REACTIVE OXYGEN SPECIES AND TELOMERE DYSFUNCTION: INVESTIGATING THE UNDERLYING MECHANISMS OF AGING AND RELATED DISEASES

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ABSTRACT

Carrie-Ann Jessica Olivia Gordon: Reactive Oxygen Species and Telomere Dysfunction: Investigating the underlying mechanisms of Aging and Related Diseases.
(Under the direction of Michael B. Jarstfer)

Atherosclerotic cardiovascular disease is the leading cause of morbidity and mortality in western countries. The underlying mechanism of the disorder remains unknown, but ROS and telomere dysfunction have been independently implicated in atherogenesis, and are common elements among the major risks factors. However, the relationship between ROS, telomere biology and atherosclerosis has not been adequately examined. Nor is it clear whether the relationship between ROS and telomere dysfunction play a causative or correlative role in atherosclerosis. Based on scientific evidence that ROS modulate telomeres, we hypothesized that telomere dysfunction is a crucial downstream target of oxidative stress in the development of atherosclerosis.

In Chapter I, we characterized the telomere biology of aortic smooth muscle cells (ASMC) from mouse models haploid deficient in mitochondrial (SOD2+/-) and cytoplasmic (SOD1+/-) superoxide dismutase. The SOD2+/- model exhibits properties associated with atherosclerosis. We found rapid telomere erosion in SOD2+/- ASMC despite elevated telomerase activity. Furthermore, we observed increased oxidative DNA damage and dysfunctional telomeres in SOD2+/- ASMC compared to wildtype (WT). Our results suggest telomere erosion was not a consequence of nuclear export of telomerase protein subunit but most likely the result of increased oxidative damage at the telomeres.

Additionally, we studied the effects of anti-atherosclerotic compounds on telomerase. In chapter III, we treated SOD2+/- and WT ASMC with NADPH oxidase inhibitors and estrogen. We found NADPH oxidase inhibitors modulate telomerase activity as well as confirmed the ability of estrogen to up regulate telomerase. Lastly, in chapter IV, we examined the effects of oxidatively modified deoxyguanosine triphosphate on telomerase activity *in vitro* and observed a modest effect suggesting telomerase is not remarkably sensitive to this nucleotide.

These findings demonstrate that chronic oxidative stress can cause telomere dysfunction in the vasculature system, particularly ASMC. Our studies provide a basis to better understand the relationship between oxidative stress and telomere dysfunction, and the roles they might play in atherogenesis. Moreover, our observation of elevated telomerase in SOD2+/- ASMC is quite promising, as it provides an opportunity to further study if telomerase promotes proliferation in SOD2+/- ASMC in a telomere independent manner.

To my family whom I love and my aunt V. Noreen Codner who passed away before this work was completed.

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Graduate School has been a rewarding experience. I learnt a lot about science, conducting research and about myself. I learnt it was okay for an experiment to not work (and many times it did not), that failure is a learning opportunity, and success can be measured in very tiny steps. This body of work is a culmination of days which stretched into nights, weeks that had no ends and most of all, the love and support a network of family, friends, advisors and colleagues.

I would first like to thank my advisor, Dr. Michael Jarstfer, who has been extremely supportive of me and my goals. When prospective students ask, what is the most important advice I can provide, my answer is always chose an advisor who is supportive of you as an individual and your career. I have been blessed to find those qualities in my advisor. Dr. Jarstfer has guided me throughout my graduate career, provided me with sound advice, reassured me when I had doubts and made time for discussion when needed. I have truly benefitted from his constant enthusiasm for science, his strong interest in training future scientists and his ability to motivate his students. For this, I will forever be grateful.

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LIST OF ABBREVIATIONS

8-oxo-dG	8- oxo-7,8-dihydrodeoxyguanosine
8-oxo-dGTP	8- oxo-7,8-dihydrodeoxyguanosine triphosphate
A	Adenine
AA	Amino acid
ALT	Alternative Lengthening of Telomeres
ASMC	Aortic smooth muscle cells
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine-5'-triphosphate
ATR	Ataxia-telangiectasia and Rad3-related
C	Cytosine
CAB	Cajal Body box
cDNA	complementary DNA
CHIP	C terminus of Hsc70-interacting protein
CST	CTC1, STN1 and TEN1
CTC1	Conserved telomere maintenance component 1
CTE	C-terminal extension domain
CTP	Cytidine-5'-triphosphate
CR	Conserved Region
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DPI	Diphenyleneiodonium
dsDNA	double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ERE	Estrogen Responsive Element
FITC	fluorescein isothiocyanate
G	Guanine
GAR1	H/ACA ribonucleoprotein complex subunit 1
GTP	Guanosine-5'-triphosphate
H/ACA	"hinge box/ACA sequence" structure
hTERT	human Telomerase Reverse Transcriptase
hTERC	human Telomerase RNA component
IFD	Insertion in fingers domain
LC	Loading control
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
mTERT	Mouse TERT
mTERC	Mouse TERC
NADPH	nicotinamide adenine dinucleotide phosphate
NHP2	H/ACA ribonucleoprotein complex subunit 2
NOP10	Nucleolar protein 10

P53	Tumor protein 53
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCI	Phenol/chloroform/isoamyl alcohol
PCR	Polymerase chain reaction
PGC	Peroxisome proliferator-activated receptor gamma, coactivator
PNA	Peptide nucleic acid
POT1	Protection of the telomeres 1
RNA	Ribonucleic acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
RT	Reverse Transcriptase
S1-4	ASMC isolated from 4 month SOD1+/- mice
S1-16	ASMC isolated from 16 month SOD1+/- mice
S2-4	ASMC isolated from 4 month SOD2+/- mice
S2-16	ASMC isolated from 16 month SOD2+/- mice
SDS	sodium dodecyl sulfate
SOD1	superoxide dismutase 1
SOD2	superoxide dismutase 2
STN1	CST complex subunit STN1
T	Thymidine
TALA	Telomere amount and length assay

TBE	Tris/Borate/EDTA
TCBP1	Telomerase Cajal body protein 1
TE	TRIS-EDTA
TEN	Telomerase essential N-terminal
TEN1	CST complex subunit TEN1
TERC	Telomerase RNA component
TERT	Telomerase Reverse Transcriptase
TRF1	Telomere Repeat Binding Factor 1
TRF2	Telomere Repeat Binding Factor 2
TIF	Telomere dysfunction-induced foci
TIN2	TRF1-Interacting Nuclear protein 2
TRBD	TERT RNA-binding domain
tTER	*Tetrahymena thermophila* telomerase RNA
tTERT	*Tetrahymena thermophila* telomerase reverse transcriptase
tTR	*Tetrahymena thermophila* telomerase RNA
U	Uracil
UNC	University of North Carolina at Chapel Hill
UTP	Uridine-5'-triphosphate
UV	Ultraviolet
VSMC	Vascular smooth muscle cell
W4	ASMC isolated from 4 month WT mice
W16	ASMC isolated from 16 month WT mice
WT	Wildtype

CHAPTER I: TELOMERE BIOLOGY AND DISEASE

A. Telomeres: Maintenance of Linear Chromosome

Linear eukaryotic chromosomes pose two primary biological problems: the end protection problem and the end replication problem.(1) The end protection problem is concerned with the chromosome termini being indistinguishable from DNA double strand breaks. If the chromosome ends are identified as DNA double strand breaks, the ends will be subjected to incorrect processing by DNA repair machinery, resulting in recombination events and end to end fusion (Figure 1.1A).(2) The second problem, the end replication problem, is concerned with the inability of DNA polymerases to replicate the distal ends of the chromosomes completely (Figure 1.1B). DNA polymerase can only synthesize DNA in the 5' to 3' direction as the enzyme incorporates nucleotides onto the 3'-OH of the existing strand. Consequently, the 3'to 5' template strand is replicated continuously, after a single initiating priming event by an RNA polymerase called primase. In contrast, replication of the 5' to 3' template strand is discontinuous. Primase synthesizes RNA primers at multiple sites along the 5' to 3' template strand which DNA polymerase extends producing short strands of DNA called Okazaki fragments. The RNA primers are then removed and replaced with

DNA. The resulting DNA fragments are then ligated, creating the lagging strand.(3) At the chromosomal termini, however, when the RNA primer used to initiate the terminal Okazaki fragment is removed, a gap is created that cannot be filled by DNA polymerase. Thus the chromosome ends are not completely replicated.(4) In absence of a mechanism to overcome the end replication problem, the chromosomes shorten with each cell cycle and genetic information can be potentially lossed.

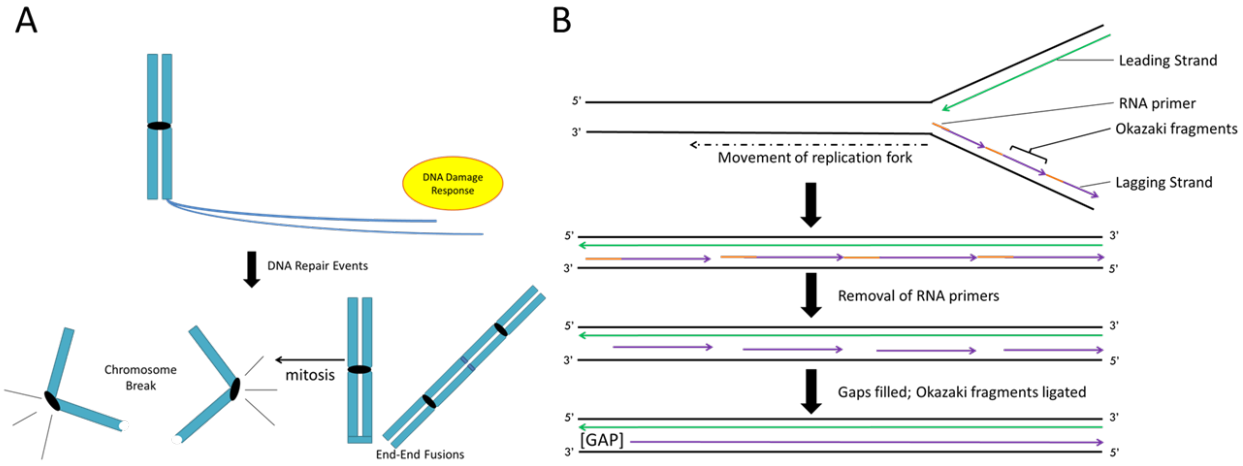

Figure 1.1. The end protection problem and the end replication problem. A) If chromosome termini are not discernible from DNA double strand breaks, they are recognized as such by the DNA damage response. Processing of the chromosome ends by DNA repair mechanisms may result in end-end fusion and increased chromosomal instability. B) The directionality of DNA polymerase allows for the leading strand to be completely replicated but not the lagging strand. In lagging strand synthesis, DNA polymerase replicates DNA using multiple RNA primers which provide 3'-OH for nucleotide addition. However, when RNA primers are removed, and the gaps filled, DNA polymerase cannot fill the gap left by the primer at chromosome termini.

Telomeres, the nucleoprotein structures at the end of eukaryotic linear chromosome, facilitate solutions to both the end replication and the end protection problems. Human telomeres contain double stranded DNA that terminates with a single stranded G-rich overhang. The double stranded portion ranges 10-15kb in length while the overhang is approximately 50–500 nucleotides long. Human telomeric sequence consists of tandem repeats of 5'-TTAGGG-3'.(1, 5) These long regions of repetitive DNA are non-coding and are genetically non-

essential. Telomeres mitigate the end replication problem as they prevent the loss of genetically relevant DNA sequences during DNA replication. Telomeres accomplish this in two ways 1) as a substrate of the enzyme telomerase, the chromosomal termini can be lengthened(6) and 2) instead of losing genetic information, it is the genetically irrelevant telomeres which are loss with each cell division(4). Furthermore, when the telomeres become critically short or otherwise dysfunctional, cells undergo cellular senescence preventing further erosion and subsequent loss of genetically relevant DNA sequences.(7)

Cellular senescence is a state of permanent proliferative cell cycle arrest with maintained metabolic activity.(8) Cellular senescence acts as a safeguard preventing damaged or otherwise unhealthy cells from continuing to proliferate and possibly transforming into cancer. Thus, senescence is considered protective against malignant transformation. There are two types of cellular senescence: replicative senescence and stress-induced premature senescence. Replicative senescence is telomere dependent and is induced when telomeres become too short to be functional. Stressed induced premature senescence occurs when damaging external stimulus such as oxidative stress causes acute cell cycle arrest without telomere shortening. Acute disruption of the telomere structure can trigger stress-induced senescence as well.(8, 9) Cells which are unable to overcome telomere shortening have limited replicative capacity, because short telomeres can cause the onset of replicative senescence.

Telomeres also solve the end protection problem. The telomeric DNA-protein complex “encapsulate” the chromosome termini, forming structures which distinguishes chromosome ends from DNA strand breaks.(2) In human cells, the G-overhang intertwines with the double-stranded region to form a ‘d-loop- t-loop’ capping structure(10) with the help

of several proteins collectively called the shelterin complex(2) (Figure 1.2). The shelterin complex is made up of 6 proteins: telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), TRF1-Interacting Nuclear protein 2 (TIN2), repressor activator protein 1 (RAP1), protection of Telomeres 1 (POT1) and TPP1. Of the six proteins, three are DNA binding proteins. TRF1 and TRF2 bind double stranded region of the telomeres while POT1 binds the single strands. Moreover, members of the shelterin complex have also been shown to inhibit DNA damage pathways directly.(5, 11, 12) Disruption of the binding of TRF2 or POT1 to the telomeres, independently elicits DNA damage response via ataxia-telangiectasia mutated (ATM) or ataxia-telangiectasia and Rad3-related (ATR) pathways respectively.(11) TRF2 is thought to inhibit the ATM pathway through its interactions with signaling kinase, Chk2 of the ATM pathway. When Chk2 encounters TRF2 at the telomeres, the kinase binds and phosphorylates TRF2. Upon phosphorylation of TRF2, the TRF2-Chk2 complex dissociates from the telomeres, thus repressing Chk2 presence at the telomeres.(12) Pot1 inhibition of ATR pathway requires interaction with TPP1.(13) Disruption of the shelterin complex results in "capping" structure being dismantled and the recruitment of DNA damage proteins to the telomeres. "Uncapped" telomeres are subjected to non-homologous end joining generating telomere-telomere fusion events and dicentric chromosome(14), and results in chromosome instability, subsequent cell cycle arrest, and apoptosis.(1, 5) In addition to facilitating the formation of a protective structure at the chromosome termini and inhibiting DNA repair mechanisms, shelterin complex also regulates the extension of the telomeres. For example, POT1 regulates telomere elongation(15, 16) In summary, telomeres and maintenance of the telomeres play important roles in chromosomal stability, proliferation, cellular survival and apoptosis.

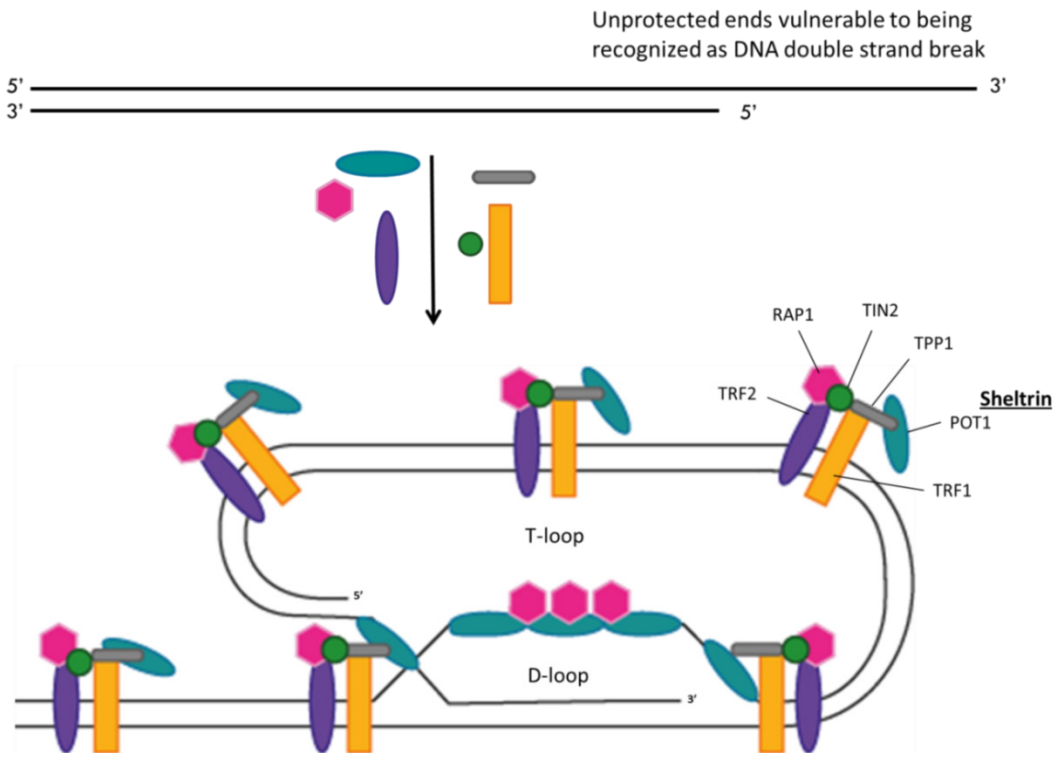

Figure 1.2. The Shelterin Complex prevents detection of chromosome termini as double strand breaks. The shelterin complex, consisting of 6 telomere-associated proteins, form a capping structure at the end of the telomere, protecting chromosome termini from being recognized as double strand break. The protein complex consists of POT1, RAP1, TIN2, TPP1, TRF1 and TRF2. The G overhang invades into the double stranded region and binds with one strand of dsDNA displacing the other creating a "displacement-loop" and T-loop structure. POT1 binds the single strand region of the displacement loop.

