorylate hTERT (at 220-GARRRGGSAS-229 and 817-AVRIRGKSYV-826 sites), a process required for its nuclear import and activity (Kang et al., 1999). Indeed, defective AKT signalling is a molecular feature of human highly differentiated T cells that have low telomerase activity (Henson et al., 2009; Plunkett et al., 2007). In addition to AKT, hTERT also binds to mTOR; this interaction can be disrupted by PP2A activity, a tumour suppressor phosphatase that dephosphorylates AKT and possibly also hTERT (Li et al., 1997). More recently, reports on the existence of AKT-independent mechanisms in controlling telomerase activity in human highly differentiated T cells that in part involve spontaneous activation of p38 MAP kinase through an unknown mechanism (Di Mitri et al., 2011) (Lanna et al.,

2013). Intervention at the point of telomerase regulating pathways may be important to restore immune cell function during ageing. We discovered that low nutrient and senescence signals converge to inhibit telomerase activity in T cells, a process triggered by the metabolic master regulator AMPK, mediated by the scaffold molecule TAB1 and exerted by the MAP kinase p38 ('Intra-sensory' pathway) (Lanna et al., 2014). **Figure 2** depicts mechanisms of telomerase regulation in mammals. Below, I describe methodology successfully used to identify signaling pathways causing telomerase dysfunction in primary human senescent T cells. Thus, I herein illuminate the genetic manipulations and telomeric-repeat amplification protocols required to modulate replicative senescence in lymphocytes, with the potential to reverse it.

2 Materials

1. 1x Dulbecco's Phosphate Buffered Saline solution (D-PBS)
2. Lymphoprep™ (Axis-Shield)
3. Magnetic Activated Cell Sorting buffer (MACS buffer, filtered and ready to use, Miltenyi Biotec)
4. $CD4^+$ T cell Isolation kit (Miltenyi Biotec)
5. LS-magnetic separation columns (Miltenyi Biotec)
6. Anti-CD27 Microbeads (Miltenyi Biotec)
7. Anti-CD28 Microbead kit (Miltenyi Biotec)

8. RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 μg/ml gentamicin, and 2 mM L-glutamine (all from Invitrogen)
9. anti-CD3 antibody (αCD3, purified OKT3 clone; Sigma-Aldrich)
10. rh-IL2 (R&D Systems, 10 νg/mL)
11. Fugene 6 (Promega) for transfections of 293 T cells
12. shRNA-expressing lentiviral systems (either commercial or designed in-house)
13. TeloTAGGG Telomerase PCR ELISA (Roche)
14. CHAPS buffer: 10 mM Tris-HCl pH 7.5, 1 mM EGTA, 150 mM NaCl, 1 mM $MgCl_2$, 0.25 mM sodium deoxycholate, 10% (v/v) glycerol, 5 mM β-mercapto-ethanol, 1% (v/v) Nonidet P-40 (NP-40), 0.5% (w/v) 3-(3-cloro-amminopropil) dimethylammonio-1-propanesulfonate (CHAPS), 0.1 mM [4-(2-aminoetil)-benzene sulfonyl fluoride hydrochloride] (AEBSF)

3. Methods

1. Isolate Peripheral Blood Mononuclear Cells (PBMC) from the blood of healthy volunteers by using standard Ficoll gradient separation (*see* **Note 1**).
2. Isolate human $CD4^+$ (or $CD8^+$) T cells from the PBMC preparation using 'negative' selection kits and by following manufacturer's instructions (Miltenyi Biotec; *see* also **Note 2**)

