TELOMERE AND TELOMERASE DYNAMICS IN HUMAN T LYMPHOCYTES

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DEDICATIONS

Dedicated to my parents, Xianjin Huang and Linying Chen,

for their unconditional love, support and encouragement.

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by

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Abstract

Advanced cancer is characterized by a phenotype that permits cells to divide indefinitely while cellular aging is characterized by cells ceasing to divide. It is believed that senescence may have evolved as an anti-cancer protection mechanism in long-lived species such as humans. Therefore, cancer and aging are essentially opposite ends of the same problem.

Telomeres are the cellular aging-clock that determines a cell's capability of proliferation. When normal cells divide, telomeres gets progressively shorter. When telomeres reach a critical short length, cells stop dividing. To compensate for the loss of telomeres, telomerase is a ribonucleoprotein enzyme complex that elongates telomeres, but its expression is restricted to

certain subsets of cell types. Investigating how telomerase is regulated in normal cells may provide insights and new approaches to block telomerase activity in cancer cells or to re-activate telomerase in aging cells.

Immune cells play central roles in defending humans from pathogens, infections, and malignant cells. Immunotherapy has shown great potential in cancer treatment and has gained increasing public attention. Thus, a better understanding of how to increase the proliferation efficiency of normal immune cells, especially in older individuals whose immune stem-like cells may becoming less efficient is important. Due to the accessibility of blood from volunteers, immune cells are a model system to investigate healthy aging.

For my doctoral research, I decided to study telomeres and telomerase dynamics in immune cells. Using novel techniques in telomere length analysis and telomerase activity measurements developed in our lab, I discovered that a subset of CD28+ T cells show robust telomerase activity and the ability to maintain telomere lengths during stimulated cell proliferation. In a centenarian study, T cells from a subset of centenarians showed stronger telomerase activation compared with many younger individuals. RNA-seq analysis revealed distinct differences between high performance centenarians and other age groups. Finally, we developed novel techniques using ddPCR for quantitating mitochondrial DNA numbers per cell and validated the differences we found in centenarian samples. Altogether, my doctoral project has added value to our current knowledge of how telomerase is regulated during immune responses and revealed its importance in longevity studies.

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LIST OF ABBREVIATIONS

6-thio-dG	6’-thio-2’-deoxyguanine
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
ANOVA	Analysis of Variance
ALT	Alternative Lengthening of Telomeres
AP1	Activated Protein-1
APC	Antigen Presenting Cell
BSA	Bovine Serum Albumin
CENT	Centenarians
CO	Centenarians' Offspring
ConA	Concanavalin A
cDNA	Complementary DNA
CTL	Cytotoxic T Lymphocyte
ddPCR	Droplet Digital Polymerase Chain Reaction
ddTRAP	Droplet Digital Telomere Repeat Amplification Protocol
dGTP	Deoxyguanosine Triphosphate
dNTP	Deoxynucleotide Triphosphate

DIG	Digoxigenin
DKC	Dyskeratosis Congenita
DKC1	Dyskerin
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
GRN163L	Imetelstat
HPLC	High–Performance Liquid Chromatography
hTERT	Human Telomerase Reverse Transcriptase
hTR / hTERC	Human Telomerase RNA Component
IFN-γ	Interferon-gamma
IL	Interleukin
IRB	Institutional Review Board
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
NFAT	Nuclear Factor of Activated T-cells

NF-κB	Nuclear Factor Kappa B
NK Cell	Natural Killer Cell
NOP10	H/ACA Ribonucleoprotein Complex Subunit 3
PARN	Poly(A)-specific Ribonuclease
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phytohaemagglutinin
PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate
POT1	Protection of Telomeres 1
Q-FISH	Quantitative Fluorescence In Situ Hybridization
qPCR	Quantitative Polymerase Chain Reaction
Rap1	Repressor/Activator Protein 1
RBC	Red Blood Cell
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species

rSAP	Recombinant Shrimp Alkaline Phosphatase
RTEL1	Regulator of Telomere Elongation Helicase 1
STELA	Single Telomere Length Analysis
TA-65	Telomerase Activator 65
TCAB1	Telomerase Cajal Body Protein 1
TCR	T-Cell Receptor
TeSLA	Telomere Shortest Length Assay
TGF-β	Transforming Growth Factor Beta
TIN2	TRF1 Interacting Nuclear Protein 2
TPP1	TIN2 Interacting Protein 1
TRAP	Telomere Repeat Amplification Protocol
TRF	Telomere Restriction Fragment (a measurement of telomere length)
TRF1/2	Telomere Repeat Binding Factor 1/2
U-STELA	Universal Single Telomere Length Analysis
WBC	White Blood Cell

CHAPTER 1

INTRODUCTION

1. An Introduction to Telomere and Telomerase Biology

1.1. Discovery of telomere and telomerase

Although first described in the late 1970s, the existence of telomeres had been predicted as early as 1938 by Herman Muller (Muller HJ 1938). Both Muller and Barbara McClintock found independently that the free ends of damaged chromosomes are highly unstable and can easily fuse together resulting in chromosomal rearrangements. They also noted that the free ends of normal chromosomes are somehow protected from fusion or rearrangements (Muller HJ 1938, McClintock 1941). Muller termed the ends of chromosomes "telomeres", which is derived from the combination of the Greek terms *télos* (end) and *méros* (part).

In the early 1960s, Leonard Hayflick and Paul Moorhead discovered that cells can only divide for a limited number of times in tissue culture(Hayflick and Moorhead 1961). This was in contrast to with Alexis Carrel (Nobel laureate) who believed that normal cells are immortal. Later Carrel's ideas were found to be incorrect and likely due to refeeding cell cultures with chick embryo extract containing some live cells. This resulted in the false belief that all somatic cells removed from the body were intrinsically immortal. Additionally, Hayflick found the limited number of divisions was very consistent and predictable, usually between 40 and 60 (population doublings) times. Later in 1974, Australian Nobel laureate Sir Macfarlane Burnet termed this finite limited number of divisions as the "Hayflick limit". As the Hayflick limit is

approached, cells gradually stop growing, become somewhat enlarged, and enter a permanent dormant stage called replicative senescence.

In 1953, James Watson and Francis Crick revealed the structure of DNA (Watson and Crick 1953), and later in 1958, DNA polymerase was discovered by Kornberg (Bessman, Lehman et al. 1958). Once the mechanisms of DNA replication were better understood, additional questions were raised by both Watson and a Russian theoretical scientist Alexy Olovnikov. Watson realized during DNA replication, due to the bi-directional DNA replication process, that the leading strand can replicate properly and completely. However, the most 5' end of the lagging template strand cannot be replicated completely, as the 5' most Okazaki fragment cannot extend an RNA primer beyond the end of the chromosome and be replaced by DNA sequence. Thus, any newly synthesized DNA molecule will have a single stranded tail after DNA duplication (Watson 1972, Olovnikov 1973). Since DNA replicates during each cell divisions, it was proposed that the lengths of chromosomes will shorten over time due to this "end-replication problem". Olovnikov further linked the chromosome shortening during each cell divisions with the limited replicative number of human cells in tissue culture (Olovnikov 1973).

Finally in 1978, Elizabeth Blackburn revealed the detailed sequence information of telomere at the end of chromosomes as $(TTGGGG)_n$ in the single-cell model organism *Tetrahymena themophila*(Blackburn and Gall 1978), which is different from the sequence in most multi-cellular organisms $(TTAGGG)_n$. Furthermore, in collaboration with Jack Szostak, they found that adding DNA end-sequences from *Tetrahymena* to both ends of an artificial minichromosome that the long-term stability could be maintained in yeast (Szostak and Blackburn 1982). This series of experiments were interpreted to suggest a striking conservation of the telomere structure and function between very distant species. Also, these results were

consistent with the previous findings by Muller and McClintock, that the "natural ends" can protect normal chromosomes from end-fusions and rearrangements while "broken ends" do not (Muller HJ 1938, McClintock 1941).

There was some support at that time that telomeres could be maintained and extended in both *Tetrahymena* and yeast (Shampay, Szostak et al. 1984). This suggested, but did not prove, that there should be a counteracting mechanism to compensate for the loss of telomeres during cell division. In 1985, Blackburn and her graduate student, Carol Greider, discovered a ribonucleoprotein complex (terminal telomere transferase) that showed enzymatic activity of adding telomeric repeats to the end of chromosomes in *Tetrahymena*. They later termed it telomerase, consisting of both a protein component, which functions as a catalytic reverse transcriptase enzyme, and a RNA component, which acts as an intrinsic guide sequence.

The discovery of telomeres and telomerase opened the door of a better understanding of human aging and cancer development. Because of their insightful efforts and outstanding contributions in telomere biology, Blackburn, Greider, and Szostack were honored with the Lasker Award in 2006 and the prestigious Nobel Prize in 2009.

1.2. Telomerase activity measurement assays

As telomerase is a critical enzyme that maintains and elongates telomeres (Blackburn, Greider et al. 1989), there was a need for an sensitive assay to detect and determine telomerase activity. In 1994 the Shay/Wright lab developed the classic method of measuring telomerase activity, called telomeric repeat amplification protocol (TRAP) (Kim, Piatyszek et al. 1994). By performing TRAP, scientists were able to measure telomerase semi-quantitatively with a small number of cells, and later even quantitatively using droplet digital PCR(Ludlow, Robin et al.

2014). The typical TRAP assay consists of three major steps: substrate extension, amplification of telomerase-synthesized DNA, and signal detection. During extension, the telomere-mimic oligo is incubated together with a cell lysis, which either does or does not contain active telomerase. Telomerase, if present in the cell lysate, will then add telomere repeats to the end of the substrate, mimicking the real process in cells. Then, PCR amplification is applied by specific primers, where telomere fragments with different lengths will be amplified. At this step, different labels can be incorporated into the newly synthesized DNA, such as radioactive ^{32}P or chemoluminescence. After this, the detection is performed based on the specific ways being used to label the DNA. The intensity of the signal usually corresponds with the relative amount of telomerase activity.

Recently the third generation quantitative PCR technique, droplet digital PCR (ddPCR) has been developed. ddPCR separates the traditional one mixture system into over 20,000 mini-systems by oil droplet formation. Instead of measuring the intensity of the signal, ddPCR collects digital data in an "all or none" pattern in every single oil droplet, i.e. mini-PCR-system. This allows quantitative measurement of the exact number of molecules or template in any given system. (Ludlow, Robin et al. 2014)

Since the TRAP assay is a PCR-based assay that includes amplification of DNA and detection of the amplicon abundance, we recently applied the novel ddPCR technique into the classic TRAP assay and developed ddTRAP(Ludlow, Robin et al. 2014). The establishment of the ddTRAP assay allows us to measure telomerase activity in a much more quantitative and accurate way, even at the single cell level, and thus, became a major technique used throughout my human T lymphocyte project.

1.3. Telomere length measurement assays

There are various methods to measure telomere length, including TRF, q-PCR, Q-FISH, STELA, and TeSLA. The strengths and weakness of these major methods are introduced respectively, as described below.

Developed in 1988, telomere restriction fragment (TRF) analysis is still the most widely used method for telomere measurements (Moyzis, Buckingham et al. 1988). Regarded as the "gold standard", TRF measures the intensity of telomere smears to determine average telomere length. When genomic DNA is digested with a combination of 4 base pair restriction enzymes, the telomere sequence TTAGGG is not and therefore, each telomere will remain intact (though of variable lengths) after digestion while the rest of the genomic DNA is cut into small fragments. Then the electrophoresisis performed using the digested DNA and the telomere sequence is detected by Southern blots. A smear of variable length of telomeres is detected by isotope or DIG-labeled probe, and the average telomere length is then determined by quantification of the Gaussian distributed intensity (Figure 1-1). While TRF has been proved to be useful for many studies, the requirement of large amount of DNA and relative long time to perform the assay limits its use. In addition, due to hybridization kinetics, the shortest telomeres are difficult to detect.

The quantitative polymerase chain reaction (qPCR) assay is a relatively easy assay with less requirement of the amount of DNA. By measuring telomere signal (T) to a single copy gene signal (S), in comparison to a reference DNA, this methods allow one to calculate the T/S ratios (Figure 1-2). This ratio is proportional to average telomere length and therefore, can be used to determine average telomere length. Since the nature of this technique can be applied to a high-

throughput format, this method is widely used in large population studies. However, since it only provides relative quantification, the variation of the results is large and also, it cannot provideabsolute telomere length values in kilobases (Cawthon 2002) and has the same limitations as TRF gels since the shortest telomeres are not detected.

Telomere length can also be measured using living or fixed cells by Quantitative Fluorescence In Situ Hybridization (Q-FISH). There are several Q-FISH methods, which are based on similar principles but with some modification for various applications (Figure 1-3). Metaphase Q-FISH uses the digital fluorescence microscopy to determine telomere fluorescence after hybridization with a fluorescent PNA probe on interphase cells or metaphase spreads(Lansdorp, Verwoerd et al. 1996). It measures telomere length at each individual chromosome end with high accuracy, but due to limits on probe hybridization, the shortest telomeres are not detected (<2 Kb). However, it is also labor-intensive as well as not being able to detect the very shortest telomeres. Flow-FISH determines telomere fluorescence in individual interphase cells by FACS technology. Since the involvement of FACS, Flow FISH can be adapted for higher-throughput and thus larger scale studies. One disadvantage of this technique is that the probe may also bind to interstitial telomeric repeats and generate some false positive result and also does not detect the shortest telomeres.

TRF, qPCR, and Q-FISH are methods that measure average telomere length, while single telomere length analysis (STELA) was designed to enable the measurement of the shortest telomere on individual chromosomes (Baird, Rowson et al. 2003). STELA measures the abundance of the shortest telomeres using a combination of ligation, PCR-based methods, and Southern blot analysis (Figure 1-4). However, it only works on a few specific chromosome ends. To resolve this problem, the Universal STELA (U-STELA) was introduced in 2010 (Bendix,

Horn et al. 2010) that is able to detect telomeres from every chromosome ends, making it possible to monitor the change of shortest telomere in cells (Figure 1-4). Nevertheless, the application of U-STELA is limited by its efficacy and accuracy of unbiased detecting telomere at different lengths. In addition, U-STELA cannot detect longer telomeres and also detect interstitial telomere repeats.

To address the need of a more sensitive, accurate and unbiased method to measure shortest telomeres and to not detect interstitial telomeric repeats, in 2017 the Shay/Wright lab has developed a novel method termed Telomere Shortest Length Assay (TeSLA). TeSLA employs an improved ligation and digestion strategy, the classic Southern blot with ultra-sensitive DIG-labeled probe, and a user friendly image-processing program to automatically measure the distribution of telomere at different lengths (Figure 1-4). This allows us to measure the abundance and unbiased distribution of telomeres from less than 1kb to ~18kb. With TeSLA, we are able to monitor subtle telomere change in a short period of time and the method provides more insights into telomere dynamics during various cellular process.

1.4. Molecular biology of telomere and telomerase

Telomere are repeated TTAGGG sequences capped at the ends of chromosomes. Besides the DNA sequence, it also requires several protein components to protect the ends of chromosomes being recognized as a DNA double stranded break. The protein complex that specifically binds to telomere region is called "shelterin complex", which consists of six major proteins: Telomere repeat binding factor 1 and 2 (TRF1, TRF2), TRF1-interacting nuclear protein 2 (TIN2), repressor/activator protein 1 (Rap1), TIN2-interacting protein 1 (TPP1) and protection of telomeres 1 (POT1) (Sfeir and de Lange 2012). When the shelterin complex binds

to the telomere region, it effectively prevent pathological DNA repair activities. Loss of any component in the shelterin complex will lead to genome instability, end fusions and cellular senescence (Sfeir and de Lange 2012). At the very end of each chromosome, the telomere will fold back and the 3' overhang of the single-stranded G-strand will insert into the double-stranded telomere DNA, which forms a loop structure called the "T-loop" (Figure 1-5). The T-loop structure must be disengaged so that DNA can be duplicated during replication, and the shelterin complex is known to be involved in this complicated process to maintain the stability of telomere during DNA replication.

Telomerase is a ribonucleoprotein that is composed of a catalytic protein component, TERT, and a template RNA component, TR or TERC (Greider 1990).The protein component in human telomerase, hTERT, was characterized in 1997(Nakamura, Morin et al. 1997), and has reverse transcriptase activity. The hTERT gene contains 16 exons that can be spliced into 22 distinct variants (Kilian, Bowtell et al. 1997, Hrdlickova, Nehyba et al. 2012). The full length transcript has all 16 exons and has functional telomerase activity, while all the other alternative spliced variants do not(Yi, White et al. 2000, Saeboe-Larssen, Fossberg et al. 2006). The major alternative splicing isoforms include minus alpha (-α), minus beta (-β), and both minus alpha beta (-α β), which are isoforms that are alternatively spliced within the reverse transcriptase domain. Currently the detailed functions of these isoforms are not well understood, but several of them have been reported to have non-canonical functions during cell proliferation and senescence(Colgin, Wilkinson et al. 2000, Yi, White et al. 2000).

The RNA component of human telomerase, hTR or hTERC, was characterized in 1995(Feng, Funk et al. 1995), and it directs the addition of new telomere repeats onto the end of

telomere. Besides hTERT and hTR, there are several other components that interact with telomerase to maintain its functionality, including TCAB1, dyskerin, and NOP10 (Egan and Collins 2012). For example, TCAB1 is required for recruitment of telomerase components in the Cajal body, where telomerase is assembled (Stern, Zyner et al. 2012).

1.5. Telomere, telomerase and human health

Since the discovery of telomerase in *Tetrahymena*, scientistsrapidly extended their efforts into human studies. In contrast with $(TTGGGG)_n$ in *Tetrahymena*, the telomere sequence in all vertebrates including humans is $(TTAGGG)_n$. At birth, telomere length in peripheral blood mononuclear cells (PBMCs) is around 10-15 kilobases. Then as the individual ages, the telomeres gradually get shorter, at the rate of 20-60 base pairs per year (Figure 1-6) (Aubert, Baerlocher et al. 2012).

A series of environmental factors can change the rate of telomere loss, including air pollution, tobacco exposure, stress, urban garbage, chronic infections and asthma(Farzaneh-Far, Lin et al. 2010, Kume, Kikukawa et al. 2012, Jiang, Dong et al. 2013, Raschenberger, Kollerits et al. 2013, Albrecht, Sillanpaa et al. 2014, Huzen, Wong et al. 2014, Rode, Nordestgaard et al. 2014, Zhao, Zhu et al. 2014). The detailed mechanism of how environmental factors affect the rate of telomere loss has yet to be elucidated, but it is reasonable to speculate that these stresses may cause cellular responses that triggers cell proliferation which leads to telomere shortening. In addition, increases in oxidative damage can lead to reactive oxygen species (ROS) that has been implicated in accelerated telomere loss.

Besides environmental factors, genetic mutations that are in gene products involved in telomere maintenance may also cause a spectrum of diseases, which are referred to as

telomeropathies (Holohan, Wright et al. 2014), such as dyskeratosis congenita, sporadic aplastic anemia, and idiopathic pulmonary fibrosis. Symptoms of telomerapathies include bone marrow failure, pulmonary fibrosis, oral leukoplakia, skin hyperpigmentation and nail dystrophy (so-called "diagnostic triad") as well as several other rare symptoms. There are a number of genes that are commonly associated with telomeropathies upon mutation, such as hTERT, hTR, Dyskerin (DKC1), TCAB1, TIN2, PARN, and RTEL1 (Vulliamy, Marrone et al. 2001, Vulliamy, Marrone et al. 2002, Armanios, Chen et al. 2005, Vulliamy, Beswick et al. 2008, Calado 2009, Calado and Young 2009, Stuart, Choi et al. 2015). The mutations frequency varies dramatically among the different genes, and it is currently not understood why some identical mutations result in very different clinical presentations.

The replicative capacity of cell is widely believed to be limited by the length of the shortest telomeres. At early human developmental stages (first 12 weeks after fertilization), telomerase is expressed in almost all cells. Later during fetal development, in a tissue specific manner, telomerase expression is turned off in the vast majority of somatic cells and only proliferating progenitor cells, and stimulated T cells maintain their telomerase activity (Wright, Piatyszek et al. 1996). To achieve unlimited proliferation, 85-90% of cancer cells re-activate telomerase. The constitutive telomerase expression enables cancer cells to maintain or even elongate telomeres to avoid DNA damage signals from a too short telomere (Figure 1-7). This results in stabilizing genomic DNA during cancer progression. Around 10-15% of immortal cancer cells remain telomerase-negative. Instead, they use a recombination-based mechanism called ALT (Alternative Lengthening of Telomeres) to counteract the loss of telomeres during proliferation and to maintain telomere length (Bryan, Englezou et al. 1997).

1.6. Therapeutic approaches targeting telomerase for cancer and aging

The constitutive expression of telomerase enables cancer cells to maintain their telomere length and proivde full potential for unlimited proliferation. Therefore, targeting telomerase or any similar method to hinder telomere maintenance specifically in cancer cells become a potential therapeutic approach for cancer treatment.

Imetelstat (GRN163L) is a 13-mer oligonucleotide that binds to the template region of hTR, the RNA component of telomerase. Since the sequence of the oligonucleotide is complementary to the active site of hTR, Imetelstat prevents telomerase access to telomeres, and thus, inhibit telomerase activity and progressive telomere shortening(Marian, Cho et al. 2010). It has been reported that Imetelstat can inhibit telomerase activity in xenograft models(Joseph, Tressler et al. 2010), and entered into phase II clinical trials for treatment of myelofibrosis (Mascarenhas, Sandy et al. 2017). Previously Imetelstat failed several large clinical trials targeting solid tumors, potentially because of hematological toxicities requiring patients coming off the inhibitor and thus telomeres regrew during the drug holiday period. In addition, it was not clear it the protocol for Imetelstat treatment was sufficient to sustain the inhibition of telomerase to drive cancer cells into crisis and apoptosis. In addition, it was reported that Imetelstat has off-target effects on the cytoskeleton (Mender, Senturk et al. 2013). Although Imetelstat shows limited promise in fulfilling its full potential in cancer treatment, it might be used together with the standard cancer therapy as a combination therapy to enhance the therapeutic effect on killing malignant cells.

Besides targeting telomerase activity, interference with the telomere elongation process is another strategy to cause telomere shortening in cancer cells. 6’-thio-2’-deoxyguanine (6-thio-dG) is a modified nucleotide that can compete with dGTP and be incorporated into telomeres during DNA replication. By incorporation, 6-thio-dG interferes with shelterin protein that bind to

telomeres that normally inhibit DNA damage responses. Therefore, 6-thio-dG can selectively kill telomerase positive cells and is currently in pre-clinical development. Initial studies have demonstrated its effectiveness in xenograft models but the efficacy in humans are yet to be conducted (Mender, Gryaznov et al. 2015, Mender, Gryaznov et al. 2015).