B. Telomerase

Telomere length is primarily maintained by a specialized ribonucleoprotein called telomerase.(17, 18) Cells with no or low levels of telomerase activity experience telomere shortening with each cell division due to end replication problem. Telomerase is conserved in vertebrates. Human telomerase activity is present in stem, germ line and embryonic cells; however, telomerase activity is not detected or is barely detectable in normal somatic tissue.(19-21) Biochemically, the minimal requirements for an active telomerase complex are the protein catalytic subunit telomerase reverse transcriptase (TERT) and the telomerase

RNA component (TERC). TERT contains a reverse transcriptase domain that uses the RNA template of TERC to extend the telomere.(22-25) Furthermore, TERC aids in catalysis by orienting the primer-template duplex in the enzyme active site.(26) In vivo, other accessory proteins are needed to reconstitute telomerase activity.(27) Members of the shelterin complex aid in the recruitment of telomerase to the telomeres and the processivity of the enzyme.(28-30) TIN2-TPP1 recruits telomerase to the telomeres.(29) TPPI-POT1 telomere complex regulates telomerase processivity though control of primer dissociation and translocation.(28, 30) The assembled telomerase complex extends the 3' end in the 5' to 3' direction. Telomerase extension of telomeres occurs in three catalytic steps: substrate binding, reverse transcription of the template, and translocation of the nascent DNA product to the start of the template (Figure 1.3). The translocation step allows for the enzyme to continue incorporating nucleotides, hence telomerase is a processive enzyme.(31) The C-strand is then synthesized by DNA polymerase. Mammalian CST trimeric complex is crucial in coordinating G-stand synthesis by telomerase and C-strand synthesis by DNA polymerase-α. Assembly of the three protein subunits of human CST, CTC1-STN1-TEN1, at the 3' overhang of the telomeres inhibits telomerase and simultaneously mediates C-strand synthesis through a physical interaction with DNA polymerase-α.(32, 33)

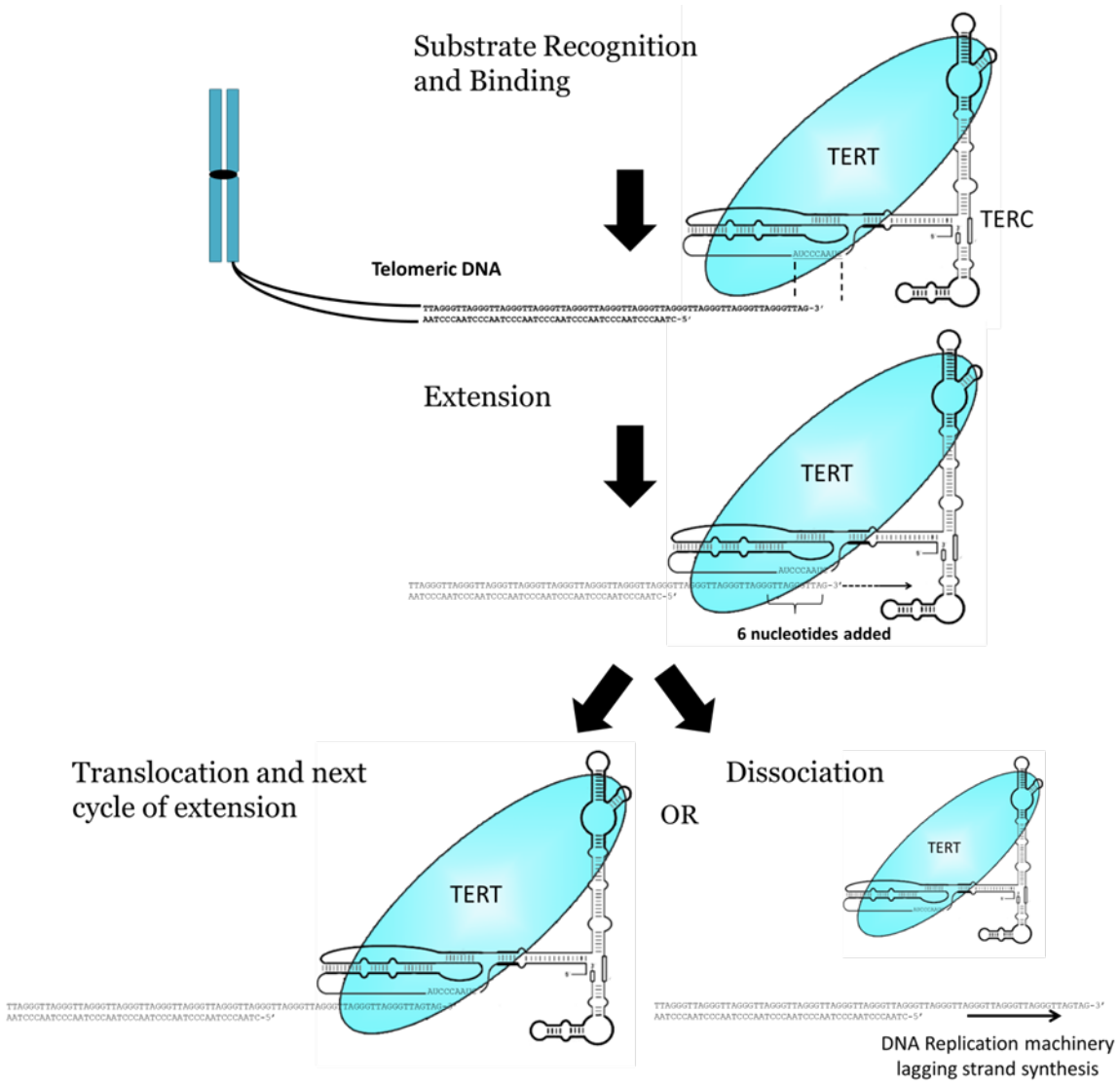

Figure 1.3. Mechanism of telomere elongation by telomerase. The assembled telomerase complex consisting of telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) polymerizes telomeric DNA. Telomerase recognizes telomeric DNA through complementary binding with the RNA template. Reverse transcription domain incorporates 6 deoxynucleotide triphosphates extending telomeric primer. After synthesis, telomerase either translocates on the DNA or dissociates from the DNA completely. Translocation allows for further elongation and subsequent processive addition until dissociation occurs.

Consequently, telomerase extension of the telomeres increases cellular proliferation capacity as it counteracts telomeres shortening and subsequent replicative senescence. Furthermore, the majority of cancer cells up regulate telomerase expression, which contributes to cancer cell immortality. In contrast, normal human somatic cells repress telomerase activity and therefore have limited replicative capacity. The difference in

telomerase activity in somatic and cancer cells suggests up regulation of telomerase is a critical step in oncogenesis.(34-36) The down regulation of telomerase during differentiation and continued repression in somatic cells coupled with telomerase re-activation during tumorgenesis, suggest that telomerase expression is tightly regulated and changes in regulation of telomerase could contribute to the development of disease.

1. Telomerase RNA Component: TERC

Human telomerase RNA is expressed in many tissues and throughout development, regardless of the presence of telomerase activity. Due to the large size of this RNA, there is difficulty attaining high resolution tertiary structure using conventional methodology and comprehensive high-resolution data to define the structure. The current models of hTERC secondary structure are based on sequence analysis, compensatory mutational analysis and footprinting (Figure 1.4).(37-39) Human TERC is a non-coding RNA transcribed by RNA polymerase II. The full length transcript, which varies substantially in length and primary sequence in eukaryotic species, is 451 nucleotides in human. Human TERC has four highly conserved domains: the core domain, conserved region 4 and 5 (CR4-CR5), conserved region 7 (CR7), and the Box H/ACA domain. Structurally, the 5' region of the RNA which includes the core domain and the CR4-CR5 domain is essential for enzymatic activity and processivity while the 3' region of the RNA, which includes the conserved CR7 region and Box H/ACA, is vital for proper cellular localization and processing.(38) The core domain consists of the pseudoknot domain, template boundary element and template. The template region is an 11 nucleotide sequence, 5'-CAAUCCCAAUC-3'. The first 5 nucleotides of the template is an alignment domain, which the enzyme uses to align the primer. The second 6 nucleotides of the template are used by TERT as the template for reverse transcription. The

template boundary element plays a significant role in ensuring that reverse transcription is limited to the six template residues. The pseudoknot binds TERT and plays an important role in catalysis; mutagenesis of the region dramatically reduces enzyme activity. TERT also binds the CR4-CR5 domain. TERT binds the core domain and CR4-CR5 domain independently and in a non-cooperative manner.(40) Together, TERT in complex with the core domain and CR4-CR5 domain of TERC, are the minimal requirements needed to reconstitute telomerase activity *in vitro*.(41)

The 3' H/ACA domain contains a hinge box and a 5'-ACA-3' sequence that is recognized by H/ACA associated RNA binding proteins: a heterotrimer consisting of Dyskerin, Nhp2 and Nop10 (nucleolar protein 10), and a fourth factor Gar1. The H/ACA domain also contains a Cajal body localization signal (CAB box).(42) The CAB box allows for binding of TERC to telomerase Cajal body protein 1 (TCBP1) and consequent localization to the Cajal bodies where the telomerase complex is packaged for delivery to the telomeres. Accumulation of hTERC in Cajal bodies appears to be dependent on the presence of hTERT, as hTERC localizes to these bodies in hTERT positive cells with telomerase activity but does not, in cells with little to no hTERT such as primary differentiated cells.(43) In close proximity to the H/ACA motif is the CR7 domain which plays a significant role in TERC accumulation through its participation in loading 2 complete sets of H/ACA proteins onto the H/ACA domain. H/ACA and C7 are essential for TERC stabilization, accumulation and proper cellular localization. Mutations in the H/ACA box and CR7 region destabilize TERC and prevent its localization to the nucleus *in vivo*.(44)

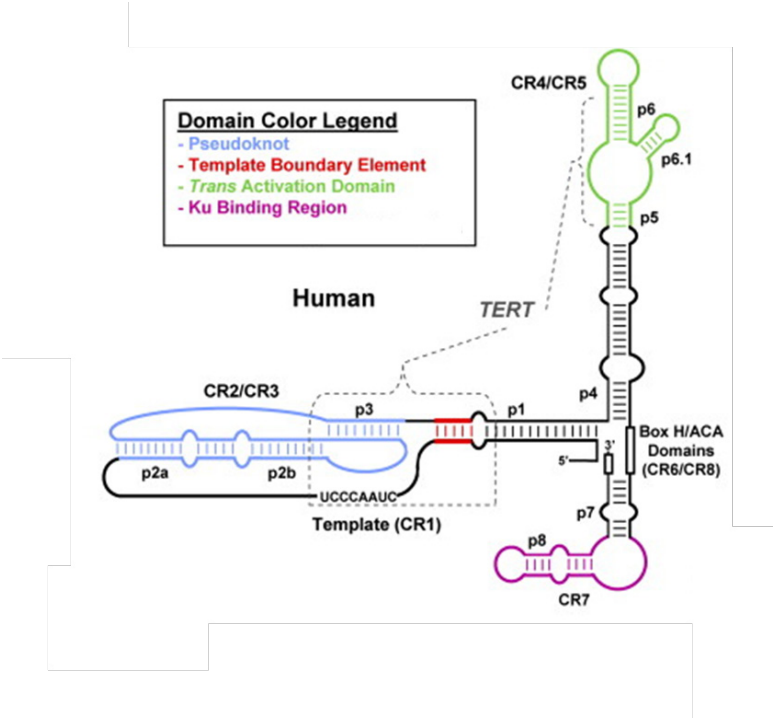

Figure 1.4. Phylogenetically derived secondary structure of human telomerase RNA component. hTERC is a 451 nucleotide RNA with four highly conserved domains: 1. the core domain which contains the pseudoknot domain, template and template boundary element, 2.conserved region 4 and 5 (CR4-CR5), 3.conserved region 7 (CR7), and 4.the Box H/ACA domain. hTERT interacts with hTERC at two separate locations, the core domain and the CR4-CR5 domain, highlighted by the "grey" dotted lines.

2. Telomerase Catalytic Subunit: TERT

Human TERT is an 1132 amino acid reverse transcriptase with four distinct domains: telomerase essential N-terminal (TEN) domain, TERT RNA-binding (TRBD) domain, reverse transcriptase (RT) domain and the TERT C-terminal extension (CTE) domain (Figure 1.5).(27)The TEN domain directs telomerase to the telomere. The TEN domain also binds and remains associated with telomeric DNA during catalysis. Interestingly, the TEN domain includes amino acids essential to telomerase activity which are not involved in DNA binding. TRBD interacts and binds TERC through an RNA recognition motif. The RT domain is the site for reverse transcription. The RT domain can be further divided into two compartments, the fingers and palm. The fingers, motifs 1-A, interacts with the telomeric DNA substrate.

The palm, motifs B-E, contains the catalytic site. The CTE domain forms the thumb which contributes to activity and cellular localization.(6, 27, 45, 46) TERT has three chief localization sites: mitochondrial signaling motif aa1-20 and two identified nuclear localization signals at aa222-240 and aa649-650. The nuclear localization signal at aa222 includes a phosphorylation site (S227).(47) TERT has a nuclear export signal in the CTE domain.(48)

Regulation of telomerase activity is closely related on the regulation of TERT. In tissues where telomerase is active, TERT is expressed and, in tissues were telomerase activity is not detected, TERT is not expressed. Therefore, expression and activation of the catalytic subunit appears to be the major limiting step of enzyme action. TERT is subjected to regulation at transcription, splicing, post-translational modification and changes in subcellular location.

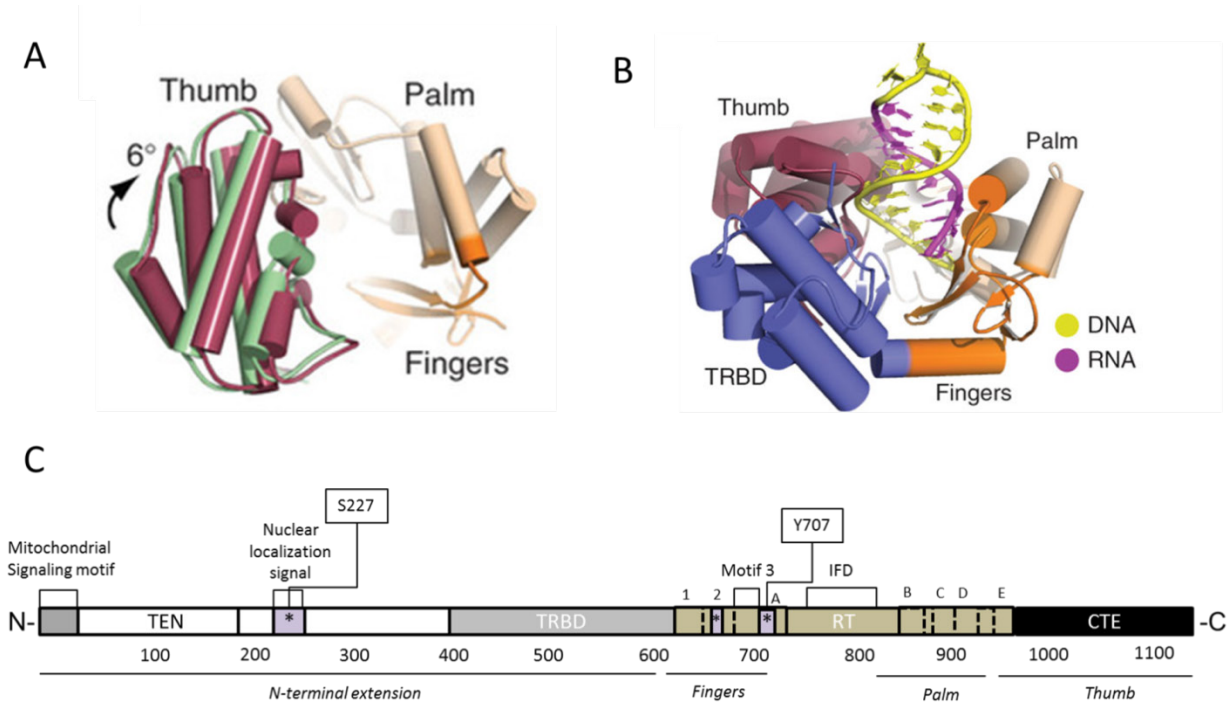

Figure 1.5. Structure of human telomerase reverse transcriptase. A) Crystal structure of free *Tribolium castaneum* TERT.(49) B) Crystal structure of *Tribolium castaneum* TERT in complex with a RNA-DNA hairpin designed to resemble RNA-template region bound to telomeric DNA.(49) C) hTERT is n 1132 amino acid protein with four distinct domains: The telomerase essential N-terminal (TEN) domain, TERT RNA-binding (TRBD) domain, reverse transcriptase (RT) domain and the TERT C-terminal extension (CTE) domain. The RT and CTE form the fingers, palm and thumb of the polymerase. Other notable artifacts are motif 3 and IFD. TERT has three primary localization sites. The first 20 amino acids form a mitochondrial signaling motif and there are two identified nuclear localization signals at aa222-240 and aa649-650. The nuclear localization signal at aa220 includes a phosphorylation site (S227). There is also a phosphorylation site at Y707 implicated in nuclear export.

i. Transcriptional Regulation.

