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Telomere length dynamics in aging mice

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Abstract

Leukocyte telomere length (TL) shortens with age and is associated with age-related pathologies. However, inherited and acquired variation in telomere length in individuals complicates clinical interpretations of TL as a biomarker of aging and age-related pathologies. Therefore, it is critical to identify a post-mitotic tissue as a surrogate marker of TL at birth. In my thesis project, I used quantitative PCR to trace TL dynamics of a variety of tissue types of inbred mice during 1st year of life. I found that TL of smooth muscle of aortic media did not shorten with age and represents birth TL. Notably, birth TL effectively offset genetic variation of TL in a genetically diverse mouse population. In addition, I further revealed that impaired collagen turnover in mice, which leads to premature aging symptoms, accelerates TL shortening. In summary, I identified that aortic media provides a powerful internal reference for birth TL with potentials to improve the accuracy of evaluating telomere shortening in individuals.

Keywords

Telomere length dynamics, T/S ratio, birth telomere length, genetic variation, inbred mice, Diversity Outbred mice, aortic media.

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List of Abbreviations

Abbreviation	Meaning
53BP1	P53 binding protein 1
ABP	ALT-associated PML body
ALT	Alternative lengthening of telomeres
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and rad3 related
BIR	Break induced replication
BMI	Body mass index
CAD	Coronary artery disease
C-FOS	FBJ murine osteosarcoma viral oncogene homolog
CHK2	Checkpoint kinase 2
CV	Coefficient of variation
CVD	Cardiovascular disease
DKC1	dyskerin gene
DDR	DNA damage response
DNA	Deoxyribonucleic acid
E2F	Transcription factor family including E2F- and DP-like subunits
ECM	Extracellular matrix
FISH	Fluorescence in situ hybridization
GWAS	Genome wide association studies
HSC	Hematopoietic stem cell
Kb	kilobases
LFS	Li-Fraumani syndrome
LTL	Leukocyte telomere length
MDC1	Mediator of DNA-damage checkpoint 1
MOV10	Moloney leukemia virus 10
NHEJ	Non-homologous end-joining

OBCF1	oligonucleotide/oligosaccharide-binding fold containing 1
PBS	Phosphate buffered saline
PCNA	Proliferation cell nuclear antigen
PD	population doubling
PML	Promyelocytic leukaemia body
PNA	peptide nucleic acid
POT1	Protection of telomeres 1
qPCR	quantitative polymerase chain reaction
Rap1	Repressor/activator protein 1
RCR	Rolling circle replication
ROS	Reactive oxygen species
SD	Standard deviation
SNP	Single nucleotide polymorphism
STELA	Single Telomere Length Analysis
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TIF	Telomere induced foci
TIN2	TRF1-interacting protein 2
TL	Telomere length
TPP1	POT1-TIN2 organizing protein
T-SCE	Telomere sister chromatid exchange
TRF	Telomere restriction fragment
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
WRN	Werner syndrome protein

Chapter 1 - Introduction

1.1 General introduction

The idea of modifying the course of aging and age-related diseases is a concept that humans naturally desire to explore with great potential for clinical application. Telomeres represent a potentially modifiable biomarker of aging that has been linked to age-related diseases (Oeseburg et al., 2010). Tremendous research efforts, with over 13000 hits on PubMed, growing continuously each day, are made to uncover the link between telomeres with aging and various age-related diseases. Telomeres protect chromosomes, but undergo attrition as DNA replicates or is insulted by environmental stresses (Oeseburg et al., 2010). In particular, shortening of telomere length (TL) in leukocytes is a well-established event in human aging (Aviv, 2012). However, an etiological link between telomeres and aging is yet to be fully elucidated. Understanding the dynamics of telomeres with normal aging in model organisms (e.g. mice) can help to uncover the role telomeres play at the organismal level with age.

1.2 Telomere structure

Telomeres are DNA-protein complexes containing repetitive units of DNA that reside at the end of each chromosome arm in eukaryotes (Figure 1.1) (Oeseburg et al., 2010). The DNA sequence of telomeres is highly conserved among vertebrates and consists of several kilobases of TTAGGG repeats (Meyne et al., 1989), ending in a 100-200 bp 3' overhang (Makarov et al., 1997). However, TL is highly variable across different species (Table 1.1).

Table 1.1 - Telomere length variation across vertebrate species

Vertebrate species	Telomere length	Reference
Human	5-15kb	(Moyzis et al., 1988)
Dogs	12-23kb	(Nasir et al., 2001)
Birds	5-20kb	(Haussmann et al., 2003)
Rats	20-100kb	(Cherif et al., 2003)
Mice	Up to 150kb	(Hemann and Greider, 2000)

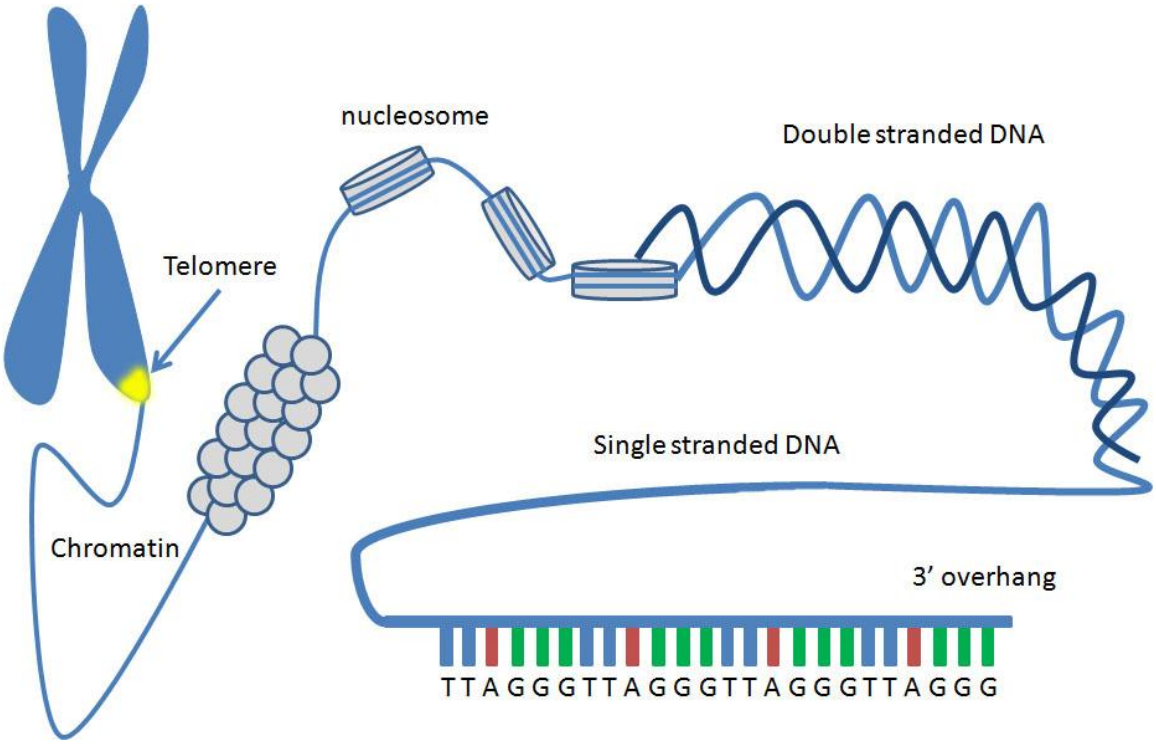

Figure 1.1 - Telomeres are located at the ends of linear DNA

Telomeres are located at the ends of linear chromosomal DNA consisting of repetitive units of TTAGGG in vertebrates.

1.2.1 T-Loop

The 3' overhang of telomere forms the secondary structure, which varies between taxa. In vertebrates it forms a T-loop structure and internal D-loop (Figure 1.2) by looping around to invade upstream telomeric DNA (de Lange, 2005). The 3'overhang is maintained during each round of DNA replication by a nuclease called Apollo (Wu et al., 2010). T-loop size is proportional to TL (Rahman et al., 2008). In fact, mice T-loop size is 18 kb long, whereas in humans it is only 3 kb (Griffith et al., 1999), reflective of their differing TLs (20-80 kb versus 10-15 kb, respectively) (Kipling and Cooke, 1990; Zijlmans et al., 1997).

1.2.2 Shelterin

Telomeres are further stabilized by a protein complex called shelterin (de Lange, 2005). Shelterin is composed of six proteins (Figure 1.2) – telomeric repeat binding factor 1 and 2 (TRF1 and TRF2), protection of telomeres 1 (POT1), repressor/activator protein 1 (Rap1), POT1-TIN2 organizing protein (TPP1, also referred to as TINT1, PIP1, and PTOP), and TRF1-interacting protein 2 (TIN2). Three of these proteins (TRF1, TRF2, and POT1) bind directly to telomeric repeats. TRF1 and TRF2 bind to double-stranded repeats via their SANT/Myb domains with high specificity and affinity and each have a flexible hinge that enables the formation of the T-loop (de Lange, 2005; Diotti and Loayza, 2011). POT1 binds directly to the single-stranded 3'overhang via its OB folds, which is important in the formation of the D-loop (Baumann and Cech, 2001; de Lange, 2005; Lei et al., 2004; Palm and de Lange, 2008). Unlike human cells that have only one POT1 gene, mice have two variants that are highly homologous (POT1a and POT1b) but have distinct roles at telomeres (He et al., 2006; Hockemeyer et al., 2006). The other three proteins (Rap1, TPP1, and TIN2) are recruited by TRF1 and TRF2 to assist in stabilizing telomeres (de Lange, 2005). TIN2 acts as the bridge between different shelterin components by binding TRF1 and TRF2 in separate domains and recruiting the TPP1-POT1 complex (Chen et al., 2008; Kim et al., 2004; Liu et al., 2004; Ye et al., 2004). Furthermore, a novel isoform of TIN2 (TIN2L) tethers telomeres to the nuclear matrix (Kaminker et al., 2009), which may play a role in chromosome stability.

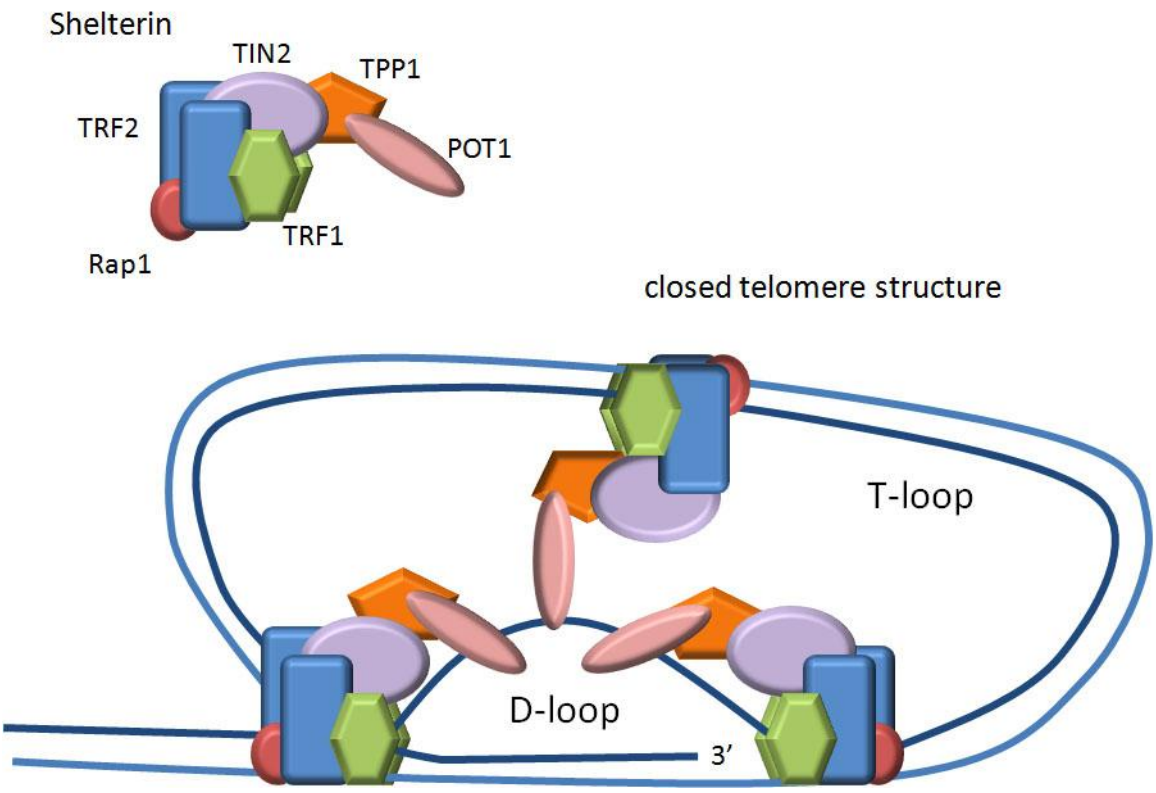

Figure 1.2 - Secondary structure of telomeres.

Protein components of shelterin complex are involved in the formation and stability of T-loop and internal D-loop secondary structures of telomeres. Image modified from (Oeseburg et al., 2010).

TPP1 binds TIN2 and POT1 in separate domains and is thought to be central in recruiting POT1 to telomeres (Chen et al., 2007; Kibe et al., 2010). Lastly, Rap1 is recruited by TRF2 and forms a tightly associated complex that is essential for Rap1's binding to telomeres (Celli and de Lange, 2005; Li and de Lange, 2003; Li et al., 2000). Overall the shelterin complex is involved in telomere stability and telomere length regulation (de Lange, 2005).

1.3 Telomere function

Telomeres are considered as a telomeric cap because of the important functions it serves for linear DNA. Over the years scientists have uncovered that telomeres play a fundamental role in the stability and mobility of the genome and prevent erosion of coding DNA.

1.3.1 Genomic stability

The chromosome ends were first proposed to be important for chromosome stability by the combined work of Barbara MClintock and Hermann Muller on their work with maize and fruit flies, respectively (McClintock, 1939; Muller, 1938). Muller coined the term telomere from the greek words *telos* (end) and *meros* (part). Both scientists observed that without telomeres, chromosomes would fuse together and break off during mitosis (Figure 1.3), which was catastrophic to cellular physiology.

Telomere dysfunction either by critically shortened telomeres or missing shelterin components can activate the DNA damage response (DDR) and signal up-regulation of cell cycle checkpoints such as p53 (Bodnar et al., 1998; Karlseder et al., 1999; Lee et al., 1998). In some cells, cell cycle checkpoints can be by passed, propagating breakage fusion bridge cycles and genomic rearrangements with risk of neoplastic transformation (Figure 1.3). Thus cells require telomeres as a way to protect natural ends from being recognized as double strand breaks, in order to suppress the DNA repair machinery and maintain genomic stability (Chan and Blackburn, 2004).

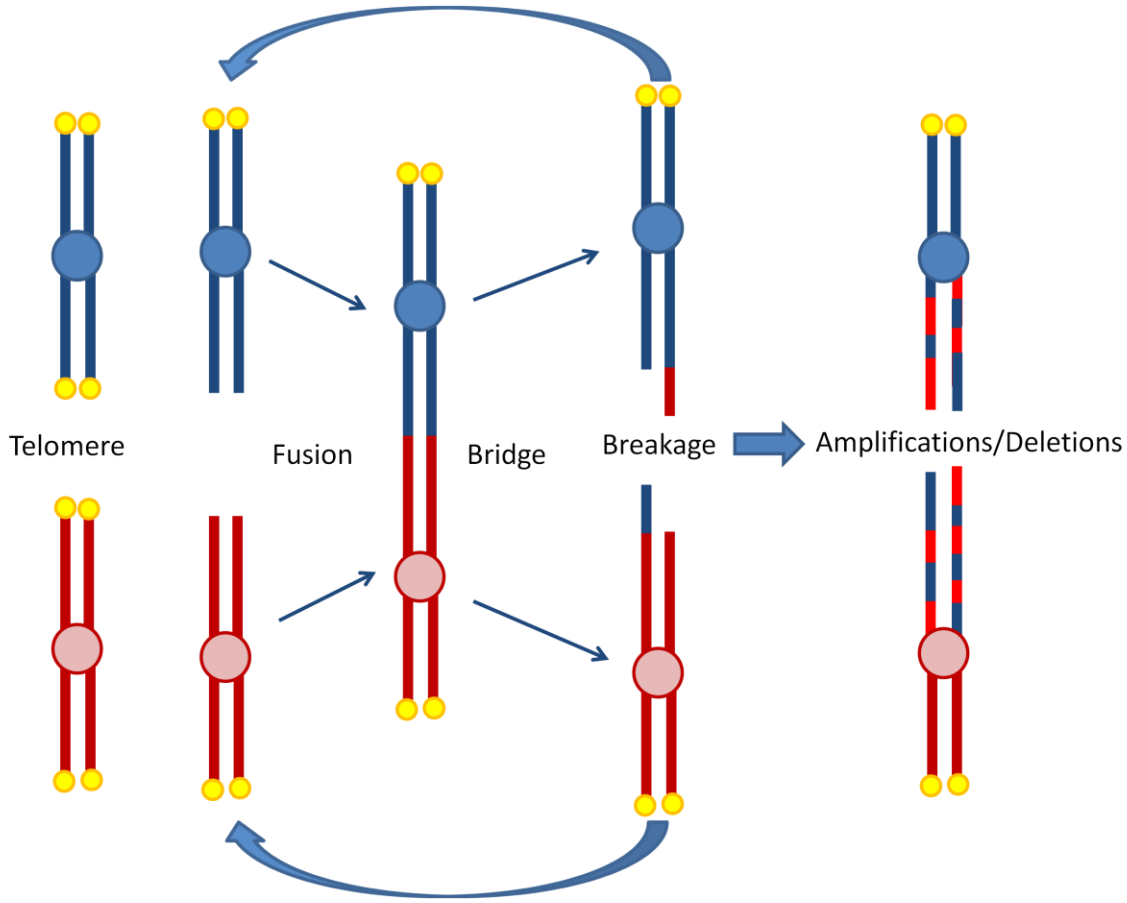

Figure 1.3 - Anaphase breakage fusion bridge cycles

Without telomeres (yellow circles) at the ends of chromosomes, a fusion event can take place between bare ends. During anaphase when chromosomes are pulled to opposite poles of the cell, DNA will break between bridged chromosomes. Anaphase breakage-fusion-bridge cycles cause genomic shuffling (amplifications and deletion events) and instability that can lead to further complications of cancer. Image modified from (Else, 2009).

Furthermore, during meiosis telomeres form bouquet structures that are integral to the coordination of the spindle apparatus to ensure proper alignment and segregation of genetic material (Maddar et al., 2001; Tomita and Cooper, 2007).

1.3.2 End-replication problem

One of the most important functions of telomeres was discovered by Olovnikov and Watson who noticed that linear chromosomes cannot replicate their very ends, termed the "end-replication problem" (Olovnikov, 1973; Watson, 1972). The end-replication problem leads to inevitable loss of telomeres at every cell division. DNA polymerase can only replicate in the 5' to 3' direction and requires a short RNA primer for initiation (Shore and Bianchi, 2009). Because DNA is double stranded, one strand will be synthesized in short "okazaki" fragments. However, at the 5' end of the lagging strand, there will be a gap due to removal of the last RNA primer (Shore and Bianchi, 2009). Currently, it is not known whether or not the last RNA primer is placed at the very end of telomeres or is variable (Sfeir et al., 2005b). Telomere ends undergo further processing following DNA replication to ensure there is a 3' overhang with a precise end sequence (Sfeir et al., 2005a). Interestingly, the length of this overhang is highly variable between different cell types and is closely associated with telomere length (Lee et al., 2008).

Initially, cells were thought to lose telomeres at a constant rate, keeping track of the number of cell divisions like a "mitotic clock" (Von Zglinicki, 2003). However, there is considerable heterogeneity in the rate of telomere shortening (Martin-Ruiz et al., 2004) and in the number of divisions a cell can undergo (Hayflick, 2003; Smith and Hayflick, 1974). Notably, the end-replication problem directly limits the number of possible cell divisions (Olovnikov, 1973), later known as replicative senescence.

1.4 Telomeres and cell senescence

There are several ways in which cells senesce but one of the main mechanisms is telomere shortening and dysfunction, specifically referred to as replicative senescence or mortality 1 (M1) senescence (Harley et al., 1990). During each round of cell division, telomeres shorten by ~50-200 bp due to oxidative stress and the end replication problem – where DNA polymerase cannot replicate the very ends of the lagging strand

(Blackburn, 2005; Vaziri et al., 1994). In cells, this shortening eventually causes telomeres to reach a critical length ("Hayflick limit") that signals a DNA-damage response (DDR), which involves protein kinases (ATM and CHK2), adaptor proteins (53BP1 and MDC1) and chromatin modifiers (γ-H2AX) (Campisi and d'Adda di Fagagna, 2007). Telomere dysfunction induced foci (TIF), defined as cells with more than 50% of 53BP1 foci colocalised with telomere repeats, are often used to identify telomere-dependent senescent cells (Jeyapalan et al., 2007). Telomere-dependent activation of DDR further leads to the activation of p53 via its phosphorylation and upregulation of its downstream target p21 (Blackburn, 2005; Campisi and d'Adda di Fagagna, 2007; Hornsby, 2002). Expression of p21 maintains pRB in a hypophosphorylated active state, which inactivates the transcriptional factor E2F. E2F is responsible for inducing the expression of genes that encode proteins responsible for cell-cycle progression (i.e. replication-dependent histones, c-FOS, cyclin A and B, and proliferation cell nuclear antigen (PCNA)). Therefore, repressing E2F activity causes cells to enter a state of permanent cell cycle arrest (senescence) (Blackburn, 2005; Campisi and d'Adda di Fagagna, 2007; Hornsby, 2002). If any of these cell cycle checkpoints are by-passed (most commonly p53), perhaps due to mutations, the cell will continue to divide and telomeres will continue to shorten until a crisis phase (M2 phase) is reached. In M2 phase, telomeres are virtually non-existent and chromosome fusion events occur due to activation of the non-homologous end joining (NHEJ) pathway, which can result in genomic shuffling as shown in Figure 1.3 (Shay and Wright, 2005).

One of the key factors in triggering senescence or apoptosis in aging cells is shortened telomeres that can no longer form a closed t-loop. In fact, inhibition of TRF2 from shelterin *in vitro* causes the activation of DDR and senescence (Celli and de Lange, 2005; Takai et al., 2003). In a similar way, human cells transfected with short telomere oligonucleotides that mimic the effect of an open t-loop also trigger a p53 dependent cell cycle arrest (Saretzki et al., 1999).