While the overall goal of treating cancer with telomerase inhibitors is to stop malignant cells from dividing and block the cells ability to maintain or elongate telomeres, aging is a completely opposite process. When a single or few telomeres get too short, cells stop dividing and go into a growth arrest state known as cellular senescence. Therefore, to maintain proliferation of older cells with short telomere, is would be important to think about ways to turn on telomerase activity, at least transiently, and elongate at least the shortest telomeres to compensate for the loss of telomere during divisions. While this could have importance in treating telomere spectrum disorders such as pulmonary fibrosis and dyskeratosis congenita, it is important to recognize that activating telomerase could increase the probability of the onset of cancer. TA-65 is a patented natural extract that is purported to very modestly activate telomerase and elongate telomeres. It is extracted from the root of a Chinese medicine called Astragalus and being used as an anti-aging product. *In vitro*, TA-65 can enhance telomerase activation during T cell stimulation (data not shown). However, this has not been fully proved *in vivo* yet. It has been reported previously that TA-65 can activate telomerase and maintain telomere lengths in mice (Bernardes de Jesus, Schneeberger et al. 2011). Our initial studies based on a small human clinical trial further indicated that TA-65 may have positive effects on maintaining telomere length in humans over a one year period, however these are only preliminary studies. Currently, a new clinical trial involving 500 volunteers is ongoing. Even with telomerase activation and some maintenance of the shortest telomeres with TA-65, this will not directly or indirectly

indicate it has anti-aging effect in humans. This will require longitudinal studies over several decades to ensure that TA-65 is safe for long-term use.

2. An Introduction to T Lymphocytes in Immune Response

2.1. The heterogeneity of immune cells

Immune cells are a highly heterogeneous population, consisting of T cells, B cells, NK cells, macrophages, dendritic cells, and other monocytes. Different subset of immune cells work together to build up the innate and adaptive immune system in humans. Immune cells are usually referred to as "white blood cells (WBC)", which is derived from the physical appearance of a blood sample after gradient centrifugation, compared with "red blood cells (RBC)". WBC are also called "leukocytes", which roots from Greek terms "leuk", meaning "white", and "cyte" meaning "cells". All WBC have a nucleus, which differentiate them with those RBC and platelets without a nucleus. After centrifugation, WBCs appear as a thin, white layer located between the RBC layer and the blood plasma. This WBC layer of cells are also referred to as the "buffy coat".

There are two major ways to categorize WBCs. One is by the appearance under the light microscope (granulocytes or agranulocytes). Granulocytes have a round nucleus with no or invisible granules in their cytoplasm, and agranulocytes have a bi- or multi-lobed nucleus with obvious granules in the cytoplasm. The other way to characterize WBC s is by hematopoietic lineage (myeloid or lymphoid). Myeloid cells are derived from the bone marrow and include neutrophils, eosinophils, basophils, and monocytes. Lymphoid cells are derived from the thymus (T cells), the bone marrow (B cells) and are the major components of the adaptive immune

response. T cells are involved in cell mediated immunity while B cells are primarily responsible for humoral immunity by producing large quantities of antibodies. The function of T cells and B cells is to recongnize specific non-self antigens, during a process known as antigen presentation. There are other subsets of T cells, called T helper cells that produce cytokines and help direct the immune responses. In addition there are cytotoxic T cells that produce granules containing enzymes that can kill pathogens. Both B and T cells can convert to memory cells and thus are maintained long term and capable of mounting a rapid and strong response to any pathogen previously encountered (e.g. acquired immunity). .

Peripheral blood mononuclear cells (PBMCs) are the population of peripheral blood cells including lymphocytes (T cells, B cells, NK cells) as well asmonocytes, and excludes RBCs and platelets that have no nucleus and thus cannot divide in the peripheral blood. Granulocytes are differentiated cells (neutrophils, basophils and eosinophils) are post mitotic and survive only a few days in the peripheral blood. PBMCs are extracted from the buffy coat after gradient centrifugation. The buffy coat is acquired by syringe, and after lysing the RBCs, the remaining cells are plated on cell culture dishes for several hours so that most of the granulocytes will be able to adhere to the dish. Thus, PBMCs that are in suspension are isolated from all the remaining cells.

After this step PBMCs is a term that commonly used in actual lab practice and consists of 30-60% CD4+ T cells, 10-20% CD8+ T cells, 10-15% B cells, 10-15% NK cells, as well as other monocytes. Since the majority of PBMCs are T cells, it is also used for the general study of T cell population. CD3+, CD4+, CD8+T cells and B cells can all be isolated through further separation of PBMCs.

CD3+ T cells refers to the whole T-cell population. CD3 (cluster of differentiation 3) is a receptor that helps to activate T cells. This antigen is presented universally on all mature T cells and no other cell types. Therefore it is used as a specific marker for T cells. CD4+ T cells are T helper cells (Th cells). This type of T cells facilitate the function of other immune cells by secreting cytokines and help regulation of immune response. CD8+ T cells are cytotoxic T cell (Tc cells, CTL). This type of T cells recognize and kill other abnormal cells, including cancer cells, infected cells (especially with virus infection), or damaged cells.

2.2. Molecular mechanism of T cell stimulation

During pathogenic events, the antigen presenting cells (APC) will process and present the antigen to the T-cell receptor, trigger T cell stimulation, and eventually eliminate the pathogen. Typically APC refers to macrophages, dendritic cells, and sometimes B- lymphocytes. APCs are central to the initial priming of T cell responses and provide all of the critical signals necessary to promote T cell activation, proliferation, and differentiation.

APCs present antigens to both CD4 and CD8 cells through Class II and I MHC pathways, respectively. There are three major signals that APC delivers to the naive T cells, to trigger activation, promote survival, and facilitate differentiation.

Signal I is T-Cell Receptor (TCR) activation. The antigenic peptide is presented by the MHC class I/II complex to the TCR/CD3 complex on the surface of T cells. This binding interactions triggers a series of downstream signaling pathway, including MAP kinases, PKC, Calcineurin, and activation of transcription factors such as NF-κB, NFAT, and AP-1.

Signal II is CD28 co-stimulatory signaling. By binding of the B7 complex with CD28 co-stimulatory receptor, it triggers cellular signaling pathway that promote survival of the T cells. Co-stimulation through CD28 is critical for complete activation.

Signal III is cytokine secretion that promotes cell differentiation. During the presenting process of antigen to T cells, APC also delivers signals that further differentiate naive T cells into more specific effector cells. For example, TGF-β promotes differentiation to Treg cells, IL-12 and IFN-γ to Th1 cells, IL-4 to Th2 cells, etc.

2.3. Comparison of stimulation methods

A variety of stimuli can achieve similar outcomes for in vitro T cell stimulation, including concanavalin A (ConA), phytohaemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA)/ ionomycin, and anti-CD3/CD28 beads.

ConA is a lectin from jackbean that can bind to various sugars, glycoproteins, and glycolipids for non-specific stimulation of T cells. It is known to stimulate four functionally distinct T cell populations and at least one subset of human T cells (Dwyer JM et al. 1981).

PHA is also a plant lectin that has carbohydrate-binding specificity on glycosylated protein including T-cell receptor (TCR). By crosslinking various surface proteins, PHA triggers T cell activation as well a variety of other signaling pathways, and affects the cell membrane in regards to transportation and permeability to proteins (Barabas E. et al. 2002 & Wang H. et al. 2013).

PMA is a small organic compound that can diffuse through the membrane and directly bind to protein kinase C (PKC). Together with ionomycin, a calcium ionophor to trigger calcium

release, PMA/ionomycin cocktail can achieve TCR-independent activation of T cells but with certain cytotoxicities (Wang H. et al. 2013).

Anti-CD3/CD28 is a cocktail of antibodies that binds to CD3 and CD28 on the surface of T cells, triggering both signaling pathway I & II that facilitate T cell activation and survival (Trickett A et al. 2003). Due to the defined function of specifically binding to CD3 and CD28 surface protein, which mimics in vivo T cell activation from antigen-presenting cells (APC), we decide to useanti-CD3/CD28-coated beads in this study to investigate telomere and telomerase dynamics in T cells during stimulation.

3. An Introduction to Centenarian Studies

The term centenarian refers to people who lives to or beyond 100 years old, and supercentenarian refers to people who lives to over 110 years old (a very rare event that only occurs in1 in about 1000 centenarian). There has been one validated case in humans where an individual that lived over 122 years. Jeanne Calment, a French lady, lived for 122 years and 165 days and died in 1997 (Robine and Allard 1998). According to the estimation in 2012 from the United Nations, there are 316,600 living centenarians worldwide. Among these, the United States currently has the highest number of centenarians with around 53,000, while Japan has the second-largest population with around 51,000 centenarians. The number of centenarians worldwide is heavily skewed towards females, with a female versus male ratio of 7-11 to 1.

Centenarians represent a very useful model to study the mechanisms of healthy aging and longevity. They reach an extreme lifespan and display a series of phenotypes that might be the result of both healthy aging and environmental factors (Franceschi and Bonafe 2003, Cevenini,

Invidia et al. 2008). There are a few studies that have provided insights into the medical conditions and functional abilities of centenarians. Schoenhofen et al. reported that supercentenarians require almost no assistance in activities of daily living and less than 15% have vascular-related disease history (Schoenhofen, Wyszynski et al. 2006). Strikingly, they also found that common diseases in younger centenarians (eg, 100–110 years), such as heart disease and stroke, are very rare among supercentenarians (eg. 110+ years). Willcox et al. also showed that most supercentenarians did not have major clinically evidence of disease up to age 105 (Willcox, Willcox et al. 2008).

Centenarians can be divided into 3 groups according to their disease onset age: survivors (onset of at least one disease prior to age 80 years), delayers (onset of at least one disease between ages of 80 and 99), and escapers (onset of at least one disease after age 100 years). The comparison of these three groups shows that supercentenarians (110+ years old) have larger proportion of escapers than younger centenarians (100-120 years old)(Andersen, Sebastiani et al. 2012). This suggests those that have long lifespan are more likely to have genetic factors to keep them away from major health-threatening disease.

Since longevity is believed to be strongly associated with familial and genetic factors (De Benedictis and Franceschi 2006, Franceschi, Bezrukov et al. 2007, Gentilini, Mari et al. 2013), centenarians' offspring (CO) may have inherited advantageous characteristics in longevity, thus providing a useful model to study healthy aging and longevity. Recent studies have reported that CO are more likely to be long-lived and have a lower risk of age-related diseases compared to matched controls(Adams, Nolan et al. 2008, Gentilini, Mari et al. 2013, Gueresi, Miglio et al. 2013). In addition, the comparison of telomere length among centenarians, centenarians' offspring, and matched control group suggests that both centenarians and their offspring have

better capability of maintaining telomere length, which may in turn contribute to improved immune responses and thus their healthy aging and increased longevity (Tedone, Arosio et al. 2014). This previous study on centenarians and their offspring was based on the results of leucocytes at resting or dormant state (without stimulation to mimic immune responses). Thus, how the immune system of centenarians respond to stimuli compared with other age groups remain unknown and is part of my dissertation research.

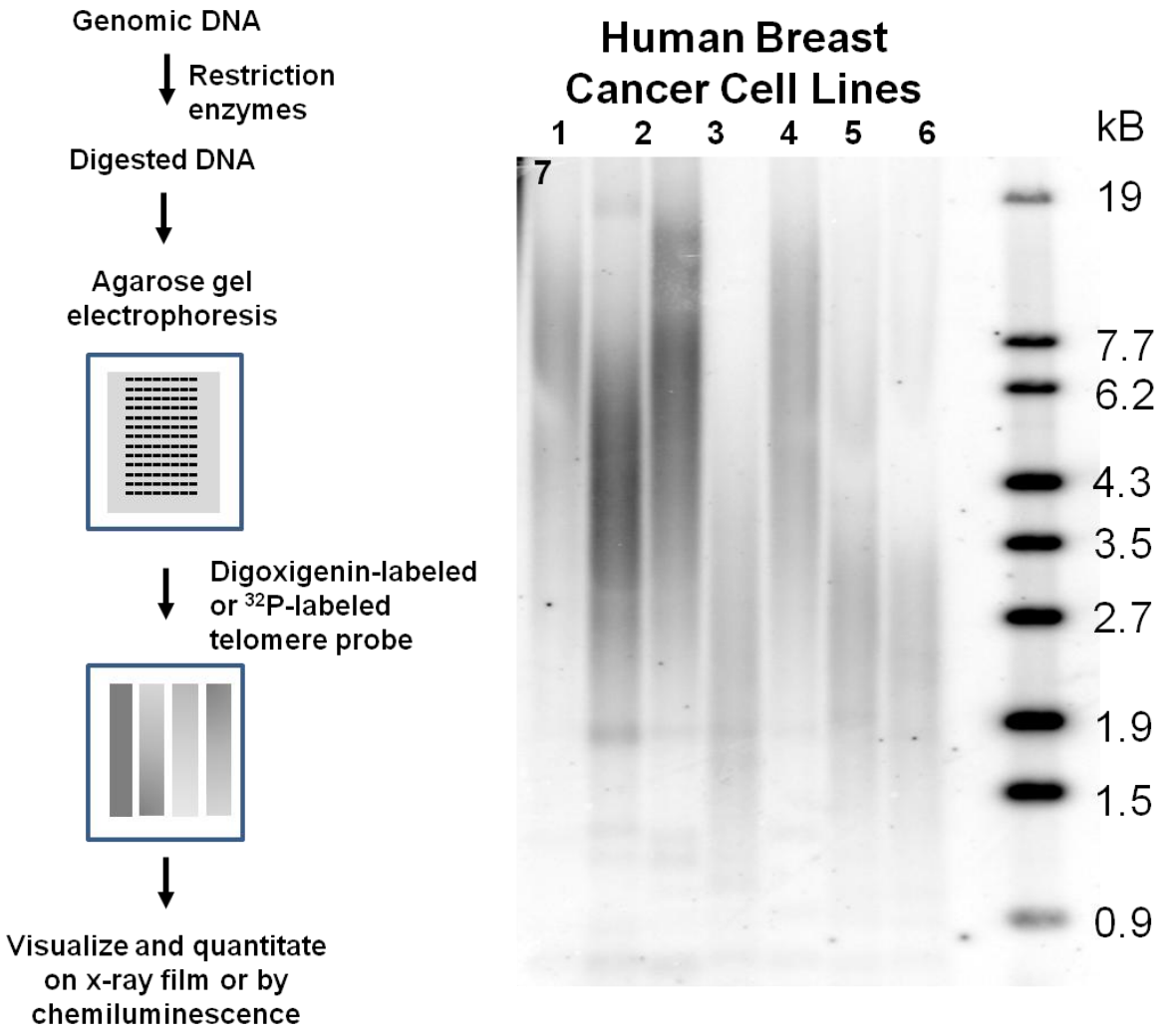


Figure 1-1. Schematic of telomere restriction fragment (TRF) analysis (left panel). Cancer cell lines show different distribution of telomere, suggesting different size of telomeres (right panel).

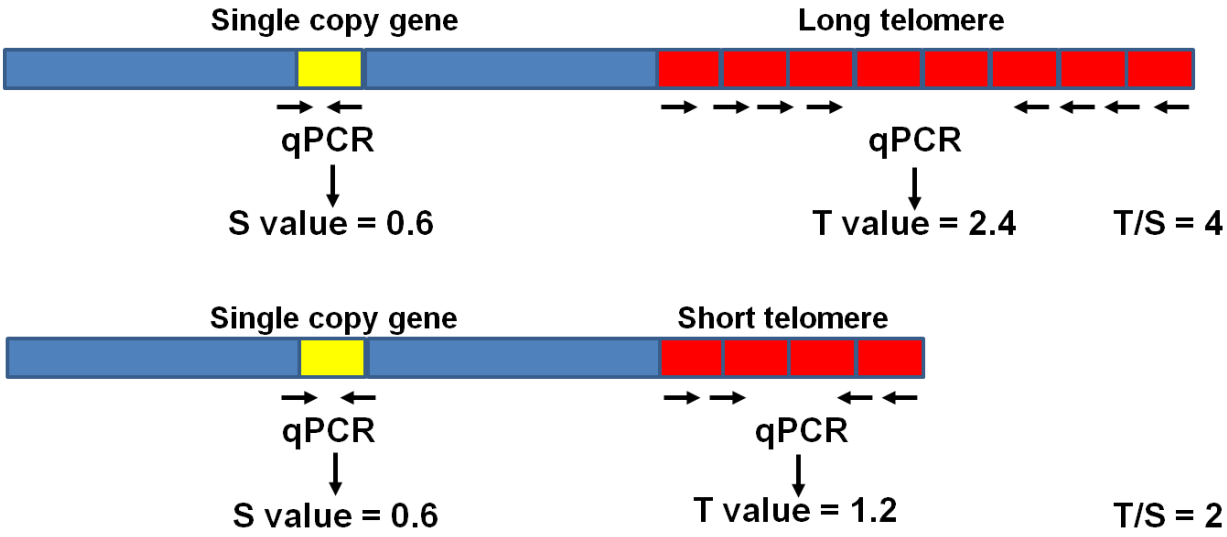


Figure 1-2. Schematic of qPCR method for telomere length measurement.

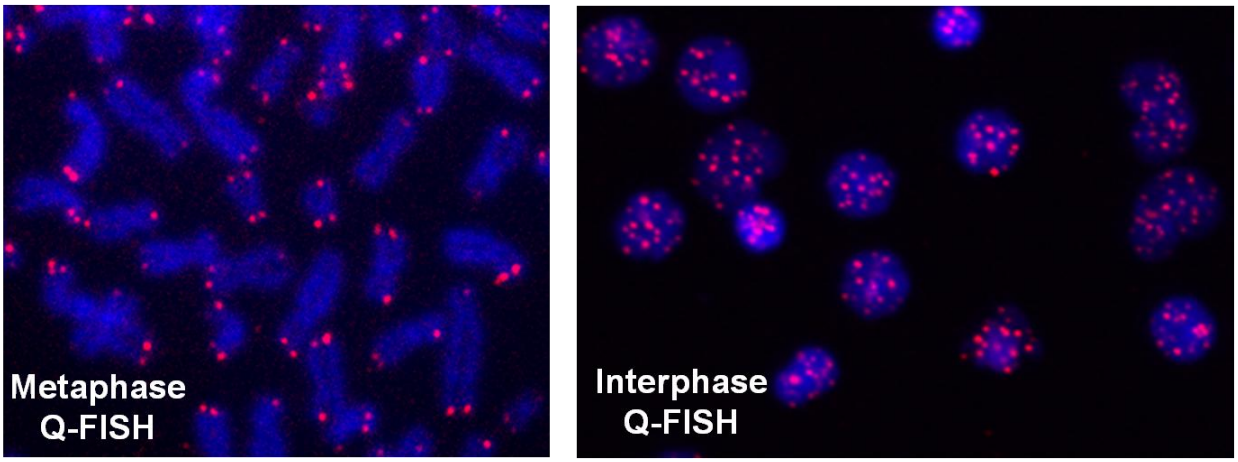


Figure 1-3. Typical images of metaphase and interphase Q-FISH. Red spots represent probe labeled telomeres.

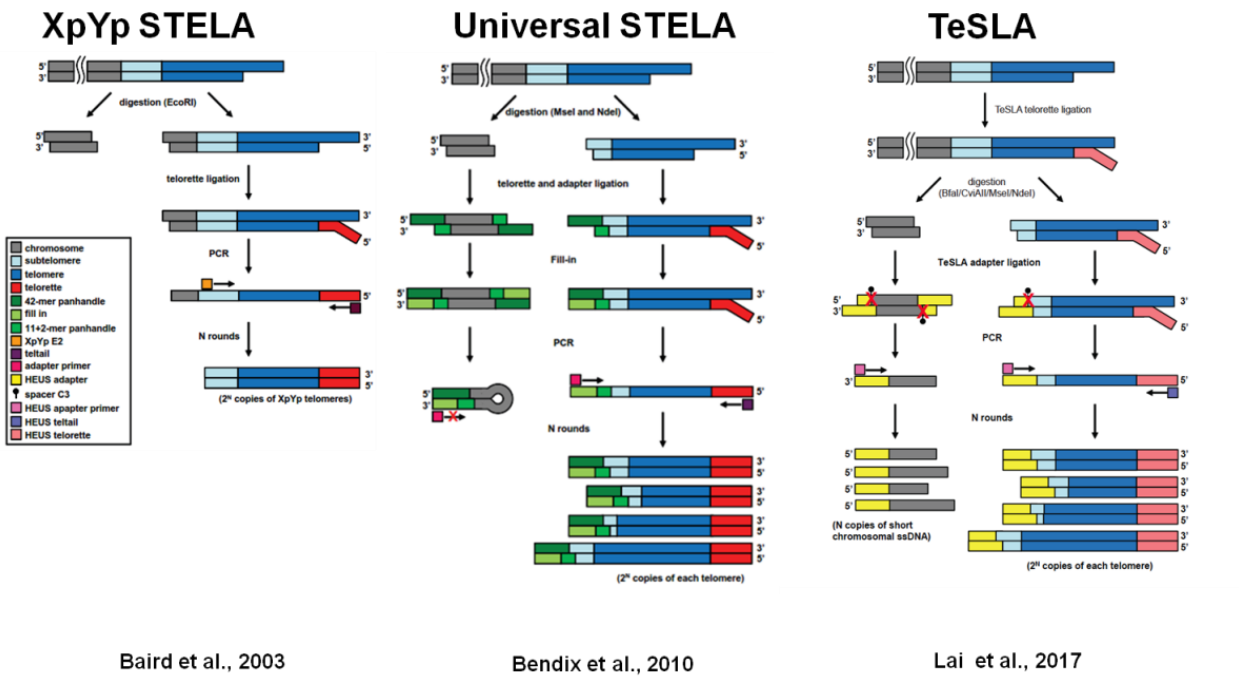


Figure 1-4. Schematic comparison of.Xp Yp STELA, Universal STELA, and TeSLA.

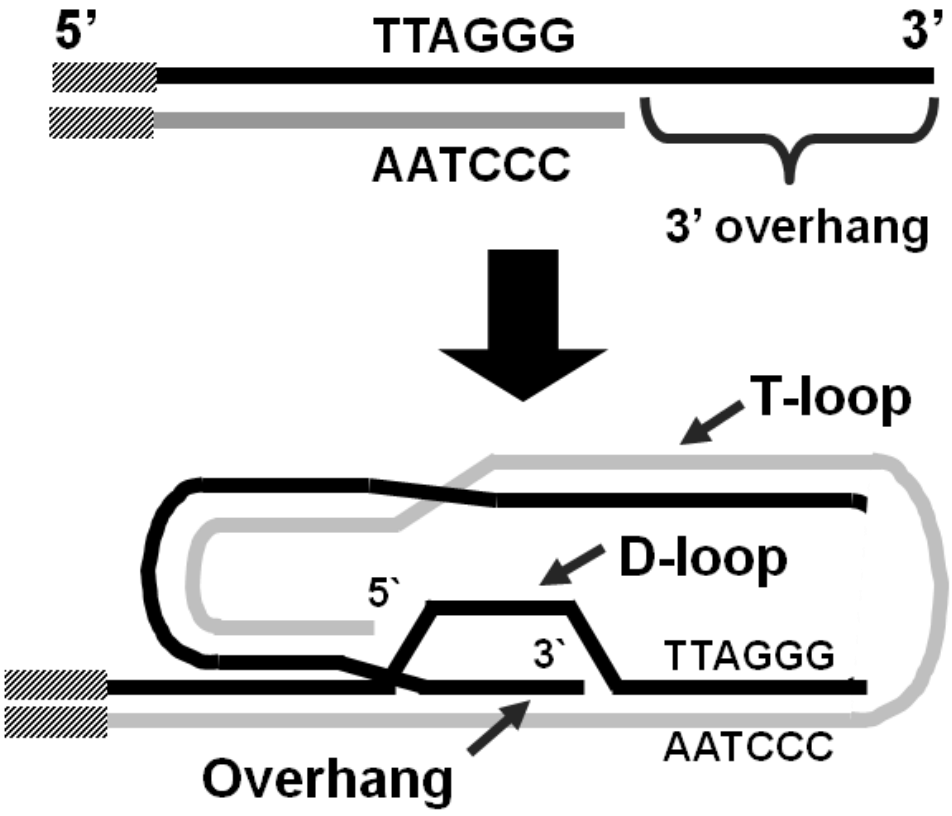


Figure 1-5. Schematic of T-loop structure at the end of telomeres.