In human, TERT is primarily regulated via transcription. Human *TERT* is a single copy gene, located on chromosome 5, which has 15 introns and 16 exons spanning about 40kb. The *hTERT* promoter does not contain a TATA or CAAT box. However, the promoter includes binding sites for transcription factors including tumor suppressor genes such as WT1, p53 and p21and oncogenes such as myc and Sp1.(50-55) Likewise Wnt/ β-catenin signaling partially regulates telomerase via β-Catenin interaction with the *TERT*

promoter.(56) *TERT* is also subjected to hormonal regulation. Estrogen can activate TERT via indirect action at the promoter region. Ligand activated estrogen receptor -α (ERα) can bind the estrogen-responsive element (ERE) in the *TERT* promoter, resulting in up regulation in telomerase.(57)

Within the last few years, it was discovered that TERT up regulation in many types of cancer was the result of *TERT* transcriptional activation via mutations in the *TERT* promoter.(58-62) In the analysis of 168 human cell lines isolated from metastatic melanomas, Horn et. al observed recurrent ultraviolet light induced signature somatic mutations in the promoter region of *TERT*. Horn et al also found that the majority of those mutations occurred at two positions in the *TERT* promoter and also generated binding motifs for specific transcription factors.(63) Two common somatic mutations identified were C228T and C250T. The mutations were believed responsible for increased *TERT* promoter activity and increased levels of TERT mRNA.(64) Chromosome remodeling is also a mechanism for transcriptional regulation of *TERT*. The *hTERT* promoter is located in a highly condensed chromatin domain and the chromatin environment and epigenetic status are believed to play regulatory role in TERT transcription.(65, 66) Furthermore, the *TERT* promoter contains a cluster of potential methylation CpG sites possibly involved in controlling *TERT* transcription. The absence of DNA methylation and the association of these unmethylated sites with active chromatin marks in the *TERT* promoter region may allow for the transcription of *TERT*.(67)

ii. Regulation via Splicing.

Human TERT mRNA has a number of splice variants, all of which identified are inactive.(52, 68) Using primers targeted at the reverse transcriptase domain, Villa et al. found

different splice variants in telomerase-positive and telomerase-negative melanomas. Splicing of TERT is believed to be non-random and regulatory. Supporting the regulatory role of splicing is the observation of a tissue-specific pattern of TERT alternative splicing which exists during human development. The tissue specific pattern of TERT is also observed in specific hormone responsive adult tissues. Moreover, the hTERTαvariant is a dominant-negative inhibitor of telomerase.(52)

iii. Post-translational Modifications.

TERT mRNA levels does not always correlate with telomerase activity. The enzyme is also regulated by post-translational modification. Post-translational modification affects enzyme activity, stability and also cellular location. Hydrogen peroxide, which represses telomerase during transcription, also suppresses telomerase post translationally via the Akt-pathway.(69) In addition, hydrogen peroxide may down regulate telomerase activity though direct action on the enzyme.(70) Apoptosis may also regulate TERT post transcriptionally. TERT has 2 non-canonical caspase sites: caspase 3 and caspase 6 sites.(71) Additionally, ubiquitination may regulate TERT through interactions with C terminus of Hsc70-interacting protein (CHIP). Interaction with CHIP, leads to decreased TERT nuclear import and increased degradation of TERT.(72) Furthermore, phosphorylation of TERT has been shown to modulate activity and regulate cellular location. C-Abl phosphorylation of TERT dramatically decreases telomerase activity(73, 74) while Akt phosphorylates TERT leading to activation of telomerase activity (75) and nuclear import (47). Two Akt phosphylation target sites were identified, serine 227 and serine 823.(75) Serine 227 is located in a bipartite nuclear localization signal and phosphorylation of the S227 is required for nuclear import (Figure 1.5).(47) TERT Nuclear Export is also modulated by phosphorylation.

Phosphorylation of tyrosine707 site by Src family kinase results in TERT being transported to the cytoplasm (Figure 1.5).(76) Notably, the tyrosine phosphatase Shp-2 can prevent Y707 phosphorylation and TERT export from the nucleus.(77)

In addition to the regulation of TERT, telomerase assembly is also highly regulated. Telomerase assemblage is a dynamic process which involves the formation of populations of pre-assembled TERT and TERC complexes kept separately during much of the cell cycle until the time needed, believed to be S-phase. Telomerase biogenesis begins with TERC binding the H/ACA heterotrimer and GAF1 and subsequently moving from nucleolus to Cajal bodies where maintained until S-phase(27). In early S-phase, TERT is imported into the Cajal body and telomerase assemblage occurs. During mid-S phase, the Cajal body localizes near the telomeres, and the telomerase complex emerges and elongates the telomeres. Once telomere elongation is complete, the telomerase complex dissociates and the components separated. Nevertheless, telomerase activity can be detected in all phases of the cell cycle. Interestingly, human primary cells which lack Cajal bodies are efficient in telomere length extension, so the Cajal bodies may not be necessary.(27) In summary, the regulation of telomerase, its assembly and action at the telomeres is complex and closely regulated system. Telomerase maybe tightly regulated to prevent uncontrolled telomerase activity which increases the likelihood of tumorgenesis.

C. Comparison of Human and Mouse Telomere Biology

Telomerase is highly conserved between human and mouse. Both holoenzymes produce the 5'-TTAGGG-3' telomeric sequence. There are substantial similarities in the sequences and motifs of mouse TERT compared to human, and both proteins are 127kDa.

(31, 78) While the telomerase RNAs differ in length (451 nucleotides in human and 397 nucleotides in mouse),(79) the primary sequences of mTERC and hTERC are about 65% similar. Both telomerase RNAs contain homologous secondary structure of template region, pseudoknot domain, H/ACA box and CR4-CR5 helices.(80) Human and mouse telomerase also have considerable differences as well. Human telomerase is more active than mouse.(80) There are 2 marked differences in mTERC and hTERC identified as being responsible for mouse telomerase lower activity.(79, 80) The differences identified are 1) mTERC having only 2 nucleotides upstream its template while hTERC has 45 nucleotides and 2) the alignment domain of the template region is 2 nucleotide in mouse and 5 nucleotides in human.(80) In addition to differences in telomerase RNA, regulation of human and mouse telomerase also differs. Telomerase is active in germ-line, stem cells and embryonic tissues in both human and mouse.(20, 81) While TERC expression remains relatively constant throughout development, human and mouse TERT are both down regulated during differentiation and proliferation.(82) However, TERT and TERT mRNA levels are still detectable in mouse differentiated tissues but not in most human differentiated tissues. In accordance, telomerase activity is readily detectable in mouse primary cultured cells but not detectable in most human normal somatic cells, exception includes hTERT being detectable at low levels in bone marrow and leukocytes.(34, 78) The presence of telomerase activity in mouse differentiated tissues is most likely responsible for mice having longer telomeres than humans. Telomeres range 2-15 kilobase pairs (kbp) in human to 10-60 kbp in the common laboratory mouse, Mus musculus.(31)

D. Non-Canonical Functions of TERT

Although, telomerase is conventionally viewed as a telomere protein and is primarily studied in relation to its role in telomere maintenance, scientific evidence suggests non-telomeric function of TERT. Recent reports have described TERT as having telomere-independent effects on transformation, stem cell biology, cell survival, DNA damage and gene expression.(83) While, telomerase is known to suppress senescence and facilitate cancer through telomere maintenance, TERT is believed to play a role in oncogenesis and promoting cell survival in cancer cells independent of its actions at the telomeres.(84) TERT can increase the efficiency of malignant transformation in ALT cells even when its ability to extend telomeres is disrupted.(85) Additionally, TERT overexpression in mice, where telomerase is widely expressed and telomeres are relatively long, resulted in increased spontaneous and carcinogen-induced cancers. Furthermore, TERT promotes cellular survival and apoptotic resistance in cancer cells via extra-telomeric function such as intermolecular interactions with tumor suppressor protein, p53 and poly(ADP-ribose) polymerase.(86, 87) In addition to its role in cancer development, TERT has effects in stem cell biology. Overexpression of TERT has been shown to increase stem cell proliferation.(88) Furthermore, TERT is believed to have a role in somatic cell reprogramming where TERT is shown to be activated. A study supporting TERT involvement in cellular reprograming, reported knocking down TERT in somatic cells decreased the efficiency of reprogramming somatic cells to induced pluripotent stem cells.(89) Mechanistically speaking, one way TERT may be involved in cellular proliferation, oncogenesis and stem cells is through its actions as a transcriptional factor. TERT can regulate numerous pathways associated with the cellular proliferation, cellular survival and the onset of cancer.(55) For example, TERT is a

transcriptional regulator of the tumorgenic Wnt/β-catenin signaling(90) and the NF-κB pathway(91) which is involved in inflammation, cell fate and immunity. TERT expression also modulates the P13K/Akt pathway (92)and pRb/E2F pathway(93), both of which are involved in cellular proliferation. The non-canonical function of TERT also extends to the action of TERT outside of the nucleus, as there is evidence that TERT localizes in the mitochondria.

1. TERT and the Mitochondria

Reports of extra-nuclear TERT being transported to the mitochondria following increased oxidative stress (94-96) disrupted the well-held convention that TERT was a nuclear protein. Prior to the last decade, TERT was studied almost exclusively as a nuclear protein. The TERT canonical role of telomere extension required its nuclear import and assemblage to form telomerase complex in the nucleus where telomeres are located. The discovery of the non-telomeric functions of TERT did not disrupt this convention, as the new roles of TERT as a transcriptional regulator or interacting with other nuclear-located proteins was based within the nucleus. The finding of TERT mitochondrial localization motif (aa1-20) and localization of the protein in the mitochondria raised questions about the distribution of TERT between the two cellular compartments and the function TERT had in the mitochondria.

TERT appears to be a predominantly nuclear protein with a major nuclear localization signal (NLS) at aa222-240 and another NLS in the C-terminal. Mechanisms involved in TERT localization are not completely understood but phosphorylation (47) and nuclear transport factors(97) are known to be involved. TERT nuclear export and mitochondrial import is mediated by ROS (Figure 1.6). Hydrogen peroxide induces nuclear export via

TERT phosphorylation at tyrosine707 and subsequent import into the mitochondria.(76, 94, 98) TERT interacts with mitochondrial import proteins including Tom 20.(99) Once in the mitochondria, TERT appears to play a protective role against oxidative stress. Mitochondrial TERT decreases ROS production, improves mitochondrial function and protects against ROS induced apoptosis.(100-102) Likewise, mitochondrial TERT binds mitochondrial DNA (mtDNA), preventing mtDNA from accumulating oxidative damage.(101) In addition to its protective role, TERT also plays a role in mitochondrial function. Mitochondrial TERT associates with the RNA component of mitochondrial RNA processing endoribonuclease *rMrP*, and this TERT- *rMrP* complex acts as an RNA-dependent RNA polymerase. TERT–*rMrP* complex produces *rMrP*-derived double-stranded RNAs that are further processed into small interfering RNAs (siRNAs) in a Dicer-dependent manner that controls the endogenous levels of *rMrP*.(103) Furthermore, nuclear TERT plays a role in mitochondrial biogenesis and function though its regulation of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha and beta (PGC-1α and PGC-1β). TERT-/- mouse model exhibited increased oxidative stress and decreased mitochondrial function via down regulation of PGC-1α and PGC-1β pathways.(100)

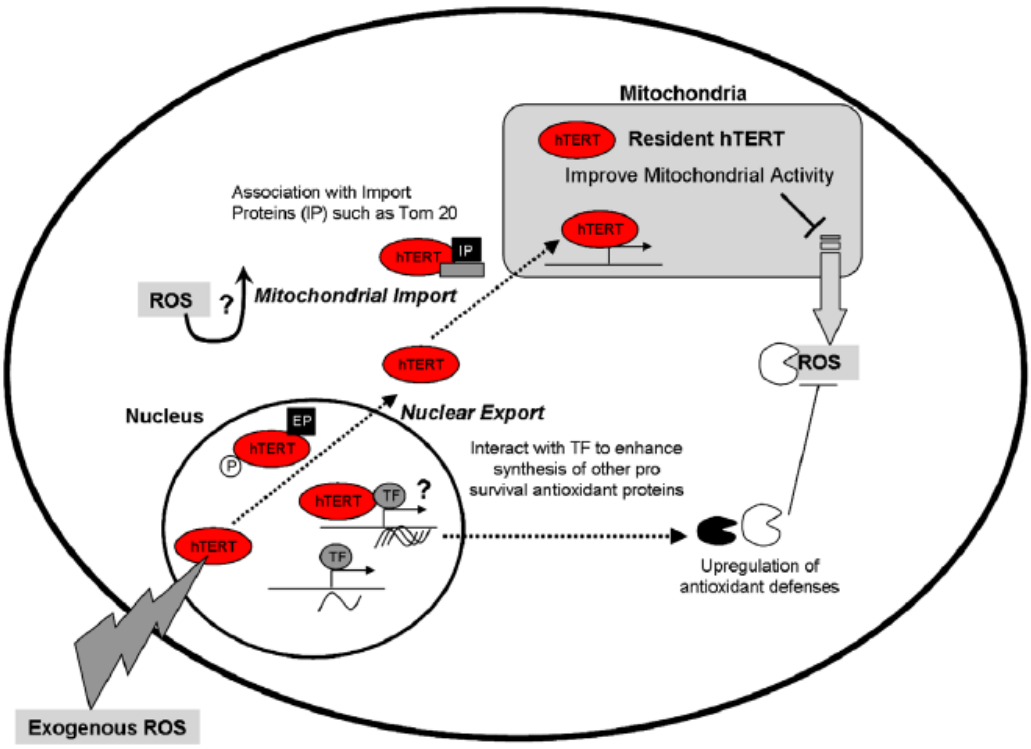

Figure 1.6. Schematic of mitochondrial TERT and feedback relationship with ROS. Oxidative stress induces nuclear export and mitochondrial import of TERT. While in the mitochondria, TERT protects mtDNA, improves mitochondrial activity and decreases ROS. Nuclear TERT also acts as a transcription factor, regulating expression of genes including those associated with mitochondrial function. Image adapted from Indran et al.(99)

E. The Roles of Telomere Dysfunction in Aging and Disease

Dysfunction of telomeres and telomere maintenance, have been implicated in the process of aging and the onset and progression of various diseases. One model for telomere involvement in aging and disease development suggests telomere dysfunction is a driver for genetic instability which results in either increased cellular aging or cancer (Figure 1.7).(5) The model stipulates that if telomeres become dysfunctional, a DNA damage response is activated and depending on whether tumor suppressor protein p53 is expressed, cells will either undergo p53 mediated cellular senescence or oncogenesis. While senescence is protective against tumorgenesis, senescent cells can also contribute to degenerative

pathologies often associated with age. Senescent cells accumulate in aged-tissues (104) and are thought to lead to pro-aging properties such as impaired tissue function and increased inflammation.(9) Accumulation of senescent cells may be the result of increased generation of senescent cells and/or decreased cellular turnover rates. Senescence protective role against cancer is partially dependent on the ability of tissues to clear the damaged and potentially oncogenic cells which have become senescent, and subsequently replace these cells to re-establish cell numbers. Thus, the accumulation of senescent cells with age could be a consequence of the cell replacement system becoming less efficient and/or the regenerative capacity of progenitor cells decreasing.(9) Furthermore, senescent cells may also contribute to ageing and disease as they undergo major changes in their secretesome that result in increased pro-inflammatory cytokines and matrix metallo-proteinases.(105) The mechanisms which contribute to aging and cancer are complex, and telomere biology plays a central role.