It is noteworthy that cellular senescence can also be induced by telomere-independent mechanisms such as damage induced by reactive oxygen species (ROS), which follows an alternate senescence pathway through the upregulation of p16 and pRB (Finkel and

Holbrook, 2000). Murine fibroblasts in culture will senesce after 15-20 population doublings despite having exceptionally long telomeres (>20 kb) when exposed to atmospheric oxygen concentrations of 20%, which is above biological levels of 3% (Hornsby, 2003; Parrinello et al., 2003). In contrast, human fibroblasts with shorter telomeres (10-15 kb) senesce between 50-60 population doublings, suggesting better protective mechanisms against ROS compared to mouse cells (Hornsby, 2003; Parrinello et al., 2003). To some degree this argues against the relationship between telomere shortening and aging, particularly in mice, however, *in vitro* studies do not fully translate *in vivo* (Varela et al., 2011). Furthermore, the shortest mice telomeres are just as short to those in human cells (Zijlmans et al., 1997), and a single short telomere is sufficient to set off the cascade of events leading to senescence (Abdallah et al., 2009; Bendix et al., 2010). Finally, it is not known whether the threshold telomere length that induces senescence in humans and in mice is different. Perhaps in mice that have an average T-loop size 6-7 times greater than in humans (Rahman et al., 2008), require longer telomeres to maintain a closed T-loop structure.

1.5 Cellular senescence and aging

Cellular senescence describes a cell that has undergone permanent cell cycle arrest and ceased to divide. This phenomenon was first observed in 1961 by Leonard Hayflick, who observed that primary human fibroblasts in culture can only divide a limited number of times (40-60 population doublings (PD)), later referred to as the "Hayflick limit" (Hayflick, 1965; Hayflick and Moorhead, 1961). The exact number of divisions a cell can undergo is dependent on cell type and organism (Campisi and d'Adda di Fagagna, 2007). For instance, stem cells and cancer cells can divide indefinitely in culture, whereas human somatic cells cannot (Itahana et al., 2004). However, murine somatic cells senesce between 15 and 20 PDs and can immortalize spontaneously in culture (Itahana et al., 2004). A senescent cell is characterized by a flattened morphology in 2D culture, altered gene expression, and secretion of various matrix-degrading enzymes (Campisi and d'Adda di Fagagna, 2007).

Cellular senescence is considered both beneficial to young organisms and harmful to old ones, a term referred to as antagonistic pleiotropy (Figure 1.4) (Jeyapalan et al., 2007).

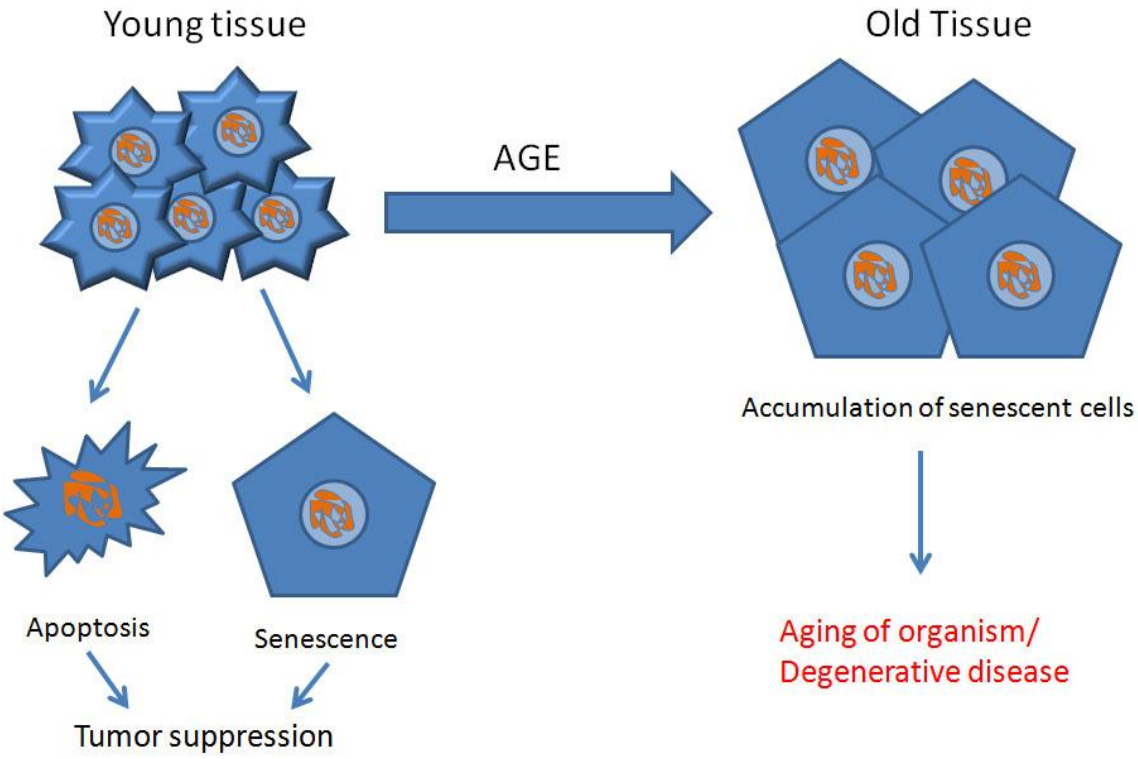

Figure 1.4 - Antagonistic pleiotropy of senescent cells in young and old individuals

Young tissue is predominantly composed of normal, healthy, fully-functional cells. Whenever stress on a cell is too great or telomeres are too short, the cell undergoes apoptosis or senescence to avoid tumorigenesis. However, as we age, old tissue is burdened by an accumulation of senescent cells, compromising tissue function. What was protective at a young age against tumors is destructive to old organisms leading to age-related pathologies. Image modified from (Chuaire-Noack et al., 2010).

In young organisms cellular senescence is said to have evolved as a tumor suppressor by giving highly proliferative cells a limited capacity to divide. In older organisms, senescent cells tend to accumulate in organs, leading to age-related phenotypes (Jeyapalan et al., 2007).

Evidence begins to accumulate that supports the link between cellular senescence and organismal aging. First, the number of senescent cells increases with age and appears to be rare in young individuals (Dimri et al., 1995; Jeyapalan et al., 2007). Furthermore, sites of age-related pathologies such as atherosclerosis and osteoporosis contain increased number of senescent cells (Chang and Harley, 1995; Price et al., 2002; Vasile et al., 2001). Moreover, the altered secretory profile (upregulation of matrix degrading enzymes, growth factors, and pro-inflammatory cytokines) of senescent cells has detrimental impacts on neighboring cells and structural components of tissue that lead to aged and diseased pathologies (Burton, 2009). Taken together, evidence suggests burden of senescent cells is connected to organismal senescence. Since telomere length is considered an endogenous marker of cellular senescence, its length is also a putative marker of biological aging.

1.6 Telomere length (TL) regulation

TL equilibrium is established by the net result of telomere shortening and lengthening events. The frequency of each of these events varies depending on the cell type and organism involved (Shay and Wright, 2007). Several of the key factors that influence telomere length dynamics in a cell are outlined in Figure 1.5.

1.6.1 Factors that cause telomere shortening

There are several factors that cause telomere shortening in a cell as listed in Figure 1.5. The two most common causes are due to the end-replication problem, discussed in section 1.3.2, and oxidative damage to DNA.

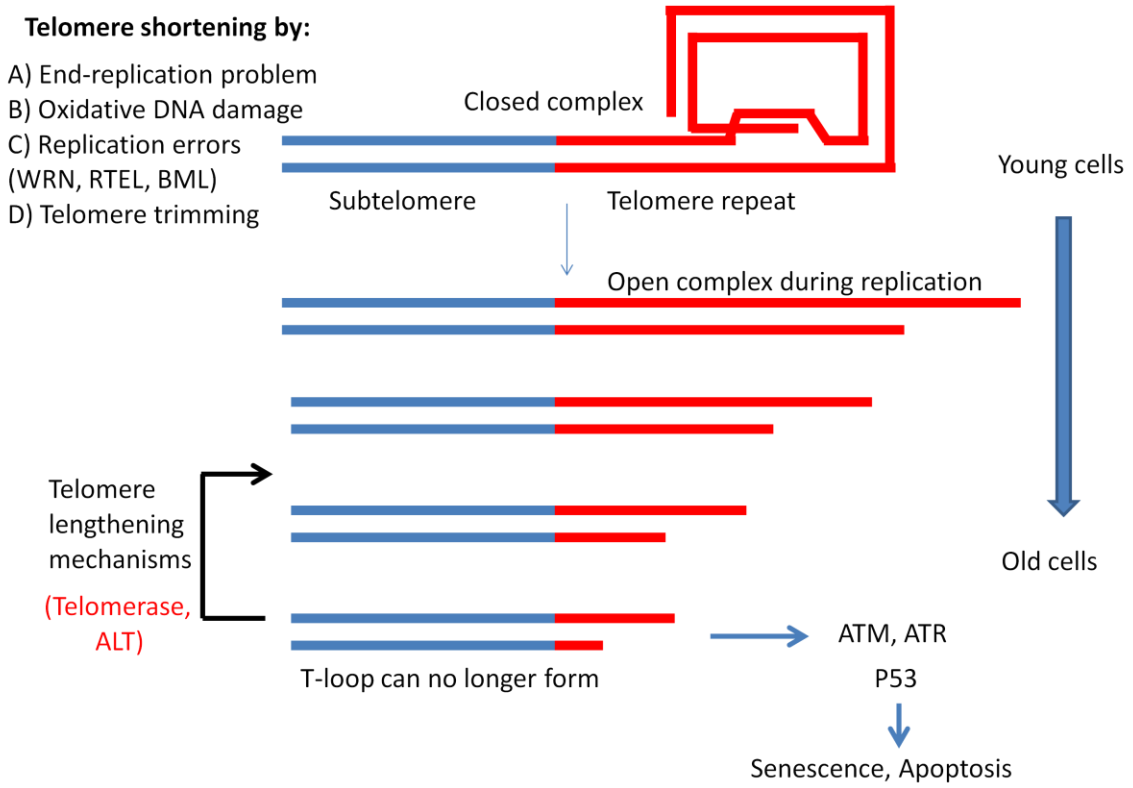

Figure 1.5 - Factors affecting telomere length dynamics in vertebrate cells

During DNA replication telomeres are in an open structure to allow DNA polymerase to extend the entire length of the chromosome. However, various factors contribute to telomere shortening that are propagated during replication such as the end-replication problem, oxidative DNA damage, replication errors, and telomere trimming. As cells divide and telomeres reach a critical length, they can no longer form a closed t-loop structure which signals cell cycle checkpoints such as ATM, ATR and p53, causing cells to enter a state of senescence or apoptosis. Telomere lengthening mechanisms, such as telomerase and alternative lengthening of telomeres can extend the replicative capacity of cells by lengthening their telomeres. Image modified from (Aubert and Lansdorp, 2008).

1.6.1.1 Oxidative DNA damage

While the end-replication problem sets the baseline of telomere shortening, oxidative stress is the best recognized modifier. The minimal rate of telomere shortening observed in culture is 10-20 bp per cell division in fibroblasts with high antioxidant capacity (Serra et al., 2003; von Zglinicki, 2002) or those treated with an agent that reduces oxidative stress in cells (Passos et al., 2007). This lower rate of attrition is likely that set by the end-replication problem, which differs significantly from the average rate of telomere attrition in human cells (50-200 bp). This difference is primarily due to oxidative stress (von Zglinicki et al., 2000).

The major source of reactive oxygen species (ROS) in cells comes from mitochondria, as a metabolic by-product. Burden of ROS in a cell induces various types of DNA damage (oxidized bases, single and double strand breaks), which accumulates over time (Fraga et al., 1990; Packer and Fuehr, 1977). Telomere shortening is largely accelerated by accumulation of oxidative DNA damage; in particular, by single strand breaks (von Zglinicki et al., 2000; von Zglinicki and Schewe, 1995). Those damages that are not repaired or replaced before the next round of DNA replication will be lost due to stalling of the replication fork. Therefore, telomere loss due to oxidative damage is dependent on DNA replication (von Zglinicki, 2002).

Cells engage antioxidant defenses to protect against the harmful effects of oxidative stress by neutralizing free radicals (von Zglinicki, 2002). The strength of these defenses varies between cell types and species. Some strains of human fibroblasts have higher antioxidant levels than others, reflective in lower telomere attrition rates (von Zglinicki, 2002). As mentioned in section 1.4, mouse cells are more prone to oxidative damage than human cells (Hornsby, 2003). Replicative lifespan can be extended and telomere shortening reduced by increasing the expression of antioxidant enzymes (Furumoto et al., 1998; Serra et al., 2003). In contrast, cell lifespan can be decreased by increasing oxidative stress or inhibition of antioxidant defenses (Kurz et al., 2004; Passos et al., 2007). The degree of damage at a given level of oxidative stress is highly variable simply due to chance, which may explain differential rates of telomere shortening

observed between populations of cell clones *in vitro* and between individuals *in vivo* (Von Zglinicki, 2003).

Telomeres are particularly sensitive to oxidative damage compared to other parts of the genome due to the presence of guanine triplet base pairs (GGG), which are major targets of ROS (Kruk et al., 1995; Oikawa et al., 2001; Petersen et al., 1998). In fact, human telomeres showed 7 times more DNA breaks than control sequences when inserted into a plasmid (Henle et al., 1999). Moreover, repair of damage at telomeres is significantly less efficient than the rest of the genome, even compared to guanine-rich interstitial sequences (Petersen et al., 1998). Therefore, telomeres contain a greater degree of oxidative damage upon initiation of DNA replication than the bulk of the genome, contributing significantly to their attrition (von Zglinicki et al., 1995). In fact, the frequency of single-stranded breaks is directly proportional to the amount of telomere loss at replication (Sitte et al., 1998). One theory for this increased damage at telomeres is an evolutionary role as sentinels to detect levels of DNA damage procured by the cell and shut it down before they become dangerously high (von Zglinicki et al., 2000).

1.6.1.2 Other factors that contribute to telomere shortening

Telomere shortening can be caused by DNA replication errors caused by mutations or malfunction of various proteins such as RecQ protein-like helicases (WRN and BML) and RTEL (Crabbe et al., 2004; Machwe et al., 2011; Sidorova, 2008; Uringa et al., 2011). These helicases function in the resolution of higher order telomeric structures (G-quadruplexes and t-loop) to allow for efficient replication of telomeres and prevent stalling of the replication fork (Machwe et al., 2011; Postberg et al., 2012).

A recently discovered and less understood mechanism of telomere shortening is called telomere trimming, which involves resolution of t-loops from telomeres by recombination. This mechanism has been observed in human and mouse cells and is believed to set an upper limit on telomere length and contribute to a length equilibrium set-point (Pickett and Reddel, 2012).

1.6.2 Factors involved in telomere length maintenance

The progressive attrition of telomeres caused by the end-replication problem and oxidative damage posed a problem for certain cell types, especially in the germ line. If telomeres continue to shorten across generations, species with linear chromosomes (eukaryotes) would soon become extinct. Cells had to develop ways to overcome this problem by mechanisms of telomere lengthening. The most common method, discovered by Nobel Prize laureates Dr. Elizabeth Blackburn and Dr. Carole Greider, is by the activity of a reverse transcriptase called telomerase (Greider and Blackburn, 1985, 1987). A second, less common method, alternative lengthening of telomeres (ALT), was discovered in telomerase negative cancer cells (Bryan and Reddel, 1997; Reddel et al., 1997).

1.6.2.1 Telomerase

Telomerase exists to circumvent the end-replication problem by catalyzing the addition of telomeric repeats *de novo* to the ends of DNA (Blackburn, 2005). Telomerase is a ribonucleoprotein composed of a core reverse transcriptase (TERT) and an RNA component (TERC), which acts as a template for addition of telomeric repeats to the 3' end of telomeres (Figure 1.6) (Blackburn, 2005).

Proper folding and stability of TERC is orchestrated by the nucleolar enzyme dyskerin (DKC1 gene) (Gu et al., 2009). Both components of telomerase are expressed at low levels, giving rise to clinical problems by haploinsufficiency in either TERT or TERC (Du et al., 2007; Mason and Bessler, 2004), or mutations in DKC1 (Mason et al., 2005). Expression and activity of telomerase are highly regulated at various levels, which include transcription, splicing, post-translational modifications, assembly, and sub-cellular localization (Gladych et al., 2011; Wojtyla et al., 2011).

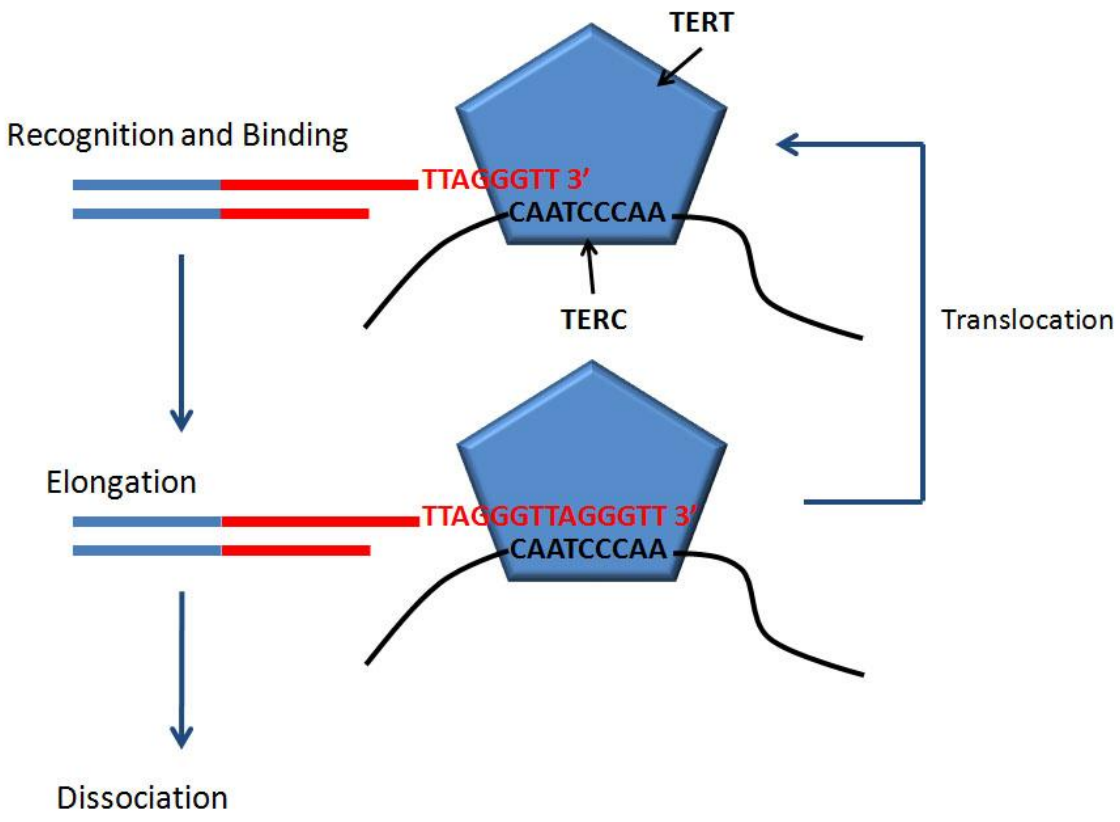

Figure 1.6 - Elongation of telomeres by telomerase

Telomerase adds repeats six nucleotides at a time in three distinct steps that involve substrate recognition and binding, elongation, and translocation or dissociation from telomere ends. Image modified from (Nicholls et al., 2011)

Many proteins function in concert to orchestrate recruitment of telomerase, telomere length itself being a signal. In fact, telomerase preferentially elongates the shortest telomeres (Britt-Compton et al., 2009), which seems to be a function of shelterin proteins (Wojtyla et al., 2011). Longer telomeres contain more shelterin proteins, which represses access of telomerase to telomere ends. However, the rate of telomere elongation by telomerase can be increased by inhibition of various shelterin components, such as POT1 or TRF1 (Wojtyla et al., 2011). The dynamics of telomerase repression and recruitment are intricate and change in different conditions. In fact, the interplay between POT1 and TPP1 acts both as a repressor of telomerase in one condition and a processivity factor in another (Wang et al., 2007). Furthermore, a novel protein (MOV10 helicase) was discovered that binds to both TERT and telomeric DNA. MOV10 is expressed exclusively in human testis and ovaries, and is thought to maintain telomerase activity in those tissues (Nakano et al., 2009).

Telomerase expression is ubiquitous in the developing embryo (Wright et al., 1996). However, during somatic differentiation telomerase is down-regulated, which is considered to have evolved as an anti-tumorigenesis mechanism (Forsyth et al., 2002). In this way, somatic cells do not retain capacity to divide indefinitely. Conversely, stem cells and germ cells maintain a degree of telomerase activity, which is highest in testes (Blackburn, 2005). This is vital so that telomere loss is not passed on to offsprings (Schaetzlein et al., 2004). Nonetheless, even somatic stem cells show telomere shortening with age with increased demand to replenish aging cells in tissue (Allsopp et al., 2001; Vaziri et al., 1994).

Telomerase expression in somatic cells also varies between species. Mice continue to express telomerase to some degree in somatic tissues (Prowse and Greider, 1995), whereas humans do not (Kim et al., 1994). Furthermore, recent evidence suggests that decreased somatic telomerase expression correlates with increased body size of a species (Gomes et al., 2011), proposing an evolutionary mechanism to offset the increased risk of cancer due to increased body mass (Gorbunova and Seluanov, 2009; Seluanov et al., 2007).

Ultimately, telomerase expression is highly associated with risk of cancer as expressed in over 85% of human cancers (Kim et al., 1994). Therefore, there is a trade-off between replicative capacity and risk of cancer in the regulation of telomerase activity, which varies between species and cell types.

1.6.2.2 Alternative lengthening of telomeres (ALT)

Alternative lengthening of telomeres involves a recombination based mechanism that is used in 15% of cancer cells lacking telomerase (Bryan and Reddel, 1997). However, anti-telomerase treatment of cancer can provoke the use of ALT (Hu et al., 2012). Two characteristic features used to detect ALT cells are the formation of ALT-associated promyelocytic leukaemia (PML) body (ABP) and long and heterogeneous telomeres (Nabetani and Ishikawa, 2011).