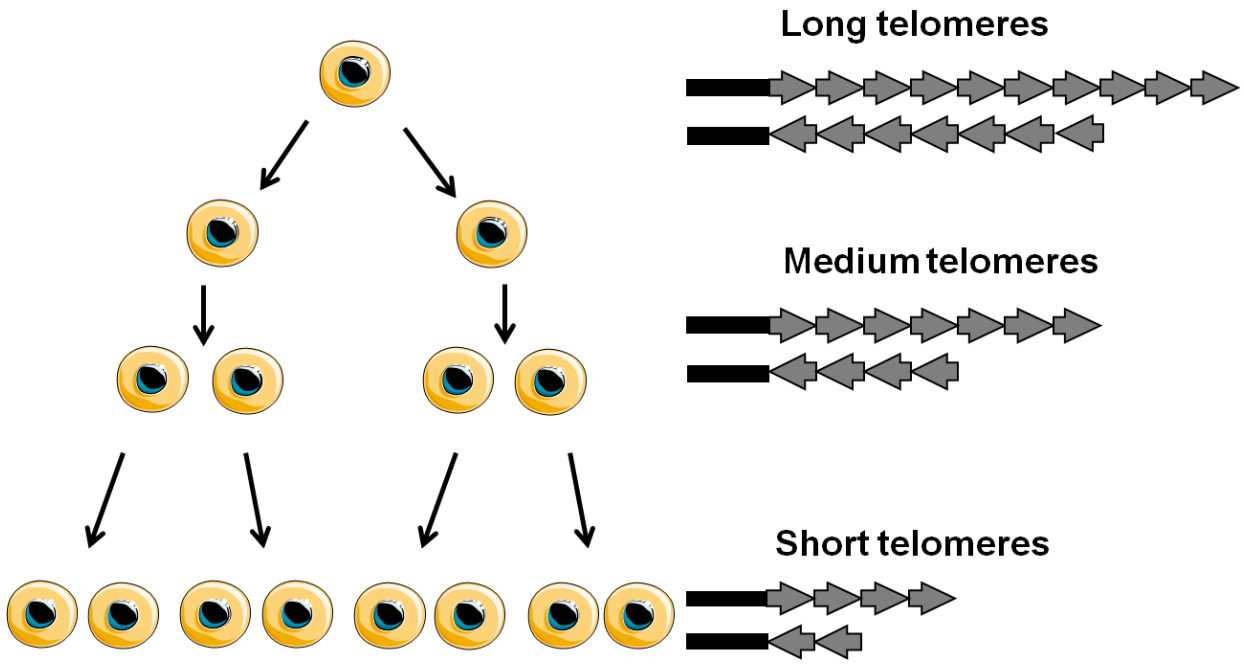


Figure 1-6. Telomere shortening with cell proliferation.

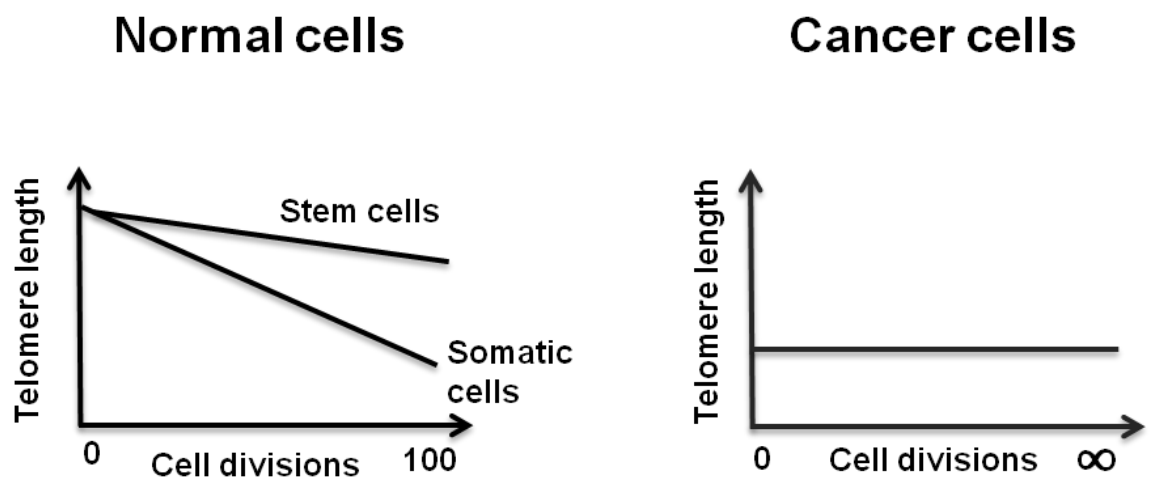


Figure 1-7. Telomere shortening in normal cells such as in stem cells and somatic cells (left panel). Telomere maintenance in cancer cells (right panel).

CHAPTER 2

TELOMERE AND TELOMERASE DYNAMICS IN STIMULATED T LYMPHOCYTES

The work presented in this chapter has been submitted for publication and is currently in review. Experiments were performed by Ejun (Elijah) Huang unless otherwise noted in the text and/or figure legends.

Introduction

Telomeres are repeated DNA sequences (TTAGGGn) that in combination with 6 shelterin proteins cap the ends of chromosomes to prevent the telomeres from being recognized as DNA damage (Blackburn and Collins 2011). The end replication problem (failure of lagging strand DNA synthesis to be fully replicated) results in the loss of DNA at the telomeres after each round of cellular DNA replication (Watson 1972). As a result, all human somatic cell telomeres become progressively shorter as cells divide. Progressive telomere shortening during each cell division finally leads to one or more critically short telomeres, initiating a DNA damage response signal that is referred to as replicative senescence(Mu and Wei 2002, Shay 2016). Previous cross-sectional studies have shown progressive telomere shortening in human lymphocytes from different age groups from newborn to individuals 90 years old (Aubert, Baerlocher et al. 2012). To compensate for telomere loss during cell division, some proliferating cells transiently express telomerase, a cellular reverse transcriptase that maintain telomeres by adding telomeric repeats to chromosome ends during DNA replication(Mu and Wei 2002, Blackburn and Collins 2011), and having a role in several essential cell signaling pathways(Cong and Shay 2008). Telomerase is a ribonucleoprotein enzyme complex consisting of a reverse

transcriptase (hTERT) and template RNA (hTR/hTERC), and is constitutively expressed in 85-90% of malignant human tumors (Kim, Piatyszek et al. 1994), while in normal cells and tissues, telomerase expression is highly regulated and restricted to some proliferating transit amplifying stem cell compartments(Huang, Wang et al. 2011).

It is known that upon mitogen stimulation, immune cells can be activated and divide rapidly. It is well established that during this process telomerase is transiently activated(Hiyama, Hirai et al. 1995). Since this earlier study, a few reports have investigated the relationship between telomerase activation, telomere dynamics, and cell proliferation capacity. For example, telomerase activity was inhibited by actinomycin D or cycloheximide(Weng, Levine et al. 1996), indicating that RNA and protein synthesis are both required for telomerase activation. Other studies have shown that telomerase is not exclusively regulated at the transcriptional level in stimulated T cells, as hTERT post-translational phosphorylation and nuclear translocation are essential to promote telomerase activity (Liu, Schoonmaker et al. 1999, Liu, Hodes et al. 2001). Although there appears to be a positive correlation between the magnitude of telomerase activity and the ability of T cells to respond to antigen-induced stimulation, it has been shown that hTERT knockdown does not affect the rate of T cell proliferation(Gazzaniga and Blackburn 2014). Furthermore, it has been shown that neither hTERT nor hTR knockdown induced increased telomere shortening during T cell stimulation (Gazzaniga and Blackburn 2014). In contrast, hTR may also have an anti-apoptotic role in human immune cells that is independent of telomerase activity, while overexpression of hTERT protein may lead to apoptosis by depleting hTR (Gazzaniga and Blackburn 2014). However, others have compared telomere lengths among populations of peripheral blood mononuclear cells (PBMCs) before and after stimulation, and

found lengthening of average telomeres after stimulation(Murillo-Ortiz, Albarran-Tamayo et al. 2013).

The studies described above employed semi-quantitative assays to measure telomerase activity and average telomere length from different cell populations(Weng, Levine et al. 1996, Liu, Schoonmaker et al. 1999, Liu, Hodes et al. 2001, Murillo-Ortiz, Albarran-Tamayo et al. 2013, Gazzaniga and Blackburn 2014). However, immune cells, especially T cell populations, consist of an extremely heterogeneous mixture of cell types (Zhu and Paul 2010). Thus, results published on heterogeneous T cell populations might not reflect the dynamics occurring in specific T cell sub-fractions. Also, monitoring subtle telomere changes is challenging due to the lack of a sensitive assay for quantifying the shortest telomere length in cells.

By taking advantage of novel techniques that can measure telomerase activity at the single-cell level using quantitative ddPCR techniques (ddTRAP)(Ludlow, Robin et al. 2014) and monitor subtle telomere changes using limited DNA input and more sensitive telomere length measurement assays (TeSLA, telomere shortest length analysis), in this report we show that only a subset of CD28+ T-cells show robust telomerase activity upon stimulation. This subset of T-cells appear to be capable of maintaining their telomere lengths while CD28- cells have significantly more short telomeres even though they proliferate at similar rates compared to CD28+ cells. This work emphasizes the importance of identifying specific subsets of T cells for the study of telomere and telomerase dynamics, and their relationship with immune responses. Investigating the function of telomerase activation during T cell stimulation may provide new insights into understanding normal immune responses (T cell in vivo proliferation), as well as T cell ex vivo expansion, which is a critical requirement for recent immunotherapy protocols.

Materials and Methods

Cell culture

Human peripheral blood samples were obtained from 10 healthy donors (20-35 years old) after informed consent and in accordance with the Institutional Review Board (IRB) at UT Southwestern Medical Center. All subjects included in this study were nonsmokers with no history of alcohol abuse or drug consumption. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation with Ficoll-Paque Plus (GE Healthcare) and T cells were further isolated by negative selection. T cells were cultured in RPMI+GlutaMAX-I with 10% fetal bovine serum, 1% penicillin, streptomycin and amphotericin B and 10ng/mL interleukin-2. Cells were stimulated 24 hours after isolation by adding Dynabeads Human T-Activator CD3/CD28 (Life Technologies) in a 1:1 ratio. The percent of live cells was determined every day by trypan blue exclusion using a TC20 Automated Cell Counter (BioRad). When the cell density reached $\geqslant 1.5 \times 106$/mL, cells were diluted with fresh complete RPMI medium to a density of below 1.0 $\times 106$/mL. GRN163L (Imetelstat) was added 1 hour before stimulation at the concentration of 2uM.

Single-cell isolation and telomerase enzymatic activity assays

Telomerase activity was determined using the telomere repeat amplification protocol (TRAP), as previously described(Kim, Piatyszek et al. 1994, Zhang, Wang et al. 2000). The droplet-digital TRAP (ddTRAP) assay was performed as described(Ludlow, Robin et al. 2014). Single-cell isolation was described previously (Wright, Shay et al. 1995). In brief, cells were

suspended and diluted in 1X PBS to 1000 or 2000 cells/mL, and then 1μL aliquots were placed on a glass slide. Drops containing single cell were identified under the microscope, and then the visually confirmed 1μL aliquots were mixed with 1μL of ddTRAP lysis-extension buffer containing NP-40 buffer (1 mM Tris-HCl, pH8.0, 1 mM MgCl2, 1 mM EDTA 10% (vol/vol) glycerol, 150 mM NaCl, 5 mM β-mercaptoethanol, 0.1 mM AEBSF), 10X TRAP buffer (200 mM Tris-HCl, pH8.3, 15 mM MgCl2), 2.5mM each dNTPs, 200mM TS substrate, and 0.4mg/mL BSA. The 2μL lysis-extension mixture was pipetted into a PCR tube, placed on ice for at least 30min incubation, followed by 40 min at 25 ℃ for telomerase extension reaction and 5 min at 95 ℃ for deactivation. After the extension reaction, the 2μL lysis-extension mixture was ready for the standard ddTRAP protocol as previously described(Ludlow, Robin et al. 2014).

DNA extraction and telomeric measurement assays

Genomic DNA was extracted using the GentraPuregen DNA extraction kit (Qiagen) according to the manufacturer's instructions. Each DNA sample was quantified on a Nanodrop (Thermo Scientific) for concentration/purity, and integrity of DNA was determined as previously indicated (Kimura, Stone et al. 2010).For DNA preparation from a small number of cells (i.e. 100 cells), T cells were resuspended in 1X PBS at the density of 1.0×105/mL. Then 1μL of cell suspension (100 cells equivalents) was mixed with 1μL of NP-40 buffer (recipe described above) and incubated on ice for 30 min to lyse the cells. Then 1μL of protease (QIAGEN) was added to the 2μL mixture and incubate at 50°C for 30 min to digest protein, followed by 70 ℃ for 15 min to deactivate the protease. The 3μL mixture was directly used for the TeSLA (shortest telomere length) assay.

Terminal restriction fragment (TRF) assays were performed as previously described (Herbert, Shay et al. 2003).

The telomere shortest length analysis (TeSLA) was performed as previously described. In brief, each telomere overhang in the genomic DNA is ligated to telorettes by T4 DNA ligase (New England Biolabs). After ligation, the genomic DNA is digested by a series of restriction enzymes including CviAII, BfaI, NdeI, and MseI (New England Biolabs) to digest all DNA but not telomeres which do not have restriction site for these enzymes. The digested genomic DNA is next treated with Shrimp Alkaline Phosphatase (rSAP; New England Biolabs) to remove 5' phosphate from each DNA fragment to improve the specificity of ligation between overhang adapters and genomic DNA fragments. For adaptor ligation,1 μM of AT adapter and 1 μM of TA adapter are mixed and ligated to the DNA fragment by T4 DNA ligase. After adapter ligation, multiple PCR reactions are performed (initial melt at 94℃ for 2 minutes followed by 26 cycles of 94 ℃ for 15 seconds, 60 ℃ for 30 seconds, and 72 ℃ for 15 minutes) using 2.5 units of FailSafe enzyme mix (Epicenter) with 1X FailSafe buffer H in 25 μl reaction containing 0.25 μM primers (adapter and TeSLA TP) and 20-40 pg of ligated DNA. PCR products are resolved on a 0.85 % agarose gel (1.5 V/cm for 19 hours). After gel electrophoresis, the Southern blot analysis to detect amplified telomeres is conducted as previously described(Lai, Wright et al. 2016).

Flow Cytometry

Surface expression of CD4, CD8, and CD28 was examined by flow cytometry. Cells were incubated with allophycocyanin (APC)-conjugated anti-CD4 (Invitrogen), PE-conjugated anti-CD8 (Invitrogen), and FITC-conjugated anti-CD28 (eBioscience) at 4℃ for 20min, washed and fixed in PBS containing 2% 7-AAD. All samples were sorted and analyzed on a FACS Aria

II SORP (5 lasers) flow cytometer. Fluorescence data from at least 50,000 cells were acquired. Data analysis was performed using Cell Quest Pro (BD Biosciences).

Statistical Analysis

Mean values and standard deviations were calculated for each time-point. Significant difference was assessed by a two-tail Student's t test, and a cutoff of p value <0.05 was employed to determine significance.

Results

Transient telomerase activity levels in stimulated T lymphocytes are comparable with cancer cell lines.

There are a variety of methods that can achieve similar outcomes for in vitro T cell stimulation, including concanavalin A (ConA)(Dwyer and Johnson 1981), phytohaemagglutinin (PHA)(Hiyama, Hirai et al. 1995, Wang, Daniel et al. 2013), phorbol 12-myristate 13-acetate (PMA)/ ionomycin(Wang, Daniel et al. 2013), and anti-CD3/CD28. Among these, anti-CD3/CD28 is a cocktail of antibodies that binds to CD3 and CD28 on the surface of all T cells, triggering both signaling pathway I & II that promote T cells to proliferate(Trickett and Kwan 2003). As the specific binding to CD3 and CD28 surface proteins mimics in vivo T cell activation from antigen-presenting cells (APC), we decided to use anti-CD3/CD28-coated beads to investigate telomere and telomerase dynamics in T cells during stimulation (Figure 2-1).

Previous reports have demonstrated that mitogen stimulated T lymphocytes transiently turn on telomerase activity for a short period of time (generally 5-10 days), even with continual

mitogen stimulation (Weng, Palmer et al. 1997). We stimulated T cells with anti-CD3/CD28-coated magnetic beads, and observed that the cell population morphologically showed cell clumping and aggregation due to rapid cell division as soon as 2-3 days after stimulation (Figure 2-2). Consistent with the morphological changes, telomerase activity, measured by the classic gel-based TRAP assay, is activated and peaks at Day 3, then gradually drops back to baseline levels in around 10 days (Figure 2-3). This same phenotype has previously been reported by several research groups (Hiyama, Hirai et al. 1995, Liu, Schoonmaker et al. 1999). We compared the classic TRAP assay with ddTRAP assay, a novel droplet digital PCR TRAP protocol recently developed (Ludlow, Robin et al. 2014). Even though measurements obtained by TRAP strongly correlated with those obtained by ddTRAP, ddTRAP assay is more accurate and quantitative without the need for gel based quantification or internal PCR controls. (Figure 2-3). We next compared telomerase activity of stimulated T cells at day 3 (peak) with other cell lines by ddTRAP and observed that the magnitude of telomerase activity in stimulated T lymphocytes is comparable with that of several well characterized cancer cell lines (Figure 2-4). Both Jurkat and SW39 cells are capable of maintaining telomere length over time. This suggests that the similar level of telomerase activity detected in stimulated T cells is potentially sufficient to promote telomere length maintenance over various cell divisions.

Telomerase activity is not required for the proliferation of T lymphocytes

Next, we tested whether telomerase activation, besides its potential function in maintaining telomeres, was necessary for the proliferation of T cells during stimulation. GRN163L (Imetelstat) is a 13-mer thio-phosphoramidate oligonucleotide that is complementary to the template region of telomerase RNA subunit (hTR/hTERC), and thus acts as a direct, competitive telomerase inhibitor (Figure 2-5). We first tested GRN163L's effectiveness of

blocking telomerase activity in Jurkat cells (an immortalized human T lymphocyte line). After treating Jurkat cells with GRN163L, telomerase activity is almost completely reduced within 24 hours, but the rate of cell proliferation is unaffected (Figure 2-6). This suggests that GRN163L can efficiently block telomerase activity in suspension cells, and telomerase activity is not required, at least initially, for the proliferation of Jurkat cancer cells. However, it was not known in normal T cells with regulated telomerase if GRN163L would affect stimulated T cell proliferation. Therefore, we tested GRN163L on normal stimulated T cells, and observed that T cells without GRN163L treatment activated telomerase to the expected level while with GRN163L treatment telomerase activity was almost completely inhibited, demonstrating that GRN163L can efficiently block telomerase activity in stimulated T cells (Figure 2-6). Importantly, we also observed that cells treated with GRN163L proliferate at very similar rates as the cells without GRN163L treatment (Figure 2-6). Our results provide further evidence that telomerase activity is not required for the short-term proliferation of normal T lymphocytes during stimulation, nor for the short-term proliferation of cancer cells (Jurkat).

Lack of telomerase activity does not change the rate of telomere shortening in T cell populations during stimulation

Although transient telomerase activation in stimulated T cells is widely believed to at least slow down the rate of telomere shortening during cell divisions, to the best of our knowledge this has never been demonstrated. It is possible that previous studies have been complicated by the lack of sensitive techniques to monitor subtle telomere length changes. The TRF (terminal restriction fragment lengths) assay is a widely used method to measure average telomere length by Southern blotting (Herbert, Shay et al. 2003). Using TRF assay, we have measured telomere length from stimulated T cells at day 0, day 5 and day 10, and could not

detect telomere shortening over the ten day period (Figure 2-7). However, the TRF assay might not be sensitive enough to detect subtle changes in telomere dynamics, especially among the shortest telomeres which are extremely important in determining the cellular replicative lifespan (Shay 2003, Shay and Wright 2011).

To further investigate telomere dynamics of T cells during stimulation, we have developed a new technique termed TeSLA (Telomere Shortest Length Analysis), an assay that measures the percentage of all the shortest telomeres and thus can monitor subtle telomere changes with much higher sensitivity. In contrast with the result from TRF, TeSLA results show that before stimulation, the percentage of the short telomeres (below 1.6kb) is ~10%. Three days after stimulation, the percentage slightly increased to 11.5%, and 10 days after stimulation the percentage has increased to 24.2%. We also measured the average lengths of the shortest 20% telomeres over the stimulation period, and observed progressive telomere shortening (2.3kb vs2.2kb vs1.5kb at day 0, day 3 and day 10, respectively) (Figure 2-8, left panel).

More strikingly, when we completely blocked telomerase activity in stimulated T cells by GRN163L treatment and performed the TeSLA assay, we did not observe a significant increase of the load of shortest telomeres as initially expected (Figure 2-8, right panel). The percentage of the short telomeres (below 1.6kb) in GRN163L treated T cells increased at a similar rate from 9.3% to 19.3%, while the average length of the shortest 20% telomeres dropped from 2.3kb to 1.6kb.Thus, the lack of telomerase activity does not change the rate of telomere shortening in T cell populations during stimulation at least over the short-term.

We hypothesized that these observations are potentially due to high T cell heterogeneity, in which only a small subset of stimulated T cells expresses robust telomerase activity. If correct

this would indicate that only sub-populations of T cells can maintain telomere length during cell divisions. When treated withGRN163L, we predicted telomerase activity of this subset of T cells would be blocked leading to more dramatic loss of telomeres during stimulation. However, when we performed TeSLA on a heterogeneous populations of T cells, the overall telomere loss of this subset of T cells would be predicted to be diluted by the large population of T cells that did not activated telomerase. If this is correct then only a subset of T cells with telomerase would be capable of maintaining telomere lengths during stimulation and this information is masked when mixed populations of T cells are measured. Therefore, an in-depth investigation on some of the sub-populations of stimulated T cells became necessary to more fully understand telomerase and telomere dynamics during T-cell stimulation.