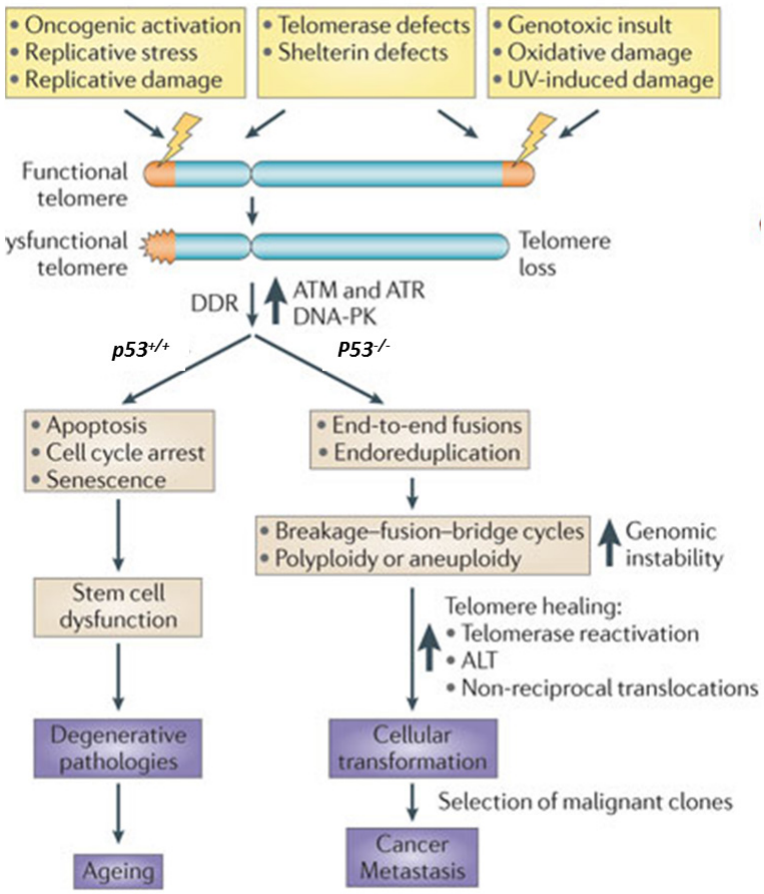

Figure 1.7. Schematic depicting model for the role of Telomere Dysfunction in aging and cancer. Many factors contribute to telomeres becoming dysfunctional including replication, disruption in telomere maintenance or external stresses such as oxidative damage. Dysfunctional telomeres, either because of shortening or uncapping, elicit a DNA damage response (DDR). The DDR is activated via ataxia-telangiectasia mutated (ATM) or ataxia-telangiectasia and Rad3-related (ATR) pathways and may drive cells towards two opposing outcomes depending on the p53 status. If tumor suppressor protein p53 is present, cell cycle arrest or apoptosis is induced. The onset of senescence or cell death results in stem cell dysfunction and subsequent tissue degeneration and organ failure. However, if the cells are p53 deficient, the damage proceeds unchecked and causes genomic instability. If a mechanism to "fix" the telomeres is achieved in these p53 deficient cells, stable malignant clones will be generated resulting in the formation of metastatic tumors. Figure adapted from Blasco 2011.(5)

Aging is considered a time-dependent functional decline. Telomeres, the protective structures at the end of the chromosomes, are particularly vulnerable to age-related deterioration.(9) Many age-related diseases such as atherosclerosis(106-108) and diabetes(109, 110) have a telomere attrition phenotype. Telomeres play an important role in

chromosome stability, cellular survival and proliferation. In humans, the length of telomeres in normal somatic cells and stem cells shortens with time. Telomerase has little to no expression in normal somatic cells and while expressed in stem cells the levels are not sufficient to completely overcome telomere loss. As these cells divide, their telomeres shorten because DNA polymerase is unable to replicate the telomeres completely. Telomeres erode in proliferating cells until telomeres become critically short and cells undergo cell cycle arrest to prevent further loss of the telomeres. This shortening of the telomeres has a profound negative effect on the functioning of tissues. Telomere erosion is a contributing factor in stem cell exhaustion,(111) one of the hallmarks of aging. Stem cells are significant in the maintenance of tissue integrity and tissue repair. Stem cell loss of function results in the reduced ability to regenerate tissue, often resulting in accumulation of tissue damage. Therefore, telomere erosion related reduction of stem cell function may contribute to the aging process. A similar effect of telomere attrition is observed for the immune system.(112) The ability of the immune system to respond to infections or vaccines diminishes with age. Immuno-senescence has been identified as one cause for loss of immunity, and telomere attrition can trigger for senescence in immune cells. Notably, telomere attrition in leucocytes is a phenotype of age-related disorders such as cardiovascular disease (CVD).(113) Not surprisingly, a study reported individuals with short telomeres were eight times more likely than those with long telomeres to die from infectious diseases and three times more likely to die from cardiovascular disease.(114) Although the association of senescence with loss of function and aging appear damaging, unlimited proliferation can also lead to human disease.

Cancer is a heterogeneous disease characterized by uncontrolled proliferation. For cancer cells to achieve unlimited replicative capacity, cells must bypass senescence and

inhibit apoptosis. Since, telomeres act as biological clocks that limit proliferative potential, cancer cells have developed mechanisms to maintain the telomeres and evade cell cycle arrest. In other words, the viability of cancer cells is reliant on the ability of these cells to maintain their telomeres. Activation of telomere maintenance is a mechanism cancer cells use to accomplish limitless replicative potential.(115) Approximately 85% of cancers achieve telomere maintenance through up regulation of telomerase and the remainder of cancers maintain their telomere length through recombination in a process called alternate lengthening of telomeres (ALT).(116) Although certain cancers have a higher incidence of ALT such as osteosarcomas with 59% of cases exhibiting ALT characteristics.(117) The central role telomere maintenance plays in oncogenesis and the reduced expression of telomerase in normal somatic cells, have made telomerase a potential target for anti-cancer therapies.(118, 119)

1. ROS, Telomere Dysfunction and Age-related Disease

The recent observations demonstrating a relationship between telomerase and reactive oxygen species (ROS) creates another layer to the telomere dysfunction model for disease and aging. Independent of telomere dysfunction, ROS has also been associated with the process of aging and age-related diseases. The free radical theory of aging states that accumulation of oxidative damage in tissues causes functional decline in biological systems contributing to aging.(120) Numerous studies reporting a complex interconnected relationship between telomere maintenance and ROS raise new questions about whether this relationship plays a causative role in the disease states associated with ROS and telomere defects. One such disease is atherosclerosis.

Atherosclerosis and the resulting cardiovascular disease (CVD) are the leading cause of death worldwide.(121) Atherosclerosis is the medical condition in which lipids, cholesterol, cellular debris and calcium build up inside arteries resulting in the narrowing of the arterial lumen. The disease is characterized by endothelial cell dysfunction and increased proliferation and migration of vascular smooth muscle cell (VSMC). Complications of atherosclerosis include stroke, heart attack, coronary artery disease, and death. Although risk factors have been identified, the causes of atherosclerosis remain poorly understood. Reactive Oxygen Species (ROS) and telomere dysfunction have been independently identified as possible contributors to atherogenesis. Telomere attrition is pronounced in regions of the vasculature most prone to developing atherosclerosis (108, 122-128) and elevated oxidative stress in common in atherosclerosis.(129-134) The existence of an interconnecting relationship between ROS, telomere dysfunction and atherosclerosis suggests that the roles of both ROS and telomere biology in disease pathology are not mutually exclusive. One possibility is that telomere dysfunction is a critical downstream target of ROS-dependent pathways which contribute to disease progression (Figure 1.8). Several reports support the hypothesis that oxidative signaling promotes telomere dysfunction causing phenotypes associated with atherosclerosis. Chronic oxidative stress and pro-atherogenic factors such as oxidatively modified low-density lipoprotein OxLDL inhibit telomerase activity, causing telomere erosion and subsequently senescence. (135)(136)(137) Moreover, anti-atherosclerotic agents activate telomerase and decrease oxidative stress.(138-141) Accordingly, increased telomerase activity has been shown to decrease the senescent phenotype in vascular cells involved in atherogenesis.(137)

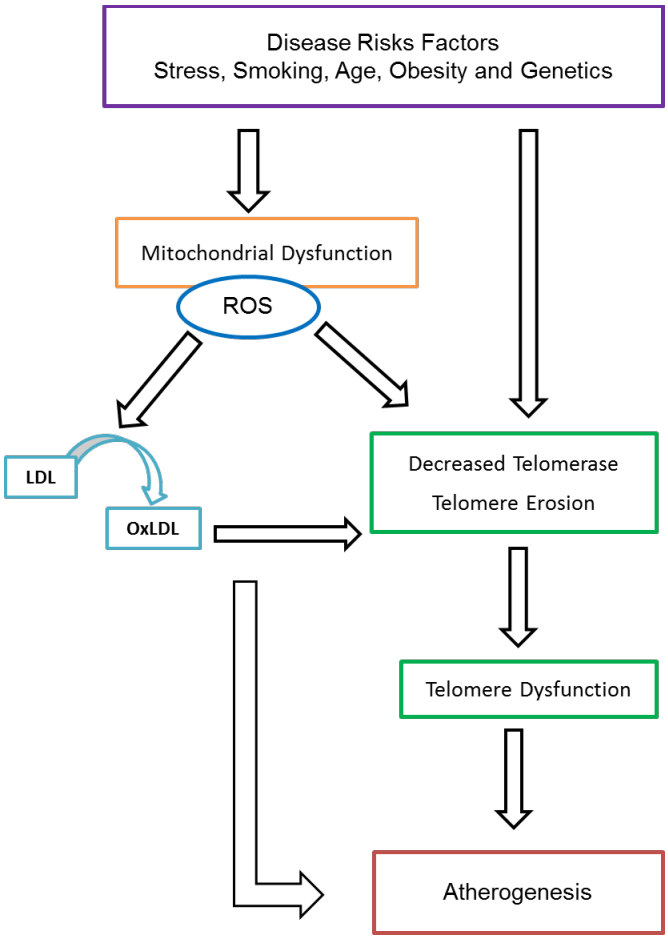

Figure 1.8. Schematic of proposed theory of Telomere Dysfunction and ROS involvement in atherogenesis. The underlying mechanisms responsible for the onset of atherosclerosis are unknown, however, ROS and telomere dysfunction have been independently identified as possible contributors. Atherosclerosis disease risk factors are known to cause elevated oxidative stress and telomere attrition, both phenotypes of the disease. Since ROS and oxidatively modified low-density-lipoproteins OxLDL are modulator of telomerase, the enzyme which elongates the telomeres, the model proposes that telomere dysfunction could be a downstream target of ROS-dependent pathways critical to disease development.

F. SOD2+/- and SOD1+/- Mouse models

In order to further the understanding of telomere dysfunction, oxidative stress and disease pathology, we examined the telomere biology of aortic smooth muscle cells (ASMC) isolated from mouse models of oxidative stress and CVD.(142-144) The models are haploid deficient in either cytoplasm superoxide dismutase (SOD1+/-) or mitochondrial superoxide dismutase

(SOD2+/-), two main cellular antioxidants. Superoxide dismutase (SOD) catalyzes the conversion of superoxide to hydrogen peroxide which is subsequently converted by catalase to oxygen and water. Thus, the localized deficiency of the enzyme allows for the accumulation of superoxide in specific cellular compartments. The SOD2+/- mouse model shows age related decline in mitochondrial function.(145) The SOD2+/- mouse also exhibits increased apoptosis in cardiomyocytes, cardiac mitochondrial dysfunction and decreased left ventricular function.(144, 146) Interestingly, Zhou et al. found that SOD2+/- mouse exhibited aortic stiffening at 16 month, but not at 4 month. SOD1+/- mouse did not have the aortic stiffening phenotype.(146) Aortic stiffening is one primary cause of CVD including increased systolic and pulse pressures, increased left ventricular hypertrophy and diastolic dysfunction, and congestive heart failure.(147) In the ASMC, SOD activity levels were reduced 50% and 42% for SOD1+/- and SOD2+/- respectively and protein levels were reduced 50% in both SOD1+/- and SOD2+/- compared to wild type (WT). Notably, SOD2+/-ASMC showed greater oxidative stress than SOD1+/-ASMC.(143) Analysis of ASMC isolated from SOD2+/- mice allowed Zhou et al to characterize the changes that occurred in the vasculature at a cellular level.(146) Studying the telomere phenotype of SOD2+/- and SOD1+/- ASMC provides a unique opportunity to study the relationship between oxidative stress, telomere dysfunction and vasculature dysfunction in a SOD1+/- model where disease phenotype did not occur and another model, SOD2+/- where disease phenotype occurred. We also have the chance to examine the telomere phenotype in ASMC before the incident of disease (4 month) and after disease developed (16 month). If shorten or dysfunctional telomere is a critical contributing consequence of oxidative stress, one would expect to see telomere dysfunction present in SOD2+/- ASMC.

G. Importance of this study

In order to create better detection methods, and preventative and therapeutic approaches, a greater comprehension of pathophysiology in disease development is necessary. Thus, the connection between ROS and telomere dysfunction and how it contributes to disease pathology must be studied. The role telomere dysfunction plays in the development of disease and progression of aging is physiologically relevant. How ROS modulates telomeres and telomere maintenance is central to our efforts of increasing knowledge of the underlying mechanisms which cause disease. Our work will contribute to the development of rational therapeutic and diagnostic approaches.

H. Overview of this Thesis

The research presented in this thesis examined the effects of oxidative stress on telomeres and telomerase and whether the relationship between ROS and telomeres contributes to CVD development. For much of this study, we used ASMC isolated from oxidative stress mouse models that are established research tools for the study of ROS and CVD. The goals are highlighted by the following specific aims of the study.

Aim 1: Determine if oxidative stress causes telomere dysfunction. In Chapter II, we examined the state of the telomeres in ASMC isolated from mouse models of elevated oxidative stress. Our study of the telomeres included measurement of telomere length, the presence of DNA damage proteins at the telomeres and whether the telomeres sustained oxidative damage.

Aim 2: Determine the effects of oxidative stress on telomerase. In Chapter II, we examined whether or not oxidative stress had any effects on the ability of the telomerase to

maintain the telomeres. In Chapter IV, we furthered our investigation by examining how oxidatively damaged nucleotides affected telomerase activity.

Aim 3: Determine if anti-atherosclerotic agents known to affect oxidative stress also have an effect on telomerase. In Chapter III, we investigated the effects of NADPH oxidase inhibitors and hormones on telomerase activity.

CHAPTER II: THE EFFECTS OF OXIDATIVE STRESS ON TELOMERE MAINTENANCE

A. Introduction

Oxidative stress can affect telomere biology in two recognized ways: by direct oxidation of the telomeres and through regulation of telomerase. Telomeres are particularly redox sensitive due to the high guanine content and oxidative stress is known to exacerbate telomere erosion. Accumulation of oxidative damage at the telomeres has been associated with telomere shortening in yeast(148), mice(149) and humans(150). The increased rate of telomere loss may be the result of increased single- and double- strand breaks(149) as well as oxidative damage at the telomeres disrupting the binding of telomere-associated proteins such as TRF1 and TRF2.(151)

In addition to direct damage to telomeric DNA, reactive oxygen species (ROS) can regulate telomerase activity and the cellular location of TERT. ROS, particularly, hydrogen peroxide, down regulates telomerase via transcription and the Akt pathway.(69) Additionally, hydrogen peroxide induces nuclear export of TERT via the Src-family pathway(76, 98) and localization of TERT in the mitochondria (94-96). Moreover, the relationship between ROS and TERT exhibits a feedback loop. TERT modulates ROS as evidenced by reports that down regulation of TERT increases cellular oxidative stress.(100, 101) Down regulation of TERT by shRNA caused an increase in mitochondrial ROS and the TERT null mice model exhibited increased oxidative stress. (100, 101) TERT might regulate cellular ROS and mitochondria dysfunction through its role as a transcriptional regulator of PGC-1α and PGC-

1β pathways.(100) PGCs are master regulators of mitochondrial biogenesis and function as well as cellular metabolism.

Given their interrelationship, the roles ROS and telomere dysfunction play in disease pathology may not be mutually exclusive. The relationship between ROS and telomere dysfunction could be part of the underlying mechanisms which contribute to disease. Supporting this hypothesis is the simultaneous occurrence of oxidative stress and telomere dysfunction in many diseases and the fact that many disease risk factors such as age, smoking and stress increase oxidative stress and telomere attrition rates. In cardiovascular disease (CVD) for example, both telomere dysfunction (108, 123-128) and ROS(129-133) have been implicated in disease etiology. Evidence supporting direct involvement of telomere dysfunction in CVD progression has been obtained from the telomerase negative, TERC-/- mouse model. The model exhibits several cardiovascular related phenotypes including hypertension, myocyte hypertrophy, left ventricular failure and dilatation, and sudden death.(124) Additionally, studies to determine the basis of the senescence in atherosclerosis, found vascular smooth muscle cells (VSMC) isolated from human atherosclerotic plagues had increased oxidative stress, shortened telomeres and increased senescence compared to VMSC from vessels of subjects not suffering from atherosclerosis.(137) In addition, Matthews et al. found oxidants increased telomere shortening and senescence in cultured VSMC and up regulation of telomerase rescued VSMC from senescence.(137) Interestingly, oxidatively modified low density lipoprotein (oxLDL), an initiating factor in atherogensis, inhibits telomerase activity via the Akt pathway in endothelial cells.(135) Although the relationship between ROS and telomere dysfunction is recognized in atherosclerosis, it is not clear if this relationship is correlative or causative. It is possible that telomere erosion simply

correlates with increased oxidative stress and does not play a etiological role in atherosclerotic development. The role the relationship between ROS and telomere dysfunction plays in disease development has not been sufficiently studied and is poorly understood.

In an effort to further the understanding of telomere dysfunction, oxidative stress and disease pathology, we examined the telomere biology of aortic smooth muscle cells (ASMC) isolated from mouse models of oxidative stress. The haploid deficient superoxide dismutase SOD1+/- and SOD2+/- mouse models have decreased quantities of SOD, an important antioxidant which scavenges superoxide, in the cytoplasm and mitochondria respectively.