PML body is a nuclear aggregate of PML and other proteins, which is present in many cell types and functions in various cellular processes (stress response, tumour formation, DNA repair, etc.). However, APB's are special as they include telomere DNA and chromatin, which can be readily observed (Yeager et al., 1999). The molecular mechanism of PML and how APB's are related to the ALT pathway is currently unknown. However, many proteins involved in metabolism, cell growth regulation, and particularly homologous recombination localize at APB. RecQ-like DNA helicase BLM is an APB protein that is required for growth of ALT cells (Bhattacharyya et al., 2009), whereas WRN appears dispensable (Laud et al., 2005). The MRN complex and shelterin components are also important for the formation of APB and maintenance of telomeres by the ALT pathway (Nabetani and Ishikawa, 2011).

The long and heterogeneous nature of telomeres in ALT cells represents the critical difference between ALT- and telomerase-dependent telomere lengthening. First, mean telomere length of ALT cells is ~20 kb compared to <10 kb in telomerase-positive cells (Bryan et al., 1995). Second, analysis of metaphase spreads by qFISH (discussed in section 1.7.2) revealed large variations in telomere signal between chromosome arms in

ALT cells in comparison to telomerase-positive cells (Perrem et al., 2001). Studies using tagged telomeres support a recombination based mechanism based on observations of telomere duplication from one chromosome to the next, as well as drastic elongation and deletion events (Dunham et al., 2000; Murnane et al., 1994). Moreover, high levels of telomere-sister-chromatid exchange (T-SCE) was observed in ALT cells but not telomerase-positive cells (Bailey et al., 2004; Bechter et al., 2004; Londono-Vallejo et al., 2004).

ALT cells also contain extrachromosomal telomere repeat (ECTR) in the form of telomeric circles (t-circles), determined by 2D gel electrophoresis (Cesare and Griffith, 2004; Wang et al., 2004). However, t-circles can also be observed in cells with defects in TRF2 or cells containing extensively long telomeres, such as *Mus musculus* mice (Pickett et al., 2009). T-circles are formed by resolution of the t-loop structure by intra-chromosomal recombination events, termed telomere trimming (section 1.6.1.2). Several mechanisms of recombination are proposed to be used by the ALT pathway (Figure 1.7).

Unlike break induced replication (BIR) and rolling-circle replication (RCR), integration of t-circles into chromosome ends do not cause an increase in total cellular telomeric repeats (Nabetani and Ishikawa, 2011). RCR is probably the most efficient recombination method of lengthening telomeric ends and can cause drastic telomere lengthening. BIR and RCR are not exclusive of each other and are likely governed by the same molecular mechanisms (Nabetani and Ishikawa, 2011).

Currently, little evidence suggests that non-neoplastic cells use the ALT pathway to maintain their telomeres. However, one study provides evidence that ALT may occur in endothelial, stromal and some epithelial cells upon acquisition of sufficient DNA damage (Slatter et al., 2012). Nonetheless, telomerase is the dominant mechanism when it comes to maintenance of telomeres in all cell types.

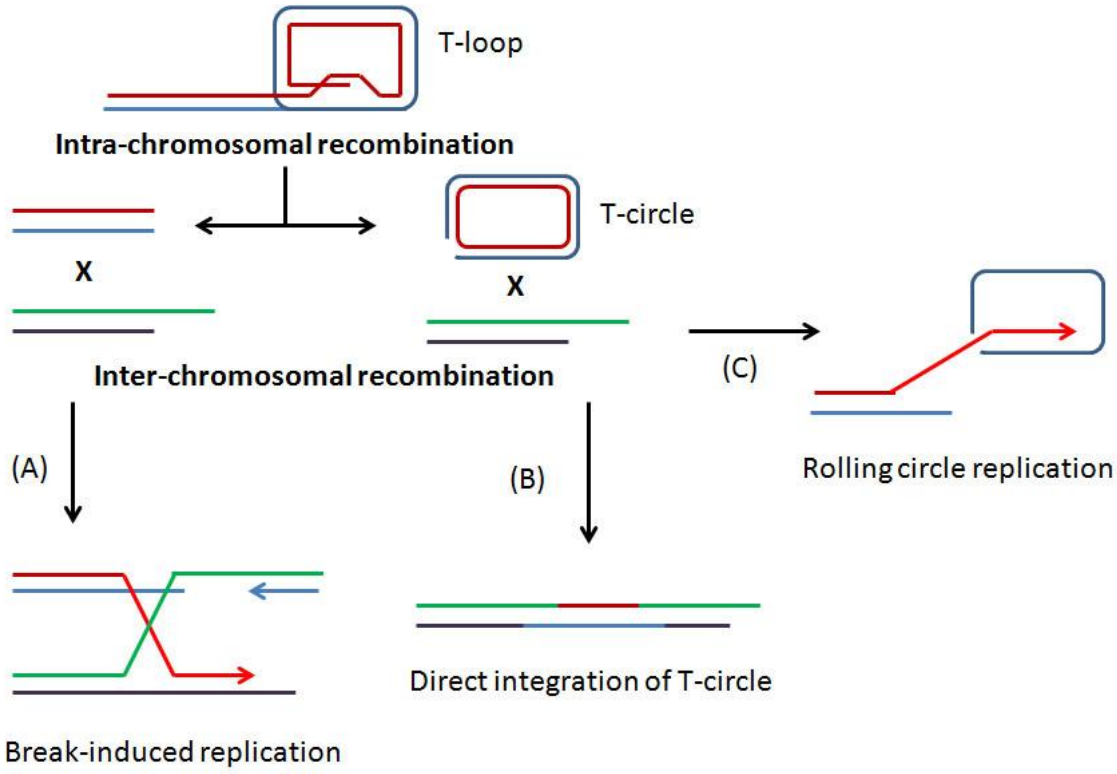

Figure 1.7 - Mechanisms of alternative lengthening of telomeres

Recombination in ALT cells may occur by intra-chromosomal recombination causing drastic telomere shortening as seen by the formation of T-circles. Lengthening by recombination can occur by three possible mechanisms: (A) invasion of chromosome ends to induce break-induced replication, (B) integration of a T-circle into a chromosome end, and (C) rolling-circle replication initiated by the 3'overhang invading proximal T-circles. Image modified from (Nabetani and Ishikawa, 2011)

1.7 Telomere length measurement

Several methods of measuring telomere lengths have been developed over the years, each with their individual caveats (Aubert et al., 2012; Lin and Yan, 2005). The main two groups of measurements are those that measure average telomere length (TRF and qPCR) and those that measure telomere lengths of individual chromosomes (Q-FISH, Flow-FISH, and STELA). All methods use binding of nucleic acid probes or primers specific to telomeric repeats. Discrepancies between telomere length measurements of the various methods arise from variability in the amount of extra-telomeric region (Figure 1.8) included in some techniques and less in others (Aubert et al., 2012). The two most popular methods in the literature are telomere restriction fragment (TRF) analysis and quantitative polymerase chain reaction (qPCR).

1.7.1 TRF analysis

TRF analysis by southern blotting was the first method to be described (Harley et al., 1990) and represents the gold standard used to validate all other techniques to date (Aubert et al., 2012; Kimura et al., 2010b; Lin and Yan, 2005). This technique uses a pair of frequent cutting restriction enzymes (i.e. *Hinf1* and *Rsa1)* to cut DNA into small fragments, specifically excluding telomeric repeats as substrates. Frequent cutting restriction enzymes are used to minimize the size and variation of extra-telomeric region (Figure 1.8). The fragmented DNA is then separated by agarose gel electrophoresis, transferred to a nitrocellulose or nylon gel and hybridized with a radioactive (^{32}P) or chemiluminescent (digoxienin) labeled probe complementary to telomere sequences. Average telomere length of the resulting smear is estimated based on a DNA ladder and normalization to a reference sample to correct for inter-experimental gel effect. The length and intensity of the smear needs to be accounted for in the calculation of average telomere length. Although this is a well established technique, variations between studies have procured over time because the technique was not standardized in terms of restriction enzymes used, DNA quantity and quality, and blot analysis. There are several main pitfalls to this method. First, a relatively large amount of pure and unfragmented DNA (0.5-10 µg) is required and small differences in telomere lengths are difficult to detect, which limits its use to broad research questions.

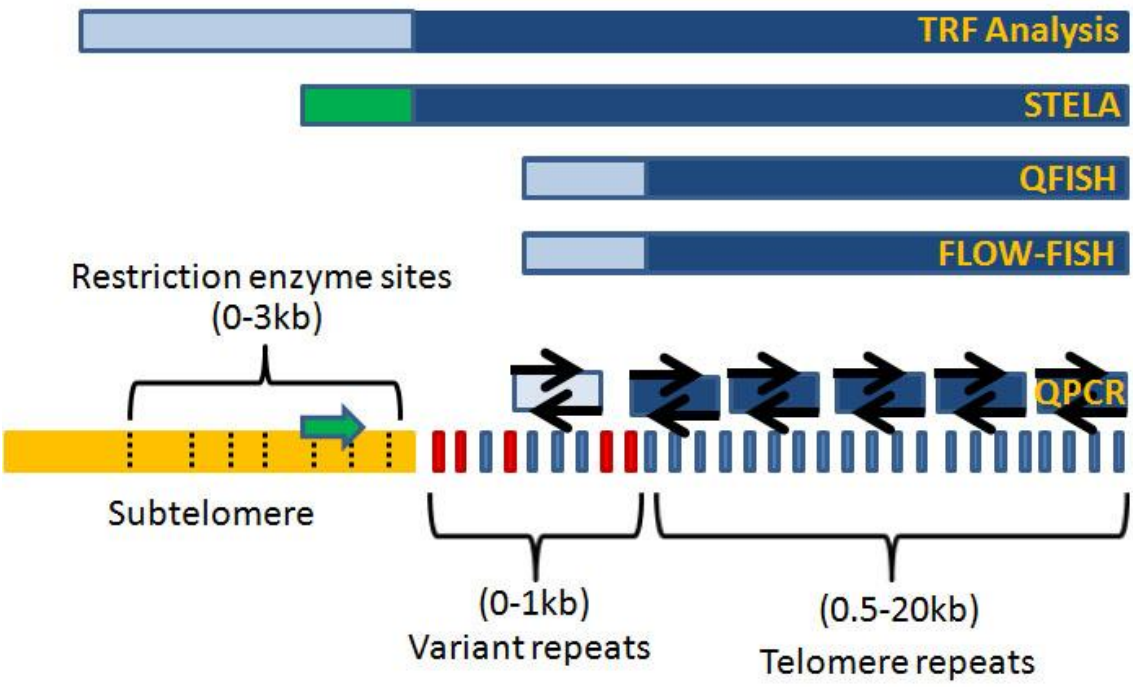

Figure 1.8 - Detection of extra-telomeric DNA with different methods of telomere length measurement

In Figure 1.8 modified from (Aubert et al., 2012), extra-chromosomal region is described as the proportion of DNA that is not considered pure telomere repeats (blue bars). In gold is the subtelomere region that contains restriction enzyme sites (dashed lines) used to digest DNA in TRF analysis. In red are the variant repeats that contain telomere-like sequences. The green box for STELA is the primer used in the subtelomeric portion that is later subtracted from the calculation of telomere repeats. The light blue boxes are regions that have variable detection.

Furthermore, TRF analysis is time consuming (5-7 days), making it less ideal for large clinical studies, although skilled technicians can process up to 130 samples per week (Kimura et al., 2010b). Lastly, large variations in telomere length (up to 5%) can be seen depending on the choice of restriction enzymes used and because of subtelomeric polymorphisms. However, despite limitations of this technique, the errors of this method are relatively small (CV=1.74%) (Aviv et al., 2011).

1.7.2 qFISH

Telomere length can also be measured by quantitative fluorescence *in situ* hybridization (qFISH), which combines use of image cytometry and metaphase spreads through use of peptide nucleic acid (PNA) probes (Aubert et al., 2012; Martens et al., 1998; Poon and Lansdorp, 2001). These probes have a greater affinity than DNA oligonucleotides and bind specifically to denatured telomere repeats. The fluorescent signal is acquired using specific qFISH analysis software and telomere lengths are measured relative to standards of known length. Karyotyping of metaphase spreads allows telomere lengths to be matched with its corresponding chromosome arm. It's the method of choice to determine telomere length of specific chromosome ends. The analysis requires 15-20 metaphases per sample to obtain reliable results due to the high variation in the technique. Some advantages are that qFISH can detect chromosome fusion events and ends with virtually no repeats (<0.5 kb). This technique is a good choice to study telomere biology in rare cell types and can be used to ask more specific scientific questions because it requires so few cells. However, some disadvantages are that qFISH cannot be used to measure telomeres in cells that cannot divide such as senescent cells and highly aberrant cells. Furthermore, the technique is labor intensive and takes a long time, and requires specialized equipment.

1.7.3 Flow-FISH

Similar to qFISH, flow-FISH also uses PNA probes, however hybridization is done with cells in suspension and median telomere length of individual cells is measured using flow cytometry (Aubert et al., 2012; Martens et al., 2000). This technique can be used to measure telomere lengths of specific cell populations (i.e. granulocytes and lymphocytes)

in a single sample by cell sorting prior to flow-fish or staining for antibodies (limited to a few cell surface markers that are retained after hybridization). A semi-automated adaptation of this technique uses a 96-well plate and robotic microdispenser to reduce tedious work and increase reproducibility. It is currently the method of choice for measuring telomere length in specific subsets of blood cells. Some drawbacks of this technique are that it requires a suspension of living cells, which is very fragile. This technique is generally limited to use of fresh blood. Like qFISH this method requires specialized equipment, is technically challenging and costly (Aubert et al., 2012; Martens et al., 2000).

1.7.4 STELA

One of the newest techniques available is Single Telomere Length Analysis (STELA), which uses PCR to amplify a specific chromosome arm of telomere (Aubert et al., 2012; Baird et al., 2003). STELA takes advantage of the fact that all chromosome arms end in a 3' overhang, which is targeted as a template to anneal an oligonucleotide linker at the 5' end of the telomere. A linker-specific primer and subtelomere specific primer is then used to amplify a precise length of a single telomere tract. PCR amplicons are then separated by gel electrophoresis, southern blotting and probed with specific subtelomere sequences. Resulting banding patterns are intricate sets of discrete bands of individual telomeres, which can be measured individually or pooled based on size according to a DNA ladder of known lengths. One drawback of STELA is its limitation to well characterized chromosome arms (XpYp, 2p, 11q, 12q and 17p) (Britt-Compton et al., 2006) because not all chromosomes have adequate sequences for the design of unique chromosome primers. Therefore, caution must be taken when analyzing results because a subset of telomere measurements may not be reflective of the overall telomere status of cells (Bendix et al., 2010). Although no specialized equipment is required, STELA is technically challenging and requires intensive initial preparation and optimization (Baird et al., 2003). Furthermore, STELA has an upper detection limit of 20kb, which limits its use mostly to human samples, and it cannot be used on model organisms with long telomeres such as *Mus musculus* strains of mice (Kipling and Cooke, 1990). Benefits of STELA are in its precision of telomere length measurement, its ability to detect subtle

changes in telomere length, and minimal starting material required (as few as 50 cells) to produce reliable results (Aubert et al., 2012). Like qFISH, STELA is well fit to study telomere lengths in rare cell populations, and it can also detect short telomere outliers, those responsible for chromosome fusion and senescence.

1.7.5 qPCR

QPCR based telomere length measurement (Cawthon, 2002) has become widely used in large clinical and epidemiological studies because of its rapid design, ease of use, and low cost. This technique relies on specific amplification of telomeric repeats (T) normalized to a single-copy gene (S) to produce an average telomere length (T/S ratio). A sample calculation can be viewed in appendix B. A special set of telomere specific primers were designed that contain mismatches every six base pairs, different in both the forward and reverse primer. These mismatches are important to minimize primer dimer formation and maximize primer-template hybridization. Telomere-specific primers bind along telomeric repeats at each chromosome arm and amplify in fragments of at least 76 bp and up to ~500 bp long. SYBR green, a dye that fluoresces upon binding to double stranded DNA, is used to monitor the amplification of DNA using Real-Time PCR. The longer the average telomeres are in a given sample, the greater the fluorescent signal.

A setback of qPCR is in the variability between experiments. Proper controls are required to minimize this variability. In every experiment, a set of inter-plate control samples and a standard curve must be produced to offset the variability (Aubert et al., 2012; Aviv et al., 2011). An important quality control of this technique is in the preparation of DNA samples. It is critical that all DNA samples are of equivalent quality so that there are no variations in amplification efficiencies. Another drawback of this technique is that it is not standardized across different laboratories, and therefore results between laboratories cannot be compared. A multiplex version of this technique has recently been developed (Cawthon, 2009) that further reduces variation, which amplifies telomere repeats and single-copy-gene in the same PCR reaction. Although qPCR technique is prone to variation, with proper controls it can produce quick and reliable results at a low cost, which is beneficial for most large-scale experimental designs. Furthermore, an impartial analysis between laboratories comparing TRF analysis and

qPCR analysis on the same set of DNA samples revealed a strong correlation between both techniques, although TRF had slightly better inter-assay variation (Aviv et al., 2011).

Choosing the proper technique depends solely on the experimental design and scientific question being asked, as each technique has its advantages and disadvantages.

1.8 Telomere dynamics in the aging population

There are key differences between human and mouse telomere biology (Table 1.2). How these differences relate *in vivo* to consequences in telomere length shortening with age and disease are currently not fully understood. However, genetically modified mice have been useful to elucidate the role telomeres play in aging and chronic illness. Key findings that link telomere biology to cardiovascular disease and cancer are further discussed below.

Table 1.2 - Differences between mouse and human telomere length dynamics

Phenotype	Mice	Humans	Reference
Inter-chromosomal variation in telomere length	*Mus musculus (*20-150 kb); CAST/Ei (15-20 kb) *Mus spretus (15*-20 kb)	5-15 kb	(Hemann and Greider, 2000; Zijlmans et al., 1997)
T-loop size	~18 kb	~3 kb	(Griffith et al., 1999)
Telomerase activity	Low levels in adult somatic cells	Absent in adult somatic cells	(Prowse and Greider, 1995)
T-circles	High frequency	Low frequency	(Pickett et al., 2011)
Immortalization in culture	Spontaneous	Rare, 1 in 10E-7	(Wright and Shay, 2000)
Telomerase processivity	Non-processive (1-2 repeats)	Processive (long tracts)	(Prowse et al., 1993)

1.8.1 Telomere length with age

Several studies have documented telomere length shortening with age in various tissue or cell types both *in vivo* and *in vitro*. The concept of telomeres as a mitotic clock came from in vitro studies, whereby fibroblasts, lymphocytes and hematopoietic stem cells

(HSCs) lose between 37 and 120 bp per population doubling (Harley et al., 1990; Vaziri et al., 1994; Vaziri et al., 1993). However, unlike human primary fibroblasts, mouse embryonic fibroblasts in culture do not show obvious telomere shortening with age, likely attributed to telomerase activity present in cultured mouse cells (Sachsinger et al., 2001). In telomerase knockout mice (TERT-/- or TERC-/-), telomeres do in fact shorten in culture. Furthermore, by inducing expression of the catalytic subunit of telomerase (TERT) *in vitro* to human differentiated cells, telomere length is maintained and replicative senescence postponed (Bodnar et al., 1998; Ramirez et al., 2001; Yang et al., 1999). One group studied telomere attrition in 14 different tissues of a large human population ranging from 0 to 104 years of age. They reveal a significant reduction in telomere length with age in all tissues studied except cerebral cortex and myocardium, thought to be due to the quiescent nature of cells in those tissues (Takubo et al., 2002). In mice, telomere length attrition has also been shown in several tissue types with age, including spleen, skin, small intestine and brain (Coviello-McLaughlin and Prowse, 1997; Flores et al., 2008). Telomere loss per year *in vivo* for most tissues appears to be within the range of telomere loss of one cell division *in vitro*. However, these results are likely skewed by telomere length maintenance and heterogeneity in cell or tissue types (Takubo et al., 2002). Several studies have calculated an average telomere shortening rate in lymphocytes (53 bp/year), granulocytes (39 bp/year) (Hoffmann et al., 2009), and pancreas (36 bp/year) (Ishii et al., 2006).

Telomere length attrition rates vary with age, showing a biphasic pattern in lymphocytes (Frenck et al., 1998; Rufer et al., 1999). Most telomere shortening tends to happen in the first few years of life, gradual shortening during adulthood, and accelerated shortening in old age. From birth to age 4, an average telomere loss of 1kb is seen and thereafter telomeres shorten by 100bp/year (Frenck et al., 1998). In specific subsets of blood cells, Rufer *et al.* determined that 1.08kb of telomere sequence was lost in lymphocytes within the first 1.5years, and 3.05kb loss in granulocytes in the first 0.5year of life (Rufer et al., 1999). There are no gender differences in telomere length at birth in both human leukocytes and mouse tissues (Coviello-McLaughlin and Prowse, 1997; Okuda et al., 2002). However, male telomeres tend to shorten more rapidly than female up until

menopause. A potential mechanism that has been proposed is related to the protective function of estrogen (Moller et al., 2009).

Telomere length dynamics with aging of hematopoietic stem cells (HSCs) is considered to be one of the most reflective cell models of biologic aging. In fact, the most prominent phenotypic consequence of defects in telomere length maintenance is bone marrow failure as is seen in dyskerotosis congenital, Werner syndrome, and aplastic anemia. Similarly in telomerase knockout mice, once telomeres are sufficiently short in late generation mice, function of the hematopoietic system is compromised (Blasco, 2005b; Herrera et al., 1999). Although telomerase is active in HSCs, telomeres shorten in culture on average 1-2 kb per month (Engelhardt et al., 1997) as well as *in vivo* (Lee et al., 2003; Vaziri et al., 1994). Seeing as telomere shortening outweighs telomerase activity, HSC resources become exhausted with aging.

On the other hand, telomere lengthening with age is also observed. Telomere length in spermatozoa is positively correlated with age such that offspring born with older fathers tend to have longer telomeres at birth. A suggested mechanism of telomere lengthening in germ cells is epigenetic modification and resistance to oxidative stress; however, it is not yet fully understood (Kimura et al., 2008).