Only a subset of CD28+ T cells show telomerase activity and are capable of maintaining telomere length during stimulation

Previous studies have demonstrated that at the single-cell level, T cells respond to antigen stimulation in an all-or-nothing manner, which is termed digital immune responses (Huang, Brameshuber et al. 2013, Wertek and Xu 2014). However, it has not been determined if there is heterogeneity of telomerase activity at the single-cell level among different T cell sub-populations. We separated single cells by dilution plating aliquots (1μL) drops on glass slides and visually confirming the presence of single cells by microscopy (Figure 2-9). Next, we measured single cell telomerase activity by using a modified ddTRAP protocol (Ludlow, Robin et al. 2014). We first tested the viability of single-cell ddTRAP in Jurkat cells, and found heterogeneity of telomerase activity in individual cells tested. As expected, the average telomerase activity of single cells was comparable to that of the whole population (Figure 2-10). Next, we applied single-cell ddTRAP on stimulated T cells from day 0 to day 10. Interestingly,

only a small proportion of T cells (~14%) showed strong telomerase activity, while the vast majority exhibited low or no detectable telomerase (Figure 2-11).

It has been previously reported that the accumulation of CD28- T cells is associated with age-related decline of immune function (Weng, Akbar et al. 2009). When CD28 is ectopically expressed in CD8 T cells, the period of telomerase activity is extended(Parish, Wu et al. 2010). Additionally, another group reported telomere length differences in resting T cells between CD28+ and CD28- cells, with CD28+ showing slightly longer than average telomeres (Murillo-Ortiz, Albarran-Tamayo et al. 2013). Considering the importance of CD28 in telomerase activation in T cells, we further separated the stimulated T cells at day 3 by flow sorting into four sub-populations: CD4+CD28+, CD4+CD28-, CD8+CD28+, and CD8+CD28- (Figure 2-12). Following single cell ddTRAP analysis, we found that only a subset of CD28+ cells, regardless of CD4+ or CD8+ expression, exhibited robust telomerase activity. In contrast,CD28- cells had low or no detectable telomerase (Figure 2-13).

We next examined the ability of CD28+ and CD28- T cells to maintain telomere length upon antigen-induced proliferation. Stimulated T cells at day 0 and day 5 were flow sorted into CD28+ and CD28-, and telomere length was measured by the TeSLA assay. At Day 0, we found no significant difference in the length of the shortest 20% telomeres (2.4kb vs 2.3kb) or the percentage of critically short telomeres (10.6% vs 10.3%) in CD28- vs CD28+ T cells, respectively (Figure 2-14). After five days of stimulation, CD28+ cells exhibited lengthened telomeres while CD28- cells underwent progressive telomere shortening (3.4kb vs 1.9kb, respectively). Over the stimulation period CD28+ cells also showed fewer short telomeres (below 1.6 kb) compared to CD28- cells (10.9% vs 16.4%, respectively) (Figure 2-14). These

results can be interpreted to indicate that a subset of CD28+ but not CD28- cells are capable of compensating for telomere loss by telomerase activity up-regulation during T cell stimulation.

Discussion

Since transient telomerase activation in stimulated T cells was first described (Hiyama, Hirai et al. 1995), various studies have investigated the role of telomerase activity in cell proliferation and telomere maintenance during stimulation (Weng, Levine et al. 1996, Liu, Schoonmaker et al. 1999). In the short term, telomerase activation correlates with cell proliferation during T-cell stimulation but the functional significance of this was unknown. Previous studies demonstrated that when hTERT was knocked down the proliferation capacity of T cells was not affected (Gazzaniga and Blackburn 2014), suggesting the coupling of telomerase activation with cell proliferation were not functionally interrelated. However, the results reported on hTERT knockdown may have been confounded by the side effects of the knock down process, off-targeted effects, incomplete inactivation of telomerase, as well as alterations in hTERT splicing variants. In the current study, we used a small oligonucleotide GRN163L (Imetelstat) to treat T cells to minimize side effects while specifically and robustly blocking the function of telomerase without affecting its transcriptional or translational processes. When we uncoupled telomerase activity with cell proliferation by GRN163L, we demonstrated that telomerase activity is not required for T-cell proliferation, at least in the short term and on a population basis.

Although there are previous studies describing telomerase and telomere dynamics in stimulated human T cells, most of the results are based on a mixture of population cell types. Since T cells are an extremely heterogeneous population of subtypes, the phenotype of the

population may not reflect the changes of specific cell types. A previous study reported telomere length differences in subsets of resting lymphocytes (Lin, Cheon et al. 2016). In stimulated T cells, it was reported that CD4+ T cells maintain telomerase activity at higher levels compared to CD8+ T cells after several rounds of stimulation(Effros 2007). In these studies all activated T cells had equal levels of telomerase activity, with no significant enrichment in telomerase activity detected among G1, S, and G2/M populations(Weng, Levine et al. 1996).

Due to the lack of accurate and sensitive measurements for both telomere length and telomerase activity, there have not been studies investigating telomere and telomerase dynamics within specific sub-populations of T cells during stimulation with more sensitive assays. By applying droplet digital PCR techniques to the classic telomerase TRAP assay, we have first addressed the landscape of telomerase activity at the single cell level in stimulated T cells, and revealed that only a small subset of T cells show very strong telomerase activity upon stimulation. We further defined that this subset of T cells are restricted to CD28+ T cells. Previous studies have reported that the accumulation of CD28- T cells is one of the prominent changes in the age-associated decline of immune function (Weng, Akbar et al. 2009). Also, sustained ectopic CD28+ expression in CD8+ T cells has been reported to delay replicative senescence, but this is an indirect measure of telomere length (Parish, Wu et al. 2010). While there is a report that telomere elongation occurs in peripheral blood mononuclear cells (PBMCs) after 72h stimulation in young aged groups (20-25 years old) using a qPCR telomere length assay(Murillo-Ortiz, Albarran-Tamayo et al. 2013), we believe this might be due to the inaccuracy or large variation of qPCR measurements for average telomere lengths. In the current study, we used the classic TRF assay to measure average telomere length changes during stimulation but detected no changes as would be expected for such a short period of cell

proliferation. In contrast, the newly developed TeSLA assay showed the accumulation of short telomeres during stimulation. We further monitored telomere changes in sub-populations and provided more direct evidence that only telomerase positive CD28+ T cells, the sub-population that has telomerase activity, are capable of maintaining their telomere length to avoid the accumulation of short telomeres during stimulation.

While telomerase activity is mainly restricted to CD28+ cells, we note that only a subset of CD28+ cells have detectable telomerase activity upon stimulation. One explanation is that telomerase activity fluctuates during cell cycle processes, but this is in contrast with previous findings that telomerase activity is not cell cycle phase restricted (Weng, Levine et al. 1996). Another possible explanation could be a further defined subset of CD28+ cells that are capable of activating telomerase to maintain telomere length during stimulation. Our present studies indicate that further elucidation of this subset of T cells showing robust telomerase activity upon stimulation may help elucidate the mechanism of T cell exhaustion with aging and age-related diseases.

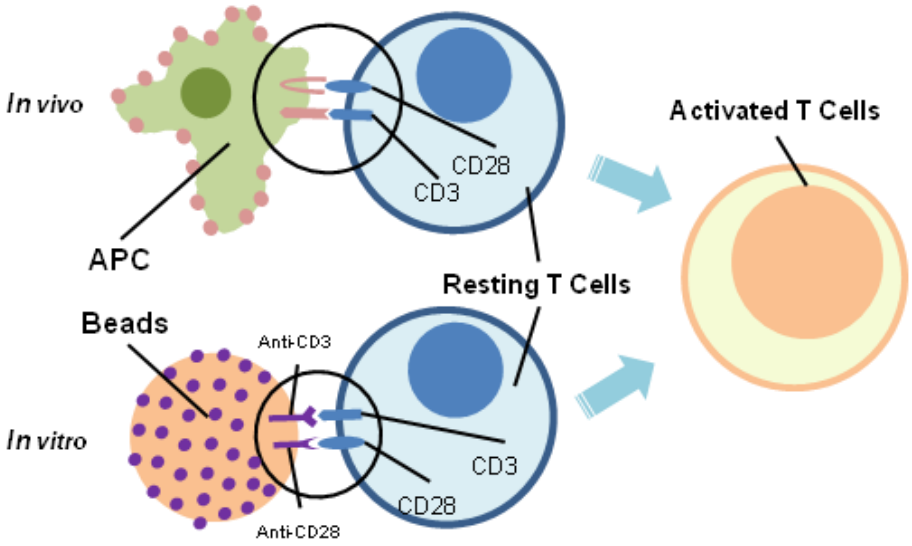


Figure 2-1. Beads activation mimics in vivo T cell activation from antigen-presenting cells (APC) by utilizing the two activation signals CD3 and CD28, bound to a 3D bead similar in size to the antigen-presenting cells.

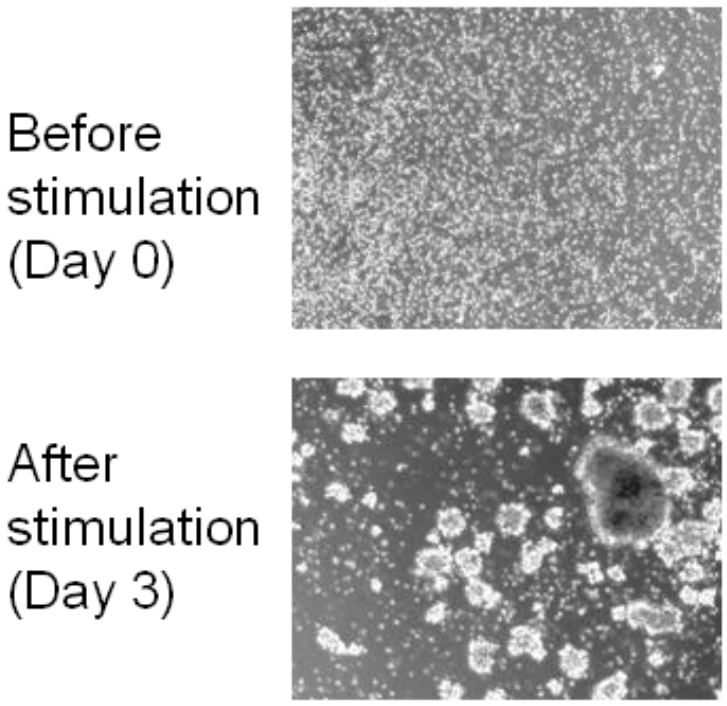


Figure 2-2. Microscopic pictures of T lymphocytes before (day 0) & after (day 3) stimulation.

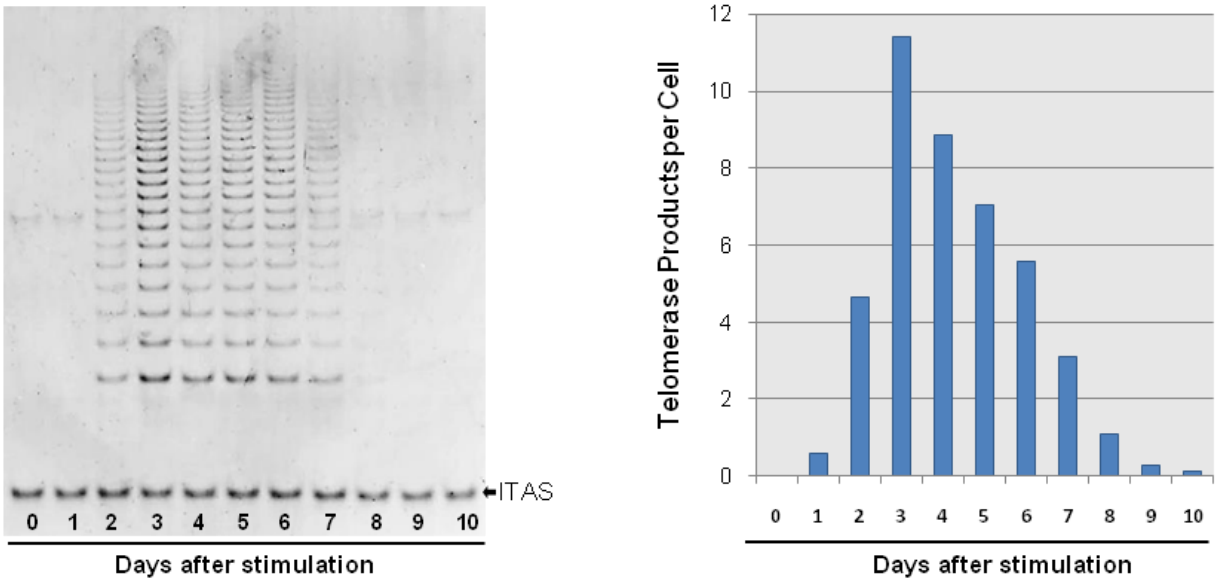


Figure 2-3. Transient telomerase activation in T cells measured by traditional gel-based TRAP assay and ddTRAP.

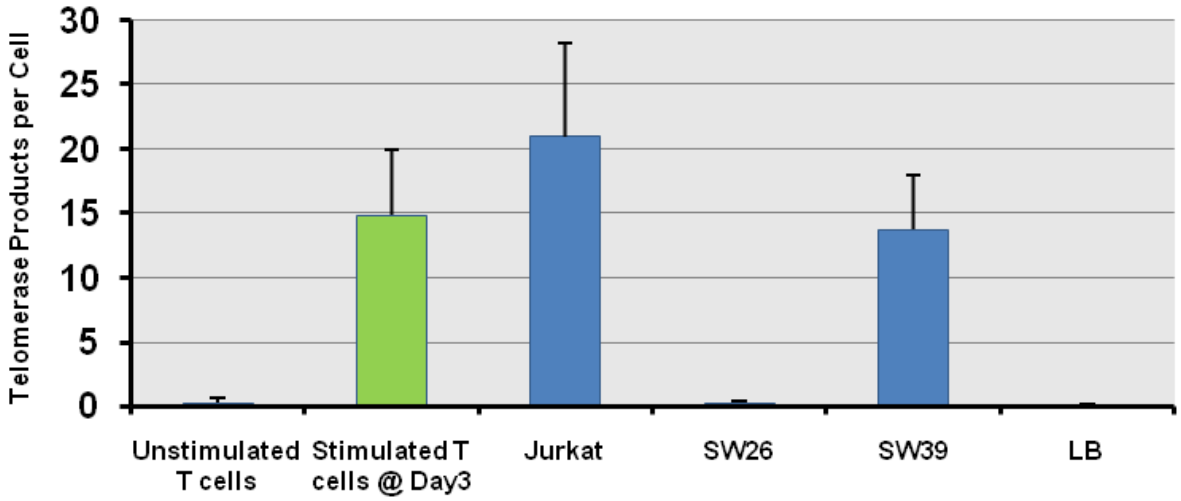


Figure 2-4. Comparison of telomerase activity among various cell types.

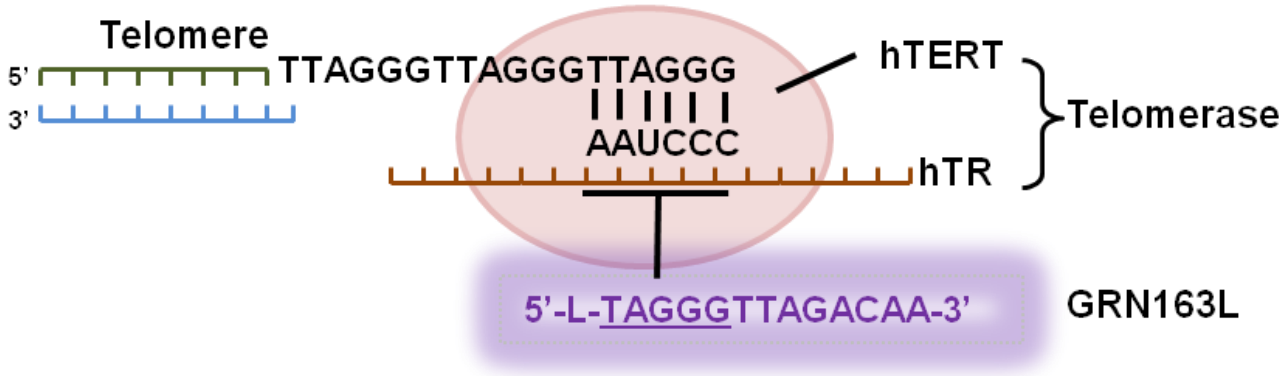


Figure 2-5. Schematic of GRN163L inhibiting telomerase activity.

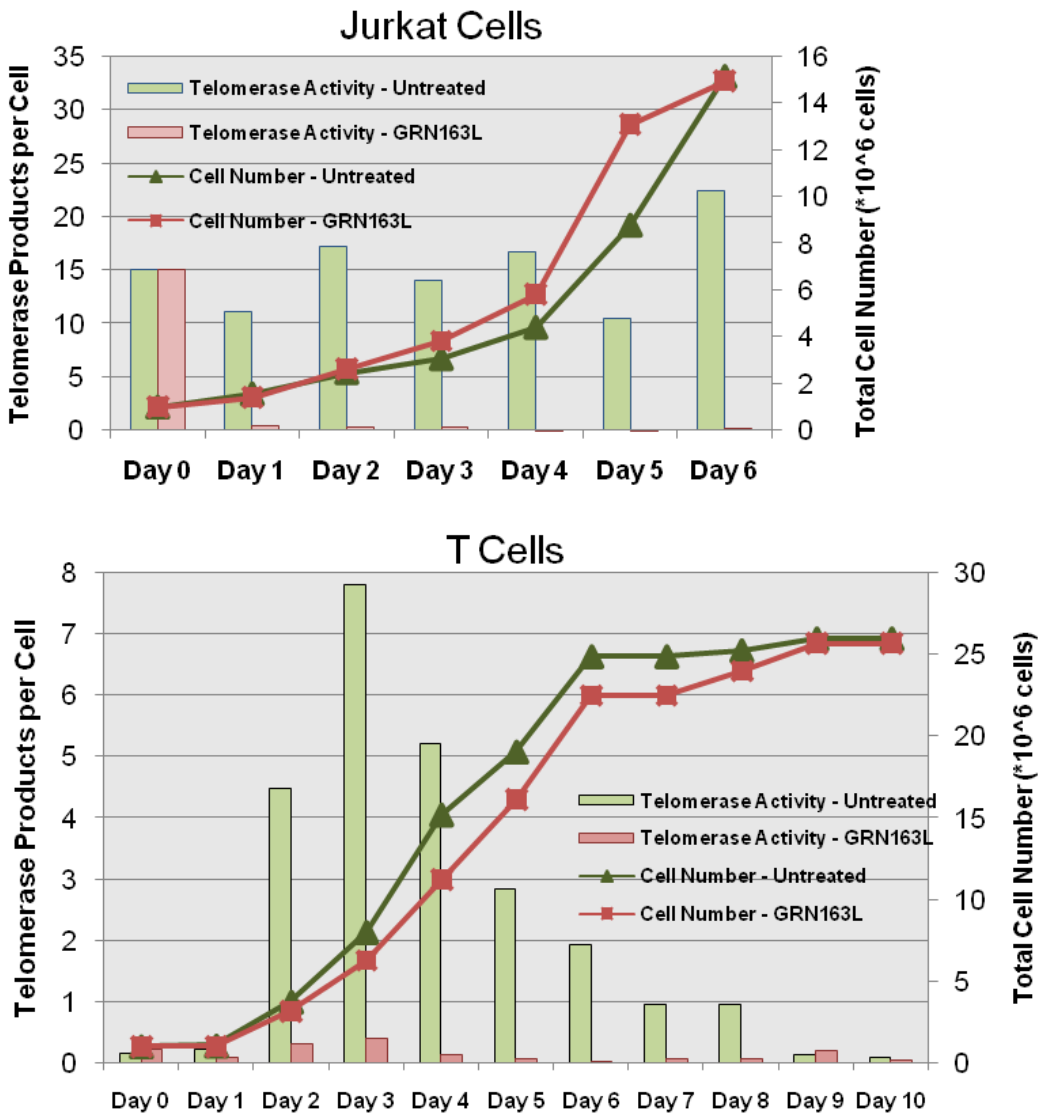


Figure 2-6. Telomerase activity and cell number growth curve of Jurkat cells (top panel) or stimulated T cells (bottom panel) treated with or without GRN163L.

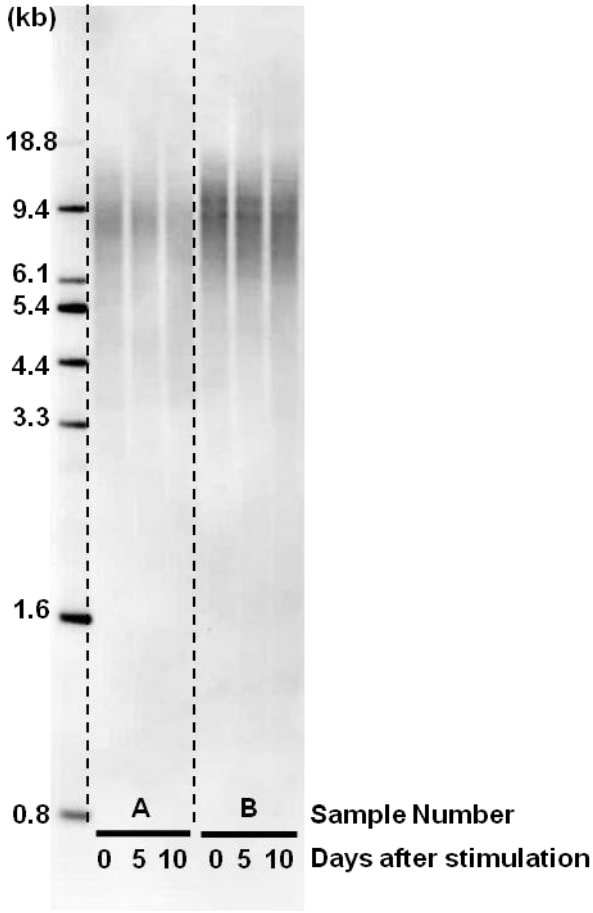
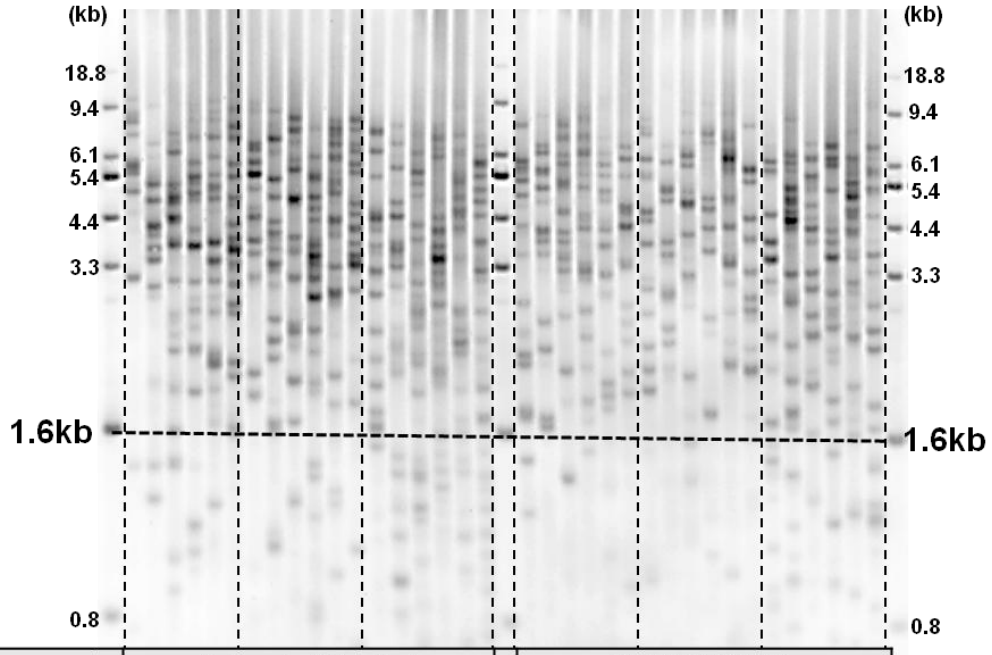


Figure 2-7. Telomere shorten during T cell stimulation (TRF assay).