B. Results

1. Telomere attrition occurs in SOD2+/- but not in SOD1+/- AMSC.

Telomere shortening has been a documented phenotype associated with atherosclerosis and other ROS associated disease. Since SOD2+/- mice exhibit arterial stiffening at 16 months but not at 4 month, we used this model to investigate if telomere attrition was present in ASMC at 4 month mice prior to expression of the disease phenotype and in the 16 month mice after arterial stiffening developed. Using Telomere Amount and Length Assay (TALA), we measured average lengths of telomeres isolated from ASMC of SOD2+/- 4 month- and 16 months old (S2-4 and S2-16 respectively) mice compared to average telomere lengths of ASMC isolated from C57BL/J6 WT 4 month and 16 month (W4 and W16) old mice. We found that average telomere length of S2-4 were slightly shorter than W4 (Figure 2.1A).We also observed that telomeres were significantly eroded in S2-16 compared to W4 and S2-4, average telomere lengths in S2-16 were approximately a third of average telomere lengths in S2-4 (Figure 2.1B). The rapid telomere erosion that occurred in

SOD2+/- ASMC did not occur in WT AMSCs as average telomere length did not vary significantly in W4 and W16.

To determine if SOD deficiency generally results in telomere attrition, we measured the average telomere lengths of SOD1+/- AMCs isolated from 4 and 16- month old mice (S1-4 and S1-16). We observed no significant decrease in average telomere length in S1-4 and S1-16 when compared to W4(Figure 2.1B). Notably, telomere attrition was not present in ASMC of SOD1+/- and WT mice with healthy arterial walls but present in ASMC of SOD2+/- mice that exhibited disease phenotype.

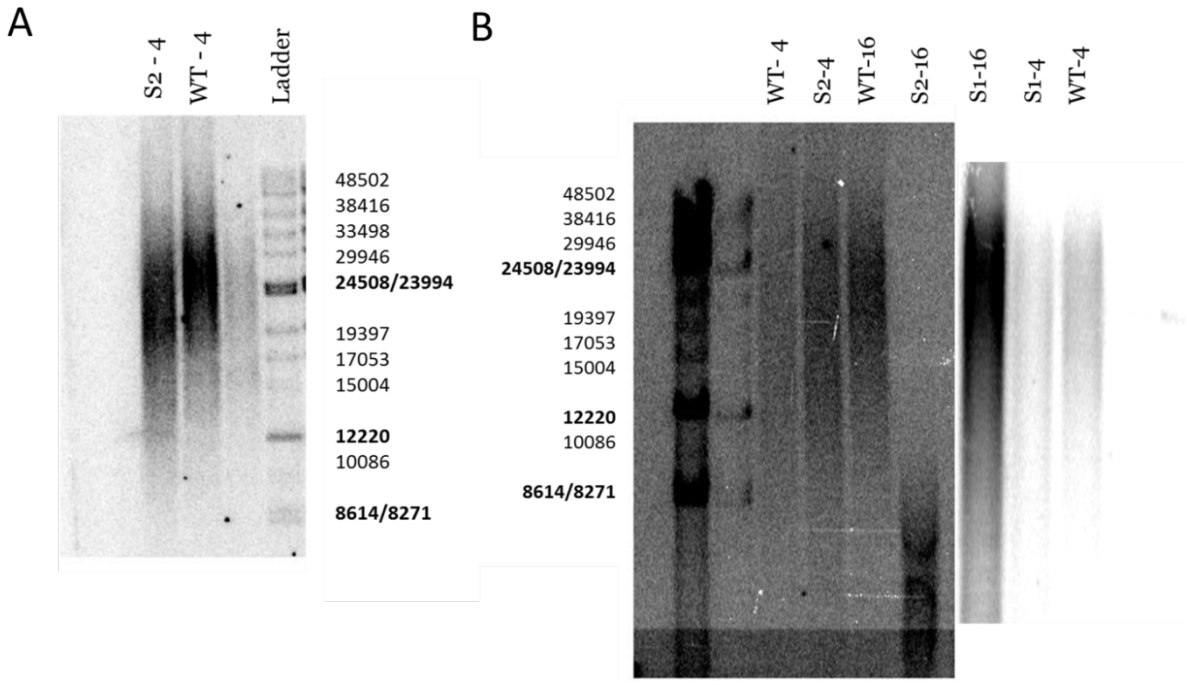

Figure 2.1. Telomere erode in SOD2+/- ASMC. (A) Telomere length of ASMC isolated from 4 month old SOD2+/- mice (S2-4) was compared to those of WT (W4) of the same age. The telomeres of S2-4 were slightly shorter than those of W4. (B) Telomeres of ASMC isolated from SOD1+/- and SOD2+/- mice aged 4 month and 16 months were compared to those of WT mice at 4 and 16 months. Telomere length of SOD2+/- ASMC isolated from 16 month mice (S2-16) was several kbps shorter than W4 and S2-4 suggesting that telomeres continued to erode with age. The lengths of the telomeres in SOD1+/- ASMC seem relatively stable as telomeres at 4 month (S1-4) seemed the same length as at 16 months (S1-16).

2. Telomerase Activity is elevated in SOD2+/- ASMC.

The observation of reduced telomere lengths in SOD2+/- ASMC led us to speculate that reduced telomerase activity was the cause of the telomere erosion. Recent studies have shown that an increase in ROS has been linked to decreased telomerase activity. Since telomerase is responsible for the maintenance of telomeres, we hypothesized that the reduction of average telomere length could be the result of decreased telomerase activity. Using the TRAP assay, we measured telomerase activity in W4, S2-4, W16 and S2-16. We found that telomerase activity was greater in SOD2+/- compared to WT ASMC isolated from both 4 and 16 month old mice. Telomerase activity of S2-4 was more than twice that in W4 while telomerase activity of S2-16 was more than six times the activity than W4 (Figure 2.2 A). In other words, telomerase activity increased with age in SOD2+/-ASMC; telomerase activity was found to be greater in S2-16 than S2-4. In contrast, telomerase activity in SOD1+/- ASMC was less than that in WT, and decreased significantly with age. Interestingly, telomerase activity in WT underwent a modest but significant decrease of about 30 % in 12 months when comparing W16 to W4 (Figure 2.2B).

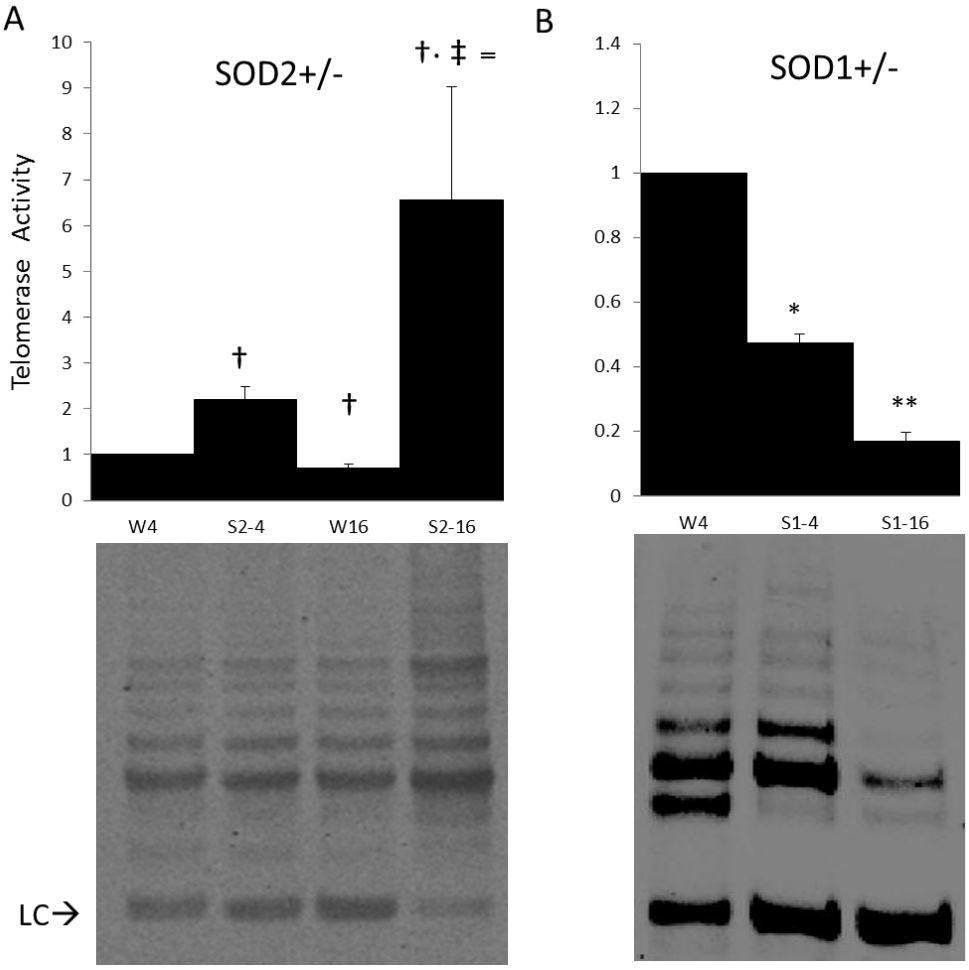

Figure 2.2. Telomerase Activity of SOD1+/- and SOD2+/- ASMC. (A) Telomerase activity of SOD2+/- ASMC isolated from 4 month (S2-4) and 16- month (S2-16) old mice compared to WT isolated from 4 month (W4) old mice. Significant increase is seen in S2-4 and S2-16, with S2-16 showing the greatest level of telomerase activity. WT isolated from 16 month (W16) was used as a control for age. W16 shows a significant decrease in telomerase activity. † notes P< 0.05 vs W4, †· notes P=0.01 vs W4, ‡ notes P<0.05 vs S2-4, = notes P=0.01 vs W16. (B) Telomerase activity of SOD1+/- SMCs isolated from 4 month (S1-4) and 16- month (S1-16) old mice compared to W4. Significant decrease in telomerase activity is seen in S1-4 and S1-16, with S1-16 showing the least activity * notes where P<0.001 vs W4, ** notes P<0.0001 vs W4.

After our discovery that telomerase activity was elevated in SOD2+/- ASMC, we speculated that the increased telomerase activity observed might be the result of oxidative stress or a cellular response to counteract the substantial loss of telomeric DNA. We also considered the possibility that if oxidative stress caused a decrease in telomerase activity, as seen in previously published reports, an increased telomerase activity could be, in part, due to

cellular compensatory response for chronic oxidative stress over time. To test if the increase in telomerase activity was a direct response to oxidative stress, we treated S2-4 and W4 cells with rotenone, a mitochondrial disruptor that causes an acute increase in superoxide levels. We then measured superoxide and telomerase activity levels. We found, rotenone treatment (200 nM for 6 hrs) further increased telomerase activity in S4 cells while decreasing telomerase activity in W4 (Figure 2.3). Furthermore, propidium iodide and annexin-FITC were used as controls to monitor apoptosis, and it was determined rotenone treatment did not induce apoptosis and therefore changes in telomerase activity were not the result of cell death.

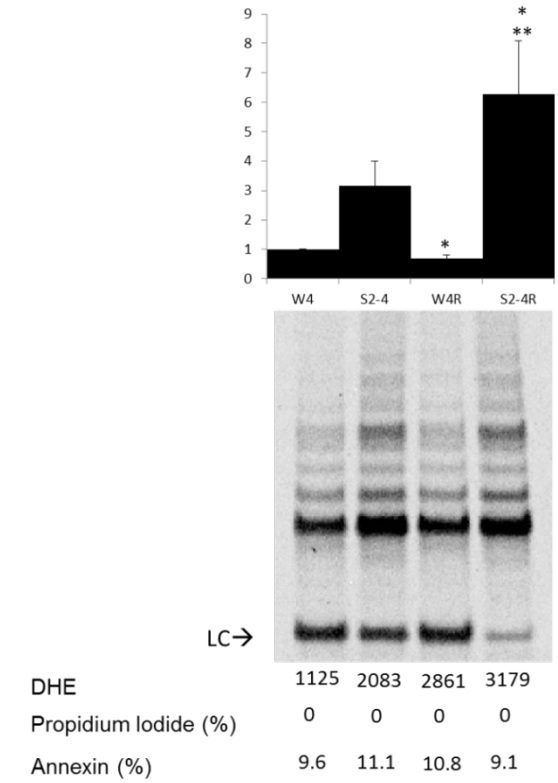

Figure 2.3. Rotenone treatment of WT and SOD2+/- ASMC. W4 and S2-4 ASMC were serum starved overnight and treated with 200nM rotenone for 6 hours. Telomerase activity for rotenone treated S2-4 (Lane4) was approximately 6 and 2 times greater than telomerase activity of untreated W4 (Lane 1) and untreated S2-4 (Lane 3) respectively. The telomerase activity for rotenone treated W4 was 70 percent that of untreated W4. (*P notes 0.1 vs W4 and ** notes P =0.14 vs S4). n=3

Several studies on the effects of ROS on telomerase have focused exclusively on the effects of hydrogen peroxide. In SOD2+/- ASMC superoxide levels are increased however, hydrogen peroxide levels are lower than in WT. This is expected since SOD2 catalyzes the conversion of superoxide to hydrogen peroxide. In an attempt to examine the effects of hydrogen peroxide on telomerase of ASMC, we treated lysates isolated from W4 and S2-4 ASMC with 25 mM hydrogen peroxide for 90 minutes. We observed a significant decrease in telomerase activity in both W4 and S2-4 consistent with published reports that hydrogen peroxide inhibits telomerase activity (Figure 2.4). This result suggests that telomerase from cells experiencing chronic oxidative stress remain sensitive to hydrogen peroxide, as expected.

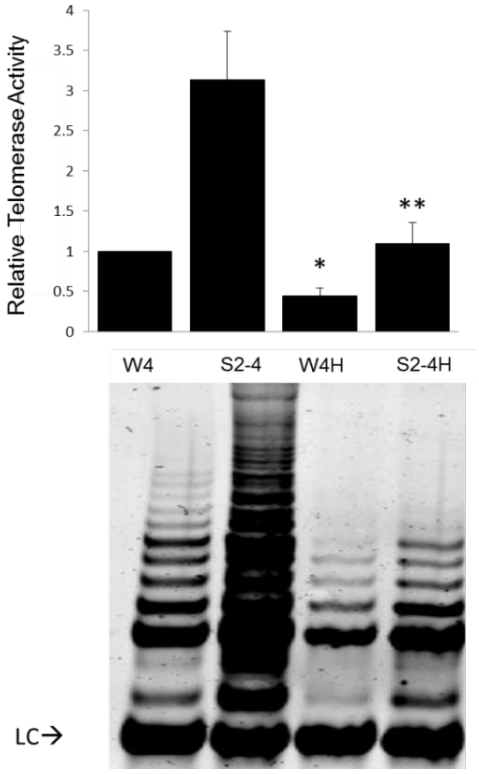

Figure 2.4. Hydrogen peroxide treatment of WT and SOD2+/- ASMC lysates. Cell lysates were treated with hydrogen peroxide for 2 hours prior to assay by TRAP. Hydrogen peroxide treatment decreased both W4 and S2-4 telomerase activity by 55 and 66 percent respectively. Telomerase activity of W4 (Lane 1) decreased 55 percent post-hydrogen peroxide treatment, W4H (Lane 3) (*P=0.003938 vs W4). Telomerase activity of S2-4 (Lane 2) decreased 66 percent post-hydrogen peroxide treatment, S2-4H (Lane 4) (**P=0.0121 vs S4). n=4

3. Telomere Erosion is not the result of Nuclear Export of TERT

In an effort to investigate the rapid telomere erosion observed in SOD2+/- ASMC despite an increase of telomerase activity, we examined telomerase location. ROS has been previously shown to increase nuclear export of TERT.(76, 98) Decreased nuclear TERT impedes telomerase assemblage, diminishes telomere-associated functions, and would explain why telomerase is unable to counter telomere shortening in SOD2+/- ASMC. Nuclear telomerase can be reduced by either increased export of TERT or decreased import of TERT into the nucleus. Importation of TERT into the nucleus is Akt-dependent, and the Akt pathway is down regulated in SOD2+/- ASMC (146) so it is plausible TERT is not imported into the nucleus. We conducted a subcellular fractionation on W4 and S2-4 and verified separation of the nuclear and cytoplasmic fractions with western blots (Figure 2.5).

We then measured telomerase activity in nuclear and cytoplasmic fractions using the TRAP assay and found that the majority of the telomerase activity resided in the nucleus of both cell types. Telomerase activity in the nucleus was approximately 3.5 times greater for S2-4 than W4 (Figure 2.6A). However, we also observed an increase of telomerase activity in the cytoplasm of SOD2+/- ASMC extracts when compared to WT suggesting there is an increase in nuclear export of TERT or reduced nuclear import, but that a substantial amount of telomerase is still present in the nucleus (Figure 2.6B).