Longitudinal studies were necessary in order to understand the true impact of telomere length with age. Using qPCR, two studies found a negative correlation of telomere length with age over a 5 and 10 year period in 500 and 959 individuals, respectively. However, in almost one third of individuals, no correlation with age has been found (Aviv et al., 2009; Nordfjall et al., 2009). Furthermore, significant decline of leukocyte telomeres seen early in life was confirmed in a study with baboons, whose telomeres were measured longitudinally over the first few years of life (Baerlocher et al., 2007). More recently, a study with birds demonstrated that telomere length at birth is correlated with life expectancy (Heidinger et al., 2012). Similarly, a genetic variant of the RNA component of telomerase (TERC) is also linked to leukocyte telomere length and longevity (Soerensen et al., 2012). Therefore, longitudinal studies are important to elucidate clear relationships with age and other confounding factors.

1.8.2 Telomere length and chronic illness

Age represents one of the greatest risk factors for chronic illness. Telomere length can be considered a function of replicative history (age) and environmental stress, two important parameters in the development of chronic illness (Oeseburg et al., 2010). A clear link between telomere length and mortality in humans has been established (Cawthon et al., 2003), but how telomere length correlates lifespan is still under debate. Nonetheless, many studies provide evidence that telomere length is associated with age-related illnesses, especially cardiovascular disease and cancer (Oeseburg et al., 2010).

1.8.2.1 Cardiovascular disease

Cardiovascular disease (CVD) is one of the main contributors to morbidity and mortality in the aging population. Telomere length is a proposed biomarker of individuals at risk for developing CVD. Several studies over the past decade have demonstrated the relationship between short leukocyte telomere length and CVD (Table 1.3).

Table 1.3 - Short leukocyte telomere length and associated cardiovascular disorders

Cardiovascular disorder	Participants	Reference
Hypertension	96 cases; 98 controls	(Bhupatiraju et al., 2012)
	327 men from FHS	(Demissie et al., 2006)
Left ventricular dysfunction	89 subjects (85 years old)	(Collerton et al., 2007)
Atherosclerosis	10 cases; 20 controls	(Samani et al., 2001)
	164 hypertensive men	(Benetos et al., 2004)
	1062 individuals from FHS	(O'Donnell et al., 2008)
Myocardial infarction	203 cases; 180 controls	(Brouilette et al., 2003)
	484 cases; 1058 controls	(Brouilette et al., 2007)
	337 cases; 337 controls	(Zee et al., 2009)
Abdominal Aortic Aneurysms	190 cases; 183 controls	(Atturu et al., 2010)
Congestive heart failure	620 cases; 182 controls	(van der Harst et al., 2007)
Aortic dissection	72 cases; 72 controls	(Yan et al., 2011)
Cardiovascular mortality	143 subjects (>60 years old)	(Cawthon et al., 2003)
	780 CAD subjects	(Farzaneh-Far et al., 2008)

FHS – Framingham Heart Study; CAD – coronary artery disease

Two major risk factors to CVD, diabetes and hypertension, have both been linked to short telomeres. One of the key studies linking short telomeres to insulin resistance and hypertension was the Framingham Heart Study (Demissie et al., 2006), later confirmed in a Chinese (Yang et al., 2009) and Indian population (Bhupatiraju et al., 2012).

Hypertensive individuals with short telomeres are more susceptible to develop atherosclerosis (Yang et al., 2009), and display increased risk of cardiovascular mortality, stroke or angina pectoris (Fyhrquist et al., 2011). Even mild activation of the rennin-angiotensin system has been linked to shorter leukocyte telomeres (Demissie et al., 2006; Vasan et al., 2008). Endothelial dysfunction, marked by cholesterol burden, inflammatory markers, and increased oxidative stress, is considered an early marker of atherogenesis (Asselbergs et al., 2005; van der Harst et al., 2006). Consistent with this, senescent endothelial cells and short telomeres have been seen in atherosclerotic plaques (Minamino et al., 2002; Ogami et al., 2004). The West of Scotland Primary Prevention Study (WOSCOPS) determined that patients were more susceptible to develop coronary heart disease if they were in the middle or lower tertile for telomere length. Patients with shorter telomeres also benefited more from treatment with statins (Brouilette et al., 2007). Furthermore, offspring of patients with coronary artery disease (CAD) have shorter telomeres than those with healthy parents (Brouilette et al., 2008), and may explain part of CAD heritability (Samani et al., 2007).

Several studies have linked short telomere length to glucose intolerance, insulin resistance and type 2 diabetes (Adaikalakoteswari et al., 2005; Adaikalakoteswari et al., 2007; Sampson et al., 2006). Even subclinical levels of insulin resistance were linked to shortened telomeres in participants of the Framingham Heart Study (Demissie et al., 2006). Patients with type 2 diabetes show signs of accelerated aging (accelerated telomere attrition, increased oxidative damage, and decreased mitochondrial DNA) compared to control subjects (Monickaraj et al., 2012).

Other cardiovascular risk factors are also associated with short telomeres. Arterial calcification is also associated with short telomeres (Mainous et al., 2010). A study with

Chinese stroke patients revealed that short telomere length is associated with ischemic stroke and post-stroke death (Ding et al., 2012). Telomere length was determined in a large prospective study of 19838 Danish participants followed for up to 19 years for incident myocardial infarction, ischemic heart disease, and death. Short telomere length was modestly correlated with increased risk of each outcome in the population (Weischer et al., 2012).

Although an etiologic role for telomere length in CVD has yet to be established, a recent study of 3271 Caucasians in the Cardiovascular Health Study aged ≥65 years old revealed SNPs in OBFC1 (a gene linked to leukocyte telomere length) were associated with CVD mortality in women (Burnett-Hartman et al., 2012a).

1.8.2.2 Cancer

A major factor influencing life expectancy is cancer, especially in long-lived species. Short leukocyte telomere length is a risk for the development of many types of cancer, including head and neck, breast, bladder, prostate, lung, kidney, ovarian (Blackburn, 2011; Martinez-Delgado et al., 2012; Wu et al., 2003).

As stated in section 1.4, dysfunction of the p53 pathway causes cells to continue to divide until a state of crisis is reached. A series of breakage fusion bridge cycles between chromosomes with dysfunctional telomeres causes genomic rearrangements that can induce upregulation of oncogenes and promote tumorigenesis (Deng et al., 2008; Hackett and Greider, 2002; Rudolph et al., 2001). Mice with enhanced p53 responses show increased resistance to cancer, however, have shorter lifespan and display age-related phenotypes sooner (Donehower, 2002; Matheu et al., 2007). In contrast, Li-Fraumeni syndrome (LFS) is characterized by a germline mutation in TP53, predisposing LFS patients to cancer. With each consecutive generation there is earlier onset of cancer caused by progressive telomere shortening in the germ cells. Therefore, telomere length has been used as a biological marker to monitor LFS patients (Tabori et al., 2007).

Tumor cells also utilize one of two mechanisms to maintain their telomeres, providing them with a means to overcome the end-replication problem. As stated earlier, 80-90% of

all cancer cells express telomerase, and the remaining use the ALT pathway (Shay and Bacchetti, 1997) or a combination of both (Venturini et al., 2012). Similar to enhancing p53, suppression of telomerase can reduce risk of cancer, but it also induces age-related phenotypes. However, reactivation of telomerase after 4 weeks reverses the degenerative phenotypes seen in telomerase-deficient mice (Bernardes de Jesus and Blasco, 2011; Jaskelioff et al., 2011). Proliferative capacity of post-senescent cells can also be extended by ectopic expression of human TERT (Counter et al., 1998). However, continued expression of hTERT in culture promotes genomic instability, perhaps due to accumulation of replication errors from an extended lifespan in culture (Roth et al., 2005; Schreurs et al., 2005).

The link between cancer and lifespan is a fine balance between protection against tumorigenesis and degeneration due to age. By combining the protective effects of enhancing p53 activity and increasing telomerase expression, the lifespan of mice can be extended and risk of tumorigenesis reduced (Donate and Blasco, 2011; Tomas-Loba et al., 2008).

1.8.3 Telomere length (TL) variation in the population

Telomere lengths are highly variable between and within individuals. Within an individual, telomeres vary in length between tissues, cells, and chromosomes (Lansdorp et al., 1996; Oeseburg et al., 2010; Zijlmans et al., 1997). However, variability between individuals is greater than that within each individual, which makes cross-sectional studies noisy, requiring large cohorts (Aviv and Levy, 2011; Kimura et al., 2010a; Takubo et al., 2002). At birth, telomeres vary in the population by 4-6 kb in blood leukocytes (Akkad et al., 2006; Frenck et al., 1998; Okuda et al., 2002), which is maintained throughout the human lifespan (Alter et al., 2007; Rufer et al., 1999). Telomere length variation in the population is a result of inherited differences, and differential rates of telomere shortening between individuals (Aviv, 2012; Chen et al., 2011). Both genetic and environmental or lifestyle factors contribute to differences in the rate of telomere shortening.

1.8.3.1 Genetic factors that influence telomere length

Genetic factors include inherited differences in birth telomere length and differences (i.e. polymorphisms, mutations) in genes involved in telomere stability and maintenance, such as shelterin and telomerase.

1.8.3.1.1 Inheritance of telomeres

Much of the variation in telomere lengths seen between individuals at birth could be the result of inherent heterogeneity between germ cells. A twin-based study revealed that telomere length is partly genetically determined. In this study, monozygotic twins had telomeres similar in length, in contrast to heterozygotic twins (Slagboom et al., 1994). Several studies have estimated that the contribution of heredity to telomere length in humans is between 36 and 80% (Andrew et al., 2006; Jeanclos et al., 2000; Njajou et al., 2007; Slagboom et al., 1994; Vasa-Nicotera et al., 2005). The precise mechanism of heritability is still unclear, with some studies suggesting maternal mode of inheritance (Nawrot et al., 2004) and others paternal (Njajou et al., 2007; Nordfjall et al., 2005; Nordfjall et al., 2010). One study suggests telomere length may be inherited as an X-linked trait, based on the fact that telomere length was correlated between parents of both sexes and offspring except between father and sons (Nawrot et al., 2004). In line with this, telomere length from umbilical cord blood of newborn babies correlated with maternal blood telomere length (Akkad et al., 2006). However, a recent large-scale study confirms that paternal influence on offspring telomere length is significantly greater than maternal contribution (Nordfjall et al., 2010). In fact, several studies confirm that age of father is positively correlated with offspring telomere length at birth (De Meyer et al., 2007; Njajou et al., 2007; Unryn et al., 2005). In the human male germline, telomere lengths are highly heterogeneous (8.8 to 16 kb) between cells, even with telomerase expression (Baird et al., 2006). Similarly, offspring of inbred mice showed variable telomere lengths in the Y chromosome (Zijlmans et al., 1997).

Many studies are using genome-wide sequencing approaches to determine genetic loci that are associated with telomere length. These studies have revealed that genetic variants of telomerase RNA component (TERC) (Codd et al., 2010; Levy et al., 2010;

Njajou et al., 2010; Shen et al., 2011; Soerensen et al., 2012) and catalytic subunit (TERT) (Atzmon et al., 2010) are associated with leukocyte telomere length and longevity. One study in families with exceptional longevity of Ashkenazi centenarians revealed an association between telomere length, longevity and a genetic variant in hTERT (Atzmon et al., 2010). Heterozygous mutation in either hTERC or hTERT genes lead to rare disorders of aplastic anemia and dyskerotosis congenital, characterized by bone marrow failure (Garcia et al., 2007). Furthermore, a recently discovered genetic variant in OBCF1 is associated with inter-individual variation in leukocyte telomere length (Levy et al., 2010) and a potential link to cardiovascular disease (Burnett-Hartman et al., 2012b). OBCF1 plays a role in telomere length regulation and can interact with shelterin protein TPP1 (Wan et al., 2009).

Insights in inheritance patterns in telomere lengths also come from studies using inbred mice over several generations. One study revealed that heterozygous knockouts in the catalytic subunit of telomerase (TERT+/-) caused subsequent shortening of telomeres over generations (Chiang et al., 2010). However, when telomerase activity was restored, telomeres stabilize at a new set-point over subsequent generations. The authors proposed that telomere length set-points can be reset based on inherited telomere lengths. Mice with short telomeres were then backcrossed to the original mice with long telomeres and found that the offspring had an average length intermediate to the parental mice, suggesting telomere lengths do not represent a simple heritable trait. Genotype alone does not determine telomere set-point, but historic events (telomerase haploinsufficiency) can influence inherited lengths (Chiang et al., 2010). Between-species crosses of *Mus musculus* (long telomeres) and *Mus spretus* (short telomeres) showed a select lengthening of the short telomeres inherited from *Mus spretus* (Zhu et al., 1998). This lengthening was mapped to a specific gene encoding the Rec helicase (Rtel) (Ding et al., 2004). The mechanism of action is not fully understood, however, genetic differences of telomere-associated proteins may account for elongation of telomeres between species. In addition, telomere elongation may only occur when recognized as critically short, which may be different for each species of mice (Chiang et al., 2010).

1.8.3.1.2 Progeroid syndromes

Much of what we know about telomeres with aging *in vivo* comes from the study of progeroid (premature aging) syndromes. These include Werner and Bloom syndrome (Rossi et al., 2010) and dyskeratosis congenita (Armanios, 2009; Walne and Dokal, 2009). These disorders are characterized by mutations in genes encoding proteins involved in telomere length stability and regulation. Accelerated telomere shortening in these disorders clinically manifests as aplastic anemia in bone marrow and fibrosis of the lung and liver, leading to premature mortality (Armanios, 2009). Mouse models have been used to study the effects of progeroid proteins on telomere length with aging by functional knockout of telomerase (TERT-/- or TERC-/-), Werner (WRN-/-), and dyskerin (DKC1) (Blasco, 2005a; Chang, 2005; Chang et al., 2004; Walne and Dokal, 2009). Mutations in shelterin components, such as TIN2, can also cause dyskeratosis congenita (Walne and Dokal, 2009).

One study generated wild-type mice with short telomeres and found that these mice displayed accelerated age-related pathologies similar to patients with dyskeratosis congenita (Armanios et al., 2009). These mice had defects in the hematopoietic and immune system, and persisted several generations of interbreeding. These findings suggest that inheritance of short telomeres even in the presence of wild-type telomerase is sufficient to cause degenerative defects with age (Armanios et al., 2009).

Accelerated aging has also been observed in mice with null mutations in the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (Espejel et al., 2004). In complex with the heterodimer Ku86/Ku70, DNA-PK plays a role in DNA repair by non-homologous end joining (Khanna and Jackson, 2001). However, these proteins also participate in telomere capping and are thought to assist in recruitment of telomerase (Espejel et al., 2002). Absence of DNA-PKcs accelerates telomere shortening and leads to earlier onset of age-related pathologies (Espejel et al., 2004).

Furthermore mice with knockouts of shelterin components, such as TRF1, TPP1 and Rap1, have helped establish *in vivo* roles in telomere maintenance and genomic stability (Donate and Blasco, 2011). For example, TRF1 knockouts lead to embryonic lethality at

the blastocyst stage, but Cre recombinase-mediated conditional knockout of epithelial cells revealed rapid induction of p53 mediated senescence, damaged foci at telomeres, chromosomal fusion events, and genomic instability. Knocking out p53 rescued some of the defects, but these mice still displayed abnormalities of human conditions linked to telomere maintenance deficiency such as nail atrophy and oral leukoplakia (Donate and Blasco, 2011).

1.8.3.2 Lifestyle factors that influence telomere length

Telomere shortening rates can be modified by several lifestyle factors that include mental stress levels, cigarette smoking and dietary intake. Baseline telomere shortening due to the end-replication problem will always exist, but efforts to live a healthy and balanced lifestyle can reduce any additional loss of telomeres (Shammas, 2011). Even poor sleep quality has been associated with shorter telomere lengths in leukocytes among midlife women, independent of perceived psychological stress (Prather et al., 2011).

1.8.3.2.1 Mental stress

The amount of stress experienced is a key factor in variations in telomere shortening among the adult population. Female house mice (*Mus musculus*) that experienced reproductive stress displayed shorter telomeres compared to controls. In male mice, crowding stress also resulted in shorter telomeres (Kotrschal et al., 2007). In humans, psychological stress has been linked to short telomeres (Lin et al., 2012). Leukocyte telomere lengths were analyzed in mother's caring for their chronically sick child and revealed that duration of care was associated with higher levels of oxidative stress, lower levels of telomerase and shorter telomeres. In fact, the level of stress perceived by the caregiver was proportional to their telomere lengths (Epel et al., 2004). Caregivers experience exaggerated anticipatory stress appraisals, which may have a negative impact on telomere length and cellular age (O'Donovan et al., 2012). Another study confirmed that high levels of perceived stress and chronic pain among adults is associated with short telomeres (Sibille et al., 2012).

Telomere shortening due to stress does not occur only in adults but also in children. More recently, Blackburn's lab revealed that socioeconomic status of parents is positively

correlated with child leukocyte telomere length. Children with parents that never attended college had telomeres 1178 bp (~6 years of additional aging) shorter than children with at least one college-educated parent (Needham et al., 2012). Moreover, exposure to violence between ages 5 and 10 resulted in significantly reduced leukocyte telomeres, providing evidence for early childhood stress and telomere maintenance (Shalev et al., 2012). However, activities that reduce stress levels, such as meditation (Epel et al., 2009; Jacobs et al., 2011) and exercise (Puterman et al., 2010), can improve telomere length and reduce rates of telomere shortening. In fact, a mindfulness intervention study revealed that metabolic and psychological stress in part may regulate telomerase activity (Daubenmier et al., 2011).

1.8.3.2.2 Smoking, obesity and dietary intake

There are several studies that link cigarette smoking to short leukocyte telomeres (Babizhayev and Yegorov, 2011; McGrath et al., 2007; Mirabello et al., 2009; Morla et al., 2006; Nawrot et al., 2010; Valdes et al., 2005). In a study of women who smoke, a pack of cigarettes a day causes an additional telomere loss of 5 bp/year, equivalent to 7.4 years of aging after 40 years of smoking (Valdes et al., 2005). Studies have also shown a dose-effect relationship between tobacco exposure and telomere length (McGrath et al., 2007; Morla et al., 2006). Accelerated telomere shortening in smokers is linked to increased oxidative stress and DNA damage (Babizhayev and Yegorov, 2011). In fact, an *in vitro* model of early mice embryos revealed that exposure to cigarette smoke condensate or cadmium caused increased oxidative stress and telomere shortening and associated with chromosomal instability and apoptosis. However, treatment with the antioxidant N-acetyl-l-cysteine (NAC) was able to greatly reduce oxidative stress and toxic effects of cigarette smoke (Huang et al., 2010).

Similar to smoking, obesity also causes an increase in oxidative stress and inflammation (Codoner-Franch et al., 2011). Short leukocyte telomere length in type-2 diabetic men was shown to correlate with triglyceride and total cholesterol levels, suggesting a link between BMI and telomeres in aging (Harte et al., 2012). A diabetes intervention study on obese individuals with glucose intolerance revealed that by promoting a healthy lifestyle leukocyte telomere length can improve over time (Hovatta et al., 2012). Even a

moderate amount of physical activity is associated with longer leukocyte telomere lengths (Du et al., 2012).

Proper dietary intake can also improve telomere length. For instance, vitamin D intake was linked to increased telomerase activity in leukocytes of overweight individuals (Zhu et al., 2011) and improved telomere length in hemodialysis patients treated with vitamin D compared to those not treated (Borras et al., 2012). Since oxidative stress accelerates telomere shortening, intake of antioxidants, such as berries, could reduce rates of telomere attrition. A study with mice showed that treatment with the antioxidant NAC effectively postponed aging of oocytes and displayed longer telomeres and higher telomerase expression than controls (Liu et al., 2012). As with mental stress, exercise can also modify telomere length attrition by improving overall health and by reducing BMI and oxidative stress levels (Shammas, 2011). In fact, exercise altered telomere maintenance and slowed age-related decrease in telomere length of the lung and heart of mice. However, exercise caused telomere shortening in skeletal muscle of mice compared to sedentary mice, suggesting its protective effects are tissue dependent (Ludlow et al., 2012).

1.8.4 Goals of this study

In humans, it is well established that telomeres shorten with age in blood leukocytes and that short leukocyte telomere lengths are associated with cardiovascular disease (CVD) (Oeseburg et al., 2010). In wild-derived *Mus spretus* mice, telomeres also shorten in spleen, a leukocyte-rich tissue (Coviello-McLaughlin and Prowse, 1997). *Mus spretus* mice have telomeres in range with that of humans, whereas commonly used inbred strains have telomeres significantly longer (Hemann and Greider, 2000). Therefore, few studies have used inbred mice to study telomere dynamics, leaving them poorly understood. It is important to understand telomere dynamics in strains that are commonly used for a wide-range of genetic studies (e.g. C57BL/6). No study to date has looked at telomere length dynamics with age in different strains of inbred mice. Furthermore, there is little information on tissue-specific differences in telomere length with age of inbred mice.

As mentioned in section 1.8.3, large inter-individual variations in leukocyte telomere length and its rate of change make use of cross-sectional studies difficult, which can only detect telomere length changes of an entire population. By knowing birth telomere length of an individual, telomere loss and rate of change could be determined. A previously defined quasi-longitudinal model in dogs uses a post-mitotic tissue (skeletal muscle) as an estimate of birth telomere length to reference against leukocyte telomere length in adults (Benetos et al., 2011). However, many cell types are considered post-mitotic, but a tissue would need to be predominantly composed of these long-living cells in order to be a good estimate of birth telomere length. No study to date has defined a tissue to estimate birth telomere length on a genetically constant model. Moreover, no study has determined if genetic diversity is the cause of telomere length heterogeneity in the population.

Goals:

1) To determine if there are strain-specific differences in telomere lengths of inbred mouse strains.

2) To determine if there is an adult tissue that can serve as a surrogate for ascertaining birth telomere length.

3) To determine if genetic heterogeneity translates to telomere length heterogeneity in a mouse population.

4) To determine if elucidating age-related associations of telomere length in a genetically heterogeneous mouse population can be optimized by referencing to a tissue with stable telomere length.

5) To determine if the extracellular matrix can influence telomere length.