3. Isolate the CD27/CD28 related subsets of human $CD4^+$ (or $CD8^+$) T cells by immune-magnetic separation ('positive' selection; Miltenyi Biotec). Importantly, the separation order will vary if working with either $CD4^+$ or $CD8^+$ T cells (*see* **Note 3**). This will result in the isolation of three separate populations of primary human T cells. Senescent T cells are $CD27^-$ $CD28^-$ lymphocytes within both the $CD4^+$ and $CD8^+$ memory pools (see **Note 4**).
4. Activate primary senescent human T cells by 0.5 μg/mL anti-CD3 antibody plus 10 νg/mL rh-IL2 (see **Note 5**) for 48 hours in complete RPMI at 37°C in a humidified 5% CO_2 incubator.
5. Transduce primary senescent human T cells using either commercial or in house-generated lentiviral particles (Escors et al., 2008). For optimal transduction efficiency, a multiplicity of infection (MOI) of 10 should be used. The genetic manipulation should target those enzymatic activities which are endogenously de-regulated in senescent T cells, as assessed by either phospho-flow or immunoblotting analysis.
6. Replace fresh complete medium with rh-IL2 every 2-3 days. For long term studies, transduced cells should be reactivated every 10 days as in step 5.
7. At one week after activation (96 hours post-transduction), prepare lysates from 2 x 10^5 cells in 200 μl of CHAPS buffer.
8. Incubate extracts 30' on ice, followed by ultracentrifugation at 13,000 rpm (26,451 g) for 20' at 4°C. Cleared supernatants are then either

stored at -80°C (for non immediate use) or immediately used as described below.

9. Measure telomerase activity using the non-radioactive TeloTAGGG telomerase ELISA kit (Roche), using 2 x 10^3 cells. Mix the cell extracts in 25 μl of ready-to use PCR Reaction mixture (provided with the kit), then add sterile water up to 50 μl final volume.
10. Transfer tubes to a thermal cycler to perform combined primer elongation/amplification PCR reactions by the following protocol (**Table 1**):
11. Add 5 μl of the amplification product to 20 μl of kit-provided denaturation reagent, then incubate for 10' at 20°C.
12. Hybridize the so-denaturated biotynilated telomere-amplicons to digoxigenin (DIG)-labeled, telomeric repeat-specific detection probes by adding 225 μl of Hybridization Buffer the transfer 100 μl of this reaction mixture to streptavidin coated micro-plates (both provided with the kit).
13. Allow biotin-streptavidin binding for 2 hours on a micro-plate shaker at 37°C.
14. Wash micro-plates 3 times using 250 μl of 1X-washing solution (provided with the kit) per well. No centrifugation step is needed.
15. Detect abundance of DIG-labeled telomere-amplicons bound to micro-plates by adding 100 μl of anti digoxigenin-antibody conjugated to peroxidase, followed by micro-plate shaking for 30' at 20°C.
16. Rinse plates by washing 5 times with 250 μl of 1X-washing solution.

17. Allow coloured-substrate development by adding 100 μl of TMB substrate (provided with the kit) per well and incubating for 10-20'.
18. Terminate the reactions by adding 100 μl stop solution (provided with the kit) per well and read on an ELISA reader as absorbance emission at 450 nM.

4. **Notes**

1. Senescent T cells accumulate during ageing or in disease (cancer, chronic viral infections or auto-immune disorders) but are also present in peripheral blood of healthy, young volunteers (between 1-20% of the total T cell compartment). We obtained similar results when studying senescent T cells isolated from either young or old donors.
2. For endogenous signaling studies direclty *ex vivo*, cells should not be re-suspended in full-medium at any time to avoid unspecific stimulation caused by the serum or other compounds.
3. $CD4^+$ T cells first lose expression of CD27 followed by that of CD28 receptors, unlike $CD8^+$ T cells in which the opposite is true. This has to be considered when isolating subsets of senescent human T cells in either cell compartment. The reason for this opposite differentiation program is not known.
4. Interrogate endogenous signaling pathways direclty without activation. Senescent human T cells exhibit a plethora of signaling abnormalities *in vivo* which can be manifested by studying signal transduction direclty *ex vivo* by either Western Blotting or phospho-flow analysis.