	Untreated Control			2uM GRN163L		
	Day0	Day3	Day10	Day0	Day3	Day10
Shortest 20% Telomeres (kb)	2.3kb	2.2kb	1.5kb	2.3kb	2.3kb	1.6kb
Telomeres Below 1.6 kb (%)	10.0%	11.5%	24.2%	9.3%	9.4%	19.3%

Figure 2-8. The accumulation of short telomeres during T cell stimulation is detected by TeSLA, and blocking telomerase activity by GRN163L does not change the rate of short telomeres accumulation.

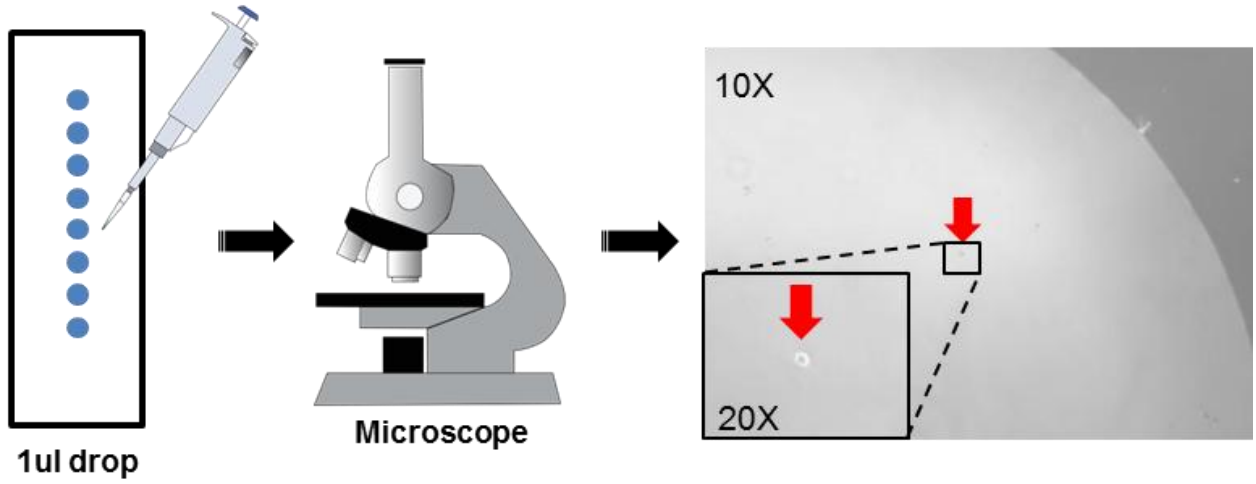


Figure 2-9. Schematic of single cell isolation procedure.

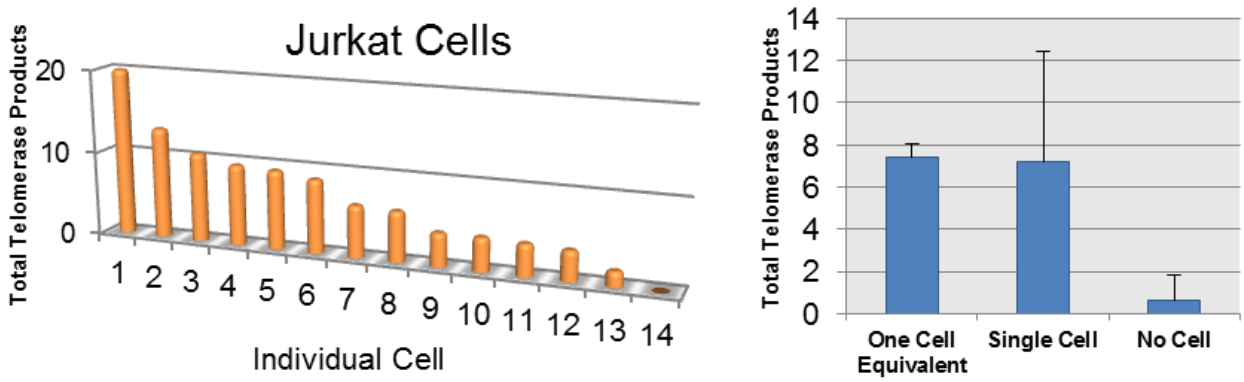


Figure 2-10. Single-cell ddTRAP reveals heterogeneity of telomerase activity level in the Jurkat cell population.

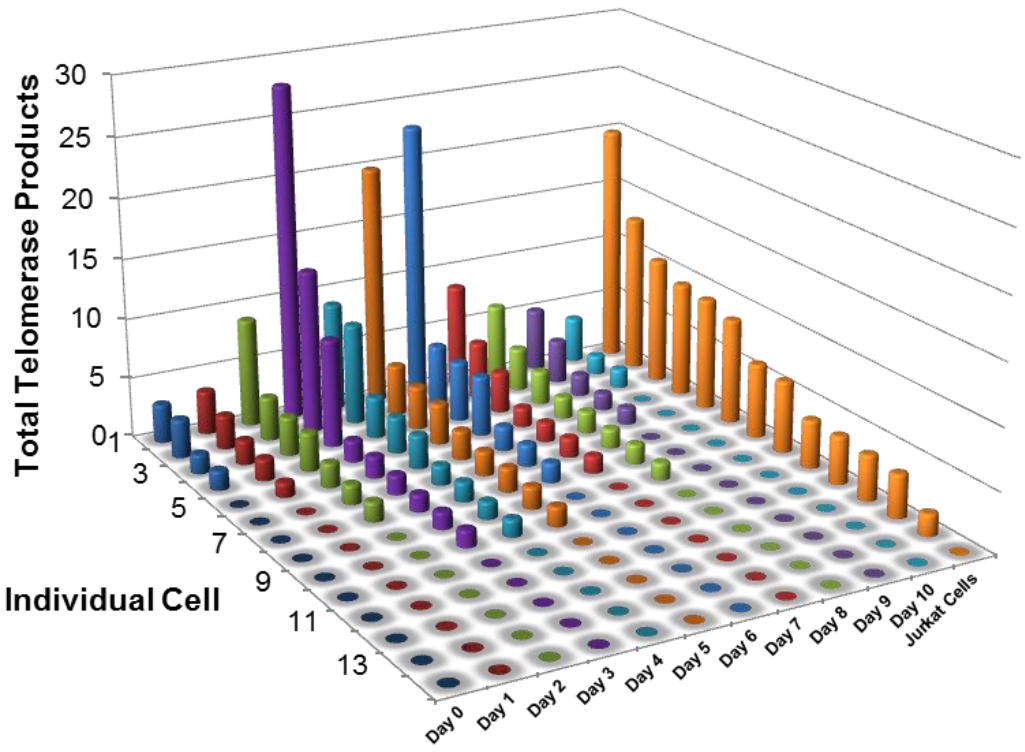


Figure 2-11. Single-cell ddTRAP on stimulated T cells from Day 0 to Day 10 shows that only a subset of T-cells population show telomerase activity.

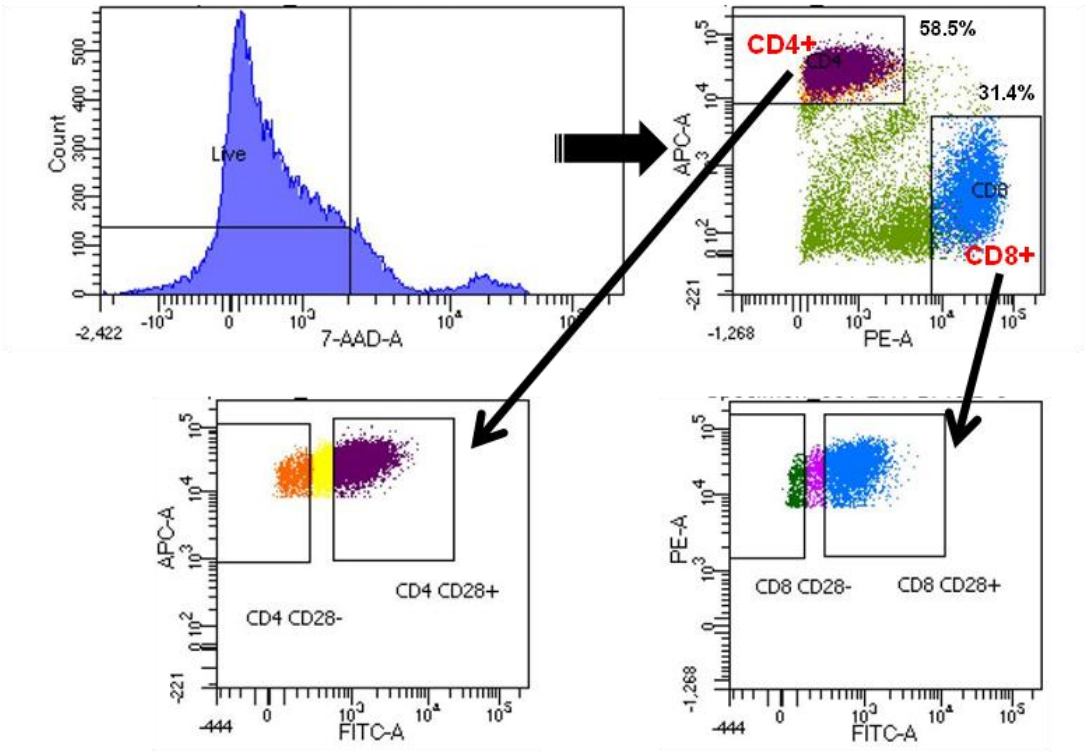


Figure 2-12. Flow sorting strategy of stimulated T cells on Day 3 to separate four populations.

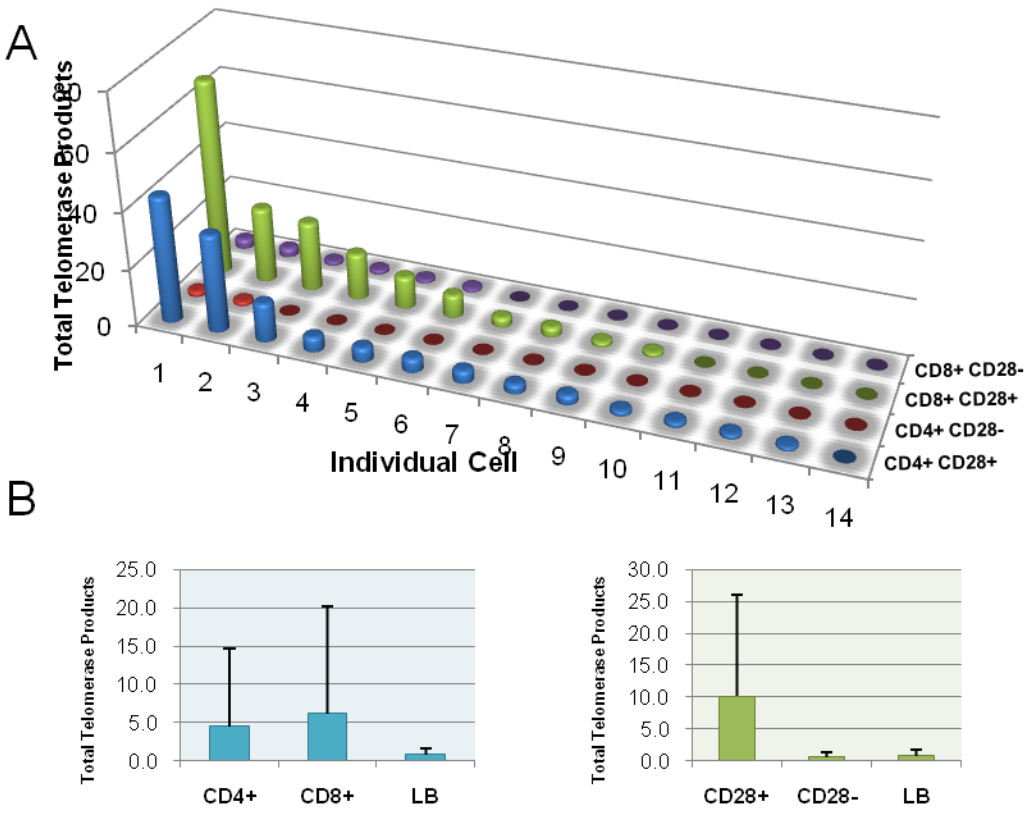
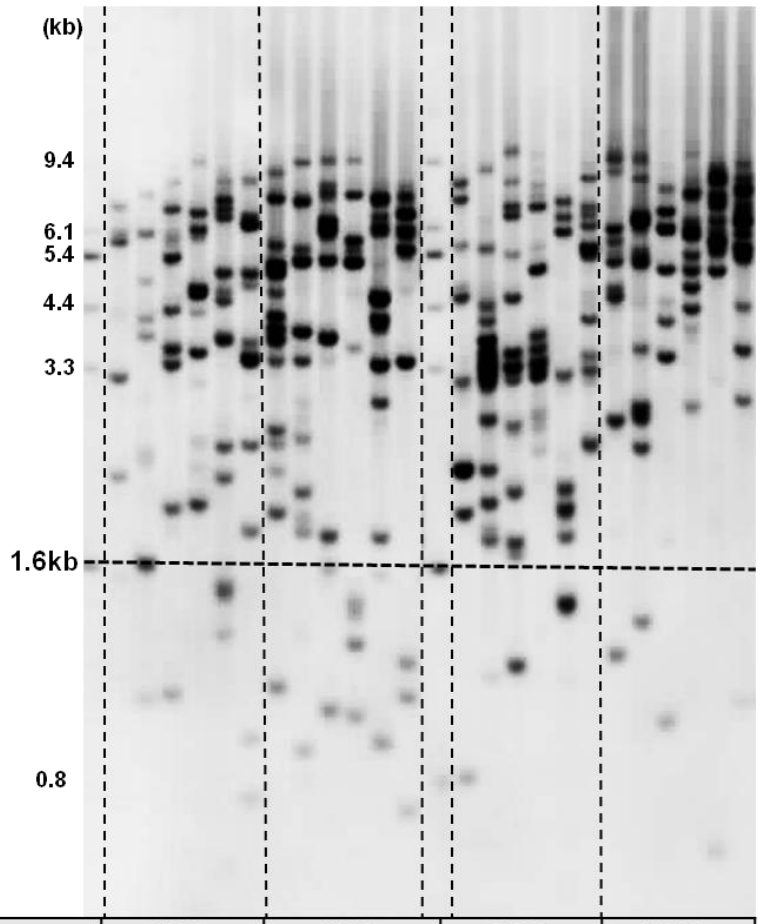


Figure 2-13. (A) Single-cell ddTRAP on four populations of T cells, i.e. CD4+CD28+, CD4+CD28-, CD8+CD28+, CD8+CD28-. (B) Cumulative analysis of (A), suggesting only a subset of CD28+ cells have shown telomerase activity.



	CD28- Day0	CD28- Day5	CD28+ Day0	CD28+ Day5
Shortest 20% Telomeres (kb)	2.4kb	1.9kb	2.3kb	3.4kb
Telomeres Below 1.6 kb (%)	10.6%	16.4%	10.3%	10.9%

Figure 2-14. CD28+ cells that show telomerase activity are capable of maintaining and even elongating telomere length, while CD28- cells without telomerase activity lose telomere length during stimulation.

CHAPTER 3

T-LYMPHOCYTES IN AGING: CENTENARIANS AND LONGEVITY STUDIES

The work presented in this chapter has been submitted for publication. Experiments were designed and performed by Dr. Enzo Tedone and Ejun (Elijah) Huang unless otherwise noted in the text and/or figure legends.

Introduction

The theory that aging is directly related to dysfunction in immunological processes leading to age-related pathologies has been reported in longitudinal studies showing significant correlations between specific changes in immune features and all-causes of mortality(Wikby, Nilsson et al. 2006). While the immune system maintains and protects our health by fighting off pathogens, most, but not all, older individuals (>65 years old) have an impaired response to pathogens such as influenza that ranks as the fifth leading cause of death in humans over the age of 65 (Murphy, Xu et al. 2013).

Thus, it is important to better understand the underlying biological mechanisms that contribute to age-related impairment of immune functions in order to develop appropriate preventative and therapeutic strategies to meet the medical and health demands of an increasing elderly population world-wide.

Some centenarians (individual who are at least 100 years old) reach what is currently the extreme limits of human life by escaping, or largely postponing, the major age-related diseases, and thus can be considered a paradigm for the study of healthy aging(Franceschi and Bonafe

2003). To assess the factors that can contribute to the healthy centenarian's phenotype, many studies of long-lived families have been carried out from several perspectives, such as genomics(Beekman, Blanche et al. 2013), epigenetics (Gentilini, Mari et al. 2013), transcriptomics (Passtoors, Boer et al. 2012) and mitochondrial DNA variability (Raule, Sevini et al. 2014), leading to the identification of characteristic profiles of both longevity and healthy aging. However, these investigations entirely focused on the study of resting immune cells (e.g. peripheral blood mononuclear cells, PBMC) isolated from peripheral blood. PBMCs consist of T-cells, B-cells, and other immune cells. T-cells, a major component of our immune responses, remain in a quiescent or dormant state when unstimulated, showing little or proliferation activity. In contrast, upon antigen-specific activation T-cells rapidly divide and exhibit dramatic changes in gene expression (over 7000 genes differentially expressed in resting vs stimulated T-cells) (Zhao, Fung-Leung et al. 2014). Activated T-cells initiate immune responses by discriminating between healthy and abnormal (e.g. infected or cancerous) cells in the body and thus represent an extremely valuable model to measure the ability of the innate immune system to fight off pathogens. Comparing stimulated T-cells in centenarians is an understudied area of research. Thus, the study of stimulated immune cells in high versus low performing centenarians might provide new insights about the mechanisms driving the age-related impairment of the immune system.

With the goal of identifying novel genes and pathways potentially involved in the process of healthy aging and longevity, this series of studies was designed to (1) characterize and compare the stimulation-induced responses in T-cells from young (23-39 years old), middle-aged (50-66 years old), old (67-83 years old) and high and low performing centenarians (100+ years

old)individuals and to (2) characterize and compare whole genome expression profiles (including splicing variants) in stimulated T-cells from the study groups listed above.

Materials and Methods

Study design and participants

We performed a cross-sectional study with PBMCs and T lymphocytes isolated from PBMCs of 114 Caucasian individuals under an Institutional Review Board (IRB)-approved protocol (certification number STU 042014-016). Briefly, we recruited 19 centenarians (100+ years old) and 95 individuals aged 23-83 years old.

The participant's age at time of enrollment was defined by birth certificates stated by local registry offices and/or dates of birth as stated on passports or identity documents. A trained multidisciplinary staff collected from the recruited subjects information regarding health status, currently used drugs, clinical history, and lifestyle. Moreover, past and current disease history was retrieved by an accurate evaluation of the participants' clinical documentations.

Subjects affected by cancer, infections or autoimmune diseases or on immunosuppressive treatment at the time of enrolment were excluded from the study. Venous blood samples were drawn from the participants under fasting conditions. All subjects gave written informed consent to participation in the study. The study protocol was approved by the Institutional Review Board (IRB) at UT Southwestern Medical Center.

PBMC isolation and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by centrifugation with Ficoll-Paque Plus (GE Healthcare) and T-cells were further isolated by negative selection. Cells were then cryo-preserved at -140 ℃ pending analysis. Cells were thawed 48 hours prior to mitogen stimulation. Thawed cells were cultured in RPMI+GlutaMAX-I with 10% fetal bovine serum, 1% penicillin, streptomycin and amphotericin B. T-Cells were stimulated by adding Dynabeads Human T-Activator CD3/CD28 (Life Technologies)in a 1:1 ratio. After 72 hours of stimulation Dynabeads were removed using a magnet and cells were cultured for a total of 10 days after stimulation. The percentage of live cells was determined every day by trypan blue exclusion using a TC20 Automated Cell Counter (Bio-Rad). The cell density was adjusted daily and when it exceeded 1.5×10^6/ml, cells were diluted with fresh complete RPMI medium to a density of 1.0×10^6/ml.

Telomerase enzymatic activity assay

Telomerase activity was assessed using the droplet-digital TRAP (ddTRAP) assay as previously described (Ludlow, Robin et al. 2014). Briefly, cells were cultured and counted and 10^5cells from each sample was pelleted and store at -80 ℃ pending analysis. On the day of the experiment, cell pellets were lysed in ice for 45 min with 40 ul of NP-40 Buffer(10 mM Tris-HCl, pH 8.0, 1 mM MgCl2, 1 mM EDTA, 1% (vol/vol) NP- 40, 0.25 mM sodium deoxycholate, 10% (vol/vol) glycerol, 150mMNaCl, 5mM β-mercaptoethanol, 0.1mMAEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride). Two microliters of cell lysate ($5x10^3$ cell equivalents) were added to a 50 ul extension reaction containing 1×TRAP reaction buffer (10×concentration: 200mM Tris-HCl, pH 8.3, 15 mM MgCl2), 0.4 mg/ml BSA, 200 nM TS telomerase extension substrate (HPLC purified, 5'-AATCCGTCGAGCAGAGTT), dNTPs (2.5

mM each) and incubated for 45 min at 25 ℃ followed by telomerase inactivation at 95 ℃ for 5 min, then cooling at 4 ℃.

The ddPCR reaction contained 2 ul of extension products (200 cell equivalents), 1 × EvaGreen ddPCR Supermix v2.0 (Bio- Rad), 50 nM TS primer, 50 nM ACX primer and $dH_2O$to a 20 ul final volume per sample. Droplets were generated exactly as reported and transferred to a 96-well PCR plate (twin-tec 96-well plate, Eppendorf, Fisher) then sealed with foil (Thermo Scientific, AB0757).PCR was performed in a T100 thermocycler (Bio-Rad) with a ramp rate of 2.5 ℃/sec between all steps. Activation of Taq polymerase (95 ℃ for 5 min) was followed by 40 cycles of 95 ℃for 30 sec, 54 ℃ for 30 sec, 72 ℃ for 30 sec, then cooling at 12 ℃.

After PCR, the fluorescencewas read on a QX200 droplet reader(Bio-Rad) as previously described. Telomerase activity values were corrected by subtracting background fluorescence (negative control) and normalized, sample by sample, based on the percentage of live cells in culture when the cell pellets were collected. Telomerase activity was expressed as the number of telomerase-extended TS molecules per cell equivalent (telomerase products per cell).