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Chapter 2 - Aortic media defines telomere length dynamics in mice

2.1 Abstract

Leukocyte telomere length (TL) shortens with age and is associated with age-related pathologies. However, inherited and acquired variations in telomere length in individuals complicate the clinical interpretation of TL as a biomarker of aging and age-related pathologies. Therefore, it may be valuable to identify a post-mitotic adult tissue as a surrogate marker of TL at birth. Many tissues are considered post-mitotic however in order for an adult tissue to have telomeres of similar length to that at birth two criteria need to be met: 1) the tissue be composed of non-replicating cells and 2) the tissue be relatively homogeneous in cell type composition. The purpose of this study was 1) to establish an adult tissue that serves as a marker for birth TL; 2) determine if use of this tissue corrects for genetic diversity; 3) characterize a mouse system that displays human-like telomere dynamics; 4) determine if a pathological state of impaired turnover of collage type I is associated with telomere shortening.

I compared TL in the first year of life in common inbred strains (C57BL/6 (N=63), 129sv (N=19), and Balb/c (N=18) to Diversity Outbred (DO) mice (N=51), a strain bred to maintain allelic heterogeneity. Mean TL was determined using quantitative PCR by measuring telomere-to-single copy gene (T/S) ratio. I found that C57BL/6 telomeres are shortest among the inbred strains tested ($P<0.0001$), with age-related shortening of telomeres in spleen ($P<0.0001$), bone marrow ($P=0.0015$), skeletal muscle ($P=0.025$), and brain ($p<0.0001$). In contrast, aortic media and heart had stable TL with age. Furthermore, adult aortic media TL was not different from aortic TL at birth. I also identified spatial heterogeneity in telomere length across the aortic wall of C57BL/6 mice (TL of intima<adventitia<media, $P<0.001$), which increased with age. DO mice were found to have long telomeres characteristic of inbred strains but more variable in length. Intra-mouse TL synchrony between spleen and aortic media in DO mice ($R^2=0.51$, $P<0.0001$) provided a means to correct for genetic diversity, revealing age-related telomere shortening of spleen referenced to aortic media in each individual mouse ($P<0.0055$). The relationship with age of the intima also improved by referencing to

aortic media TL (R^2=0.52; P=0.0003 to R^2=0.61; P<0.0001). Lastly, I determined in a collagenase-resistant collagen mutant (Col1a1r/r) mouse that telomere length of heart and skeletal muscle is significantly lower than wild-type controls (P=0.006 and P=0.008, respectively).

In conclusion: 1) human-like telomere attrition with age can be detected in some inbred mouse strains; 2) TL in aortic media may serve as an internal reference for determining rate of leukocyte TL shortening; and 3) impaired collagen turnover accelerated telomere shortening in collagen-rich tissues.

2.2 Introduction

In humans, short leukocyte telomere length (LTL) has been shown to correlate with age and age-related illnesses, in particular cardiovascular disease (CVD) (Balasubramanyam et al., 2007; Farzaneh-Far et al., 2010; Fitzpatrick et al., 2007; Oeseburg et al., 2010). Insulin resistance and hypertension are also associated with short LTLs (Demissie et al., 2006), as well as homocysteine levels (Richards et al., 2008), all of which are risk factors for CVD. Furthermore, short LTL is associated with type-2 diabetes mellitus (Salpea et al., 2010; Sampson et al., 2006; Zee et al., 2010) and many forms of cancer (Hou et al., 2009; Jang et al., 2008; McGrath et al., 2007; Risques et al., 2007; Willeit et al., 2010). Several genetic variants in telomerase RNA (TERC) have been linked to increased risk of both cancer (Hills and Lansdorp, 2009; Jones et al., 2012; Sheng et al., 2012; Varadi et al., 2009) and CVD (Burnett-Hartman et al., 2012; Zee et al., 2011), suggesting a genetic link between LTL and chronic illness. Recently, a longitudinal study revealed early life LTL as a predictor of lifespan in birds (Heidinger et al., 2012), implicating birth telomere length as a determinant of biological fitness with potential for risk assessment of chronic illnesses.

LTL in the adult can be expected to be a product of both birth telomere length and rate of telomere shortening and there are large variations in LTL in the population (Aviv, 2011). In newborns, LTL varies 4-6 kb between individuals and this variability sustains throughout human lifespan (Frenck et al., 1998; Rufer et al., 1999). Furthermore, telomere length attrition is variable among individuals (Chen et al., 2011). Since most

human studies are cross-sectional, only a snapshot of LTL at a single time is known (Aviv, 2011). Therefore, it would be valuable to know birth telomere length of an individual, in order to determine the rate of telomere shortening for that individual. Recently, work has been done in canine and macaques to use certain tissues as an internal reference or marker of birth telomere length, such as skeletal muscle and adipose tissue, chosen because of their post-mitotic nature (Benetos et al., 2011; Gardner et al., 2007). However, to date no study has assessed in detail differences in telomere lengths in a variety of tissue types on a genetically constant model to determine which tissue, if any, best represents birth telomere length.

Although many cell types *in vivo* are considered post-mitotic, the ideal marker of birth telomere length would likely be tissues with cell types that are long-lived and seldom renewed. Myocytes of the cardiovascular system such as cardiomyocytes and smooth muscle cells (SMC) as well as the aforementioned skeletal muscle are candidates as a marker for birth telomere length. Smooth muscle and skeletal muscle of healthy adults show little cell turnover post-natally with an average age of 15.9 and 15.1 years, respectively (Spalding et al., 2005). Cardiomyocytes have an average age of 6 years younger than the individual (Bergmann et al., 2009), although only represent 20% of the human myocardium (Rubart and Field, 2006). Although neurons may be the longest living post-mitotic cells, particularly from the cerebellum with an average age of 2 years younger than the individual (Spalding et al., 2005), they are not practical as a tissue to biopsy for telomere measurements.

All tissues are composed of a variety of different cell types at different proportions. Unless cell sorting is used, which is time consuming and costly, for a tissue to be a surrogate for birth tissue with respect to telomere length, it would have to be almost entirely composed of post-mitotic cells. Therefore, two criteria need to be met for a tissue to serve as a surrogate for birth telomere length: 1) composed of a long-lived cell type and 2) homogeneous population of said cells**.** The aorta may be a good candidate since the media layer can be isolated mechanically and represents a relatively homogeneous population of vascular SMC.

In this study, I used inbred mice to assess telomere dynamics. Inbred mice provide a powerful tool to control genetics, isolate a trait of interest and selectively modify gene function. Moreover, understanding telomere dynamics of inbred mice may allow us to capitalize on the plethora of disease models readily available, short lifespan and short replication time. Finally, it's important to understand telomere dynamics of commonly used inbred strains in order to make inter-study comparisons regarding disease and genetics.

Inbred mice have been the most common animal models in lab for decades, but there is very limited knowledge on their telomere length dynamics with age. Murine telomere dynamics were originally characterized in wild-derived mice, *Mus spretus*, because their telomeres are closer in length to those of humans (10-15 kb) than established inbred strains (Prowse and Greider, 1995). *Mus musculus* strains, such as C57BL/6, Balb/c and 129, have telomeres on average between 30-80kb, producing large smears on agarose gels that are difficult to resolve and detect differences (Kipling and Cooke, 1990). Moreover, unlike primary human fibroblasts, primary mouse embryonic fibroblasts cells are not prone to telomere shortening as dividing in culture, partially attributed to the activation of telomerase and spontaneous immortalization (Sachsinger et al., 2001). Telomeres of inbred mice have thus been scrutinized as representing a poor model to study telomeres. However, since the development of quantitative PCR (qPCR)-based telomere length measurements, average telomere length can be easily and accurately determined with minimal starting material compared to the gold standard southern blotting technique (Aviv et al., 2011; Cawthon, 2002).

I used qPCR to evaluate the relationship of mouse strain and tissue on age-dependent telomere length dynamics in mice. Using C57BL/6 mice and a unique strain of Diversity Outbred mice that has human-like genetic diversity, I established telomere length in aortic media as a surrogate for birth telomere length, and internal reference for determining age-dependent attrition in spleen cells (leukocytes).

2.3 Materials and methods

2.3.1 Animals and tissue

All mice used in this study were male except for C57BL/6 neonates, which were a mix of male (N=9) and female (N=2). Inbred mice (C57BL/6 (N=60), 129sv (N=10), Balb/c (N=17), and Diversity Outbred (N=49)) were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice at 15.3 months (N=3) of age were purchased from Charles River Laboratories (Sherbrooke, ON). Collagenase-resistant collagen mutant (Col1a1r/r) mice (N=5) and wild-type controls 129P1/re (N=8) were bred and maintained at Western University (London, ON). Wild-type 129 mice used throughout the study include both the 129sv and 129P1/re, so are referred to as simply 129 in some contexts. Note that N number may vary between groups in experiments because samples were extracted from banked tissues.

Mice were anaesthetized and blood was collected in 0.5M EDTA from the left ventricle. Mice were then perfused with 1xPBS, and tissue (heart, liver, spleen, skin, aorta, skeletal muscle, and brain) was collected and flash frozen in liquid nitrogen and stored at -80^{o}C until further use. DNA from blood and bone marrow was immediately isolated following tissue extraction.

2.3.2 Tissue preparation

Prior to DNA isolation, all tissues were first thawed in 1xPBS. For the heart, the left ventricle was sampled, and skeletal muscle was sampled from the adductor muscle. Whole blood was processed using a red blood cell lysis buffer (Qiagen Inc., Mississauga, ON) to ensure purity of its DNA. The aorta of a subset of C57BL/6 mice (N=23) was dissected into its three layers (intima, media, and adventitia) prior to DNA isolation. After the adventitia was peeled off with forceps, the aorta was cut in half longitudinally in a small pool of 1xPBS, and the intima was careful scraped off using L-shape forceps. For mice at 4 and 10 months, the endothelium was pooled from two or three mice, and for mice at 14 months the endothelium was from individual aortas. The medial layer of a subset of those aortas (N=12) was further segmented into four partitions: 1) lower abdominal located infra-renal; 2) upper abdominal located supra-renal and below

diaphragm; 3) lower thoracic, the lower half between the diaphragm and mid aortic arch; and 4) upper thoracic, the upper half of the former.

2.3.3 DNA isolation and quantitation

DNA from all tissue samples was isolated using the DNA mini or micro kit (Qiagen Inc., Mississauga, ON) depending on sample size/volume; for tissue less than 5 mg and blood less than 50 µL, the micro kit was used. SPRET/Ei DNA was acquired from Jackson laboratory (Bar Harbor, ME) from spleen of 1 month old male mice.

DNA quantification was done using the picogreen kit (Invitrogen, Burlington, ON). Briefly, lambda DNA provided with the kit was serially diluted by 2-fold in 1xTE from a concentration of 2000 ng/µL to yield an 8-point standard curve. Standards and samples were loaded onto black Nunc-Immuno 96-well plates (Sigma-Aldrich, Oakville, ON) at a 100x dilution in working picogreen solution (200-fold dilution of stock). A non template control was included as a blank. The excitation and emission wavelengths were set to 485 nm and 530 nm, respectively, and fluorescence was measured using a SpectraMax M5 fluorescent plate reader (Molecular Devices Corp., Sunnyvale, CA). Data were exported to Microsoft excel to calculate the initial concentration of each sample based on the equation of the standard curve. Concentrations above 30 ng/µL were diluted in 1xTE buffer to an intermediate working concentration of 10 ng/µL and quantified a second time with picogreen. All samples were diluted to a final working concentration of 4ng/uL in 1xTE buffer and stored at -80^{o}C until further use.

2.3.4 Telomere length measurement by qPCR

An average telomere length was determined using a qPCR approach as previously described by Cawthon (Cawthon, 2002) and optimized for mice by Callicott (Callicott and Womack, 2006). Average telomere length is calculated based on a standard curve of a reference sample by measuring the quantity of telomeric repeats to single-copy gene (T/S ratio) as shown in appendix B.

The sequence of the telomere-specific forward (tel1b) and reverse (tel2b) primers (Sigma-Aldrich, Oakville, ON) was 5’ CGG TTT GTT TGG GTT TGG GTT TGG GTT

TGG GTT TGG GTT 3' and 5' GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT 3', respectively. The single-copy gene, acidic ribosomal phosphoprotein PO (36B4), was used to normalize for cell count. The sequence of the 36B4-specific forward (36B4u) and reverse (36B4d) primers (Sigma-Aldrich, Oakville, ON) was 5' ACT GGT CTA GGA CCC GAG AAG 3' and 5′TCA ATG GTG CCT CTG GAG ATT 3', respectively.

Each reaction sample contained 5 µL of RT^2 SYBR green master mix with ROX (SAbiosciences, Mississauga, ON), 300 nM of both tel1b and tel2b primers or 300 nM of 36B4u and 500 nM of 36B4d primers, and topped off with nuclease-free water to yield a total volume of 10 µL. To determine the optimal amount of DNA to use, an array of DNA quantities (10 ng, 5 ng, 1 ng, and 300 pg) was tested on 5 samples of DNA from the same spleen and the DNA quantity (5 ng) with consistent results and the lowest coefficient of variation (CV) was chosen (Figure 2.1). A non-template control was included on each plate as a negative control for non-specific amplification. Genomic DNA from spleen of an individual mouse was used as reference DNA by serially diluting 25 ng by 1.68 fold in 1xTE buffer to yield a 7-point standard curve. Six to eight samples acted as inter-plate controls and were included in each experiment to normalize for inter-experimental variability as previously described (Aviv et al., 2011). The T/S ratio of these inter-plate controls varied by 12.83±3.4% (CV±SD) based on the average of 7 qPCR experiments.

The assay was prepared in triplicate on two separate optically clear 384-well plates and samples were matched well-to-well. The plate designated to measure telomeres had the following thermal profile: 95°C hot start for 10 min, followed by 30 cycles of denaturation at 95°C for 15 sec, and anneal-extension at 56°C for 1 min. The thermal profile for 36B4 was: 95°C hot start for 10 min, followed by 35 cycles of denaturation at 95°C for 15 sec and anneal-extension at 60°C for 1 min. The experiment was carried out using a 7900HT Real-time PCR machine (Applied Biosystems, Burlington, ON).

Following the PCR reaction, the threshold and baseline were manually adjusted as necessary using SDS 2.4 software (Applied Biosystems, Burlington, ON) to optimize the

standard curve to yield a slope between -3.1 and -3.6 and an R^2 greater than 0.98. The data were exported to Microsoft Excel and a T/S ratio and standard deviation was calculated for each sample.

2.3.5 Statistical analysis

All data were analyzed using Graph Pad Prism version 5.0. Telomere lengths with age (Figure 2.2B,C,D,2.3,2.7C,D,E,F,2.8A,B,C) and correlation of telomere lengths (Figure 2.7B) were analyzed using linear regression, and the equation of the best fit line, R^2, and P value were reported on each corresponding graph. Telomere lengths of different strains (Figure 2.2A) were compared using One-Way ANOVA and Tukey's post-test. Telomere lengths of neonatal and 1-6 month old C57BL/6 mice in both aorta and heart (Figure 2.4) and wild-type versus mutant Col1a1r/r mice (Figure 2.9) were compared using two-tailed student t-tests; aorta and heart at a given age were also compared with a paired student t-test. Aortic layers (Figure 2.5) at various time points were compared using Two-Way ANOVA and Bonferroni post-test. The variance in telomere lengths of each strain were compared using Bartlett's test of equal variance (Figure 2.7A). Reliability of T/S ratio measurements by qPCR using different DNA quantities (Figure 2.1) and telomere lengths of different aortic regions (Figure 2.6) were compared using repeated measures One-Way ANOVA followed by Tukey's post-test.

2.4 Results

2.4.1 Optimization of qPCR telomere length measurement for mice

To optimize the reliability of qPCR measurement of telomere length in mice, a concentration analysis of different DNA quantities was performed. My goal was to determine a range of DNA quantities that produce stable average telomere length measurements (T/S ratios). A single spleen from a 1-month-old C57BL/6 male mouse was divided into five pieces and DNA was isolated and quantified for all five samples separately. Reactions were set up with varying amounts of DNA (300 pg, 1 ng, 5 ng, and 10 ng) loaded in triplicate on each 384-well plate for each sample in a single experiment (Figure 2.1).

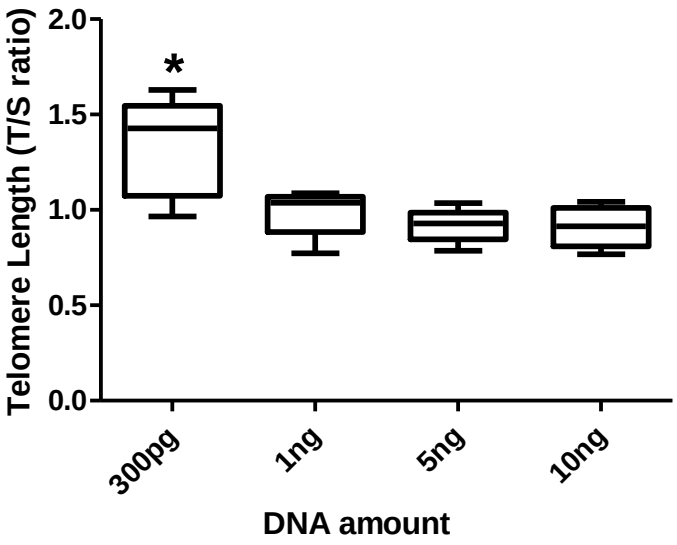

Figure 2.1 - Reliability of T/S ratio measurements using different DNA quantities

T/S ratios were determined from a qPCR reaction using different quantities of DNA, harvested from five tissue samples of a spleen from a 1-month-old male C57BL/6 mouse. The data were plotted using whisker box plot to show the spread of data. Whisker box plots graphically display the spread of data by grouping in its five-number summaries: sample minimum, lower quartile, median, upper quartile and sample maximum. T/S ratio is stable when between 1-10 ng of DNA was loaded in each reaction well. Five ng of DNA was chosen to carry out all qPCR-based telomere measurements for this thesis.
*$P<0.0001$

This revealed that T/S ratios were consistent from 1 to 10 ng of DNA. However, 300 pg produced T/S ratios that were significantly higher than the other concentrations. These data suggest <1 ng of DNA for the qPCR reaction is too low for determining a reliable T/S ratio.

The coefficient of variation for telomere measurements of each 300 pg, 1 ng, 5 ng, and 10 ng of DNA loaded were 19.52%, 12.66%, 9.73%, and 11.78%, respectively. Therefore, 5 ng was chosen to carry out qPCR telomere length measurements, as this represented a balance between a small amount of DNA and reproducible T/S ratios.

2.4.2 Telomere length changes with age are strain specific

I next began to characterize telomere length dynamics with age in mice. First, I measured and compared telomere lengths of spleen DNA from inbred (C57BL/6 (5.1±1.84 months), Balb/c (5.8±2.1 months) and 129 (6.3±2.5 months)) and wild-derived SPRET/Ei mice (1 month). SPRET/Ei mice are known to have telomeres similar in length to human telomeres, so were used as a reference for short telomeres. This assessment revealed that SPRET/Ei telomeres were significantly shorter than telomeres of the three inbred strains ($P<0.0001$) (Figure 2.2A). Furthermore, telomeres from the spleen of C57BL/6 mice were significantly shorter than those of 129 mice ($P<0.0001$), which were shorter than those of Balb/c mice ($P<0.05$). These findings indicate that inbred mice had long telomeres and displayed strain-dependent differences in telomere length.

I next determined if telomere shortening with age could be detected in inbred strains of mice and, if so, if genetic background influenced telomere length dynamics with age. Spleen is a leukocyte-rich tissue, is highly proliferative, and has previously shown telomere shortening with age in *Mus spretus* mice (Coviello-McLaughlin and Prowse, 1997). Therefore, I measured telomere lengths (1-16 months) in whole spleen of inbred mice (129, Balb/c, C57BL/6). C57BL/6 mice displayed significant telomere shortening with age ($P<0.0001$) (Figure 2.2B). In contrast, Balb/c displayed an increase in telomere length with age ($P=0.001$) (Figure 2.2C), and 129 mice showed no age-related changes in telomere lengths (Figure 2.2D). These findings indicate that murine telomere length

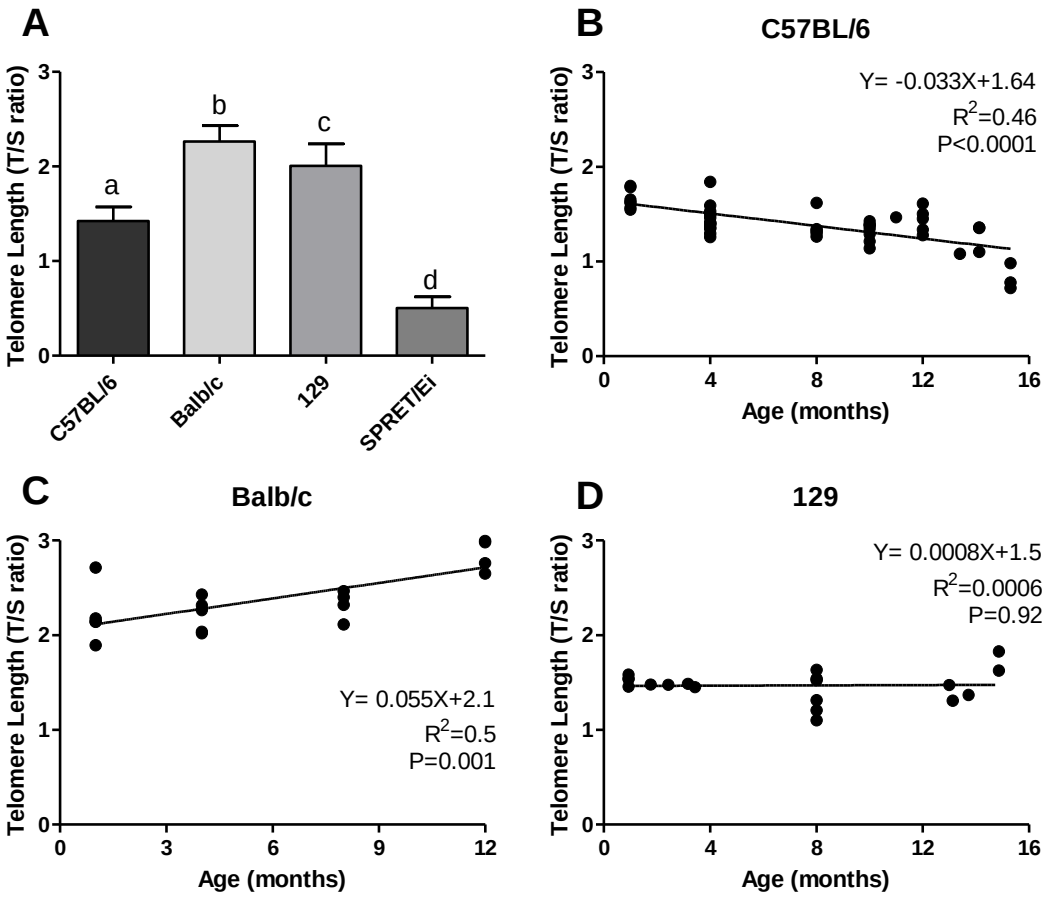

Figure 2.2 - Inbred mouse strain differences in spleen telomere lengths

A) T/S ratios (+SD) were measured in whole spleen DNA of adult inbred mice: C57BL/6 (5.1±1.84 months; N=18), Balb/c (5.8±2.1 months; N=9), 129 (6.3±2.5 months; N=9), and wild-derived mice (SPRET/Ei) (N=3) (For different letters $P<0.001$, except for b and c $P<0.05$). Telomere length changes with age were also measured in the spleen of **B)** C57BL/6 (1-16 months; N=49), **C)** Balb/c (1-12 months; N=18), and **D)** 129 (1-16 months; N=19). These mice include the subset measured in panel A. The equation of the best fit line, R^2, and P-value are reported on each graph.

dynamics with age are strain-specific. As well, they show that C57BL/6 mice can serve as an animal model that displays telomere shortening with age.