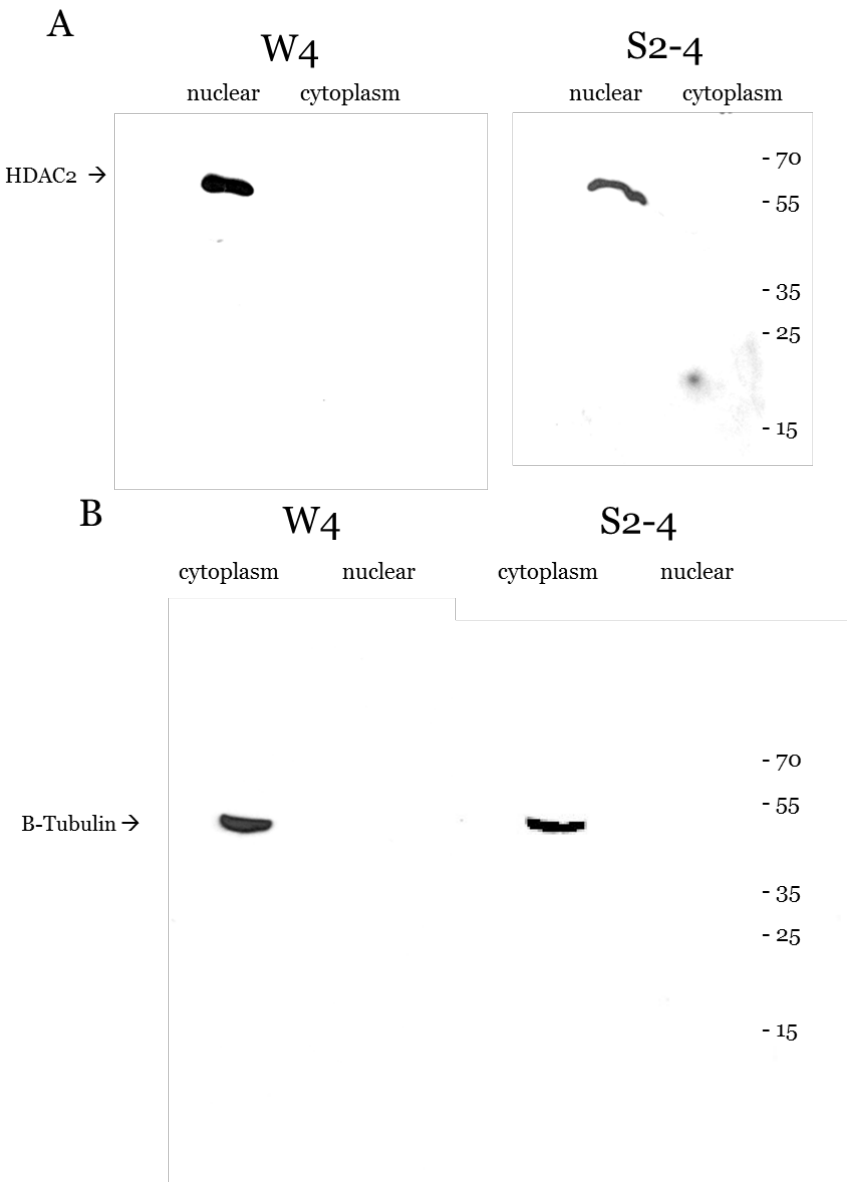

Figure 2.5. Subcellular fractionation. Western blots were used to verify subcellular fractionation was successful. The marker used for the nucleus was HDAC2 and the marker used for the cytoplasm was β-tubulin. A) Western blot has a bright single band in the nuclear lanes for W4 and S2-4 at approximately 60 kDa, and no corresponding band in the cytoplasmic fraction. B). Western blot for β-tubulin shows a single band at approximately 50 kDa in the cytoplasm fraction, and no corresponding bond in the nuclear lane.

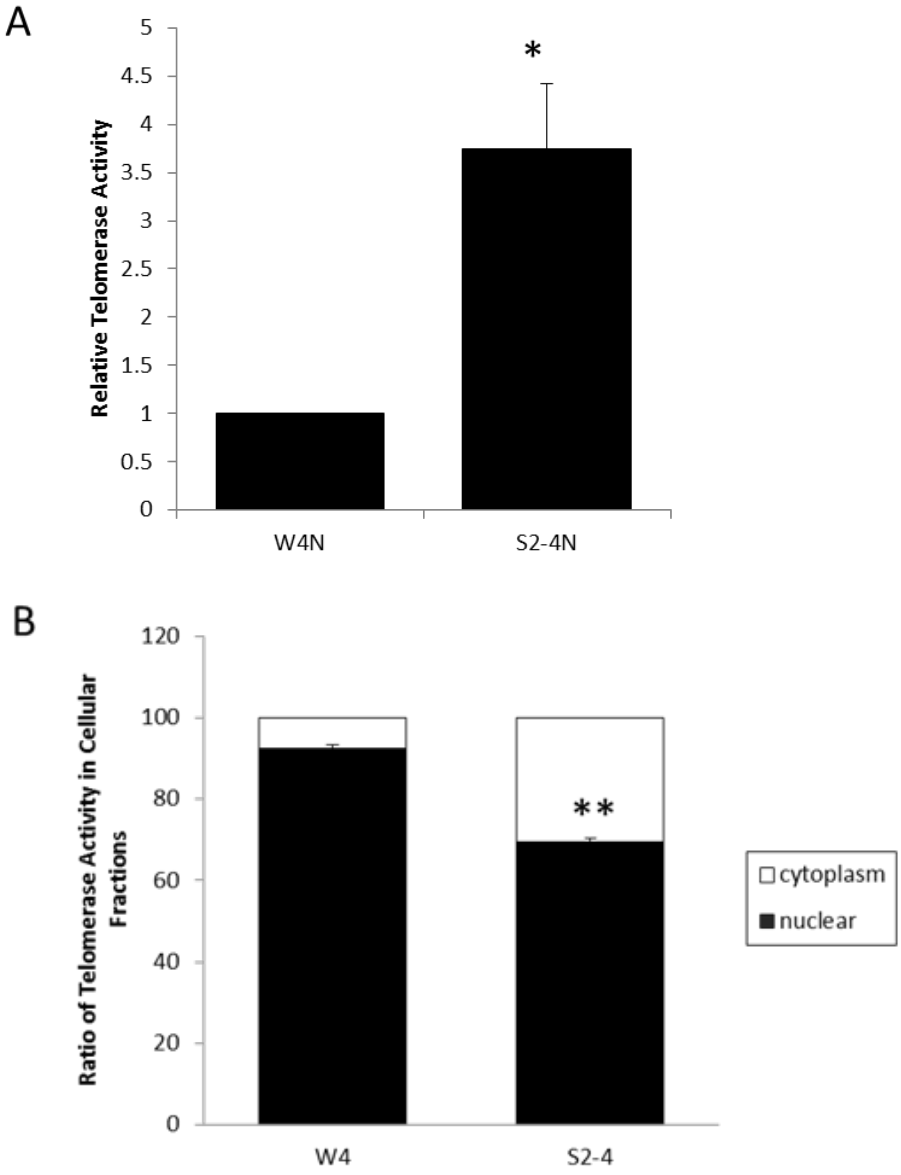

Figure 2.6. Telomerase Activity in ASMC subcellular fractions. A) Relative telomerase activity in the nuclear fractions of W4 and S2-4. Telomerase activity in nuclear fraction of S2-4 is approximately 3.5 times that in W4. (*P=0.007 vs W4), n=5. B) The ratio of telomerase activity assayed in nuclear to cytoplasmic fractions of W4 and S2-4 cells. Approximately 7.5% and 30.5% of telomerase activity assayed was present in the cytoplasmic fractions of W4 and S2-4 respectively. (**P=2.49321E-08 vs W4), n=8 for W4 and n=5 for S2-4

4. Expression of mTERT mRNA and mTERC

ROS has been shown to repressed telomerase expression at transcription (69) so we wondered if oxidative stress in SOD2+/- ASMC regulated telomerase activity at the

transcriptional level. We used Real-Time PCR (RT PCR) to investigate telomerase associated RNA levels. We hypothesized that increased telomerase activity is the result of increased telomerase expression at the transcriptional level. The minimal catalytic core of telomerase consists of mTERT and mTERC. Using RT PCR, we conducted relative quantification studies of mTERT mRNA and mTERC levels. We found that mTERTmRNA and mTERC levels were increased in S2-4 but were unexpectedly decreased in S2-16 compared to W4 (figure 2.7B). The mTERT mRNA and mTERC levels remained unchanged in age control W16 compared to W4 (data not shown) while decreased in S1-4and S1-16 samples (Figure 2.7A).

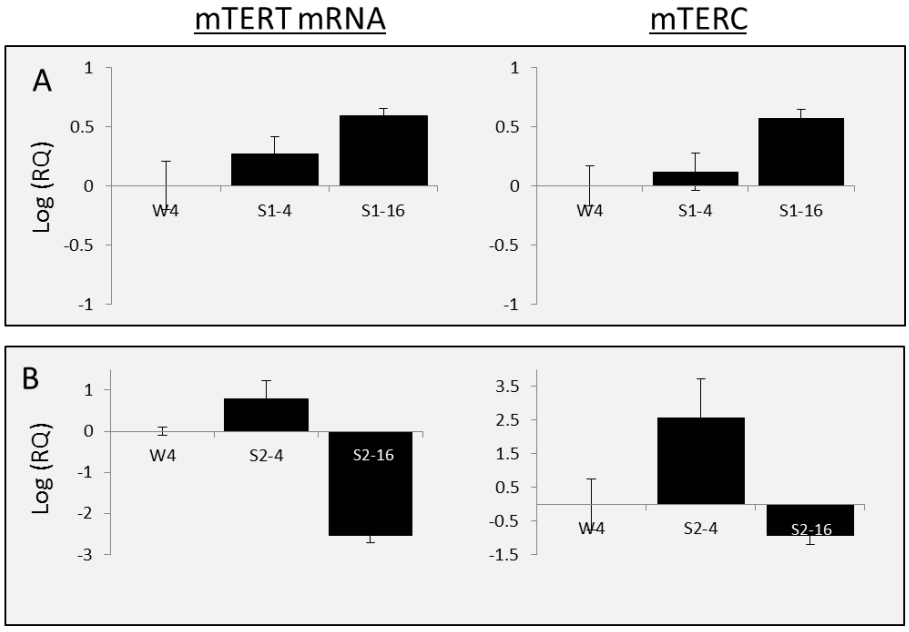

Figure 2.7. Real Time PCR data for telomerase associated RNAs in SOD1+/- and SOD2+/- ASMC. Relative Quantification of mTERT mRNA and mTERC levels for (A) S1-4 and S1-16 (B) S2-4 and S2-16 (C) age control W16, all compared to W4.

5. Telomere Dysfunction is the result of Increased Oxidative Stress

We found that telomeres in SOD2+/- ASMC were remarkably shortened despite telomerase being up regulated and located in the nucleus. One possible explanation for this observation is that telomerase is unable to extend the telomeres because they have accumulated substantial oxidative damage and are no longer proper substrates for the enzyme. Telomeres are especially susceptible to oxidative damage due to the G-rich nature of the telomeric DNA sequence. Exposing the telomeres to ROS can generate 8-oxoguanine lesions (8-oxo-dG) that may disrupt binding of telomere binding proteins to the telomeres.(151) Furthermore, accumulation of 8-oxo-dG at the telomeres results in telomere attrition.(148, 149) Moreover, the presence of 8-oxo-dG lesions in the GGG triplet of the telomere has been reported to disrupt intramolecular G quadruplexes and inhibit telomerase activity depending on the location of the lesion in the telomeric sequence.(152) Thus, oxidative damage to the telomeres could explain the inability of telomerase to extend the telomeres.

Since oxidative damage to telomeres can disrupt telomere association with the shelterin complex, telomeres will not be able to form the protective cap structure that prevents chromosome termini from being recognized as DNA double strand breaks. Therefore, the telomeres become dysfunctional. Dysfunctional telomeres elicit a DNA damage response and can be measured by the presence of DNA damage repair proteins at telomeres.(153) Telomere dysfunction is characterized by formation of foci where DNA damage response factors including 53BP1, γ-H2AX, Rad17 and Mre11 are recruited to the telomeres. The domain of telomere-associated DNA damage factors are referred to as a Telomere Dysfunction-Induced Focus (TIF).(153)

We used fluorescence co-localization to determine presence of TIFs in S2-4 ASMC. The DNA damage protein we detected was γ-H2AX using a primary antibody for the protein and a fluorescently labeled secondary antibody. We probed for the telomeres using fluorescently labeled PNA oligonucleotide complimentary to the 5'-TTAGGG-3' sequence (Figure 2.8A). We found that γ-H2AX expression was greater in S2-4 compared to W4. γ-H2AX was detected in 61% of S2-4 and in 35% of W4 cells analyzed (Figure 2.8B). Additionally, S2-4 ASMC was more likely than W4 to have 5 or more γ-H2AX foci. Approximately 23% S2-4 had 5 of more γ-H2AX foci versus 4% of W4 (Figure 2.8C). The occurrence of TIFs was also greater in S2-4 than in W4. TIFs were present in 43% of S2-4 counted versus 5% of W4 (Figure 2.8D) and only 7% of S2-4 had 5 or more TIF per cell (Figure 2.8E).

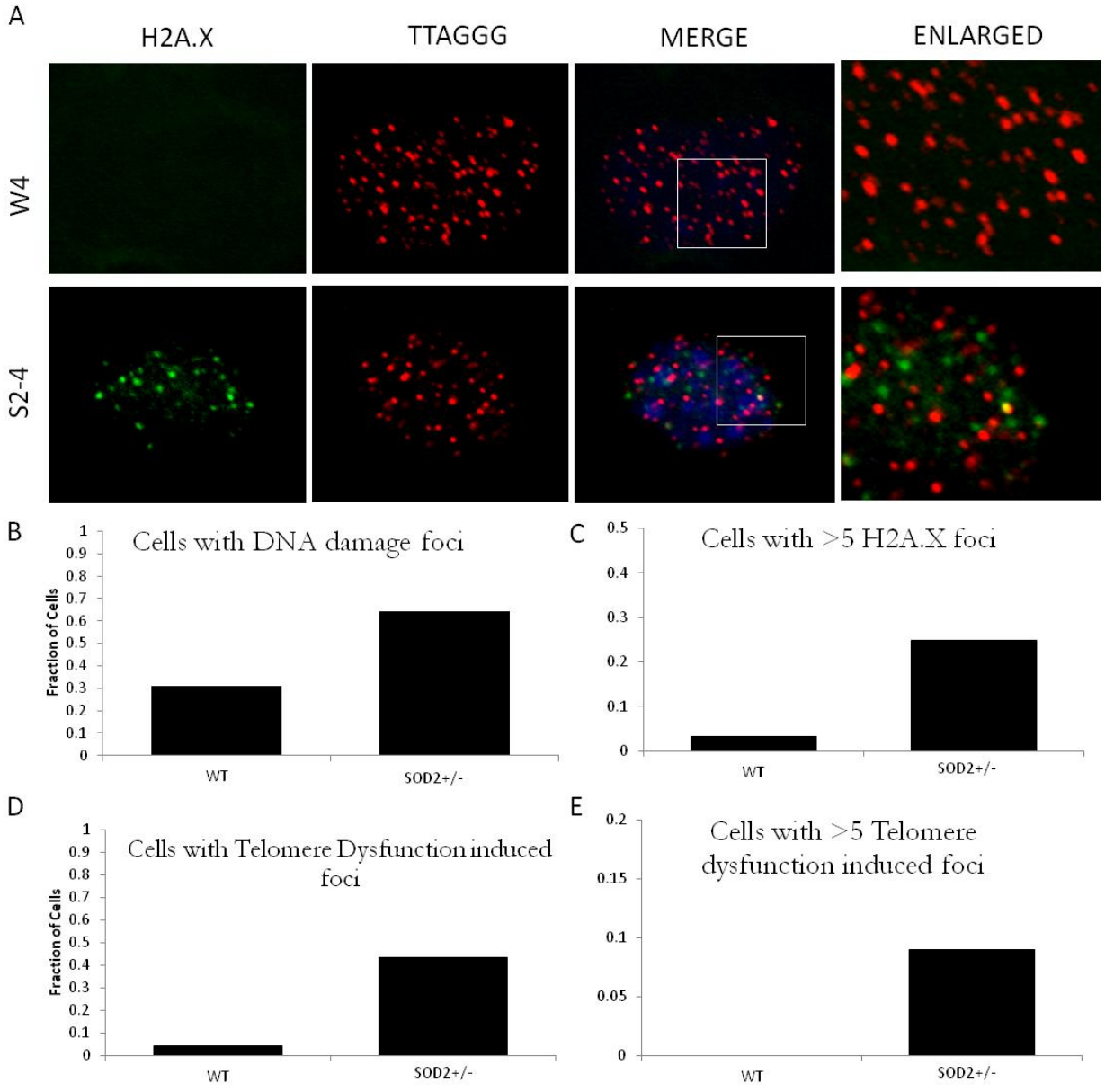

Figure 2.8. DNA damage and telomere dysfunction induced foci. A) Fluorescence co-localization of γ-H2A.X and TTAGGG. DAPI is used to identify the nucleus. Telomere-Induced Dysfunction Foci are regions where overlap of signals for H2A.X and telomeres. B) Fraction of WT and S2-4 ASMC that expressed γ-H2A.X was 0.35 and 0.62 respectively. *P=0.007 vs WT-4 C) Fraction of WT and S2-4 ASMC that expressed ≥5 γ-H2A.X foci was 0.04 and 0.23 respectively. **P=0.015 vs WT-4 D) Fraction of WT and S2-4 ASMC that had TIFs were 0.05 and 0.43 respectively. †P= 0.006 vs WT-4 E) Fraction of WT and S2-4 ASMC that expressed ≥5 TIFs were 0 and 0.07 respectively. Total of 251 cells for S2-4 and 330 cells for W4 were analyzed.