Mitochondrial DNA copy number quantification

Extraction and purification of total genomic DNA was performed as previously described (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2692272/). Briefly, 2.0 ×105 cells were pelleted and resuspended in 250 µl of SDS lysis buffer. Samples were vortexed then boiled for 10 minutes. Samples were allowed to cool to room temperature then treated with 2.5 µl of RNAse A (10mg/ml) at 37 ℃ for 2 hours. Next, 2.5 µl of proteinase K (10mg/ml) was added and samples were incubated at 55 ℃overnight. The next day, samples were boiled for 10 minutes, followed with DNA precipitation by adding 0.1 volumes of 3M sodium acetate and 2 volumes of 100%

ethanol at -20 ℃ overnight. Centrifugation was performed at 4 ℃ and 1500g for 15minutes. Pellets were washed once in 70% ethanol and resuspended in 50 µl diH20. Aliquots of 1ng DNA per µl were made for each sample and stored at -20 ℃.

Shortest telomere length measurement

Genomic DNA was extracted using the GentraPuregen DNA extraction kit (Qiagen) according to the manufacturer's instructions. Each DNA sample was quantified on a Nanodrop (Thermo Scientific) for concentration and purity, and integrity of DNA was determined as previously indicated.

The Telomere Shortest Length Assay (TeSLA)was performed exactly as described (Lai et al, 2017). Briefly, 50 ng of genomic DNA was added to a final volume of 20 µl of ligation buffer containing 1000 units of T4 DNA ligase (New England Biolabs), 1X Cut Smart Buffer (New England Biolabs),1 mM ATP and 1 nM of TeSLA telorettes (TeSLA Telo 1-6) (Lai et al, 2017) and incubated at 35 ℃ for 16 hours followed by heat inactivation at 65 ℃ for 10 min. After ligation, genomic DNA was digested using a set of restriction enzymes (2 Units each of CviAII, BfaI, NdeI, and MseI, New England Biolabs)as reported (Lai et al, 2017) and then treated with 1 unit of Shrimp Alkaline Phosphatase (rSAP, New England Biolabs)at 37 ℃ for 60 min in a final volume of 50 ul. This mixture was subsequently heat inactivated at 80 ℃ for 20 min and 10 ul of sample were added to 10ul of adapter ligation mix (1 µM AT adapter, 1 µM TA adapter, 1mM ATP, 1X Cut Smart Buffer and 2000 units of T4 DNA Ligase) and incubated at 16 ℃ for 16 hours. After adapter ligation the sample was heat inactivated at 65 ℃ for 10 min and subsequently diluted to a concentration of 20pg DNA/ul (1:25 dilution). For each sample analyzed we performed seven independent PCR reactions (94 ℃ for 2 min followed by 26 cycles

of 94 °C for 15 sec, 60 °C for 30 sec and 72 °C for 15 min) using a total of 25 µl mix containing 40 pg DNA, 2.5 units of FailSafe enzyme (Epicenter), 1X FailSafe buffer H (Epicenter) and 250nM primers (adapter and TeSLA TP) (Lai et al, 2017). PCR products were run on a 0.85 % agarose gel (1.5 V/cm for 19 hours) followed by Southern blot analysis to detect amplified telomeres as previously described [26]. Southern blot images were finally analyzed by using MATLAB-based software to automatically and accurately detect and size annotate the telomere bands including the percentage of shortest and average telomere length.

Statistical Analysis

Statistical analysis was performed by using the software GraphPad Prism 7.0. Telomerase activity, mitochondrial DNA copy number data and cell proliferation growth rate were compared between the study groups by univariate ANOVA, followed by Bonferroni's correction. Correlations were performed by linear regression analysis. $p<0.05$ was used as the threshold value for statistical significance.

Results

Stimulated T cells from centenarians display robust telomerase activity and unimpaired division capability

Telomeres are tandem repeats that cap the end of linear chromosomes to protect the ends from degradation and prevent chromosome fusion (Blackburn and Collins 2011). Telomeres get progressively shorter with each cell division even in telomerase positive proliferating human stem cells that eventually leads to replicative senescence (Shay 2016). As a result, telomere

length declines with age (Aubert, Baerlocher et al. 2012) and is considered a marker of mortality risk (Epel, Merkin et al. 2008) and exposure to various age-related pathologies including cancer (Shay 2016), dementia (Honig, Schupf et al. 2006, Tedone, Arosio et al. 2015) and cardiovascular diseases (Epel, Merkin et al. 2008).

Our previous study showed that a subset of centenarians undergo a slower rate of telomere shortening compared to the normal population (Tedone, Arosio et al. 2014). To test whether centenarians' T-cells display increased telomerase activity, we employed a quantitative assay, ddTRAP (Ludlow, Robin et al. 2014) that allows measurements of telomerase activity at a single cell level. Unstimulated T-cells showed extremely low levels of telomerase activity and no significant differences were found between the study groups (Figure 3-1A). Consistent with previous studies (Hiyama, Hirai et al. 1995), upon T-cell stimulation telomerase activity is transiently upregulated and generally peaks 3 days after stimulation and slowly declines over a period of 10 days (Figure 3-1A). Therefore, we calculated the total telomerase activity over a 10 day period following stimulation by measuring, for each study group, the area under the curve (AUC) (Figure 3-1B). In our study population, telomerase activity from stimulated T-cells gradually decreased with age, until 83 years of age (Figure 3-1A/B). Interestingly, telomerase activity in stimulated T-cells from our pool of 19 centenarians was significantly higher compared to 67-83 year old individuals and was comparable to that of 50-66 year-old individuals (Figure 3-1A/B).

In addition, stimulated T-cells from centenarians showed an increased proliferation rate compared to stimulated T-cells from 67-83 year old subjects (Figure 3-1C). Also, no significant difference in number of population doublings was found between stimulated T-cells from centenarians and both 50-66 and 23-39 year-old individuals (Figure 3-1C).

Since telomerase is believed to prevent or at least slow down the extensive telomere shortening during rapid T-cell expansion, we investigated whether telomerase activity correlated with cell proliferation rate. We found a significant correlation between telomerase activity 3 days after stimulation(peak) and the number of cell divisions occurring during the 10 days following stimulation (Figure 3-1D). This suggests that telomerase activity at day 3 after stimulation might be considered as a biomarker of stimulation-induced response in T-cells.

The combined results indicate that in our study population stimulated T-cells from centenarians express higher levels of telomerase activity and proliferate more than old individuals (67-83 years old).

Mitochondrial DNA copy number declines with age but is increased in both resting and stimulated T-cells from centenarians

Mitochondria function is crucial for antigen-induced stimulation of immune cells and recent reports show that mitochondrial DNA copy number per cell correlates with age, telomere length and all-causes of mortality (Kim, Kim et al. 2013, Mengel-From, Thinggaard et al. 2014).

We first measured the amount of mitochondrial DNA in unstimulated PBMC using ddPCR. In our study population, mitochondrial DNA copy number declined with aging, until 83 years of age (Figure 3-2A). However, unstimulated PBMC from centenarians expressed a significantly higher mitochondrial DNA content compared to PBMC from 67-83 year old individuals and a similar content compared to 50-66 year old individuals (Figure 3-2A).

To address the question whether immune cells from centenarians maintain an increased mitochondrial DNA content when stimulated and actively dividing, we employed a new highly quantitative assay developed in our laboratory (ddMito) that allow measuring mitochondria DNA copy number at a single cell level. Similarly to telomerase activity, upon mitogen stimulation, de-novo synthesis of mitochondrial DNA copy number increased by 3 days and then gradually decreased (Figure 3-2B). Stimulated cells from young (23-39 years old)showed similar levels of mitochondrial DNA compared to 50-66 years old individuals, while cells from 67-83 years old subjects had significantly lower levels (Figure 3-2B). Even though the peak of mitochondrial DNA copy number per cell (day 3 after stimulation) was not different between 67-83 years old individuals and centenarians, the latter significantly maintained high levels of mitochondrial DNA for a longer time period (day 5: 581 $\pm$203 vs 866 $\pm$246; day 7: 427 $\pm$199 vs 686 $\pm$201 in 67-83 years old vs centenarians respectively) (Figure 3-2B).Mitochondrial DNA copy number peaked (day 3 after stimulation) and significantly correlated with the cell proliferation rate (indicated as the total number of cells at day 10 after the stimulation) (Figure 3-2C).

Since the catalytic subunit of telomerase (TERT) has been reported to bind to mitochondrial DNA exerting a protective function against oxidative stress-induced damage (Haendeler, Drose et al. 2009), we next investigated whether mitochondrial DNA copy number correlated with telomerase activity. We plotted mitochondria DNA copy number and telomerase activity data from all samples (all day points) and found a very significant correlation (Figure 3-2D).

These results together can be interpreted to suggest that on average centenarian T-cells may have more efficient mitochondria biogenesis and maintenance mechanisms which, in turn, may contribute to a less age-related impaired response to antigen-induced stimulation.

Whole genome expression profiles comparison

We next sought to identify genes that potentially played key roles in the age-related impairment of the T-cell response to stimulation as well as genes that might be associated with longevity and healthy aging. Thus, we compared whole genome expression profiles (RNA-sequencing) of stimulated T-cells from different aged groups, including centenarians. We first performed a Principal Component Analysis (PCA) to investigate whether in stimulated T-cells there exist specific expression profiles defining young, old and centenarians (Figure 3-3). Both young and old individuals exhibited very different gene expression profiles (Figure 3-3). Interestingly, the centenarian group was heterogeneous, with some centenarians clustering together with the young and some centenarians clustering with the old individuals (Figure 3-3). We compared those two sub-groups of centenarians based on the previously collected information about health status and based on our data on both T-cells telomerase activity and mitochondrial DNA copy number. The centenarians that clustered with the young subjects exhibited better cognitive and physical performances (respectively measured by Mini Mental State Examination test and Lawton Instrumental Activities of Daily Living test (Lawton and Brody 1969, Folstein, Folstein et al. 1975). These "high performing" centenarians had a lower number of diseases and had both higher telomerase activity and mitochondrial DNA copy number compared to the "low performing" centenarians. Moreover, we measured both the average telomere length and the length of the shortest 20% telomeres and found that high performing centenarians (who escaped or largely postponed age-related diseases) had also longer telomeres compared to the low performing centenarians (Figure 3-4).

We next investigated differences in gene expression among the study groups. When compared to young healthy individuals, old subjects exhibited more genes differentially expressed compared to the pools of centenarians (all subjects) (2925 vs 471 genes differentially expressed respectively) (Figure 3-5). Also, within the centenarian group, high performing centenarians had a significantly smaller number of genes differentially expressed compared to the low performing centenarians (77 vs 2612 genes differentially expressed respectively) (Figure 3-6).

Discussion

The prevalence of cancer in centenarians is reduced compared to the normal population. The work presented in this chapter may lead to the identification of a characteristic or more consistent pathway of telomerase regulation in centenarians which might, in turn, lead to new insights about the mechanisms of healthy aging or cancer initiation and progression. We have little knowledge about the regulation of telomerase activity in normal cells. Telomerase is greatly increased by day 3-4 after T-cell stimulation, but even while cells continue to rapidly proliferate for an additional several days, telomerase is downregulated. One significance of these studies is the insights we gained into what regulates telomerase enzyme activity in normal human stem-like cells. RNA-seq analyses have recently been conducted at different time points after T-cell stimulation. Bioinformatics analyses should provide new insights into aspects of regulated telomerase in normal cells versus less well regulated telomerase in cancer cells. We believe that regulating telomerase activity in proliferating normal stem cells is critical to prevent the early

onset in long-lived species such as humans. However, additional studies will be required to identify how cell proliferation and telomerase is regulated in normal cells.

Additionally, we collected serum from the enrolled subjects, including centenarians. To date, as far as we could determine, no studies have been reported on the relationship between the age and the effects of human serum on human immune cell function. Moreover, because blood is such an integral component of the native immune cell environment, T-cells represent a physiologically relevant compartment in which to investigate the influence of age-dependent circulating factors on cellular functions.

Gaining a better understanding of immune cell responses associated with human aging and overall health span has important translational implications. The project has identified subset of genes whose expression is regulated by telomere length/aging, directly leading to a progressive impairment of T-cell responses to endogenous and external stimuli. These genes (biomarkers of the immune cell performance) may lead to novel insights into healthy aging and to the discovery of therapeutic targets playing important roles in delaying the age-related decline of the antigen-induced responses in T-cells. This research bridged the gap between basic biomedical research and clinically-relevant findings, treatments, diagnostics and prevention.

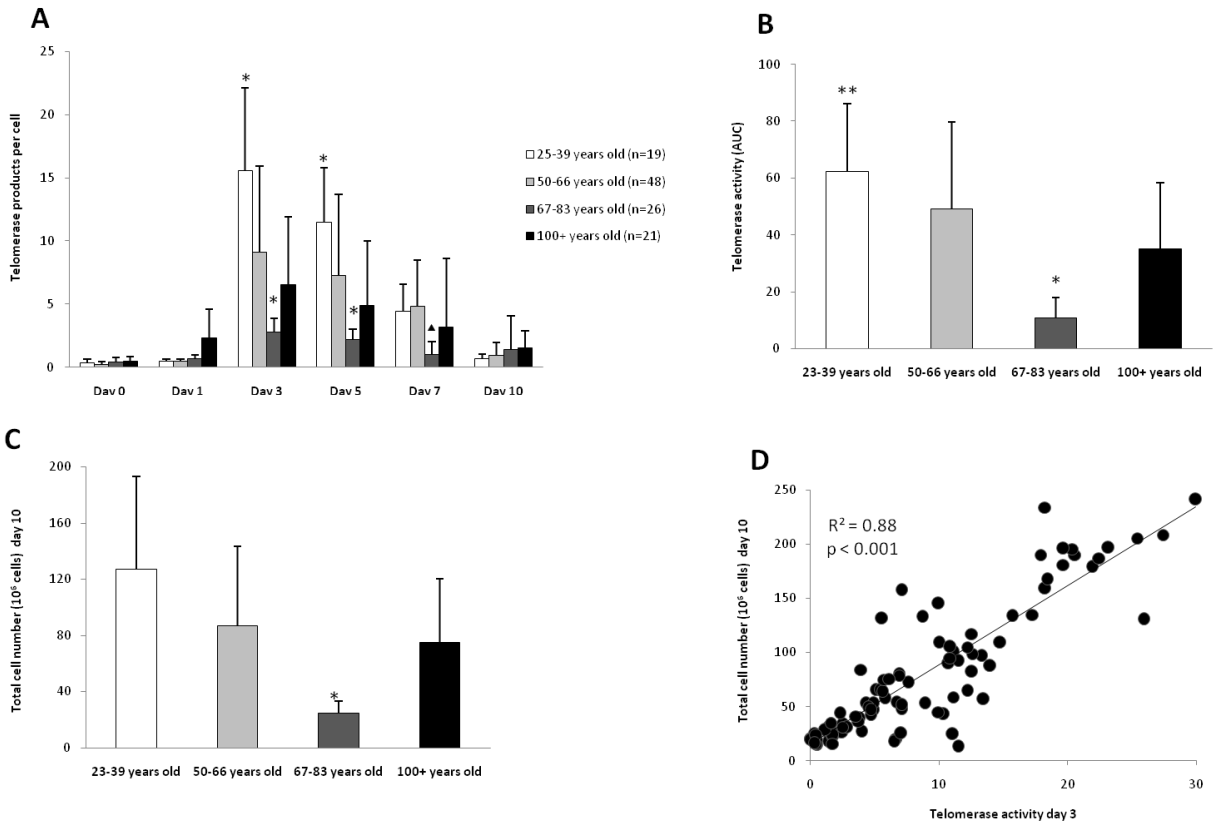


Figure 3-1. Stimulated T cells from centenarians express higher telomerase activity compared to 75-85 years old and similar levels compared to 55-65 years old individuals. (A) Telomerase activity over time in stimulated T-cells, * p ‹ 0.05 vs each of the other groups; triangul: p ‹ 0.05 vs 23-39 years old & 50-66 years old; (B) Telomerase Activity (Area Under the Curve) * p ‹ 0.05 vs each of the other groups ; ** p ‹ 0.05 vs centenarians. (C) Comparison of population doublings between each group. * p ‹ 0.05 vs each of the other groups; (D) Correlation between tel act day 3 e PD D0-D10.

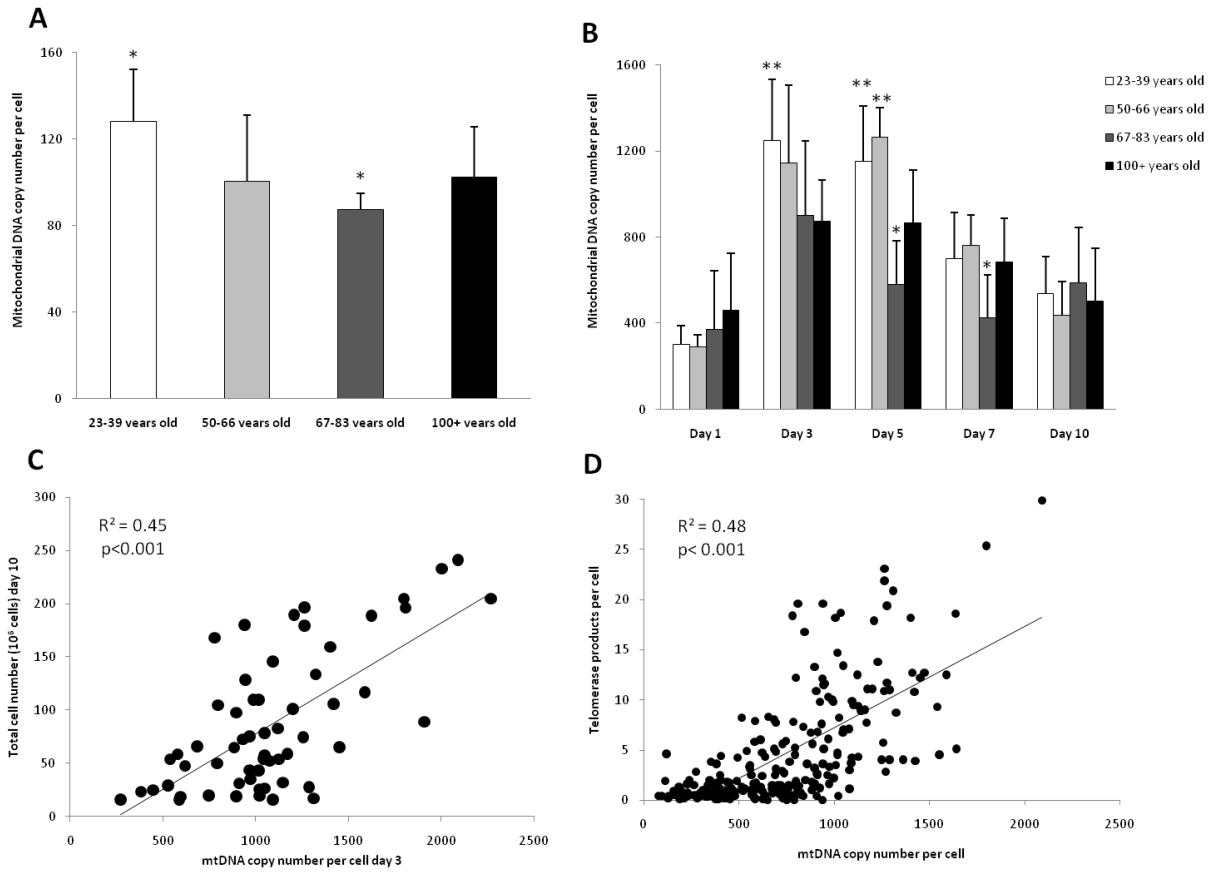


Figure 3-2. Mitochondrial DNA copy number declines with age but is increased in centenarians' T-cell. (A) MtDNA copy number in unstimulated T-cells. * p ‹ 0.05 vs each of the other groups. (B) MtDNA copy number in Stimulated T-cells. * p ‹ 0.05 vs each of the other groups; ** p ‹ 0.05 vs both 67-83 years old and centenarians. (C) Correlation between PD D0-D10 and mtDNA copy number at day 3; (D) Correlation between tel act day 3 e PD D0-D10.

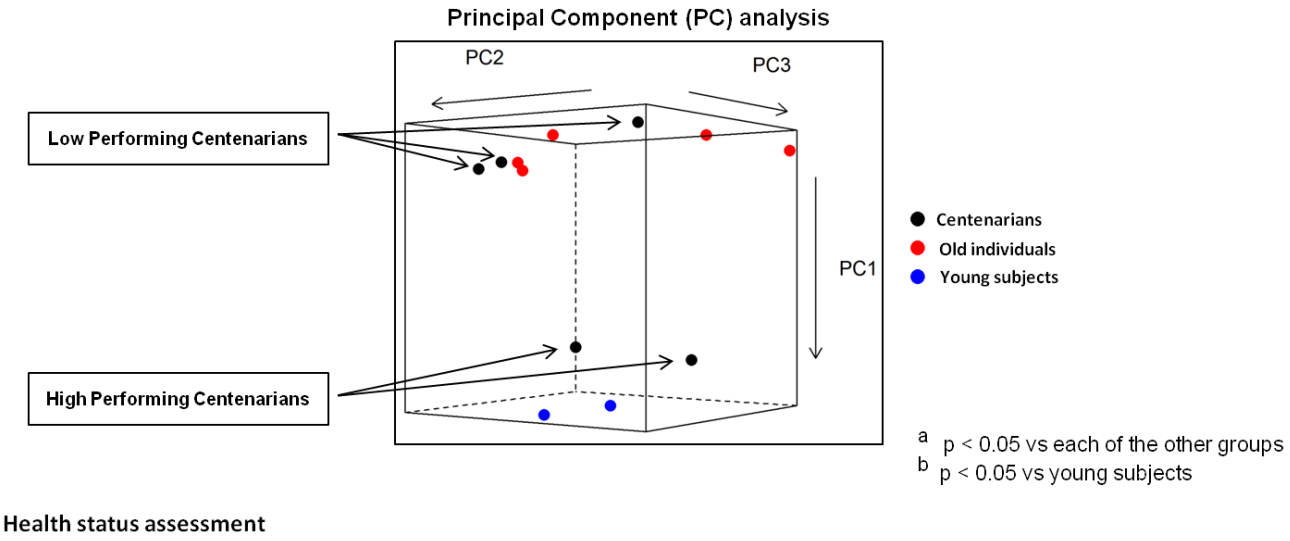
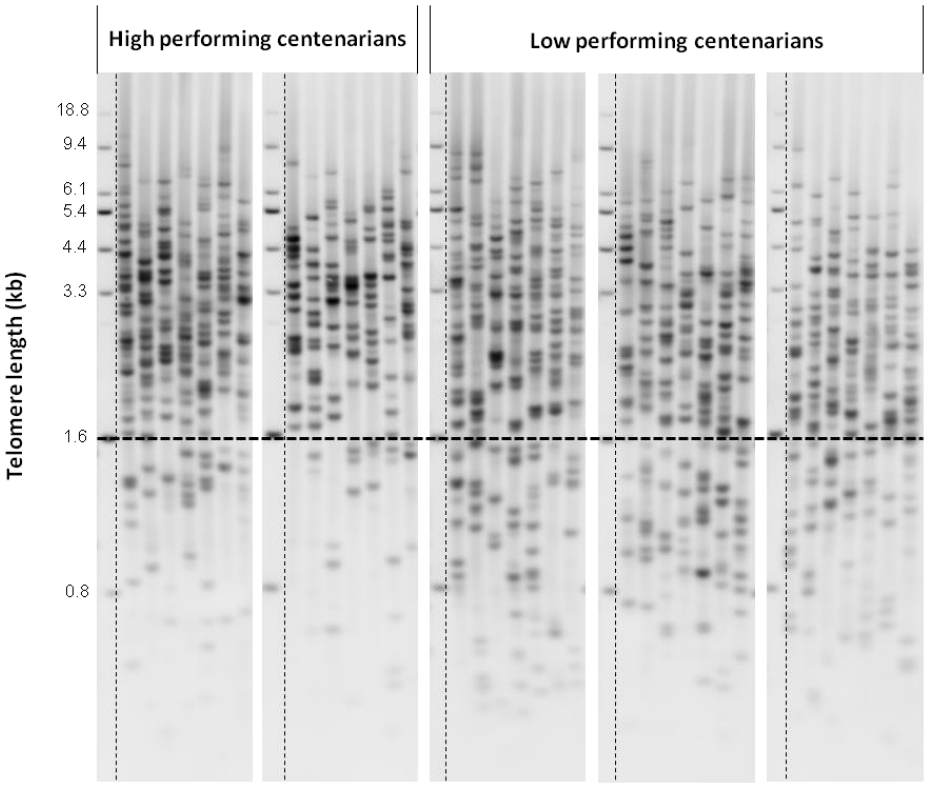


Figure 3-3. Principal Component analysis (PCA). Some centenarians ("High Performing") exhibit a significantly more youthful gene expression profile and a better health status.