2.4.3 Age-related changes of telomere lengths in C57BL/6 mice differ among tissues

To approximate birth telomere length, a tissue must have a stable telomere length with age. However, little is known about the rate of telomere attrition in solid organs, particularly in mice. Therefore, having identified detectable age-related shortening of leukocytes in C57BL/6 mice, I used this strain to compare telomere dynamics among different tissues.

I selected tissues rich in muscle, as these are generally considered post-mitotic tissues, as well as brain and bone marrow. Telomeres in DNA harvested from bone marrow showed significant telomere shortening over 1 to 16 months of age ($P=0.0001$) (Figure 2.3). Interestingly, telomere attrition was also detectable over this time in skeletal muscle ($P=0.023$) and also in brain ($P<0.0001$). In contrast, age-related declines in telomere length were not detected in heart, aortic media and blood leukocytes.

2.4.4 Aortic media telomeres are stable in early life development

Telomeres in humans (Frenck et al., 1998), baboons (Baerlocher et al., 2007) and pigs (Jiang et al., 2004) are shown to shorten within the first few months of birth, in part because of rapid growth during that time. Given the relative stability of telomere length in the heart and aortic media, I assessed for this drop in these tissues. Respective tissue was harvested from neonates and compared with the average telomere length from 1-6 month-old C57BL/6 mice. In neonates and post-natally, the aortic media had telomeres that were significantly longer than that of heart (neonates: 1.76 ± 0.28 vs. 1.42 ± 0.22, $P<0.0013$; postnatal: 1.71 ± 0.27 vs. 1.21 ± 0.22, $P=0.0002$) (Figure 2.4). Interestingly, there was no difference in aortic telomere length at birth with that of aortic media of older mice ($P=-0.63$). However, heart telomeres at birth were significantly longer than that from heart harvested from older mice ($P=0.019$). Thus, aortic media displays remarkable length stability throughout neonatal growth and development and during adult aging.

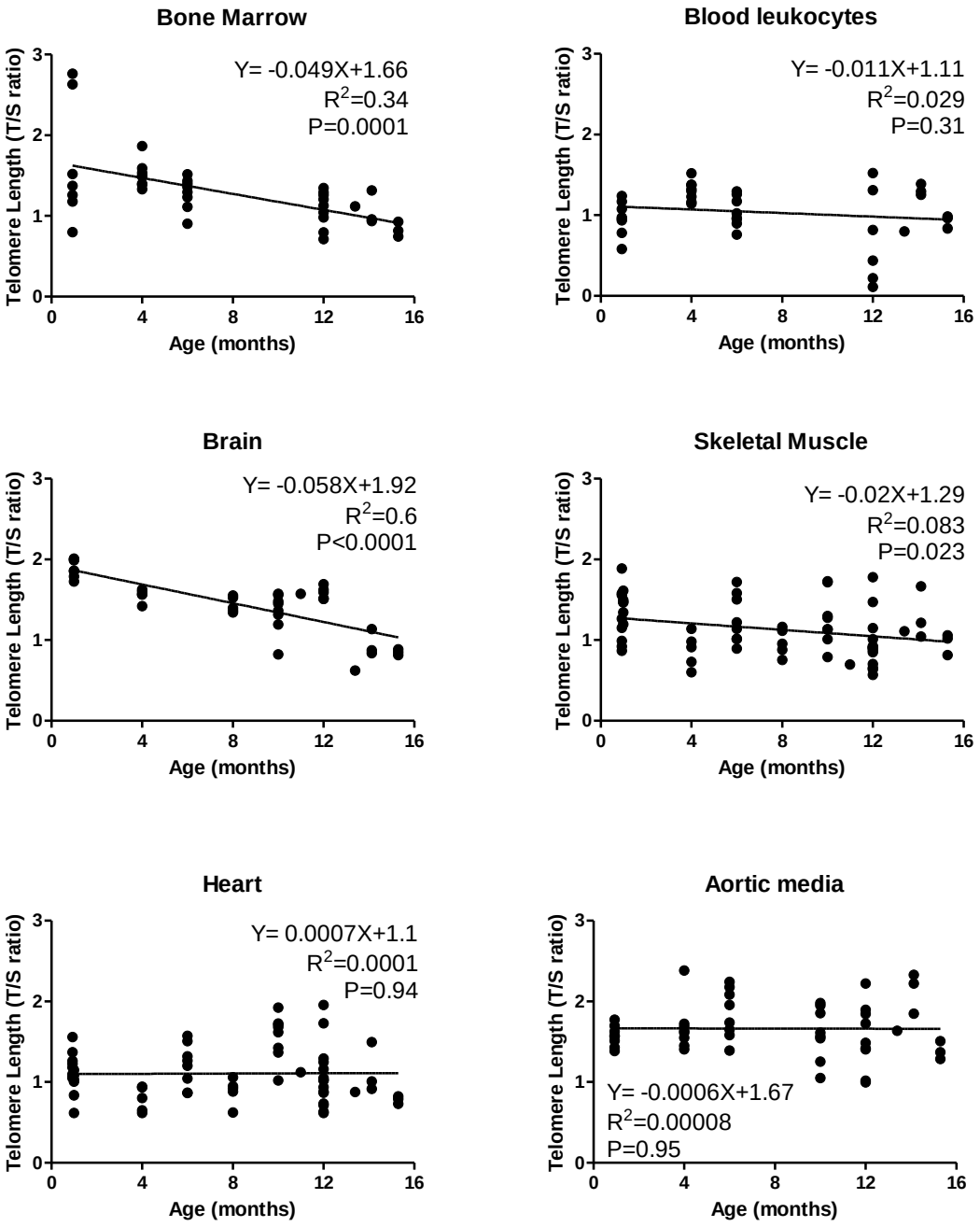

Figure 2.3 - Telomere length dynamics with age in different tissues of C57BL/6 mice

T/S ratios were measured in different tissues (bone marrow (N=40), brain (N=38), aortic media (N=48), heart (N=63), skeletal muscle (N=62), and blood leukocytes (N=37)) harvested from C57BL/6 mice at different ages (1-16 months). The equation of the best fit line, R^2, and P-value are reported on each graph.

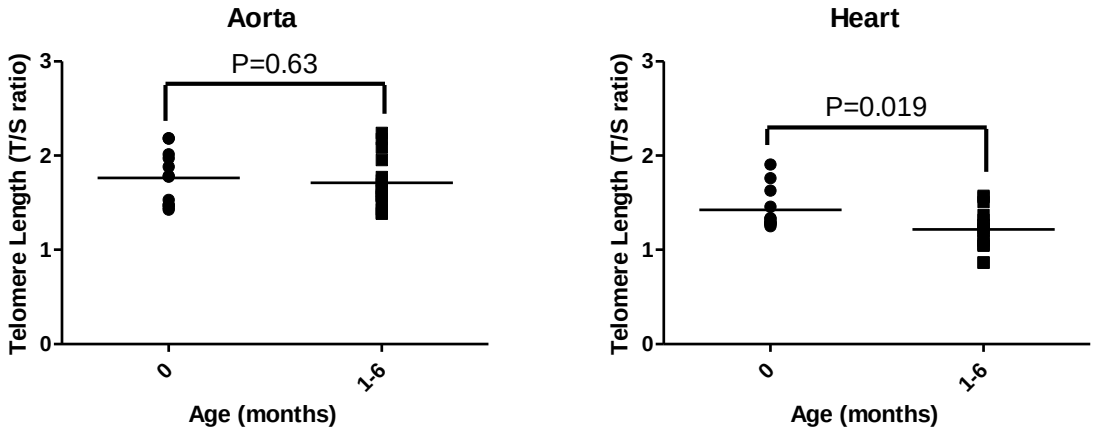

Figure 2.4 - Telomere length changes in early life of C57BL/6 aorta and heart

T/S ratios were measured in aortic media and heart in neonatal (1 day old) C57BL/6 mice (N=12) and compared to post-natal (1-6 months old) C57BL/6 mice (N=16). P-values are reported on each graph.

2.4.5 Telo-mapping identifies age-dependent, spatial telomere length gradients in the aortic wall

The aorta is derived of 3 distinct layers and each comprised of different cell types. To determine if variations in telomere length dynamics could be identified within these layers, I isolated each layer individually. The intima was scrapped off the vessel, the media and adventitia were dissected apart, and DNA was harvested from each layer separately. At 4 months, there was no difference in telomere length among the intima, media and adventitia (Figure 2.5). However, at 10 and 14 months, telomere length in the endothelial layer had declined and was now significantly less than that of the aortic media ($P<0.001$). Telomere length of the adventitial layer was also significantly less than the aortic media, but greater than the intimal layer, at 10 and 14 months ($P<0.01$). Thus, an age-dependent spatial gradient within the aortic wall evolved.

2.4.6 Telomere length along the length of aortic media reveals shorter telomeres in the infra-renal aorta

Different regions of the aortic media are composed of smooth muscle cells of different developmental origins (Majesky, 2007). Therefore, I assessed whether there were differences in telomere length in the aortic media along the length of the aorta. For this, the aorta of 4-14 month old mice was partitioned into four segments. Telomeres of the infrarenal abdominal aorta were significantly shorter ($P=0.0014$) than the other three levels of the aorta (Figure 2.6). However, no differences in telomere length between upper thoracic, lower thoracic, and supra-renal abdominal aorta were detected.

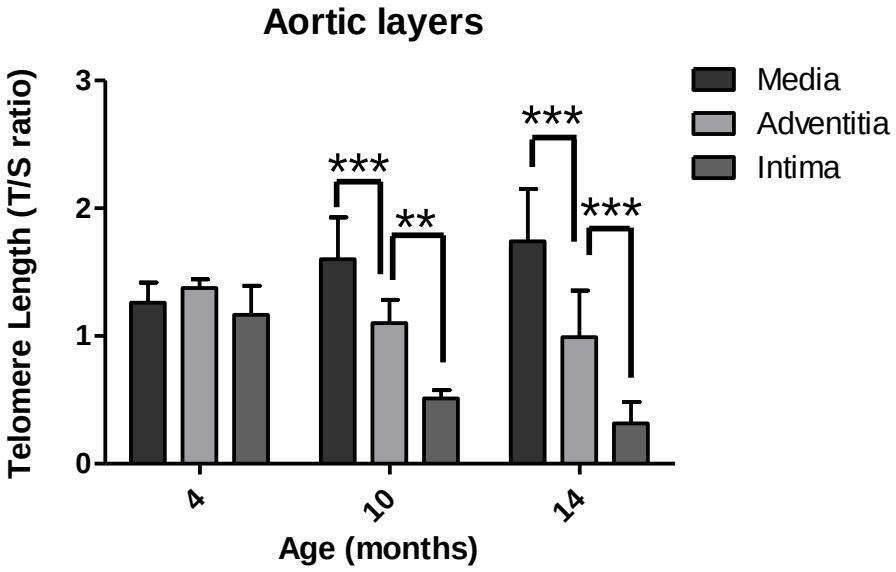

Figure 2.5 - Telomere lengths of different layers in the aorta of C57BL/6 mice

T/S ratios (+SD) were measured in different layers (intima, media, and adventitia) of the aorta at 4 (N=8), 10 (N=8) and 14 (N=7) months of age in C57BL/6 mice. The intima of thoracic aorta was pooled from 2 mice at 4 months (N=4) and 2-3 mice at 10 months (N=3) to yield enough DNA. At 14 months, the intima from the entire aorta was pooled from individual mice (**P<0.01, ***P<0.001).

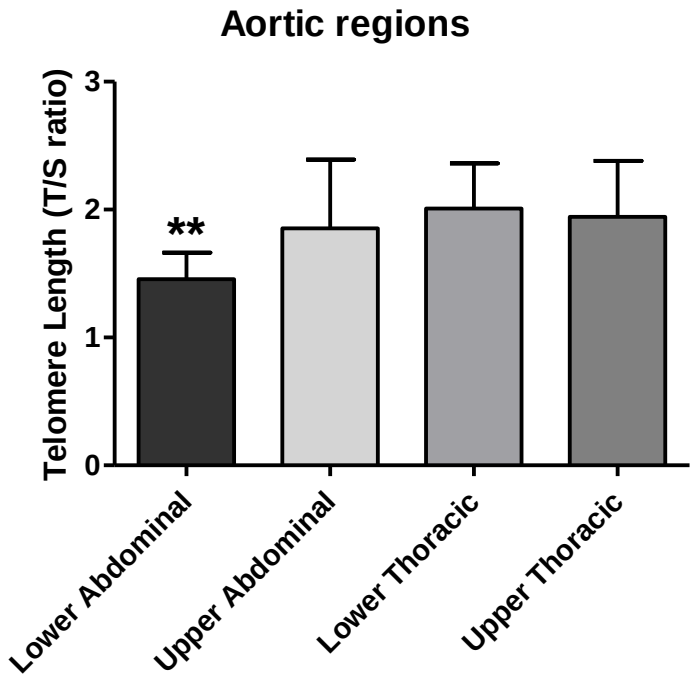

Figure 2.6 - Telomere length in different regions of the aortic media in C57BL/6 mice

T/S ratios (+SD) were measured in different regions (lower and upper abdominal, and lower and upper thoracic) of the media layer from aortas in 4-14 month old C57BL/6 mice (N=11; **P=0.0014).

2.4.7 Aortic media referencing reveals age-dependent telomere attrition in spleen and aortic intima of Diversity Outbred mice

Having identified the aortic media as a highly stable tissue with respect to telomere length, I next sought to determine if this stability, and similarity to birth telomere length, could be used to reference telomere length across a population of mice with genetic diversity, akin to the human population. To address this, I measured telomere lengths in Diversity Outbred (DO) mice, mice that are bred to maximize and maintain allelic heterogeneity in the population. Furthermore, the DO population has been proposed to have human-like genetic diversity and has been used for high resolution genetic mapping (Svenson et al., 2012).

I first determined whether this genetic diversity would translate into changes in telomere length, as well as length heterogeneity, compared to inbred mice. As shown in Figure 2.7A, mean telomere length of spleen DNA from DO mice (1-9 months old) was not demonstrably different from those of inbred strains (1-9 months old). DO mice had telomeres significantly longer than C57BL/6 ($P<0.001$) and shorter than Balb/c ($P<0.05$). These results indicate that outbreeding and genetic heterogeneity, in and of themselves, do not result in shortening of telomere lengths compared to inbred strains. However, the inter-mouse variability of telomere length within DO mice was significantly greater than that for each inbred strain evaluated and baseline variation. Thus, genetic diversity in mice translates to diversity in telomere length.

I next determined if there was synchrony between aorta and spleen telomere lengths in DO mice. Spleen and aortic media were harvested from 49 separate DO mice and revealed an intra-mouse telomere length synchrony between aorta and spleen ($R^2=0.44$, $P<0.0001$) (Figure 2.7B). This synchrony suggests that genetic variation is a major determinant of telomere length in individuals. I then determined the relationship of telomere length with age in spleen and aortic media in DO mice, between 1 and 12 months of age. There was no obvious significant change in telomere length with age for either spleen (Figure 2.7C) or aortic media (Figure 2.7D).

I then referenced spleen telomere length using two different approaches, one previously modeled on dogs (Benetos et al., 2011) using subtraction (Figure 2.7E) and the other using division (Figure 2.7F). Both methods revealed a significant age-dependent decrease in spleen telomere length relative to aortic media telomere length (P=0.0055 and P=0.0029, respectively).

Moreover, we also used aortic media telomere length as a reference to assess the relationship between intima telomere length and age. This was done using a mix of C57BL/6 and DO mice between 1 and 16 months of age. I found that the correlation coefficient for the relationship improved modestly from an R^2=0.52 (P=0.0003) to R^2=0.58 (P=0.0001) and 0.61 (P<0.0001), based on subtraction and division, respectively (Figure 2.8). Therefore, aortic media telomere length may provide a powerful tool to more powerfully identify age-related changes in telomere lengths in leukocytes and the vasculature, and DO mice are a good model to study the effects of genetic diversity on telomere length dynamics with age.

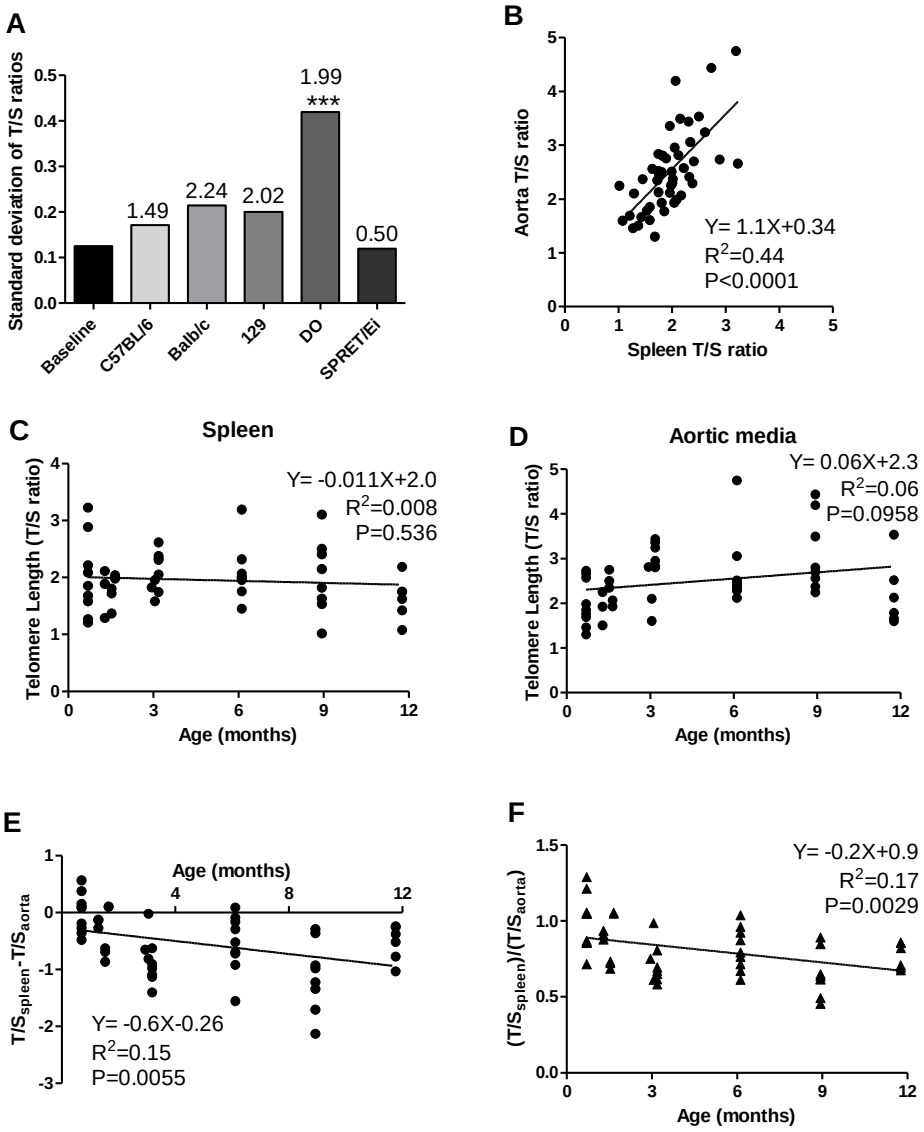

Figure 2.7 - Spleen telomere length referenced to aortic media telomere length as a function of age in DO mice

A) Standard deviation of T/S ratios in spleen of 1-9 month old inbred mice (C57BL/6 N=25; Balb/c N=14; 129 N=14; SPRET/Ei N=3) and DO mice (N=34) with mean T/S indicated above each bar. Baseline is the variation in T/S ratio from 10 tissue samples of an individual spleen, representing the baseline variation derived from experimental processes (***P<0.0001). Note that only T/S ratios of DO mice display a significant increase in standard deviation compared to baseline variability. **B)** Synchrony of spleen T/S ratio vs. aortic media T/S ratio in DO mice (N=49). **C)** Spleen T/S ratio with age in DO mice (N=49). **D)** Aortic media T/S ratio with age in DO mice (N=49). Referencing of spleen to aortic media T/S ratios by **E)** subtraction and **F)** division plotted against age (1-12 months). The equation of the best fit line, R^2, and P-value are reported on each graph.