6. Oxidative damage may be the cause of rapid telomere erosion

The TIF assay determined that telomeres were increasingly dysfunctional in the oxidative stress environment of SOD2+/- ASMC. To explore potential mechanisms for this dysfunction, we asked if the increase in telomere dysfunction was due to the accumulation of 8-oxo-dG lesions. To determine if DNA in SOD2+/- ASMC exhibited increased 8-oxo-dG lesions, we used a FITC-conjugated peptide that binds 8-oxo-dG. Our initial attempts to use FACS analysis failed (data not shown) due to high background fluorescence. Our attempts to used florescence microscopy had mixed success. We could detect 8-oxo-dG legions in S2-4 and not in W4 ASMC suggesting that DNA in S2-4 exhibited increased oxidative damaged than WT (Figure 2.9). However we had problems reproducing the results. A more robust assay will be needed to clarify this preliminary observation.

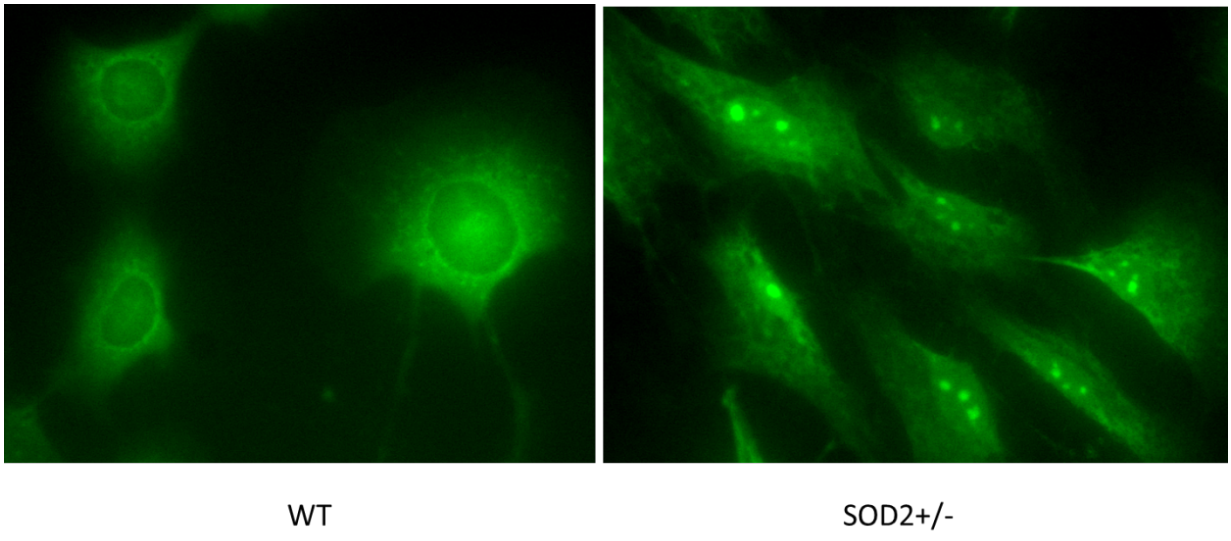

Figure 2.9. Detection of 8-oxo-dG lesions in WT and SOD2+/- ASMC. Using a FITC-conjugated probe for 8-oxo-dG, we observed evidence that S2-4 accumulated oxidative DNA damage. We found FITC signal signifying 8-oxo-dG foci in S2-4 but not in W4.

C. Discussion

There is a growing body of evidence that oxidative stress, telomere dysfunction and aging are interconnected. However, the relationship among all three is poorly understood. Using the hypothesis that telomere dysfunction is a downstream target of oxidative stress in age-related disease development, we analyzed the telomere phenotypes in two models associated to increased oxidative stress in an effort to better understand the relationship between telomere dysfunction and ROS, and their contribution to aging and disease progression.

One of the main goals of this study was to determine if telomere dysfunction was a downstream target of ROS during ROS-induced cardiac dysfunction. The first step in determining that goal was to determine if oxidative stress in ASMC resulted in telomere dysfunction. We predicted that if telomere dysfunction is crucial to the development of CVD, we would observe telomere dysfunction in the SOD2+/- ASMC since that SOD2+/- mouse model developed aortic stiffening. We decided a good measure of telomere dysfunction was the average length of the telomeres. In addition, published reports suggest shorter telomeres are associated with CVD. We used ASMC isolated from young healthy WT mice as our reference, and found that telomeres rapidly eroded in SOD2+/- ASMC, and that this erosion correlated with age. Telomere lengths in S2-16 were drastically shorter than those present in S2-4. Indeed, the rate of erosion we observed in SOD2+/-ASMC in 1 year is significant. We observe a loss of about 10kb over a 12 month period in telomerase positive SOD2+/- ASMC, when the rate telomeres shorten in telomerase negative (mTERC-/-) mice was measured to be 4.8 ± 2.4 kb per generation.(154) The appearance of shorter telomeres in S2-4, also gave insight into whether telomere dysfunction could contribute to changes in the vascular

function. If telomere dysfunction was part of the underlying mechanisms of atherosclerosis, then telomere shortening would be present in ASMC prior to the onset of aortic stiffening seen in 16 month SOD2+/- mice. If however, telomere shortening was a consequence of the disease, then it would be only be present after the disease had developed and not in S2-4 isolated from healthy vessels. Our results showing that telomere shortening began in ASMC before any measurable change in the function of vasculature happened, is consistent with the hypothesis that telomere dysfunction is involved in disease progression. Several reports have suggested a causal relationship between telomere dysfunction induced cellular senescence and age-related CVD development.(106, 125, 155, 156) There is evidence which supports our hypothesis that telomere dysfunction plays a causative role in CVD. Many CVD risk factors such as oxidative stress are known to accelerate telomere dysfunction, leading to increase cellular senescence or apoptosis in tissues and resulting in tissue dysfunction and disease. Also, telomere attrition is most pronounced in areas known for developing atherosclerosis such as the carotid artery and aortic wall.(107, 157) Additionally, higher levels of senescent cells are found in VSMC, endothelial cells, monocytes and macrophages of aged arteries and atherosclerotic plagues.(122, 136, 137, 158) It also remains plausible that cellular senescence is a contributing cause of CVD, and telomere dysfunction is just one of the many mechanisms that induce senescence in the vascular tissue.

While the telomere erosion we observed in SOD2+/- ASMC is consistent with the literature accounts of shortened telomeres present in VSMC involved in atherosclerosis, our results are not conclusive evidence that telomere dysfunction plays a causative role in development of the disease. There are alternative explanations for the occurrence of telomere attrition observed in SOD2+/- ASMC. Telomere length is believed to be correlated with

biological age, and the rapid telomere shortening observed in SOD2+/- ASMC may signify increased cardiovascular aging and not be involved in disease progression. Furthermore, many factors that affect cardiovascular age also affect telomere dysfunction, such as oxidative stress, so reduced telomere length may be reflection of increased cardiovascular age. Moreover, the observation of telomere erosion in the SOD2+/- model is significant because while cardiac dysfunction has been observed in SOD2+/- model, the condition of the telomere had not been characterized in the model. So prior to this study, it was not determined if the model had the phenotype of telomere shortening in its vascular system similar to that observed in humans suffering from CVD.

It is also important to note that the observed telomere attrition in SOD2+/- ASMC occurs while telomerase activity is elevated. We verified that telomerase activity can be up-regulated by an acute increase in mitochondrial dysfunction and superoxide, and not just in response to the erosion of the telomeres. Although ROS has been shown to regulate TERT via transcription (69), we found that mTERT mRNA and mTERC levels did not account for the increase in activity. Our discovery that mTERT mRNA levels did not correlate with telomerase activity suggests that telomerase activity is not regulated strictly at the transcriptional level in SOD2+/-ASMC but is likely up-regulated by post-translational modification, for example, Akt-phosphorylation is known to enhance telomerase activity.(75) Notably, we observed elevated telomerase activity despite the reported decreased Akt phosphorylation(146) in SOD2+/- ASMC, suggesting telomerase activity is likely up regulated by another mechanism. One possible explanation for telomerase up-regulation is the lower level of hydrogen peroxide levels in SOD2+/- ASMC.(146) Hydrogen peroxide has been shown to inhibit telomerase directly and via post-translational modification of TERT.

Lower levels of hydrogen peroxide may translate to decreased inhibition of telomerase. We treated cellular extracts with hydrogen peroxide verifying the ability of hydrogen peroxide to inhibit telomerase activity in the SOD2+/- ASMC. Additionally, the activity levels of catalase, the enzyme that converts hydrogen peroxide to water in SOD2+/- ASMC, was confirmed to be the same as WT.(143) Our data suggests that telomerase can be up- and down-regulated by different forms of ROS. We showed an increase in superoxide caused telomerase activity to increase while hydrogen peroxide treatment caused a decrease in telomerase activity. Although not explored in this paper, perhaps degradation of mTERT is reduced in SOD2+/- ASMC. TERT has been how to have 2 caspase sites (71) and is also subjected to regulation by ubiquination.(72, 159) Further studies on telomerase regulation will need to be conducted to explore these possibilities. It is important to note, that TERT up regulation may contribute to the increase in proliferation in SOD2+/- ASMC. TERT has been shown to increase proliferation independent of action at the telomeres (88, 160) and SOD2+/- ASMC exhibited increased proliferation.(143) This relationship also warrants further exploration.

Importantly, our data showed that the increase in telomerase is unable to counteract the loss of the telomeres. Two possible explanations for the absence of telomerase extension of the telomeres can be envisioned. One is that ROS-dependent pathways reduced TERT localization in the nucleus and the second is that oxidative damage of the telomeres prevents the telomeres from being extendable by telomerase and decreases protection afforded by the shelterin complex. We were able to conclude that the former possibility of ROS induced reduction of nuclear TERT was not responsible for the rapid telomere erosion. We observed a greater than three-fold increase of nuclear telomerase activity in S2-4 compared to W4.

Though, we also did observe an increase in telomerase activity in the S2-4 cytoplasmic fractions. This increase in cytoplasmic TERT may be due to ROS increased nuclear export of TERT(76, 94) and/or ROS inhibition of the Akt dependent nuclear import of TERT. Import of TERT into the nucleus requires Akt-mediated phosphorylation of the reverse transcriptase(47) and it has observed that the Akt pathway is downregulated in aged SOD2+/- ASMC (146). Our results imply that nuclear TERT import could be partially disrupted since there is an increase in cytoplasmic TERT accumulation. Cytoplasmic TERT may play a beneficial role during oxidative stress owing to the ability to enter the mitochondria and improve mitochondrial function. Mitochondrial localized TERT has been found to decrease ROS and protect mitochondrial DNA (mtDNA) from oxidative damage by binding mtDNA. It is possible that the increase in oxidative stress in the SOD2+/- model induces increase in cytoplasmic TERT as a mechanism of decreasing oxidative damage. We however, did not specifically examine TERT presence in the mitochondria during our investigation.

The second and in our opinion, more likely possibility for explaining telomere attrition with increasing telomerase activity is oxidatively damaged telomeres are poor substrates for telomere binding proteins and telomerase. Telomeres are nucleoprotein structures where telomeric DNA is in complex with telomere-associated proteins that help facilitate the folding of telomere ends to protect the ends of linear chromosomes from being recognized as DNA double strand breaks. These proteins are unable to assemble allowing for incorrect repair (and breakage) of the chromosomal ends, and as a result increase genetic instability and apoptosis. Consistent with this, an increase in apoptosis was observed in SOD2+/- ASMC.(146) Since telomeric DNA is particularly sensitive to ROS due to the guanine-rich nature of telomere sequence. Exposure of telomeres to ROS can produce 8-oxo-

dG, and these lesions have been shown to disrupt binding of telomere binding proteins to the telomeres, leading to telomere dysfunction.(151) Furthermore, the presence of 8-oxo-dG lesions in the GGG triplet of a telomeric primer has been reported to affect the formation of intramolecular G quadruplexes and decrease the ability of telomerase to extend the primer.(152) Accumulation of 8-oxo-dG leads to increased telomere damage including increased single- and double- strand breaks and telomeric DNA erosion.(148, 149)

To determine if oxidative damage to the telomeres was responsible for the erosion, we analyzed telomere dysfunction and 8-oxo-dG lesions. We found that S2-4 had higher occurrence of DNA damage signal, telomere dysfunction and oxidative DNA damage than W4. Our results confirmed reports that DNA damage and telomere dysfunction increased with oxidative stress. Furthermore, the accumulation of oxidative damage at the telomeres may be the cause for the telomere erosion observed in SOD2+/- ASMC. Accumulation of oxidative damage at the telomeres has been shown to increase telomere attrition.(148, 149)

While we report that rapid shortening of telomeres is present in SOD2+/- ASMC from the SOD2+/- model suffering aortic dysfunction, we also report that telomere erosion was not present in SOD1+/- ASMC isolated from a model with healthy aorta. A possible explanation for the oxidation-induced telomere erosion not being present in SOD1+/- ASMC but being observed in SOD2+/- ASMC is SOD1+/- ASMC has significantly lower oxidative stress than SOD2+/- ASMC.(143) Greater oxidative stress likely means greater oxidative damage and telomere dysfunction. Another explanation for the difference in telomere dysfunction observed in SOD1+/- and SOD2+/- ASMC is the existence of relationship between mitochondrial dysfunction and aging. There are studies showing that mitochondrial dysfunction and mitochondrial oxidative stress specifically have an effect on aging.

Mitochondrial dysfunction is one of the hallmarks of aging.(9) Using mouse models where localized catalase was overexpressed in the nucleus, peroxisome or the mitochondria, only the model with overexpression of mitochondrial catalase showed significant increase in life span.(161) Additionally, a relationship exists between telomere biology and mitochondrial function. TERT, independent of the telomeres, regulates mitochondrial function through PGC-1α and PGC-1β and also by localizing in the mitochondria and binding mtDNA(100, 101). This leads to a model in which a relationship between telomere dysfunction and mitochondrial dysfunction combine to contribute to aging and related disease states.

An important finding from our study is the occurrence of telomere attrition with increased telomerase activity due to mitochondrial oxidative stress. A similar discovery was made by Cardin et al. in a study examining the role of oxidative damage and telomere dysfunction in the progression of Barrett's esophagus, a gastro-esophageal reflux disease associated with adenocarcinoma.(150) Cardin et al. saw increased oxidative damage, increased telomere shortening with increased telomerase activity in a subset of patients suffering from Barrett esophagus. Although studies have not been conclusive as to why telomeres erode drastically in the presence of telomerase, the enzyme which extends them, the phenotype is now being associated with chronic stress. Rats suffering from chronic stress have shortened telomeres but also exhibited elevated telomerase activity.(162) In accordance, shorter telomeres with increased telomerase activity appear to be associated with poor ability to deal with stress including lack of social support.(163) Notably, patients with metabolic syndrome exhibit mitochondrial dysfunction(164), chronic stress and increased risk of developing CVD(165), also exhibits elevated telomerase levels(166) and shorten telomeres(167). The SOD2+/- mouse model may be a useful tool to use for further analysis

in understanding the mechanisms which prevent telomerase from counteracting loss of the telomeres and the role that may play in development of some disorders.

D. Conclusion and Future Work

In this chapter we reported the results of our characterization of telomere biology in the ASMC of mouse models of oxidative stress, SOD1+/- and SOD2+/-mice. We found telomere lengths were stable in SOD1+/- ASMC and telomerase activity decreased with age more dramatically than in WT. Interestingly, we observed rapid telomere erosion with increased telomerase activity in SOD2+/- ASMC. Telomere erosion was not the result of increased nuclear export of TERT but most likely the result of increased oxidative damage at the telomeres. We showed that there was an increase in telomere dysfunction in SOD2+/- ASMC and our preliminary data suggests there is also an increase in oxidative damage. However, a more reliable and specific method will be needed to detect telomeric oxidative damage so that we can produce more conclusive results. Possible methods we can explore includes a co-localization assay similar to the TIF, using an antibody for 8-oxo-dG and the telomere PNA oligo probe and counting areas where 8-oxo-dG and telomere signals overlap at telomeric oxidative damage. Furthermore, an experiment determining shelterin proteins binding to the telomeres would also be important to determine if oxidative damage has made the telomeres poor substrates for telomere binding proteins. We could conduct a co-localization experiment with PNA probe for the telomeres and a commercially available anti-body for TRF2. Signal overlap between telomeres and TRF2 would be counted as TRF2 at the telomeres and no signal overlap would be counted as “free” protein. An alternative

approach would be to conduct a chromatin immunoprecipitation with antibodies for shelterin proteins such as TRF2 and determine the amount of proteins associated with telomeric DNA.

Our study also found endogenous ROS increased the presence of telomerase in the cytoplasm in S2-4, but it was not determined if that telomerase was present in the mitochondria. Numerous studies have reported exogenous ROS inducing nuclear export of TERT and subsequent mitochondrial import.(76, 94, 98) Isolation of the mitochondria from SOD2+/- ASMC, and testing telomerase activity would be a useful experiment to determine the exact cellular location of TERT due to endogenous oxidative stress.