Average telomere length	3.29 kb	3.23 kb	2.94 kb	2.79 kb	2.47 kb
Telomeres shorter than 1.6kb	17.4 %	17.1 %	26.4 %	29.8 %	31.7 %
Length of the shortest 20% telomeres	1.78 kb	1.81 kb	1.30 kb	1.14 kb	1.26 kb

Figure 3-4. Comparison of telomere lengths between high performing and low performing centenarians using TeSLA. High performing centenarians have less critically short telomeres compared to low performing centenarians.

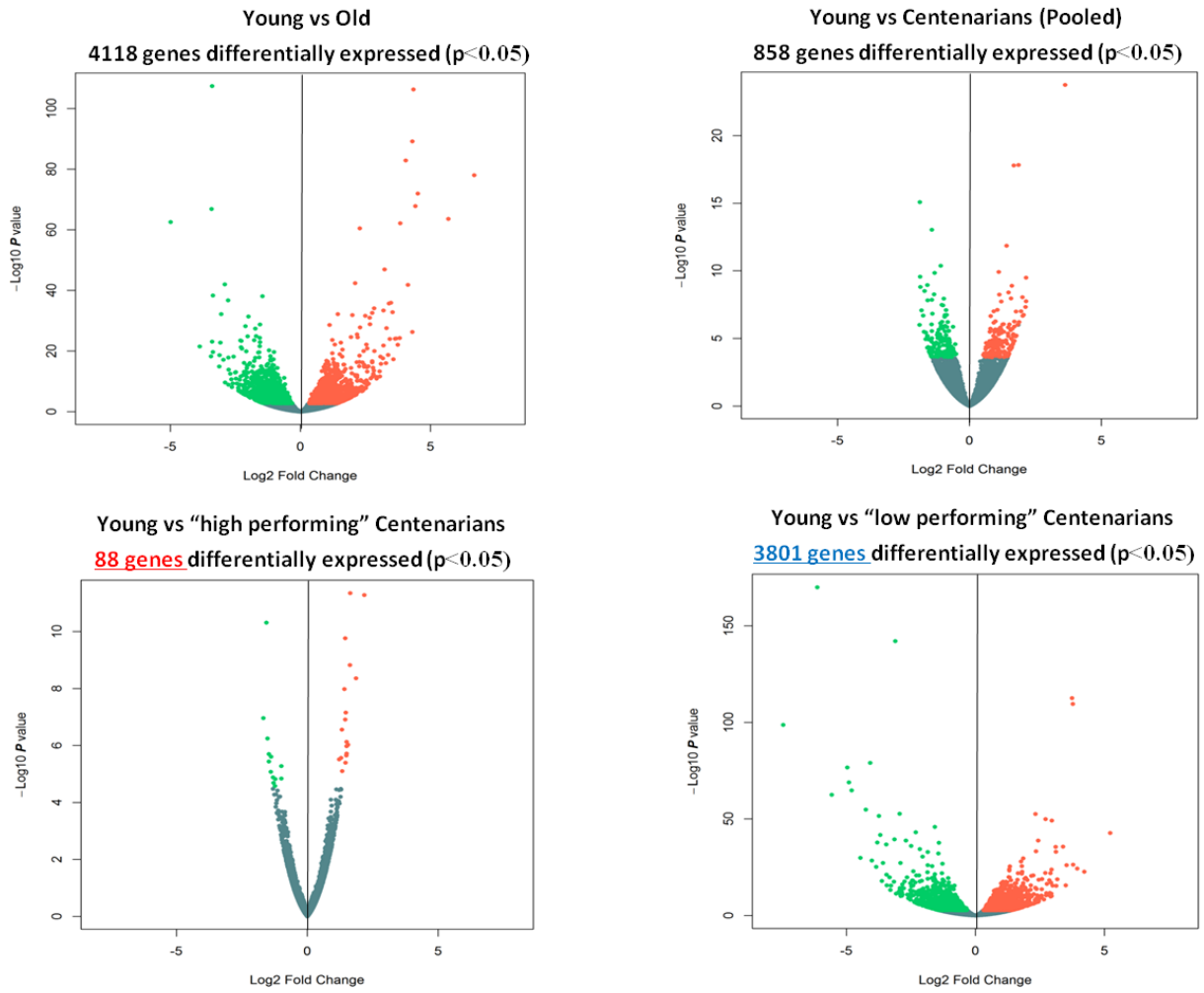


Figure 3-5. Volcano plots of young individuals, old individuals, "high performing" centenarians, and "low performing" centenarians. High performing centenarians have a global gene expression profile similar to that of young healthy individuals.

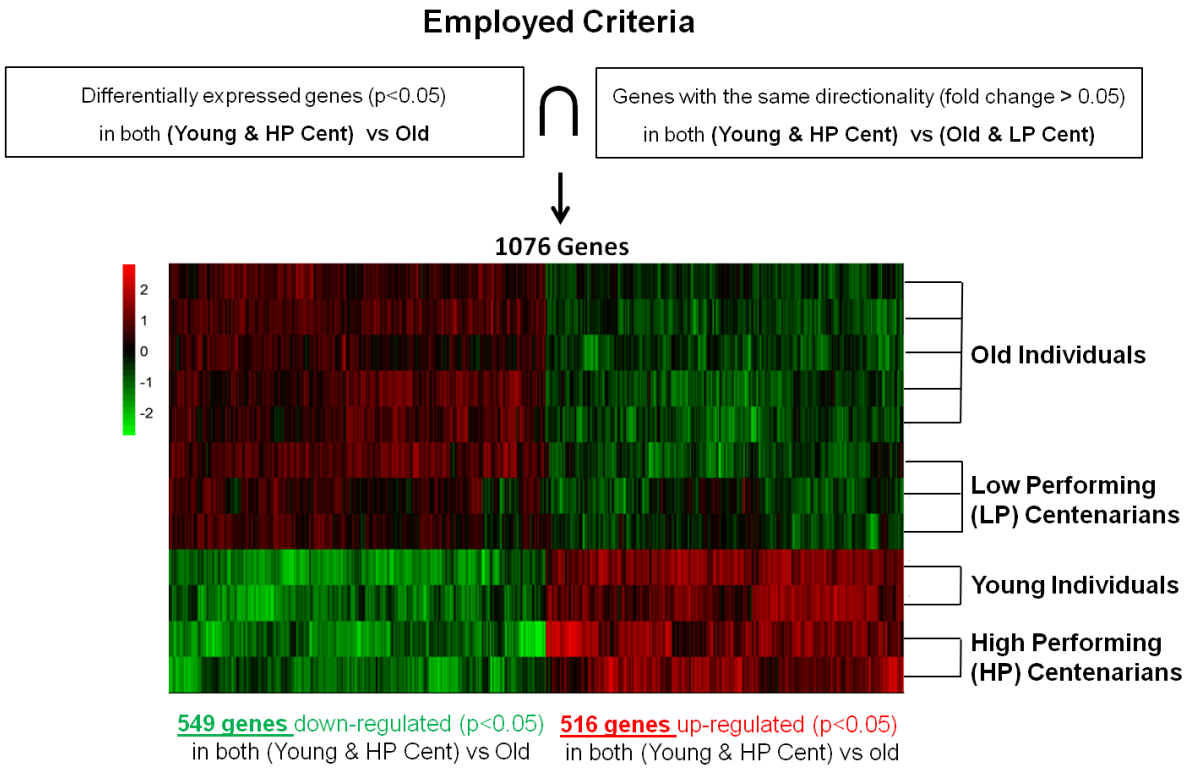


Figure 3-6. Gene expression analysis on young individuals, old individuals, "high performing" centenarians, and "low performing" centenarians. Identification of genes potentially involved in healthy aging and healthy T-cell response to stimulation.

CHAPTER 4

T-LYMPHOCYTES IN AGING: MITOCHONDRIA STUDIES

The work presented here were designed and performed by Ryan O'Hara & Ejun (Elijah) Huang unless otherwise noted in the text and/or figure legends.

Introduction

Human mitochondrial DNA (mtDNA) is a circular 16kb genome which encodes thirteen essential protein components of the electron transport chain. mtDNA is present at hundreds to thousands of copies per cell, with copy number varying widely between cell types or in response to stimulus.

Since it is now possible to extract DNA, RNA, and protein from small number of cell pellets, it was our goal to be able to use cells pellet (100,000 cells) for both cellular telomerase activity and mtDNA content. Previously, it has been shown that highly sensitive and accurate gene expression data could be obtained from crude lysates, therefore we reasoned that it should be similarly feasible to quantify mtDNA content in such a manner.

Inhibitors and different PCR efficiencies between reference and target genes can represent major hurdles for accurate quantification by qPCR. Since these problems could only be exacerbated by using unpurified DNA and because of our lab's previous experience with droplet digital PCR, we developed ddTRAP (Ludlow, Robin et al. 2014) for quantitating telomerase activity from single cells, and now decided to develop a ddPCR platform for quantitating mtDNA using our limited number of cells from centenarians.

It has become increasingly clear in cancer, as well as in normal physiology, that small subsets of cells may play crucial and varying roles within a larger population (DeGennaro, Savir et al. 2016, Mendenhall, Driscoll et al. 2016, Saito, Nishikawa et al. 2016, Franzen, Zirkel et al. 2017, Ibrahim-Hashim, Robertson-Tessi et al. 2017, Morandi and Indraccolo 2017). As such, the demand has increased for single-cell techniques to help characterize potentially diverse subpopulations in a heterogeneous mixture (Payne, Cree et al. 2015). The capability of using extremely small amount of cells or even single cells for mtDNA copy number measurements, enabled us to pursue various of key questions in aging and cancer using our centenarians samples.

Materials and Methods

mtDNA quantification from cell lysate

10^5cell pellets were collected and stored at -80 ℃ pending analysis. We found that dilution of cell cultures to around 10^6 cells per µl was ideal to obtain accurate and reproducible cell counts. Cell pellets were lysed on ice using µlNP-40 lysis buffer (Figure 1A). Samples were vortexed every 15 minutes for a total of 45 minutes. Once lysed, 10 µl of lysate (containing 2.5×10^4 cell equivalents) was added to 9 µl of TNES buffer and 1 µl of proteinase K (25mg/ml). Proteinase K digestion was run at 50 ℃ for 15 minutes, followed by 100 ℃ for 10 minutes, then cooled to 12 ℃. Following proteinase K treatment, samples were diluted 1:250 to obtain the final concentration of 5 cell equivalents per µl. After dilution, samples were then prepared for ddPCR. Note that ddPCR is sensitive to oversaturation of template molecules and that optimal sample dilution will vary between cell types as a function of mtDNA content per cell.

ddPCR

The ddPCR reactions contained 1 µl of sample (5 cell equivalents), 1X EvaGreen ddPCR Supermix (Bio-Rad), 100nM forward, and 100 nM reverse primer for a total volume of 20 µl. Samples were then partitioned into droplets using a droplet generator according to the manufacturer's instructions (QX100 drop generator, Bio-Rad) and the emulsions (approximately 40 µl) were transferred to a 96-well PCR plate (twin-tec 96-well plate, Eppendorf) and sealed with foil (Thermo Scientific, AB0757). The ddPCR reaction was 95℃ for 5 minutes, 40 cycles of 95℃, 54℃, and 72℃ for 30 seconds each, concluded by a hold at 12℃. The ramp rate between all steps was 2.5℃/second. After PCR, fluorescence was measured using a droplet reader (QX200, Bio-Rad), with 17-20 thousand droplets on average being read per sample. The threshold for positive droplets was determined by the software's analysis of droplet clustering across all samples and confirmed manually using a negative, cell-free control (Figure 1B). The final output was given by the software as a concentration of starting template molecules per µl and could be converted to molecules per 20µl reaction (the original pre-emulsion volume). Finally, mtDNA per reaction was either normalized to cell equivalents per reaction (Figure 1C) or to nuclear DNA content (NCOA3).

Single-cell mtDNA quantification

After trypsinization, cells were washed twice with PBS and then resuspended in PBS at a density of 10^6 cells/ml. At this point, 10^5 cell pellets were made to be simultaneously run as pooled control samples. The remaining cell suspension was further diluted to approximately 2 cells per µl. This was then pipetted onto a glass slide in 1µl aliquots. As confirmed by microscopy, droplets containing exactly one cell were pipetted into PCR tubes containing 0.05µl proteinase K (25mg/ml), 0.45µl TNES buffer, 1µl NP-40 lysis buffer, and 7.5µl MS2 RNA in water (16pg/µl). The final 10µl reaction was run in a thermocycler at 50℃ for 30minutes, 100℃

for 10 minutes, then 12 ℃ for simultaneous lysis and proteinase K treatment. 1 µl of this reaction was then used to perform ddPCR as described above (0.1 cell equivalents). We found that dilute quantities of MS2 RNA helped mitigate excessive quantities of DNA from sticking to tubes during single cell reactions.

mtDNA quantification from purified DNA

Extraction and purification of total genomic DNA was performed as previously described (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2692272/). Briefly, 2.0×10^5 cells were pelleted and resuspended in 250 µl of SDS lysis buffer. Samples were vortexed then boiled for 10 minutes. Samples were allowed to cool to room temperature then treated with 2.5 µl of RNAse A (10mg/ml) at 37 ℃ for 2 hours. Next, 2.5 µl of proteinase K (10mg/ml) was added and samples were incubated at 55 ℃overnight. The next day, samples were boiled for 10 minutes, followed with DNA precipitation by adding 0.1 volumes of 3M sodium acetate and 2 volumes of 100% ethanol at -20 ℃ overnight. Centrifugation was performed at 4 ℃ and 1500g for 15minutes. Pellets were washed once in 70% ethanol and resuspended in 50 µl diH_20. Aliquots of 1ng DNA per µl were made for each sample and stored at -20 ℃.

qPCR

All qPCR reactions contained 1 µl of sample (1ng DNA), 1X Ssofast EvaGreen Supermix (BioRad), 10 µM forward, and 10 µM reverse primer to a final volume of 20 µl. qPCR was performed using a Light cycler 480 (Roche) at 95 ℃ for 5 minutes, 40 cycles of 95 ℃ for 15 seconds and 60 ℃ for 60 seconds, followed by melting curve analysis. Samples measured in qPCR represent the average of three technical replicates, unless otherwise indicated. In order to convert threshold cycles to absolute molecules, dilution series of known quantities of purified D-

Loop and NCOA3 amplicons were performed concurrently with samples. Exponential regressions created from these dilution series were then used to calculate absolute molecules of mtDNA per diploid genome.

Preparation of purified amplicons

One nuclear and three mitochondrial primer pairs were chosen, targeting NCOA3, the D-Loop region, MT-ND1, and Leucine UUA/G. These primers were confirmed for specificity by gel and by qPCR melting curve analysis. PCR amplicons of these four primer pairs were then purified from 1% agarose gel using Qiagen Gel Extraction kit and concentrations were taken with Qbit High Sensitivity DNA kit. Each amplicon was then diluted to 1 billion molecules per μl, aliquoted, and stored at -20 ℃ pending analysis.

Statistical analysis

Statistical analysis was performed using unpaired, two-tailed Student’ s t-test. Significance was denoted as follows: * $p \leqslant 0.05$; ** $p \leqslant 0.01$; *** $p \leqslant 0.001$. All error bars represent standard deviation of no fewer than three replicates.

Results

Workflow and experimental design

To keep up with the growing demand for large epidemiological studies, our proposed method and workflow has been optimized with high-throughput application in mind (Figure 4-1A).Using ddPCR technology, our proposed method captures the robust difference in mtDNA

copy number that can be seen between resting, stimulated, and transformed lymphocytes (Figure 4-1B). Raw ddPCR output can then be transformed into absolute numbers of mtDNA molecules per cell equivalent with a high degree of precision (Figure 4-1C). In order to reliably quantify ddPCR measurements in terms of absolute molecules, we evaluated the linearity and slopes of three different mitochondrial primers targeting the D-Loop region, MT-ND1, and MT-TL1.The results indicated that measurements taken from ddPCR accurately reflected absolute molecules per reaction in a nearly to 1-to-1 ratio (Figure 4-2). Since large mtDNA deletions do occur in some parts of the mtDNA genome, we focused on the mtDNA origin of replications since deletions in the D-Loop region have never been reported (Bai and Wong 2005). Thus, the primer pair targeting the D-Loop region was employed in subsequent experiments.

As a first proof of principle, we measured the mtDNA content of T-lymphocytes from 22 healthy donors for two weeks following CD3/CD28 stimulation. Our proposed method showed a dramatic upregulation of mtDNA copy number in antigen-stimulated lymphocytes beginning at day 2 and peaking between days 3 and 6 (Figure 4-1D). This data was consistent with previous studies reporting a significant increase in mitochondria biogenesis and mtDNA content following T-cell activation (D'Souza, Parikh et al. 2007, Dimeloe, Burgener et al. 2017).Additionally, we measured mtDNA copy number in a variety of well characterized normal and transformed cell lines and showed different mtDNA copy number characterizing each cell line (Figure 4-1E).

Comparisons with previously established techniques

Next, we wanted to compare measurement of mtDNA copy number by ddPCR with that of an established qPCR method from purified mtDNA (West, Khoury-Hanold et al. 2015).To this end, total genomic DNA from BJ fibroblasts was purified as previously described and mtDNA

copy number was normalized to a single copy nuclear gene. Figure 4-3A shows the tight linearity between both ddPCR and qPCR. It is important to note that the absolute copy numbers of each method differed by a factor of around 1.5. This may due to the fact that qPCR is more sensitive to differing efficiencies between target and housekeeping genes (Regier and Frey 2010, Kiselinova, Pasternak et al. 2014), but may also reflect potential difficulties that ddPCR has in the inclusion of bulky nuclear DNA within droplets. We then evaluated if the use of total cell lysate as opposed to purified DNA would have any bearing on mtDNA quantification. Working in ddPCR, we obtained an R^2 of 0.9972 and a slope of 0.9663, indicating that both purified DNA and cell lysate gave nearly identical mtDNA copy number in terms of absolute molecules(Figure 4-3B).

Finally, we wanted to assess mtDNA measurements by our proposed method directly with that of standard qPCR and ddPCR methods. To accomplish this, for both qPCR and ddPCR, we followed the standard practice of normalizing mtDNA to a nuclear target using purified total genomic DNA. In the comparison with qPCR, the resulting R^2 was 0.9999 (Figure 4-3C). Likewise, the correlation between our method and ddPCR was very strong, with an R^2 of 0.9977 (Figure 4-3D).These results showed that our technique very highly correlated with the standard techniques in the field and is vastly easier to perform.

Detection of 10% differences between samples

mtDNA copy number is becoming widely recognized as a promising prognostic biomarker in both cancer (Hu, Yao et al. 2016) and age-related diseases (Malik, Shahni et al. 2009, Pyle, Anugrha et al. 2016), where small differences in copy number may have large impacts on phenotypic conditions and metabolism alterations. Currently, qPCR is used as the

gold standard for assessment of mtDNA copy number. We next compared the limit of sensitivity, or the smallest discernable difference between samples, using our ddPCR protocol with that of qPCR. To accomplish this, we employed mitochondrial and genomic amplicons to simulate DNA samples that differed in mtDNA copy number by changing the input ratios of 10-60% from a baseline control. In order to determine sensitivity, samples were tested for significance against the baseline control using a student's t-test. Additionally, the standard error of the estimate (σ_{est}) was calculated as a measure of the overall variability in each assay.

We found that qPCR could distinguish between samples whose copy number differed by 50% or more (Figure 4-4A). The σ_{est} for qPCR detection of mtDNA copy number was 21.3%, reflecting how even moderate deviations in Ct can produce large changes in relative abundance calculations. This variability is further exacerbated by the need to use a reference gene, as this compounds error from not only the target but the reference as well. In contrast, ddPCR performed on the same samples could detect significance at30% from the baseline, with a σ_{est} of 13.1% (Figure 4-4B). Additionally, ddPCR quantification more closely resembled the true sample ratio that was prepared. Together these highlight that ddPCR is generally much more accurate both terms of technical variability, as well as absolute quantification. This data is in line with previous reports showing a superior sensitivity of ddPCR over qPCR (Zhao, Xia et al. 2016).

Moving forward, we tested the limit of sensitivity in our proposed method using cell lysate from stimulated lymphocytes. Strikingly, we were able to distinguish samples with only a 10% difference between them to a high degree of significance (Figure 4-4C).Unlike both the standard qPCR and ddPCR assays, our proposed method forgoes the use of a nuclear reference gene and thus eliminates the compounded error caused by reference variability. The advantage of this is further highlighted by the σ_{est} of only 3.77%. In summary, our technique could be used to

distinguish between samples whose copy number was far too similar for standard practices in the field to detect any significant difference.

Detection of mtDNA copy number in single cells

In order to assess the limit of detection in our proposed method, we created a 10-fold dilution series from BJ fibroblasts that extended down to fractions of a cell equivalent. Surprisingly, mtDNA copy number could be accurately measured using as little as one hundredth of a cell equivalent based on a dilution series, giving a corresponding R^2 of 0.9936 (Figure 4-5A). Since our assay was able to quantify mtDNA well below that of a single cell equivalent, we next investigated if we could reliably quantify mtDNA from individual cells.

In order to accomplish this, we employed a slightly modified version of our protocol to look at copy number in individual H1299 cells. Each single cell reaction contained 0.1 cell equivalents, well within the limits of detection for our protocol. The high sensitivity of our assay allowed us to generate very stable technical replicates for each individual cell (Figure 4-5B). Interestingly, mtDNA copy number varied widely from cell to cell, showing that it is possible to detect differences in even a homogeneous population. This intercellular variation could potentially be explained by cell cycle dependent mtDNA fluctuations (Van den Bogert, Muus et al. 1988, Margineantu, Gregory Cox et al. 2002, Trinei, Berniakovich et al. 2006) or by potentially hard-to-detect subpopulations (Kondo, Setoguchi et al. 2004). As would be expected, the average copy number of the single cells assayed was roughly equal to that of the pooled controls (Figure 4-5C), showing that these intercellular differences are unlikely to reflect methodological artifacts.