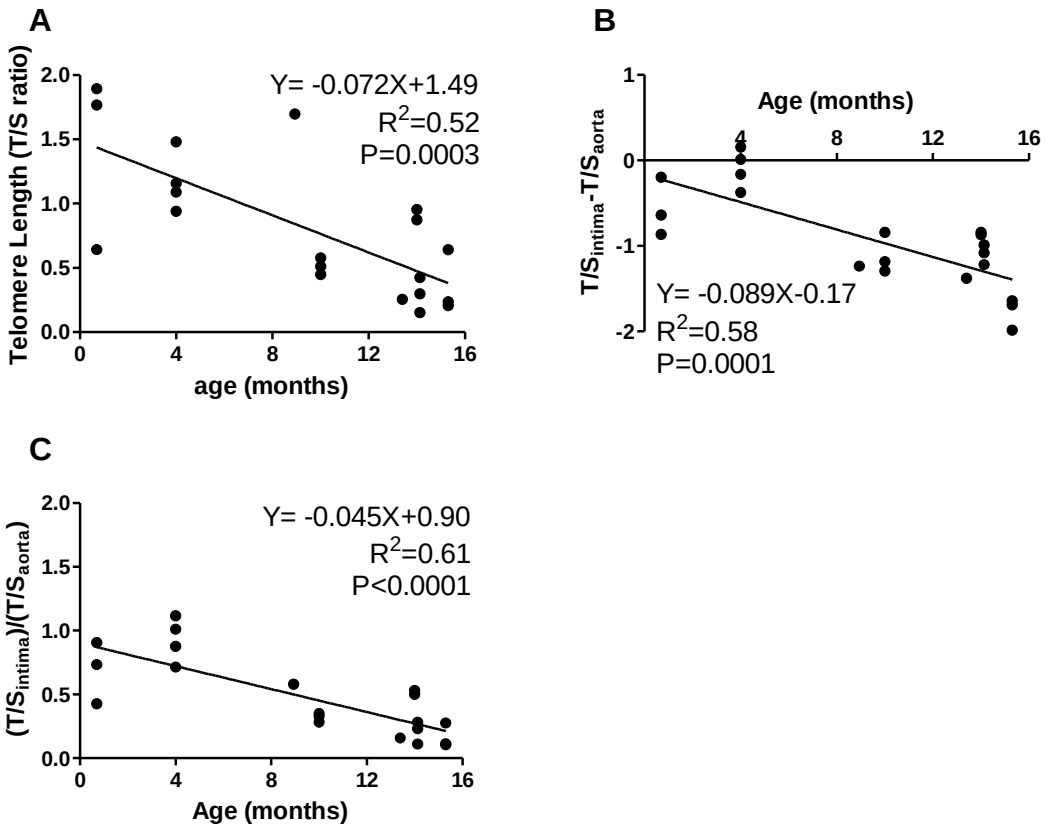

Figure 2.8 - Intima telomere lengths normalized to aortic media telomere lengths with age

A) T/S ratios of genomic DNA harvested from the intima of the aortic wall with age (0.7-15.3 months) in a mix of C57BL/6 (N=14) and DO (N=6) mice. Intima T/S ratio was referenced to aortic media by **B)** subtraction and **C)** division. The equation of the best fit line, R^2, and P-value are reported on each graph.

2.4.8 Telomere length shortening is induced by impaired collagen turnover

Finally, having developed techniques to establish telomere length in mouse tissues, I sought to determine if a pro-senescence stimulus would lead to telomere shortening in tissues. Cardiac and arterial aging is characterized in part by senescent cells. Another feature of cardiac disease and aging is abnormal remodeling of the extracellular matrix, predisposing individuals for cardiovascular disease. With age, type I collagen shows reduced turnover and increased cross-linking, leading to its accumulation in vascular tissue (Jacob, 2003). During remodeling of vascular tissue, collagen fibrils are cleaved between Gly^{775}-Ile^{776} (in the α-1 chain) and Gly^{775}-Leu^{776} (in the α-2 chain) by matrix metalloproteinases (Birkedal-Hansen et al., 1993). One model of abnormal collagen remodeling is the collagenase-resistant collagen mutant (Col1a1r/r) mice used in our lab. These mice have a targeted base substitution of proline at residue 776, leading to defective type I collagen turnover (Liu et al., 1995). Furthermore, these mice display many premature aging phenotypes such as skin ulcerations, hair loss, high blood pressure and predisposition to vascular senescence, suggesting that impaired collagen turnover is causative to organismal aging (O'Neil C., Vafaie F., Pickering J.G., unpublished data). Therefore, I decided to use this mouse model in order to determine if telomere shortening in mice could be detected in a pathological state.

I examined telomere lengths in several tissues of old Col1a1r/r mutant and age-matched wild-type mice. I chose tissues both abundant in collagen type I (heart, skeletal muscle and aorta) as well as those lacking in this ECM protein (spleen and brain). Figure 2.9 shows that telomere lengths in heart and skeletal muscle of Col1a1r/r mice were significantly shorter than in age-matched wild-type mice ($P=0.006$ and $P=0.008$, respectively). A trend was observed in the aorta, but not statistically significant. In contrast, spleen and brain showed no difference between mutant and wild-type mice. My data suggested that defective collagen turnover in collagen-enriched tissues may accelerate telomere attrition rate.

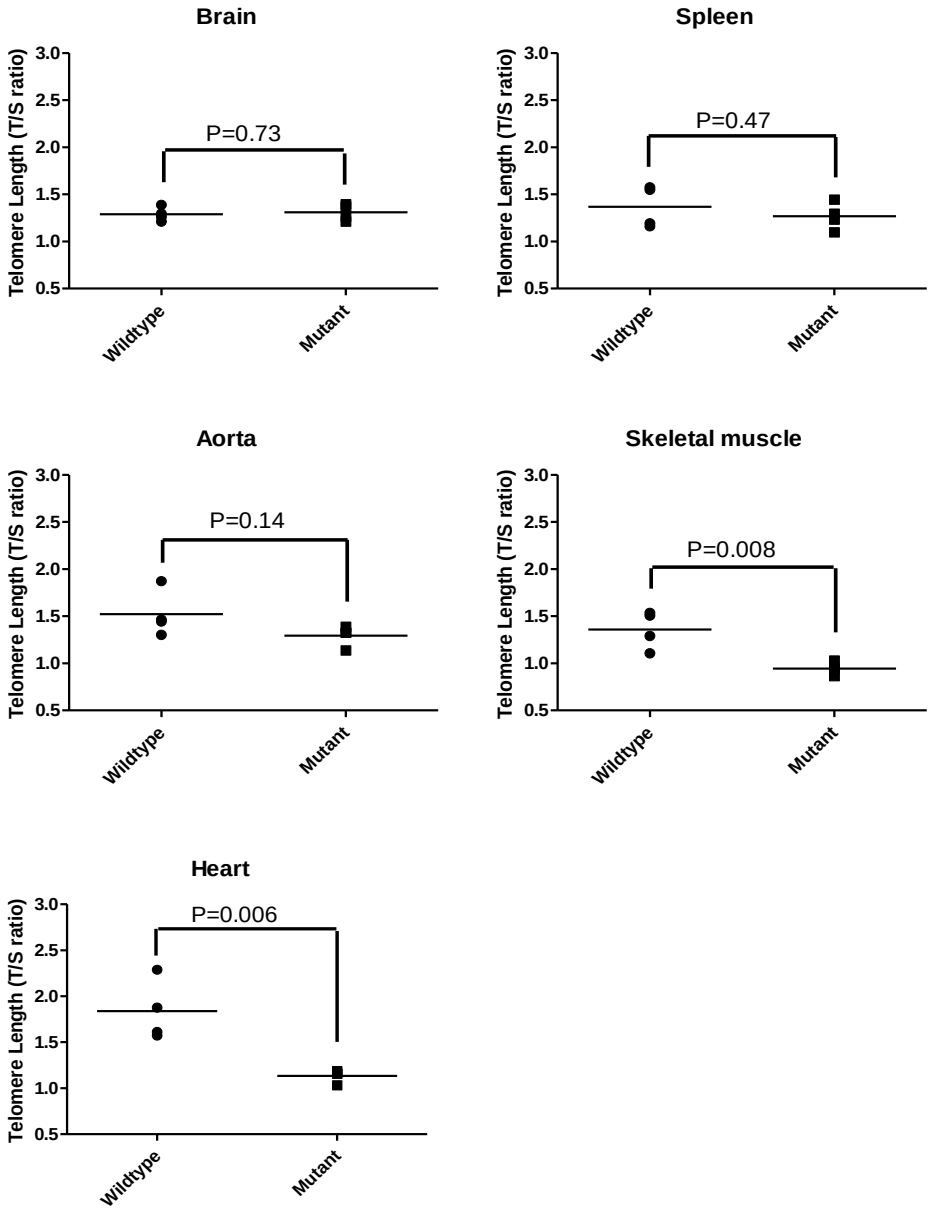

Figure 2.9 - Telomere length in different tissues of aged collagenase-resistant mice

T/S ratios were determined in different tissues (heart, skeletal muscle, aorta, brain, and spleen) of old (12-14 months) Col1a1r/r mutant (N=5) and wild-type (N=4) col1a1 mice. P-value is reported above each graph.

2.5 Discussion

I have found that age-related changes in telomere length in mice are detectable but are strain-specific. I also determined that aortic media telomere lengths are highly stable with age and approximate telomere length at birth. Using aortic media, I showed that age-related changes in telomere length can be uncovered in a DO mouse model system that entails human-like genetic diversity. Finally, I showed that impaired collagen turnover can lead to significant telomere shortening *in vivo*.

2.5.1 Strain-specific differences in telomere length dynamics

Few studies have studied strain-specific differences in telomere length set-point in mice. In this study, I have shown that C57BL/6 mice have shorter telomeres than other inbred strains (129 and Balb/c) and, as previously reported (Callicott and Womack, 2006; Prowse and Greider, 1995), SPRET/Ei mice telomeres are significantly shorter than C57BL/6 (Figure 2.1A).

There are only a few previous reports on telomere length differences among inbred mouse strains. In line with our study, one report established that telomeres in tissues of Balb/c mice were longer than those in C57BL/6 mice (Kipling and Cooke, 1990). However, another study reported that in liver, telomeres of 129sv mice were shorter than those in C57BL/6 mice (Hemann and Greider, 2000). This contrasts with what I observed. Discrepancies between the studies could be explained by different telomere length detection methods. Whereas Hemann and Greider used TRF analysis, I used qPCR. TRF analysis detects subtelomeric regions, and the length detected is dependent on restriction enzyme cut sites, which may differ between strains. Furthermore, the 129 strain used in this study is a combination of two specific substrains (129sv and 129P1/re). However, as both substrains showed similar telomere lengths in my study (data not shown), this is unlikely to be the cause of the discrepancies.

Telomere length of mesenchymal embryonic fibroblasts (MEF) from pure C57BL/6 mice has been found to be shorter than that from 129sv:B6 (Canela et al., 2007), supporting my data that C57BL/6 telomeres may be shorter than 129sv mice. Furthermore, telomerase knockout mice on a pure C57BL/6 background have shorter telomeres than the equivalent

mutants with a mixed 129sv:B6 background and show pathologies sooner (Herrera et al., 1999). These results support our finding that 129 background may have longer telomeres than C57BL/6 background.

Currently, no study has looked at strain-specific changes in telomere length with age. I detected that spleen from different inbred strains exhibit differential telomere length dynamics with age. C57BL/6 mice showed age-related telomere shortening (Figure 2.2B), 129 mice showed no telomere length change with age (Figure 2.2C), and Balb/c telomeres lengthened with age (Figure 2.2D). Similar to C57BL/6 in our study, a previous study using wild-derived *Mus Spretus* mice showed significant telomere shortening at a rate of -0.046kb per month in spleen (Coviello-McLaughlin and Prowse, 1997). It would be expected that tissues with a high cellular turnover like spleen have shortening of telomere lengths with age. Balb/c and 129 mice may have a lengthening mechanism at play, such as telomerase activity, to elongate and maintain their telomere lengths. In fact, there is evidence that Balb/c mice may have active telomerase in spleen, whereas C57BL/6 mice do not (Prowse and Greider, 1995).

Taken together, my data indicate strain-specific differences in telomere length set-point and regulation over time. Of the strains studied, C57BL/6 displayed age-related shortening of telomeres in a leukocyte-rich tissue.

2.5.2 Tissue-specific differences in telomere length dynamics of C57BL/6 mice

After identifying a strain that shows age-related telomere shortening in a leukocyte-rich tissue, I evaluated telomere length dynamics in a variety of tissue types. I looked at tissues considered post-mitotic, such as muscle-rich tissues and brain, as well as those with a high cellular turnover (bone marrow and blood). I observed that age has differential impacts on different tissues in C57BL/6 mice (Figure 2.3).

2.5.2.1 Blood leukocytes

In somatic tissue with a rapid cell turnover like blood leukocytes, one would expect telomeres to shorten with age. Interestingly, I did not detect statistical age-related

shortening in blood leukocyte telomeres of C57BL/6 mice between the ages of 1 to 16 months (Figure 2.3). In contrast, a previous study with C57BL/6 mice showed significant shortening in blood lymphocytes between 6 and 18 months of age (Rota et al., 2007). In humans, telomeres of both blood lymphocytes and granulocytes are well established to shorten with age both cross-sectionally (Oeseburg et al., 2010) and longitudinally (Aviv et al., 2009; Chen et al., 2011). The fact I see no telomere shortening in blood leukocytes raises the question of the validity of using C57BL/6 as a model for human shortening with age. However, in the same human longitudinal studies, there is also evidence of no change or even telomere lengthening in blood leukocytes of specific individuals. Using TRF analysis, 68 out of 561 participants in the Bogalusa Heart Study showed a gain in telomere length over a five year follow-up period (Aviv et al., 2009). Notably, longitudinal evidence in wild-caught *Mus musculus* mice also showed telomere lengthening with age by qPCR in blood lymphocytes collected at 3 and 12 months of age (Ilmonen et al., 2008). Discrepancies between studies may be in part due to various sources of inbred mice (suggesting genetic drift) and different detection and analysis methods. Furthermore, blood cells come from various sources (thymus, bone marrow, lymph nodes, and spleen), which may confound the validity of comparing telomere lengths at different times.

It is also important to note that, in my study, telomere length of blood cells in young mice (1-4 months of age; N=15-20) was significantly lower than those of other organs, including spleen and bone marrow (T/S=1.13±0.24 vs. 1.52±0.17 and 1.57±0.51, respectively, $P<0.01$). This suggests that murine blood cell telomeres may undergo shortening early in life. In fact, I have preliminary evidence in leukocytes of C57BL/6 neonates suggesting this is the case (data not shown). This speculation is interestingly consistent with well-established biphasic shortening of leukocyte telomeres with age in humans and large mammals (Baerlocher et al., 2007; Rufer et al., 1999), but warrants further experimental efforts. Telomere lengths of C57BL/6 leukocytes could be followed longitudinally to confirm C57BL/6 mice as a model for human telomere shortening with age. However, caution must be taken that extraction of blood does not contribute to increased telomere shortening due to increased cell turnover to replace blood cells.

2.5.2.2 Bone marrow

It is well known that stem cell compartments are responsible for replenishing aged somatic cells in tissue and that stem cells require telomerase activity to maintain their telomere lengths (Flores et al., 2006). In this study, bone marrow exhibited a steady decrease in telomere length from 1-16 months of age in wild-type C57BL/6 mice (Figure 2.3). This result suggests that telomerase activity in murine bone marrow is not high enough to completely prevent telomere erosion in such a highly proliferative tissue. This conclusion is supported by another study showing telomere shortening in stem cell compartments of C57BL/6 mice (Ferron et al., 2009; Flores et al., 2008). Furthermore, hematopoietic stem cells in humans had also been suggested to shorten with age (Aviv and Levy, 2011) as a result of high cellular turnover.

2.5.2.3 Brain

Neurons are considered truly post-mitotic, undergoing little cell turnover after birth if any (Bergmann et al., 2009; Spalding et al., 2005). Therefore, telomere lengths derived from brain are expected to be stable with age. Surprisingly, in my study, brain exhibited a steady decrease in telomere length with age (Figure 2.3). This is probably explained by the fact that I assessed whole brain. Thus, the age-related telomere shortening may be reflective of microglial cell turnover, as seen in rats (Flanary and Streit, 2004). Moreover, our finding on brain is also consistent with previous reports in wild-type *Mus spretus* and C57BL/6 mice (Coviello-McLaughlin and Prowse, 1997; Ferron et al., 2009; Flores et al., 2008).

2.5.2.4 Heart

Cardiomyocytes are reported to have little turnover throughout adult life (Bergmann et al., 2009) and may represent a good candidate surrogate for birth telomere length. Consistent with this, I found no change in telomere length with age between 1 and 16 months of C57BL/6 mice (Figure 2.3). In contrast, another study showed significant telomere shortening in cardiomyocytes between 6 and 18 months of age in wild-type C57BL/6 mice (Rota et al., 2007). In CAST/Ei mice, telomeres significantly shortened in the heart of sedentary mice in the first year, whereas exercise was protective against

such shortening (Ludlow et al., 2012). Wistar rats showed extensive telomere shortening only in the heart among other tissues measured (Hastings et al., 2004). These studies suggest that telomeres in the heart may not be stable with age. Although I saw stable telomere lengths with age in the heart, it was nonetheless highly variable. Furthermore, it's possible that most telomere shortening events occur between 1 and 2 years of age in C57BL/6 mice, as suggested in another study (Flores et al., 2008). Notably, we also observed a significant shortening event of heart telomeres between 1 day and 1 month of age (Figure 2.4), suggesting it may not be an ideal choice for estimating birth telomere length.

2.5.2.5 Skeletal muscle

Unlike cardiac muscle, skeletal muscle showed a significant decrease in telomere length with age (Figure 2.3). Another study using C57BL/6 mice showed telomere shortening in the diaphragm but not the tibialis anterior muscle of wild-type mice with age (Lund et al., 2007). In CAST/Ei mice, unlike cardiac tissue, exercise induced telomere shortening of the gastrocnemius, whereas sedentary mice showed no significant shortening in the first year (Ludlow et al., 2012). Furthermore, in humans, endurance runners have reduced telomere length in the vastus lateralis muscle, possibly because of increased load on the skeletal muscle (Rae et al., 2010), whereas power-lifters have been reported to have longer telomeres than non-athletes (Kadi et al., 2008). Therefore, telomere shortening may be dependent on muscle type and mechanical stress applied to induce regeneration during post-natal life. Nonetheless, two studies have used skeletal muscle as an indicator of telomere length at birth (Benetos et al., 2011; Gardner et al., 2007) while one review indicates to do so with caution (Kadi and Ponsot, 2010). At least in mice, certain muscle types may be more reflective of neonatal telomere length than others. In addition, the heterogeneous cell population in skeletal muscle tissues, including myocytes, fibroblasts, vascular cells and blood cells (Biressi and Rando, 2010), could contribute to the large telomere length variability observed in our study.

2.5.2.6 Aortic media

The aorta was of particular interest because of the relative ease with which a homogeneous population of smooth muscle cells (SMC) can be isolated, by harvesting only the medial layer. A homogeneous cell population could help reduce variations in telomere length through eliminating potential differences in telomere lengths between cell types. Since SMCs do not replicate very often, except during injury or vascular disease (McCarthy and Bennett, 2000), we were interested to see if the aortic media showed a stable telomere length with age in wild-type mice. In C57BL/6 mice, we saw a stable telomere length with time in the aortic media (Figure 2.3), and no significant change with age in DO mice (Figure 2.7D). There is one study with C57BL/6 mice showing telomere length in the aorta decreasing between 6 and 18 months of age (Werner et al., 2009), but they use whole aorta sections. I have shown that the adventitia and endothelium can differentially contribute to the results (Figure 2.5).

2.5.3 Aortic media has stable telomeres during early life development

In order for a tissue to be a good indicator of birth telomere length, it would have to maintain long and stable telomere lengths throughout the lifespan of an individual. Skeletal muscle has previously been proposed as a good indicator of birth telomere length (Benetos et al., 2011), however, here it showed signs of post-natal telomere shortening in our study (Figure 2.3). Therefore, I looked at telomere lengths in the two myocyte abundant tissue types showing no signs of post-natal shortening (cardiac and smooth muscle) at birth compared to early life. I found that aortic media maintained stable telomeres from birth to adulthood in these mice, whereas heart dropped significantly after birth (Figure 2.4). No other study has looked at telomere length changes in early life of mice. However, a study using Wistar rats demonstrated a 38% drop in telomere length between newborns and adults in cardiac tissue (Hastings et al., 2004). Moreover, in my study, aortic media had the longest telomeres at birth and post-natally compared to heart.

Thus, aortic media displays stable telomere lengths during early life development and adult aging. These results suggest that aortic media represents a surrogate for birth telomere length.

2.5.4 Telo-mapping reveals telomere length heterogeneity in the aorta

Different telomere length dynamics with age were observed in three circumferential layers of mouse aorta (Figure 2.5). I identified that aortic intima, media, and adventitia began with the same length of telomeres at early adulthood (4-months old). However, by 10 months the intimal layer had the shortest telomeres. This may be reflective of hemodynamic and oxidative stress endothelial cells endure (Davies, 2009; Forstermann, 2010), exacerbating cellular turnover and telomere shortening. Also, telomere length in the adventitia was shorter than that in the media, likely reflective of the heterogeneity in the fibroblast population embedded in it, which have variable proliferation rates compared to medial SMC cells (Stenmark et al., 2006). According to a previous study in human abdominal aortas, the medial and intimal layers show no significant difference in telomere length (Okuda et al., 2000). The difference between human and mice telomere lengths in aorta layers may be reflective of differences in their cardiovascular physiology. For example, murine hearts beat up to 10 times faster than human hearts, imposing a greater degree of hemodynamic shear stress on mouse vessel walls (Ceconi et al., 2011). Also, mouse aortic composition is different from humans – humans have a sub-endothelial layer of SMCs that is not present in mice (Schwartz and Benditt, 1972; Schwartz et al., 1995).

Of note, I observed that aortic medial telomere length was significantly less in the infra-renal abdominal aorta compared to other segments above this (Figure 2.6). Different regions of the aortic SMCs are of different developmental origins (Majesky, 2007), which may contribute to the segmental variation in telomere lengths observed here. This finding also raises the intriguing possibilities that shorter telomere length in the infra-renal abdominal aorta compared to other aortic regions contributes to its higher susceptibility to vascular diseases, such as atherosclerosis and aortic aneurysms (Guo et al., 2006). In fact, patients with abdominal aortic aneurysms have shorter telomeres in

both aortic biopsies and blood leukocytes than age-matched controls (Atturu et al., 2010; Wilson et al., 2008). Furthermore, a recent study shows that endothelial and smooth muscle cells in patients with abdominal aortic aneurysm have shorter telomeres and increased oxidative stress than controls matched for age and clinical history (Cafueri et al., 2012). Therefore, it is possible that the vascular wall ages more readily in the abdominal portion of the aorta and telomere length may represent a valuable indicator of its diseased or aged state.