5. Upon transduction, fresh medium with new rh-IL2 should be replaced every 2 days.

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Figure legends

Figure 1. Telomerase function. The telomere-telomerase complex is shown. Telomerase is a RNA dependent DNA polymerase that extends telomeric DNA at each cell division, preventing telomere shortening. Telomerase function requires both a catalytic subunit (hTERT) and a RNA component (TERC) that directly binds to the single strand 3' overhangs at the end of eukaryotic chromosomes. Details of telomerase function are provided in the text.

Figure 2: Molecular tuning of telomerase. An overview of both transcriptional (*left*) and post-translational mechanisms (*right*) regulating the catalytic subunit of telomerase hTERT is shown. An extract (sites comprised between -200 and +78) of the telomerase catalytic sub-unit hTERT gene is reproduced, depicting the main transcriptional activators (c-MYC, Sp1, NFkB, and ERK) and repressors of hTERT expression (p53, E2F1, phosphorylated Rb and HDAC). Various regulatory elements have been identified within the hTERT promoter that directly or indirectly interacts with transcriptional regulators; the role of both E-box sites binding to c-MYC and Sp1-binding sites between the E-boxes is described in the text. NFkB promotes hTERT transcription but the precise binding on the hTERT promoter has not been identified. ATG in bold indicates gene hTERT start. The post-translational tuning of telomerase is less well characterized; AKT can directly phosphorylate hTERT promoting its nuclear import; mTOR binds to hTERT and promotes its activity through an unknown mechanism; NfKB promotes

hTERT nuclear import; PP2A inhibits telomerase activity by de-phosphorylating both AKT and hTERT. AMPK inhibits telomerase via TAB1-dependent p38 signalling, a process defined by the convergence of senescence and low nutrient deprivation signals in T cells ('Intra-Sensory' Pathway). The downstream signals regulating this pathway have not been identified.

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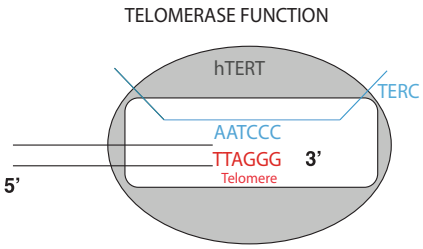

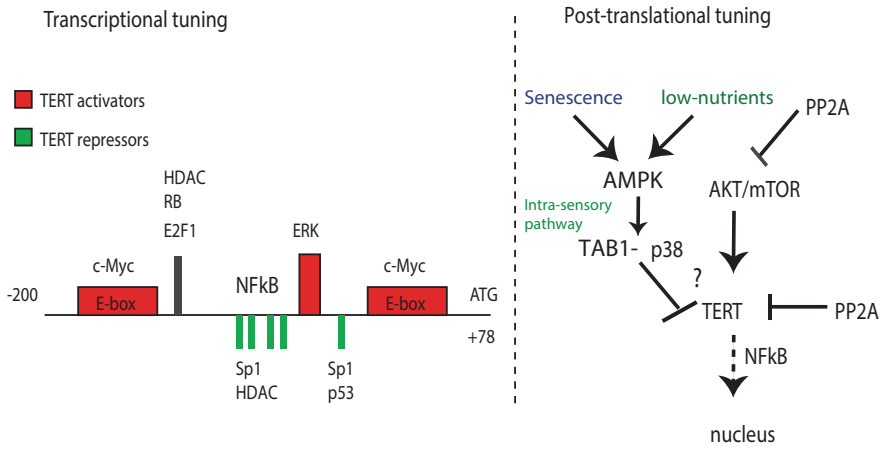
Transcriptional tuning
TERT activators
TERT repressors
HDAC
RB
E2F1
ERK
c-Myc
E-box
NFkB
c-Myc
E-box
-200
ATG
+78
Sp1
HDAC
Sp1
p53
Post-translational tuning
Senescence
low-nutrients
PP2A
AMPK
AKT/mTOR
Intra-sensory
pathway
TAB1-
p38
?
TERT
PP2A
NFkB
nucleus