Discussion

Human mitochondrial DNA (mtDNA) is a circular non-nuclear DNA which encodes many polypeptides that are subunits of enzyme complexes of the oxidative phosphorylation system and are involved in many cellular signaling pathways involved in metabolism. The number of mtDNA copies in a cell varies from hundreds to thousands depending on cell type and current cell activity. It has also been reported that the mtDNA copy number is a biomarker for various diseases and aging processes. Therefore, there is an need for methods that can easily and accurately measure mtDNA copy numbers for both basic research and in diagnoses of disease.

In this study, we combined the droplet digital PCR technique with a new-developed DNA extraction method, and created a novel technique to measure mtDNA copy number in a very accurate and sensitive manner. Using this technique, we are able to detect even 10% differences of mtDNA copy numbers, making this technique very robust and reproducible.

Additionally, we further improved the protocol to fully exploit the potential of this technique, especially to maximize its strengths in accuracy and sensitivity, and applied this technique at the single cell level. Being able to measure mtDNA copy number in a single cell greatly enhanced our ability to study some highly heterogeneous populations. For example, our previous study has shown that during T cell stimulation, only a subset of cells can activate telomerase and maintain telomere length. Going forward the next question to address is if mtDNA content is determining telomerase activity levels in fractionated subset of T-cell. Also, in our centenarian studies, we compared the whole T cell population. Since there is clear heterogeneity of T cells, it will be important in the future to determine whether a subpopulation

of T cells from centenarians have higher numbers of mtDNA copies, one of the future directions we are pursuing.

Since mtDNA copy number is well-known to be a biomarker for many diseases as well as aging processes, the novel technique we have developed to measure mtDNA copy number in a much more accurate and sensitive way will provide opportunities to conduct new research discoveries in these related fields.

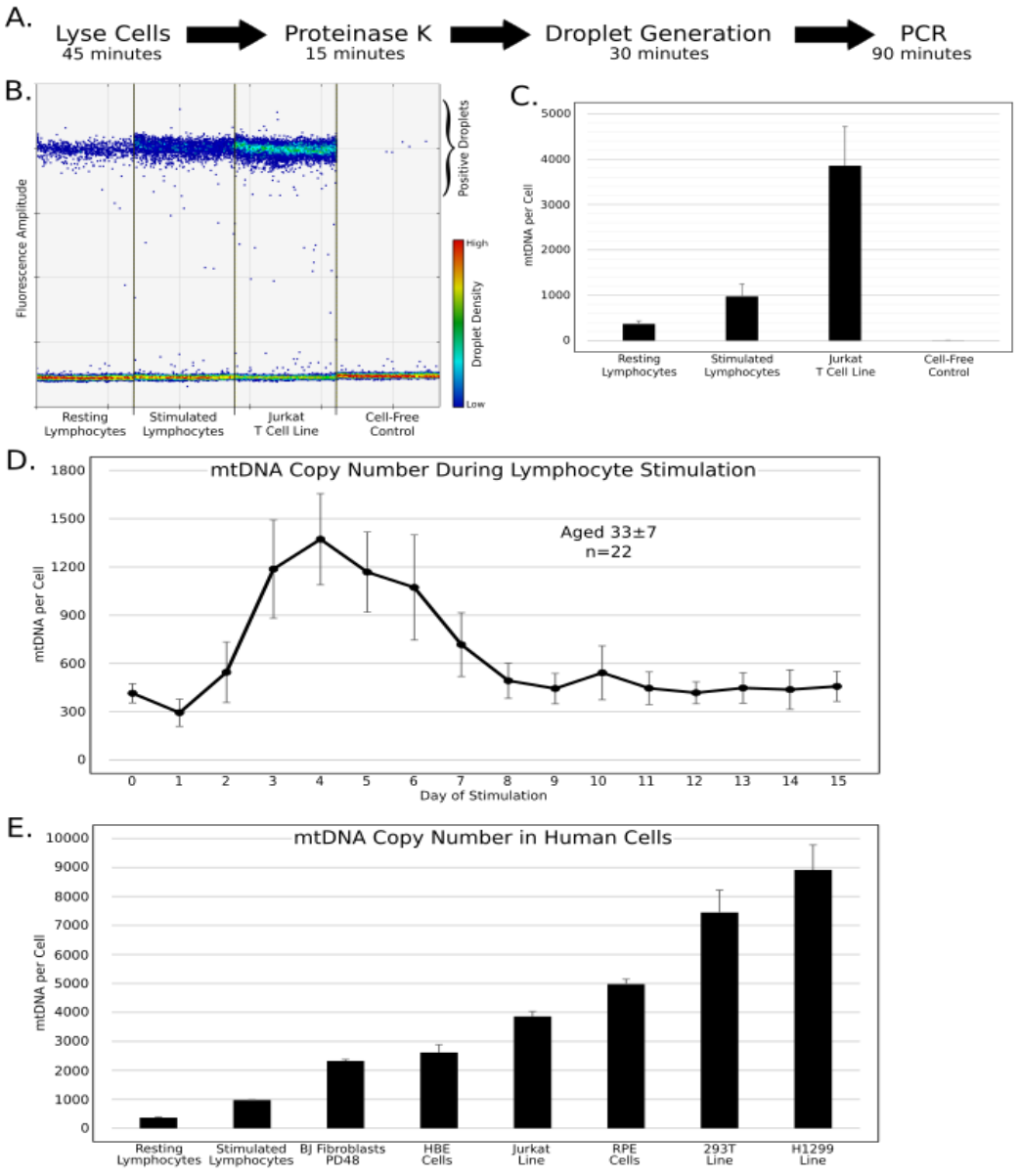


Figure 4-1. Optimized workflow and mtDNA quantification in different cell types. A. General workflow of our method for the quantification of mtDNA on a per cell equivalent basis by ddPCR. B. ddPCR output showing resting and stimulated T lymphocytes, Jurkat T leukemia cells, and a cell-free (lysis buffer) control. All samples were diluted to a final input of 5 cell equivalents. C. Quantification of ddPCR results. mtDNA copy number was calculated per cell equivalent. D. T lymphocytes from 22 healthy donors were stimulated at day 0 by anti-CD3/CD28 beads and mtDNA per cell equivalent was measured during stimulation. Error bars show standard deviation between the 22 individuals. E. Absolute mtDNA molecules per cell equivalent were measured in the indicated primary and transformed lines. (HBE: human bronchial epithelial, RPE: retinal pigment epithelium, 293T: human embryonic kidney line, H1299: non-small cell lung cancer line). Error bars show standard deviation of technical replicates.

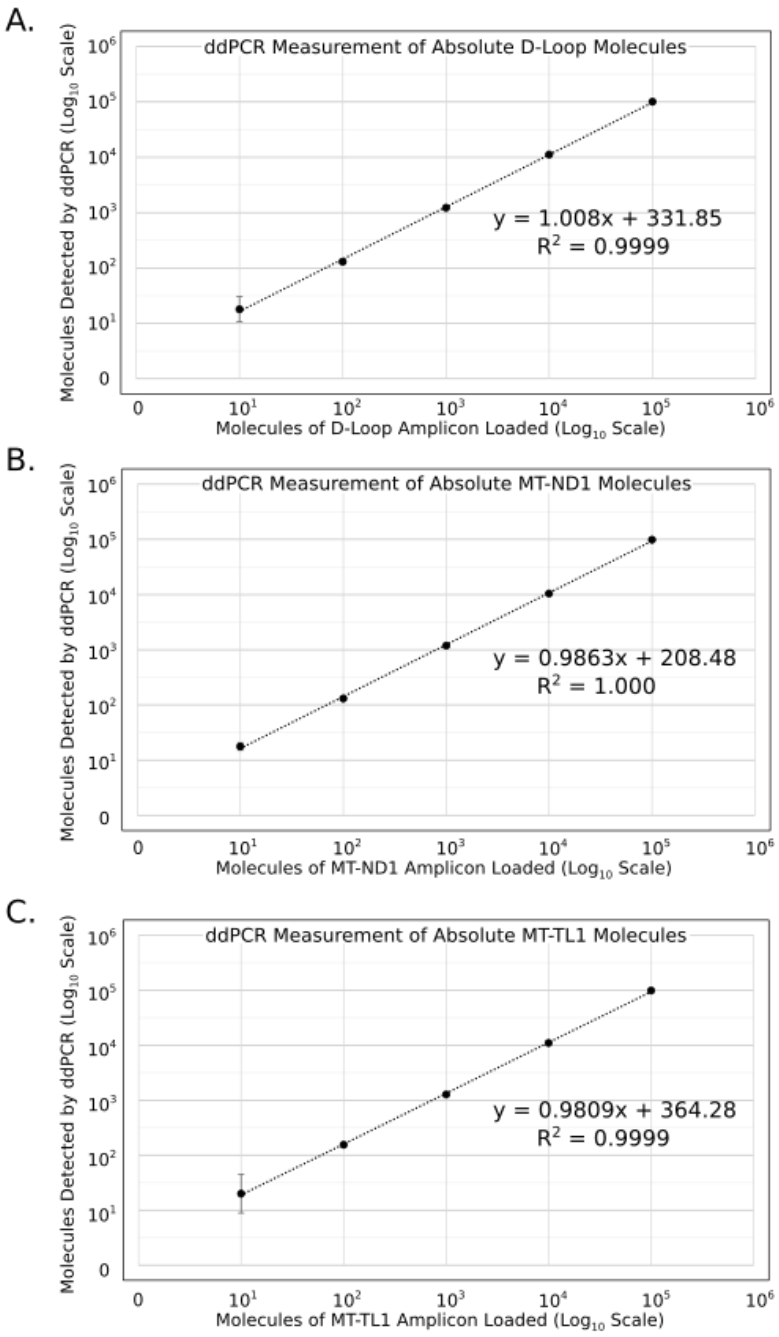


Figure 4-2. Absolute quantification of mtDNA amplicons in ddPCR. All amplicons were measured by ddPCR in 10-fold dilution series. Axis are displayed on Log scale. Error bars show the standard deviation of four technical replicates. A. D-Loop. B. MT-ND1, NADH Dehydrogenase Subunit 1. C. MT-TL1, Mitochondrially Encoded TRNA Leucine 1 (UUA/G).

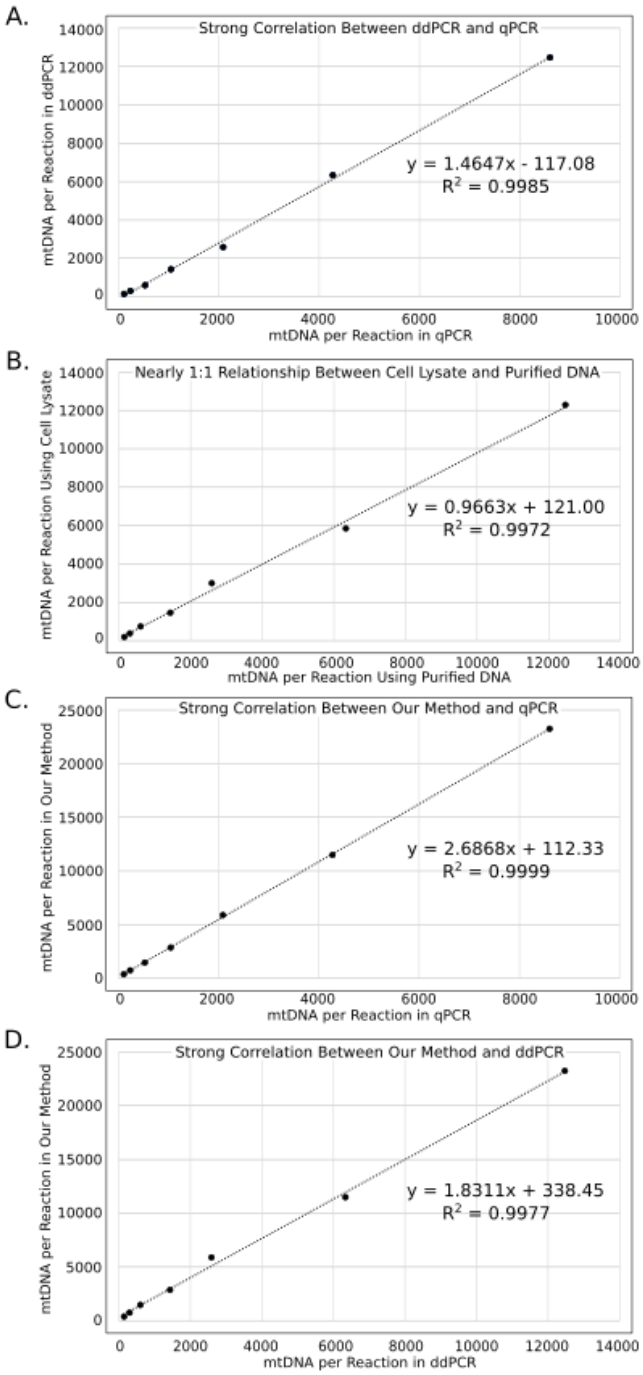


Figure 4-3. Comparison between methods. All method comparisons were performed using BJ fibroblasts. Data points are the average of two experiments run in triplicate. Error bars display the standard deviation of the six replicates. A. Comparison between mtDNA quantification by either ddPCR or qPCR (using purified DNA and normalized to genomic DNA content). B. Comparison performed in ddPCR using either cell lysate or purified DNA (both normalized to genomic content). C. Comparison between our method (normalized to cell equivalents) and qPCR. D. Comparison between our method and ddPCR.

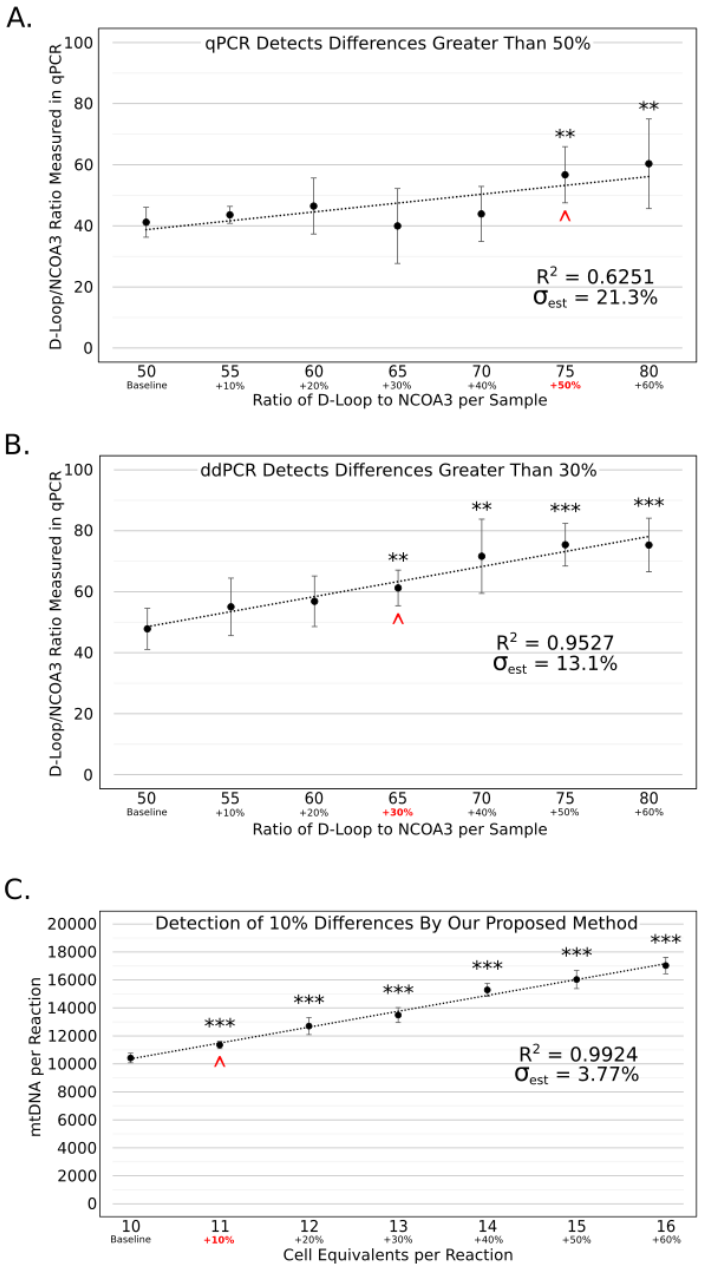


Figure 4-4. Ability to detect minor differences between samples in qPCR, ddPCR, or our method. All data points are the average of two experiments performed in triplicate. Error bars represent the standard deviation of these six replicates. In order to quantitate the ability of an assay to distinguish between two samples, a student's t-test was performed between each sample and the baseline. displays the standard error of the estimate as a percentage, normalized to the population mean. A. qPCR determination of D-Loop/NCOA3 ratio using purified amplicons. The minimum detectible difference was seen at 50% above the baseline. B. ddPCR determination of D-Loop/NCOA3 ratio using the same samples. The minimum detectible difference was seen at 30% above the baseline. C. mtDNA molecules (D-Loop) per 20µl reaction were measured using our method. All samples were distinguishable from the baseline, showing a minimum detectible difference of 10% or less from the baseline.

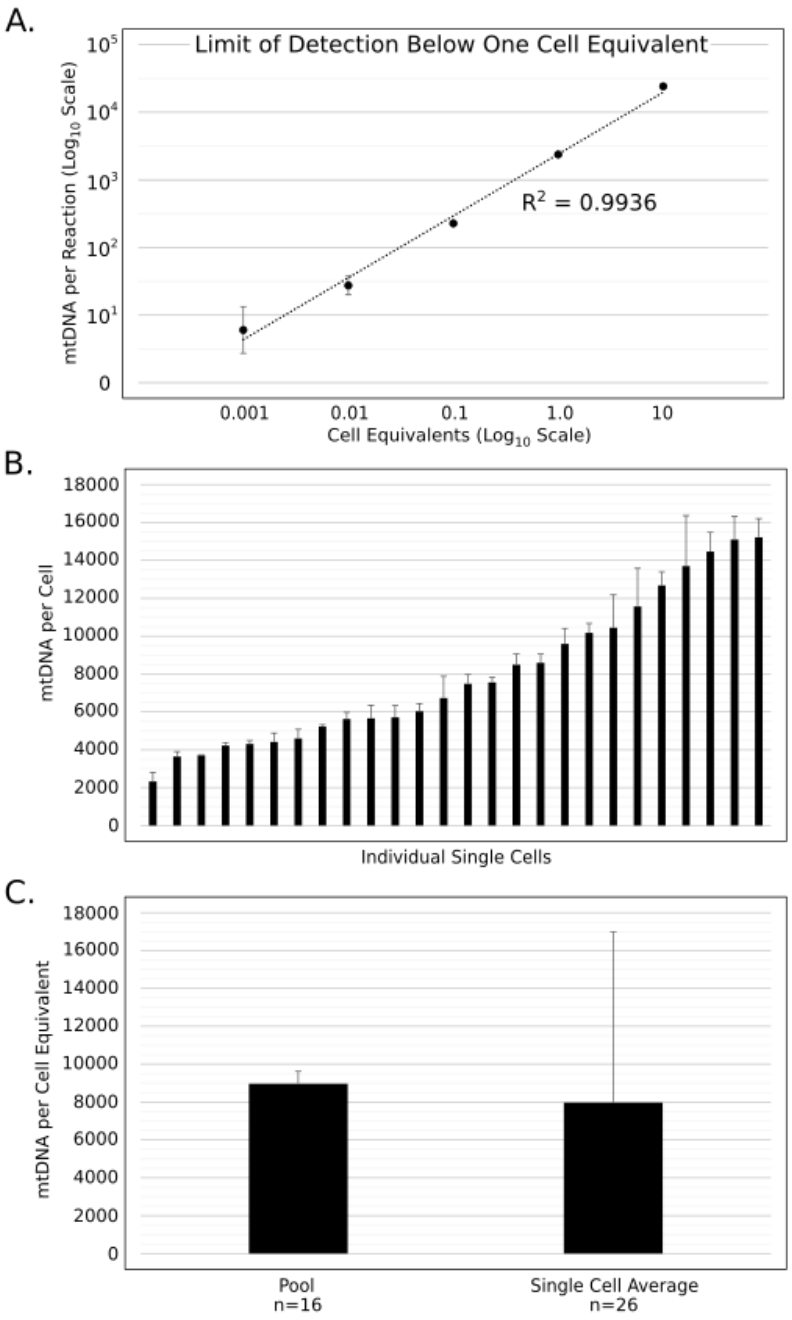


Figure 4-5. Limit of Detection and Single Cell Analysis A. 10-fold dilution series in BJ fibroblasts. Data points show the average of two experiments run in triplicate. Error bars display the standard deviation of replicates. B. mtDNA from single H1299 cells was quantified. Error bars show the standard deviation of technical replicates of each individual cell. C. Shown are the averages of a pooled cell control and of 26 single cells.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

Taken together, my doctoral project on T lymphocytes have made the following key conclusions:

- ✓ Telomerase activity is NOT necessary for the proliferation of T cells during stimulation.
- ✓ Telomeres shorten during T cell proliferation, but only a subset of CD28+ T cells show strong telomerase activity, and are capable of maintaining their telomeres.
- ✓ High-performing centenarians show robust telomerase activity and increased expression of CD28, indicating less age-related impairment of immune responses.
- ✓ High-performing centenarians show similar gene expression pattern with younger individuals, while low-performing centenarians are more similar with old individuals, suggesting there are certain genes that are involved in human healthy aging and longevity.
- ✓ Measuring mtDNA copy number at the single cell level with great accuracy and sensitivity using ddPCR may lead to new research opportunities for disease diagnosis and health status monitoring.

For centuries, mechanism of aging is a topic that is of popular interest. Dating back to 210 BC in China, the Emperor of the Qin Dynasty is the first recorded human who tried to develop the "Elixir of Life". Although these attempts failed, it reflects the long-term fear of aging

throughout human history. Entering the 21st century, as the health status of human beings has been greatly improved due to improved sanitation, vaccines and antibiotics, human life span has been lengthened dramatically. Increased longevity however has resulted in increases in both neurodegenerative disease and cancer, and these are becoming major causes of death. Although at first aging and cancer seems completely different topics, aging and cancer are actually two phenotypes that harmoniously tie into one process -- cell proliferation. During this process, the dynamics of telomerase and telomeres may be a key component for the regulation of cell proliferation in normal and cancer cells.

Therefore, my doctoral research has focused on elucidating the dynamics of telomerase and telomeres in immune cells. Taking advantages of the novel techniques developed in our lab, I was able to investigate how telomerase and telomeres behave during T cell stimulation. In addition, by studying centenarian samples and comparing them with other age groups, we have gained some insights into potential genes related to longevity, that might allow us to counteract some aspects of aging in the future.

As immunotherapy becomes an important method to have better outcomes in treating patients with advanced cancer, it is well established that T cell proliferation is the key step in this promising therapy. Thus, my doctoral research work has shed some novel insights into the mechanisms of proliferation in subsets of T cells and related this to telomerase activity and telomere maintenance. This work has added some value to this field that will hopefully improve therapeutic efficiency and patients experiences of immunotherapy in the near future and potentially to healthier aging in the normal population.

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