2.5.5 Diversity Outbred mice as a murine model for telomere biology

Diversity Outbred mice are bred specifically to maintain maximum genetic heterogeneity in its population. I determined that inter-mouse variability in telomere length is correspondingly heterogeneous in the DO population, significantly greater than inbred strains (Figure 2.7A). Evidently, genetic determinants play a fundamental role in telomere length set-points of individual mice. A helicase called Rtel has been shown to be critical in determining the set-point of telomeres in newborn mice (Ding et al., 2004). Therefore, mice with diverse genetic backgrounds, like DO mice, may have diverse telomere length set-points at birth. Another study showed that inbred lines also have an innate heterogeneity in telomere length established in the parental germ-line (Zijlmans et al., 1997). Nonetheless, my study showed that there was greater variability in telomere length set-point in mice with greater genetic diversity.

I further showed that DO mice maintain long telomeres characteristic of inbred strains from which they were derived (Chesler et al., 2008). This suggests that outbreeding does not induce significant telomere shortening. Hemann and Greider discuss that wild-derived strains have short telomeres because they were recently inbred, whereas the appearance and maintenance of long telomeres in established strains is likely due to extensive breeding of a small isolated colony (Hemann and Greider, 2000). Another study argues that inbreeding may in fact induce telomere lengthening in mice (Manning et al., 2002). Here I showed that extensive outbreeding of a large population of genetically heterogeneous mice derived from established inbred strains does not restore telomere lengths to human-like lengths, as proposed in wild-derived mice (Hemann and

Greider, 2000), but rather maintains long telomeres. Furthermore, Hathcock *et al.* reports that by crossing *Mus spretus* with Balb/c results in the lengthening of the short telomeres from *Mus spretus* (Hathcock et al., 2005), suggesting that long telomeres may be dominant to short ones. The inbred lines used to make the Collaborative Cross (CC) strains and DO mice contain both long and short telomere lengths. Evidently, the short telomeres from CAST/Ei mice (Hemann and Greider, 2000) were not selected for during the outbreeding of these mice, further suggesting that long telomeres may be dominant to short ones.

2.5.6 Correcting for telomere length heterogeneity in Diversity Outbred mice

With aortic media (specifically supra-renal) representing a potential candidate for birth telomere length, I wanted to dissect the contributions of age and genetic variation to telomere length dynamics using the DO mouse model. I showed that an intra-mouse telomere length synchrony exists between spleen and aorta in individual DO mice (Figure 2.7B), independent of inter-mouse variation in the DO population. Tissue telomere length synchrony has been previously shown in macaques (Gardner et al., 2007), humans (Gardner et al., 2007; Kimura et al., 2010; Wilson et al., 2008), dogs (Benetos et al., 2011), and wild-derived mice (Prowse and Greider, 1995). Furthermore, such synchrony has allowed the genetic variation of telomere length in a population to be accounted for by subtracting telomere length of leukocyte from that of skeletal muscle, thereby strengthening the association of telomere length with age (Benetos et al., 2011; Gardner et al., 2007). My data indicate that aortic media may be a good choice, potentially better than skeletal muscle.

With uncorrected data, DO mice showed no detectable telomere shortening in either spleen (Figure 2.7C) or aorta (Figure 2.7D) with age. The DO spleen data is inconsistent with C57BL/6 spleen data, which shows statistical telomere shortening with age (Figure 2.2B). This inconsistency may be due to the genetic diversity and large inter-mouse variability in telomere lengths of DO mice. For instance, in one generation of DO mice telomeres could be long, whereas in the next they could be shorter. Therefore, it's difficult to make any conclusive interpretations of telomere lengths with age in cross-

sectional studies of a genetically diverse population. To offset genetic variability, I applied two methods of referencing: subtraction (Figure 2.7E) and division (Figure 2.7F) between spleen and aorta. I found improvements in the relationship of telomere length with age, both becoming statistically significant. Both referencing methods have different biological implications. Subtraction yields total spleen telomere length shortening since birth, with the assumption that aortic media estimates birth telomere length. Division normalizes telomere length to its presumed starting length, yielding relative telomere length shortening.

Furthermore, these two referencing methods of subtraction and division also showed an improvement in the age-related telomere shortening of the intima in a mixed population of C57BL/6 and DO mice (Figure 2.8). Therefore, aortic media may act as a powerful internal reference of birth telomere length to uncover age-related shortening of both leukocytes and the endothelium.

2.5.7 Impaired collagen turnover accelerates telomere attrition

Finally, cardiovascular disease is an age-related phenotype that is in part characterized by reduced arterial compliance and cardiac function, as well as accumulation of cross-linked collagen fibrils (Jacob, 2003; Kovacic et al., 2011; Lakatta and Levy, 2003a, b). In this study I revealed in a mutant collagen mouse model that has the same build-up of extracellular matrix proteins, such as collagen type-I, in our heart and blood vessels that leads to cardiovascular disease also shows a telomere length reduction compared to healthy mice (Figure 2.9). Only those tissues that have an abundance of collagen (skeletal muscle and heart) showed a significant reduction in telomere length. Therefore, telomere length may be associated with age-related changes in extracellular matrix proteins, such as collagen type I, that lead to cardiovascular disease.

In summary, these studies indicate that considerable data on telomere length can be obtained from standard laboratory mice. Moreover, I propose that the aortic media has unique attributes as a surrogate for birth telomere length.

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Chapter 3 - General Discussion

The major findings of this thesis are:

1) Mouse telomere lengths are strain-specific in both their set-point and rate of attrition

2) Aortic media telomere length is stable in early life development and adult aging in C57BL/6 mice

3) Telomere lengths are heterogeneous across the aortic wall in C57BL/6 mice (intima<adventitia<media)

4) Genetic diversity translates to telomere length diversity in mice

5) Aortic media can act as a surrogate for birth telomere length to uncover age-related relationships by referencing to leukocyte-rich tissues in a genetically diverse mouse population

6) Impaired collagen turnover increases telomere length attrition in mice

3.1 Limitations and immediate next steps

First, an understanding of telomerase activity could inform findings of telomere length in mice. For example, telomerase could be a contributing factor to aortic media telomere lengths. Measuring telomerase activity in aortic media at different ages would determine if telomerase activity contributes to its stability of telomere length with age or if minimal cell turnover is the main cause. Furthermore, aortic media telomere lengths with age could also be measured in a telomerase knockout model. As well, telomerase activity could explain strain and tissue differences observed in my study.

Another key finding that merits further study was my observation that intima telomere lengths shorten faster than those of the adventitia and aortic media. It would be interesting to see if tissue-specific overexpression of telomerase could compensate for this telomeric loss in the intima. Furthermore, would this compensation decrease the

atherosclerotic burden of ApoE knockout mice, seeing as endothelial dysfunction is a precursor to atherosclerosis? Moreover, it would also be interesting to see how the telomere length gradient across the aortic wall is affected in a stressed system, such as mice injected with angiotensin over time.

Moreover, the three layers of the aorta were isolated mechanically as best as possible, but there was likely some cross-over between layers. However, it's unlikely that the amount of cross-over was enough to skew the telomere length measurement in any way. However, Q-Fish could be performed on specific cells in the aorta to confirm that smooth muscle cells have longer telomeres than endothelial cells in aged mice.

Lastly, I determined that impaired turnover of collagen in tissue has a negative impact on telomere length with age. The composition of the extracellular matrix (ECM) plays an important role to the health and function of tissue at a cellular level (Jacob, 2003). In fact, the ECM contributes to the regulation of smooth muscle and endothelial cell function (Hultgardh-Nilsson and Durbeej, 2007; Sottile, 2004). Increased mechanical stress through an accumulation of collagen content may create an environment that induces high cellular turnover, resulting in increased telomere attrition. Further studies are necessary to assess the role of various ECM proteins on cell turnover and the rate of telomere attrition. Telomere length may be a valuable marker for vascular aging and pathogenesis. Notably, the aorta telomere lengths were unaffected by impaired collagen turnover. In this experiment, I used whole aorta, and the collagen content varies between layers, which may have skewed the results. Furthermore, a greater N number may be necessary to detect statistical differences between the mutant (col1a1r/r) and wild-type mice.

3.2 Future implications

I discovered that aortic media is a stable indicator of birth telomere length. Birth telomere length has been shown to be a predictor of lifespan in birds (Heidinger et al., 2012). It may be possible to use aortic media telomere length at a given age to predict lifespan to some degree of accuracy. A large population study in humans would be necessary to correlate aortic media telomere length to lifespan of the aging population

using mortality as the endpoint. Such a study would validate the accuracy of using aortic media in humans as an indicator of birth telomere length, assuming like in birds, birth telomere length can predict lifespan in humans.

Human studies are necessary to validate use of aortic media as an internal reference for birth telomere length to track telomere length changes in leukocyte-rich adult tissues. In humans, access to aortic biopsies poses a potential problem, which may be limited to patients undergoing surgery. However possible alternatives to aortic media may be the umbilical cord or veins that are more accessible. Another possibility is use of a fragment of the internal thoracic artery of patients undergoing bypass surgery. Future studies will need to confirm the validity of such alternatives. Another issue in human studies is vascular health status. For individuals in a diseased state, their aortic SMC's may have undergone proliferation and been exposed to increased oxidative stress and inflammation, which would effectively shorten their telomere lengths (Cafueri et al., 2012; Okuda et al., 2000). Studies will need to be performed that establish parameters for the use of aortic media as an estimate of birth telomere length in humans.

Ultimately, if birth telomere length can be established in human adult tissue there are implications for tracking telomere loss, telomere rate of shortening, and potential for lifespan prediction. All of these readouts will allow investigators to further elucidate how telomeres are associated with age and age-related illnesses for an individual and the human population.

3.2.1 Model for estimating birth telomere length

I determined telomere length dynamics with age in a variety of post-mitotic tissues (brain, skeletal muscle, heart, aortic media) and tissues with high cellular turnover (spleen, blood, bone marrow). Not only was aortic media telomere lengths the most stable with age, but it tended to maintain the longest telomeres of all tissues measured.

These results may in part be reflective of the cell composition of each tissue type. In fact, I noticed higher inter- and intra-mouse variability of telomere lengths in some tissues compared to others; heart and skeletal muscle was often high (data not shown). Telomere

length variability between tissue types may be dependent on how homogeneous the cell population is in type, age and distribution in a given tissue sample. For instance, a study by Rota *et al.* showed that the mouse heart is composed of myocytes heterogeneous in age and function, such that telomere lengths varied from 14 to 58 kbp with the largest and oldest myocytes having the shortest telomeres (Rota et al., 2007). This heterogeneity of myocytes in age likely contributes to the relatively high variability seen in telomere lengths of heart samples in our study. Another study by González-Suárez *et al.* measured telomere lengths of metaphase spreads using qFISH and showed significant amount of variation within a given tissue (Gonzalez-Suarez et al., 2005), suggesting that tissues are composed of individual cells with varying telomere lengths. Moreover, telomere lengths of keratinocytes in the basel layer of skin had less variation than telomere lengths in kidney sections (Gonzalez-Suarez et al., 2005), further supporting the notion that different tissue types have greater variation in telomere lengths than others.

Previous studies have shown that skeletal muscle may be a good choice as a surrogate marker for birth telomere length in adults (Benetos et al., 2011; Gardner et al., 2007). However, skeletal muscle is composed of a variety of different cell types, including a proliferating population of satellite stem cells (Biressi and Rando, 2010). Estimating birth telomere length requires that the tissue sampled be composed predominantly of post-mitotic cells as shown in figure 3.1. This would effectively reduce any noise or skewing effect caused by specific cell populations in tissues that have undergone extensive proliferation post-natally.

3.2.2 Diversity Outbred mouse model to study genetic diversity and telomere length dynamics

The Diversity Outbred (DO) mice showed extensive variation in telomere lengths compared to inbred mice. Thus, DO mice may prove to be a valuable tool to study the effects of genetic heterogeneity on telomere length dynamics in genome wide association studies (GWAS) to identify critical modulators of telomere length. In fact, a recent study demonstrated that DO mice can be used for high resolution genetic mapping with minimal number of mice (Svenson et al., 2012).

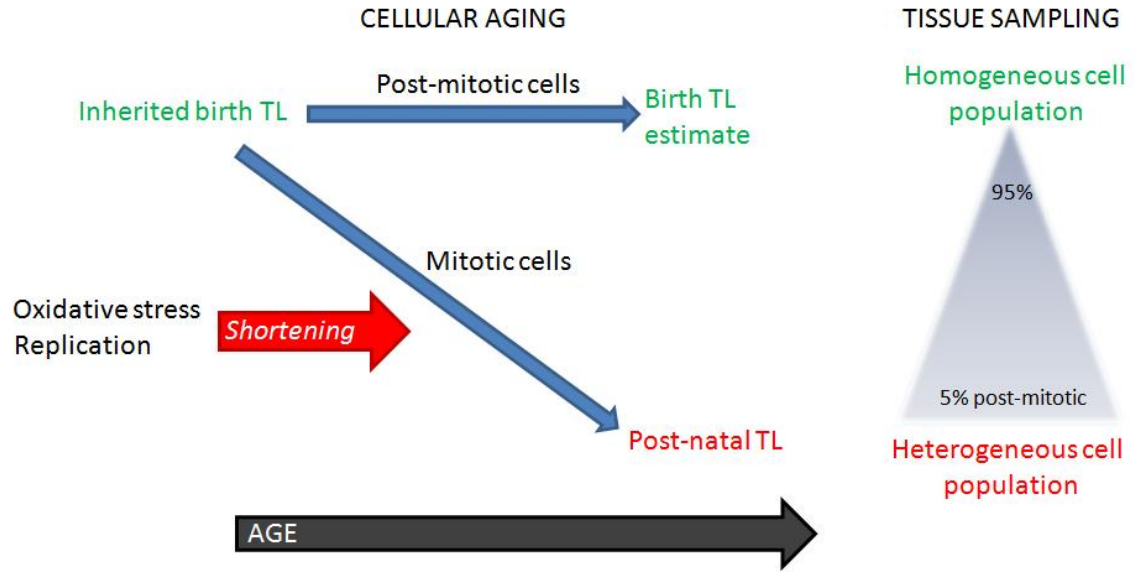

Figure 3.1 - Estimating birth telomere length

Post-mitotic tissues are composed of both post-mitotic cells and mitotic cells in different proportions. Mitotic cells undergo proliferation and telomere shortening events, whereas post-mitotic cells do not divide and show no significant telomere shortening. A tissue predominantly composed of post-mitotic cells will have the most accurate representation of birth telomere length. However, the greater the proportion of mitotic cells in the tissue sample, the further away it will be from true birth telomere length.

Furthermore, these mice showed diverse physiologies from plasma lipid and glucose levels, hematological parameters, to body composition (Svenson et al., 2012), and as I have also shown in telomere lengths. It would be interesting to see if DO mice born with short telomeres have a predisposition to age-related illnesses compared to those born with long telomeres.

The implications of having a good estimate of birth telomere length, such as aortic media, may allow investigators to take a snap-shot of an individual's leukocyte telomere length at a given age relative to their starting point. Referencing in this way might enable investigators to determine telomere loss and rate of telomere shortening of an individual. Although actual telomere length is an important readout, both telomere loss and rate of shortening may also be important readouts to further establish an etiological role of telomeres with aging and disease. Those two readouts could also be applied to GWAS studies in large populations of individuals to determine genetic variants that contribute to telomere attrition with age. One study in particular looked at 743 SNPs in 43 telomere biology genes in 3646 participants of the prostate, lung, Colorectal, and ovarian screening trial and Nurse's health study and found no strong associations with telomere length (Mirabello et al., 2010). They propose it may have been due to a lack of diversity and/or evolutionary conservation. The DO mice may provide the allelic variation and statistical power necessary in order to detect SNP variants associated with telomere length. Therefore, DO mice have extensive genetic and telomere length heterogeneity with potential to further elucidate the complexity of aging and its association with telomere length.

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Appendices

Appendix A: Statement of permission for the use of animals for experimental research

All animal experimentation was conducted in compliance with the animal use protocol 2010-244 held by Dr. J. Geoffrey Pickering, principal investigator at the Schulich School of Medicine and Dentistry and the Department of Biochemistry at the University of Western Ontario in London, Ontario, Canada.

Appendix B: Sample calculation of qPCR telomere length measurement (T/S ratio)

Relative telomere length by quantitative PCR (qPCR) was determined by measuring the factor by which each experimental sample differed from a reference DNA sample in its ratio of telomere repeat copy number to single gene copy number (Cawthon, 2002). In theory, this ratio should be proportional to the average telomere length. The quantity of telomeric repeats and single copy gene is best described from the original paper by Cawthon. "The quantity of telomere repeats in each experimental sample was measured as the level of dilution of an arbitrarily chosen reference DNA sample that would make the experimental and reference samples equivalent with regard to the number of cycles of PCR needed to generate a given amount of telomere PCR product during the exponential phase of PCR amplification. Similarly, the relative quantity of the single copy gene in each experimental sample was expressed as the level of dilution of the reference DNA sample needed to match it to the experimental sample with regard to the number of cycles of PCR needed to generate a given amount of single copy gene PCR product during the exponential phase of the PCR. For each experimental sample the ratio of these dilution factors is the relative telomere to single copy gene (T/S) ratio (Cawthon, 2002)."

First, a standard curve for both telomeric repeats and single copy gene (36B4) was generated using an arbitrarily chosen reference DNA sample by serial dilution (Figure 4.1). The reference DNA sample can be from a single individual or pooled from many. In this thesis, I chose spleen DNA of an individual mouse as the reference DNA sample because spleen is concentrated in cells and therefore DNA is easily extracted in large amounts.

Second, a Ct value is determined for each well by quantitative PCR. Ct value is defined as the fractional cycle number at which accumulating fluorescence in a given well crosses a set threshold. This threshold can be manually adjusted or automatically set several standard deviations above baseline fluorescence. In this thesis, I manually adjusted the threshold to optimize the reaction efficiency and achieve a slope between -3.1 and -3.6 for both the telomere and 36B4 standard curve.

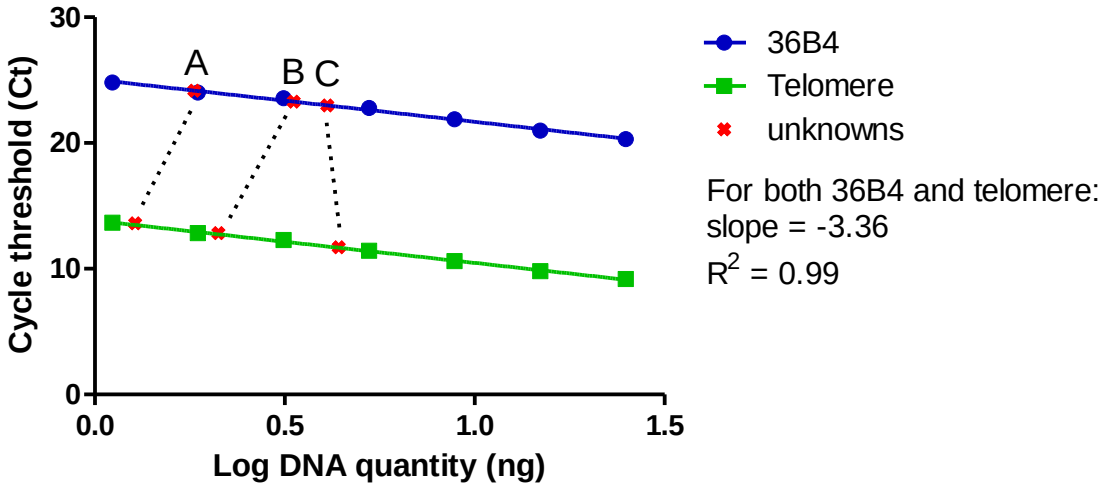

Figure 4.1 – Standard curve generated to determine a T/S ratio.

Serial dilution of an arbitrarily chosen reference DNA sample was used to generate a standard curve for both telomeric repeats and a single copy gene (36B4). Experimental samples (unknowns) are quantified based on matched Ct values relative to standard curve for telomeric repeats or 36B4.

Furthermore, baseline fluorescence is automatically set between cycle 3 and 15. However, amplification of telomeric repeats results in a fluorescent signal that passes the cycle threshold well before cycle 15. Therefore, I manually adjusted the baseline fluorescence for telomere qPCRs to be between cycle 2 and 6.

Third, a plot of Ct versus log (amount of input target DNA) is linear, which allows simple relative quantitation of unknowns by comparison to both the telomere and 36B4 standard curves. For this thesis, telomere (T) PCRs and single copy gene (S) PCRs were always performed in separate 384-well plates. A standard curve was included in each plate. Applied Biosystems SDS software v2.4 was used to generate the standard curve for each plate and to determine the dilution factors of reference DNA corresponding to the T and S amounts in each sample. A T/S ratio for each experimental sample was then determined. An example derivation of T/S ratios of three unknown samples (A, B, and C) from figure 4.1 can be seen in table 4.1.

Table 4.1 – Sample calculation of T/S ratio

Unknown sample	Log T quantity (ng)	T quantity (ng)	Log S quantity (ng)	S quantity (ng)	T/S ratio
A	0.1	1.26	0.3	2.00	0.63
B	0.4	2.51	0.5	3.16	0.79
C	0.7	5.01	0.6	3.98	1.26

If the T/S ratio is equal to 1, the experimental sample has an average telomere length equivalent to the reference DNA sample. If T/S ratio is above or below 1 then the experimental sample has an average telomere length respectively greater or less than the reference sample. T/S ratios of samples within a laboratory can be compared relative to each other, but comparisons of T/S ratios between laboratories cannot be made unless converted to kilobases using universal standards (currently do not exist). It is important to note that qPCR generated T/S ratios may not be directly linear with absolute telomere length as measured in kilobases by Southern blotting. One study impartially compared the qPCR and TRF analysis of the same leukocyte DNA samples between laboratories (Aviv et al., 2011). Results indicated that the two methods are correlated, but may deviate from linearity. Addition of a quadratic term improved the fit between both methods. This result suggests that when comparing T/S ratios, differences must be

interpreted with caution. Differences in T/S ratios may not be directly proportional to actual telomere length differences. However, differences in T/S ratios can still be statistically identified and interpreted as such.

Lastly, a standard curve slope between -3.1 and -3.6 represents reaction efficiency between 90-110%, which is deemed acceptable. Variations in reaction efficiencies between experiments can cause variations in T/S ratios. These variations were accounted for by using inter-experimental plate controls. These controls were seven arbitrary samples of DNA that were included in each plate. An average T/S ratio for each control was determined based on seven experiments. The average T/S ratio for each control was used as a baseline for individual runs to determine a factor by which T/S ratios deviated for a given run. The ratio between baseline and experimental T/S ratio of each control was determined and the average of these seven values was used as the factor to normalize T/S ratios of a given experiment.

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