While we successfully characterized the telomeres and telomerase activity, our study suffered from a few weaknesses such as our inability to detect TERT. During this study, we attempted several times to use Western blotting to examine TERT protein levels. Unfortunately, we were unable to find a commercially available antibody specific to the protein and with low background. Other options include producing our own antibody in house, or hiring a company to produce the antibody for us. Another weakness was our inability to determine by which pathway(s), superoxide regulated telomerase activity. We used previous reports of decreased Akt-phosphorylation and hydrogen peroxide in SOD2+/- ASMC(143) to rule out Akt pathway and identify the possibility of hydrogen peroxide involvement. Obtaining TERT protein levels and doing further investigation on pathways implicated in the regulation of TERT would improve our understanding of the effects of oxidative stress on TERT subunit. Furthermore, an exploratory study concerned with whether or not TERT is partially responsible for the increased proliferation observed in SOD2+/- ASMC would be beneficial to this research. TERT has been identified as regulating proliferation in a telomere-independent manner through its regulation of cellular pathways

that promote cell survival and proliferation. Thus SOD2+/- ASMC provides an opportunity to study the functions of TERT not related to the telomeres in a cellular environment where TERT is up regulated but is unable to extend the telomeres.

E. Method

1. Cell Culture

Mouse aortic smooth muscle cells were isolated from 4- and 16- month old C57BL/J6 wild-type (WT), superoxide dismutase 1 haploid deficient (SOD1+/-) and superoxide dismutase 2 haploid deficient (SOD2+/-) mice. SOD1+/- and SOD2+/- mice were backcrossed at least 8 times into C57BL/J6 background. ASMC were derived from several mice. ASMC were cultured in Gibco® Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS at 37 ºC in a 5 % CO_2 incubator. AMSCs were a gift from Marschall Runge, PhD, MD (McAllister Heart Institute, UNC-CH).

2. Telomere Amount and Length Assay (TALA)

Telomere length was measured using a modified version of TALA.(168) Cells were lysed in genomic digest buffer (0.01 M TrisCl pH 8.0, 0.1 M NaCl, 25 mM EDTA pH 8.0, 0.5 % SDS) supplemented with 200 µg proteinase K and 10 µg rnase A at 37 ºC overnight. Genomic DNA was extracted from cell lysates by phenol-chloroform extraction followed by ethanol precipitation. DNA samples were dissolved in ddH_2O and stored at -20 ºC. DNA concentration was determined using UV-Vis. A restriction digest was performed on 2.5 µg isolated DNA using 25 U HinfIII, 10 U RsaI and 10 U HaeIII in Buffer #2 (all from NEB), at 37 ºC for 3 hours. The digested DNA was mixed with 0.8 pmoles of ^{32}P labeled 5'-

CCCTAACCCTAACCCTAA-3' probe. The mixture was denatured at 98 ºC for 6 minutes, hybridized at 55 ºC for 1 hour and cooled at 4 ºC for at least 5 minutes. The hybridized telomeres were then separated from the un-hybridized probe and digested non-telomeric DNA by gel electrophoresis on a 0.5 % agarose gel for 18 hours at 37 volts in 1x TAE buffer (40 mM Tris-acetate 1 mM EDTA). A radiolabelled ladder (Fermentes Lambda Mix Marker 19) was used as a reference. The gel was dried in the GelAir Dryer (Biorad) and exposed to a phosphorimager screen overnight. The resulting autoradiograph was imaged on a phosphorimager (Storm 860) and the intensity of the signal determined using ImageQuant.

3. Telomere repeat amplification protocol (TRAP)

Endogenous telomerase activity was determined by a modified Trap assay.(169) Cells were lysed using CHAPS lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% Glycerol) and clarified by centrifugation (10,000 × g, 30 min at 4 ºC). Protein concentrations of the clarified cell lysates determined using the Coomassie Plus Assay kit (Pierce). Primer extension reactions contained 0.5 µg cell extracts or 0.125 ng subcellular fractions, 0.1 µg TS primer (5′-AATCCGTCGAGCAGAGTT), TRAP buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween20, and 1 mM EGTA) and 50 µM of each dNTP. Reactions were incubated at 30 ºC for 30 min. Telomerase extension reactions were then amplified with 0.1 µg ACX primer (5′-GCGCGG$(CTTACC)_3$CTAACC), 0.1 µg NT primer (5′-ATCGGCTTCTCGGCCTTTT), 0.5 units Taq DNA polymerase, 0.5µl Taq buffer (Invitrogen), dNTPs to a final concentration of 50 µM and 0.001 amol TSNT template (5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT) used as a loading control.

The reactions were heated to 95 ºC for 5 minutes and then subjected to 33 PCR cycles at 95 ºC for 30 s, 60 ºC for 30 s, 72 ºC for 60 s. Reaction products were separated on a 12.5 % nondenaturing polyacrylamide gel, stained with SYBR Green I (Molecular Probes), and imaged on a phosphorimager (Storm 860). Reactions were quantified using ImageQuant and the product intensity for each reaction was normalized to the TSNT internal standard.

For H_2O_2 treatment, cell lysates of WT and SOD2+/- ASMC were treated with 25 mM H_2O_2 for 90 minutes prior to telomerase extension reactions.

4. Rotenone Treatment

Mouse ASMC were treated with 200 nM rotenone for 6 hours. Before treatment, ASMC were grown to 50% confluence and serum starved for 12-16 hours. Superoxide levels were detected by assessing dihydroethidium (DHE) oxidation. Cells were then incubated with 5-10 μM Dihydroethidium (DHE) for 30-45 min at 37 ºC. Cells were rinsed twice with PBS before fluorescence was measured (excitation at 480 nm and emission at 567 nm) using microscopy (Olympus IX81 microscope with emission filter >600-700nm). Apoptosis was determined using Annexin V-FITC kit (Immunochemistry Technologies, LLC) and fluorescence microscopy.

5. Real Time PCR

Total RNA was extracted with RNeasy Mini Kit (Qiagen), following the manufacturer instructions. RNA quality was verified by denaturing agarose electrophoresis and quantity was determined using Nanodrop UV-Vis Spectrometer. cDNAs were synthesized from 1 μg total RNA with a mixture of random hexamers and oligo(dT) primers

using SuperScript III (Invitrogen). PCR reactions were carried out in triplicates for each cDNA sample in the ABI PRISM 7500 Real Time PCR System. (Applied Biosystems). Primers used for mTERT(82), mTERC(170) and endogenous control 18S RNA(82) were previously published.

Table 2.1. Sequences of primers for RT-PCR

	Forward Primer (5'->3')	Reverse Primer (5'->3')
mTERT	TTCTAGACTTGCAGGTGAACAGCC	TTCCTAACACGCTGGTCAAAGGGA
mTERC	CCTAACCCTGATTTTCATTAGCTGTGGG	GAGGCTCGGGAACGCGCGGTGGCCC
18S rRNA	TAGAGGGACAAGTGGCGTTC	CGCTGAGCCAGTCAGTGT

6. Subcellular Fractionation.

All centrifugation occurred at 4 ◦C. ASMC were grown on 10 cm plates to 80-90% confluence. Ice cold subcellular fractionation buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and proteinase inhibitor) was added to the plate, the plate was scraped and the cellular mixture immediately transfer to a microcentrifuge tube chilled on ice. Lysate was then homogenized by passing the cellular mixture through a 1 ml syringe with a 25G needle 10 times. Homogenized lysate was incubated on ice for 20 minutes. To separate the nucleus from the cytoplasm, the cellular lysate was centrifuged at 720G for 5 minutes resulting in a nuclear pellet and cytoplasm supernatant. The cytoplasm supernatant was concentrated to 50 µl using vivaspin500 GE spin column. The nuclear pellet was washed with 500 µl subcellular fractionation buffer and the supernatant discarded. The nuclear pellet was washed again by re-dispersing in 500 µl subcellular fractionation buffer, passing the mixture through a 25G needle 10 times, and centrifuging at 720G for 10 minutes. The nuclear pellet was then lysed in 25 µl Chaps lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM Benzamidine, 5

mM β-mercaptoethanol, 0.5 % CHAPS, 10 % Glycerol) supplemented with proteinase inhibitor (Roche) and 0.01 mM DTT.

Protein concentrations of cellular fractions were determined using the Coomassie Plus Assay kit (Pierce). Fractionation was verified by Western blot and telomerase activity was measured using a modified TRAP assay.

7. Western Blot.

Approximately 40 μg of each subcellular fraction was separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with non-fat dry milk (5% w/v) in Tris-buffered saline containing 0.1 % Tween-20 (TBST) for 1hr, the membrane was incubated with primary antibody overnight at 4°C. The primary antibodies used were anti-HDAC2 (Millipore) diluted 1:500 to detect nuclear fractions and anti-β-tubulin diluted 1:5000 detect cytoplasm. After sufficient washing, the membrane was incubated with secondary antibody (Thermo Scientific) for 1 hour at room temperature. Anti-rabbit secondary antibody was used to detect HDAC2 and anti-mouse was used to detect β-tubulin. Blots were developed using the ECL chemiluminescence detection reagent (Amersham Bioscience).

8. Telomere Dysfunction Induced Foci

W4 and S2-4 ASMC were grown to 50% confluency on sterilized coverslips. Coverslips were previously sterilized by autoclaving and/or soaked in 70 % ethanol. The cells grown on the coverslips were washed with twice with phosphate buffered saline (PBS) and fixed with 2% paraformaldehyde in PBS for 10 minutes. The coverslips were then

washed three times with PBS, for 5 minutes each and incubated in blocking solution (3 % goat serum, 1 mg/ml BSA, 0.1 % triton X100 and 1 mM EDTA pH 8.0) for 30 minutes. Cells were incubated in anti-gamma H2A.X (phospho S139) antibody (abcam), diluted 1:200 in blocking solution, for an hour. Coverslips then were washed three times with PBS for 5 minutes each and the cells incubated Alexa Fluor® 488 Donkey Anti-Rabbit IgG (H+L) Antibody (Molecular Probes®) diluted 1:200 with blocking solution. Coverslips were washed three times with PBS for 5 minutes each, and cells fixed with 2 % paraformaldehyde for 5minutes. Following second fixation, coverslips were washed twice with PBS. Cells were dehydrated in a series of 5 minute incubations with 70 %, 95 % and 100 % ethanol. Coverslips were air dried and incubated in prewarmed denaturation solution (70 % formamide, 2x SSC) at 75˚C for 10 minutes. Cells were then incubated in hybridization solution (70 % formamide, 0.5 % blocking reagent Roche®, 10 mM $NaHPO_4$, 10 mM NaCl, 10 mM Tris-Cl pH7.5, 0.2 µM conjugated PNA probe Cy3 5'-CCCTAACCCTAACCCTAA-3'), overnight, in a dark humid chamber. Cells were washed twice with wash solution I (70 % formamide, 10 mM Tris-Cl pH7.5, 0.1 % BSA) for 15 minutes each. Cells were then washed three times with wash solution II (0.1 M Tris-Cl pH7.5, 0.15 M NaCl, 0.08 % Tween), for 5 minutes each. Cells were dehydrated in a series of 5 minute incubations with 70 %, 95 % and 100 % ethanol. Coverslips were air dried and mounted in anti-fade solution containing 0.2 µg/ml DAPI. Images were acquired using Olympus BX61.A sum total of 330 W4 and 250 S2-4 ASMC were used for analysis.

12.5 % Paraformaldehyde stock: 1 g of paraformaldehyde was dissolved in 6.5 ml of water heated in the microwave(somewhere between 70-90 ˚C). NaOH was added until paraformaldehyde dissolved completely. Approximately 1 ml of 10 x PBS was added to

solution for a final volume 8ml. Stock solution was store at 4 ˚C for up to a month. 2 % paraformaldehyde solution was made from stock diluted in 1 x PBS.

10 % (w/v)Blocking Reagent Roche was made by dissolving Blocking reagent in 1 x maleic acid buffer with shaking and heating in microwave periodically until dissolved. Solution was stored in 4˚C for short term with long term stored in -20 ˚C. For 2x maleic acid buffer, 2.32 g Maleic acid, 1.75 g NaCl was dissolved in 80ml of water. The pH was adjusted to 7.5 with NaOH pellets. Then, the volume was also adjusted to 100 ml with H_2O for a final concentration 200 mM Malic acid, 300 mM NaCl. The acid buffer was filter-sterilized and stored at room temperature. 1x maleic acid buffer was made by diluting 2x maleic acid buffer 1:1 with H_2O.

9. Detection of 8-oxo-dG lesions.

W4 and S2-4 ASMC were grown to 50 % confluency on sterilized coverslips. The coverslips were washed twice with PBS and fixed with 1 % paraformaldehyde in PBS on ice for 10-15 minutes. The coverslips were then washed twice with PBS, each wash was 5 minutes. Cells were re-suspended in cold 70 % ethanol for storage in -20 ˚C. At the time of the assay, coverslips were washed in PBS, and then in 1x Wash Solution. 50 µl 1:20 diluted FITC conjugate was added to coverslips, and incubated in dark humid chamber, for 30-60 minutes. The coverslips were then mounted, and images taken using florescence microscopy, excitation of 495 nm and barrier filter of 515 nm.

CHAPTER III: THE EFFECT OF ANTI-ATHEROSCLEROTIC AGENTS ON TELOMERASE

A. Introduction

Humans have evolved intricate strategies for using oxygen and minimizing the harmful effects of reactive oxygen species (ROS). Equilibrium exists between ROS and the antioxidants which neutralize them, in order for cells to properly function. An imbalance in ROS and antioxidants, shifting towards ROS results in oxidation stress.(171) Oxidative stress often causes increased oxidative damage to biological macromolecules such as DNA, RNA, lipids and proteins(172) and is a contributing factor in the development of diseases such as cardiovascular disease (CVD).(132, 173) Considering the role ROS plays in CVD development, antioxidants were once thought to be promising drug candidates for CVD. Theoretically, the therapeutic use of antioxidants would restore the balance to the equilibrium between ROS and antioxidants, and improve clinical outcomes for CVD patients. However, small molecule antioxidants such as vitamins E have had little success in clinical trials.(174, 175) A partial explanation for the lack of clinical benefit of antioxidant vitamins is large scale counteraction of ROS, itself, can be harmful. ROS is not only damaging to cells but also has a beneficial role regulating many biological processes which ensure the proper cellular functioning.(176) Therefore, any therapeutic strategy used to neutralize ROS has to take into account the crucial role ROS plays in cellular function. One new strategy being developed for CVD treatment is targeting specific forms of ROS. An example is the use of inhibitors of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase for the

treatment of atherosclerosis.(177)

Excessive production of the oxygen radical, superoxide anion has been linked to atherosclerosis. Oxidation of low density lipoprotein (LDL) to oxidized low density lipoprotein (oxLDL) is a major contributor to the onset on atherosclerosis. Oxidation of LDL has been shown to be mediated by superoxide production in cells of vasculature.(178-181) Additionally, expression of superoxide dismutase (SOD), a superoxide scavenger, inhibits oxidation of LDL and protects against oxLDL toxicity(182, 183). SOD catalyzes the one electron dismutation of superoxide into hydrogen peroxide(184):

$$2 \bullet O_2^- + 2H_3O^+ \rightarrow O_2 + H_2O_2 + 2H_2O$$

NADPH oxidase is a major source of superoxide in vascular tissue and many studies suggests it has a causative role in atherogenesis.(130, 171, 185) P47phox, a subunit of NADPH oxidase, was shown to be required for atherosclerotic lesion progression in ApoE(-/-) mice. The same study showed ASMC isolated from p47phox(-/-) mice had decreased superoxide levels and proliferation response to growth factors.(186) NADPH oxidase converts atmospheric oxygen (O_2) to superoxide ($\bullet O_2^-$):

$$NADPH + O_2 \rightarrow NADP^+ + H^+ + \bullet O_2^-$$

Disruption in NADPH oxidase activity has been shown in animal models to lessen the occurrence of atherosclerotic lesions. Transgenic mice expressing non-functional NADPH oxidases in monocytes/macrophages or vascular wall, had lower levels of superoxide and atherosclerotic lesions.(187) Treatment of atherosclerotic mouse model with the NADPH oxidase inhibitor, apocynin, decreased the progression of atherosclerosis. Kinkade et al observed apocynin treatment resulted in decreased lesion size and superoxide levels in the

mice.(188) Thus, targeting NADPH oxidase has become a forerunner in potential therapeutic agents as application of once promising antioxidants were not successful.(175, 177)

Based on the evidence of NADPH oxidase inhibitors having anti-atherosclerotic properties, for this study we investigated whether NADPH oxidase inhibitors had any effect on telomerase. We utilized two NADPH oxidase inhibitors, diphenyl iodonium (DPI) and 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo[4,5-d]pyrimidine (VAS2870) (Figure 3.1). DPI is a classical NADPH oxidase inhibitor. The compound is non-specific, and inhibits a variety of targets including nitric oxide synthase, the enzyme which catalyzes the production of nitric oxide.(189) Additionally, DPI inhibits two superoxide producing enzymes, NADPH oxidase and xanthine oxidase.(189) DPI is a potent NADPH oxidase inhibitor. The IC_{50} for NADPH oxidase is 0.9 μM (rat peritoneal macrophages)(190) and Ki for time-dependent inhibition of NADPH oxidase by DPI is 5.6 μM (human neutrophils membrane).(191) It is important to note, DPI may also induce oxidative stress by inhibiting antioxidant pathways such as pentose phosphate pathway.(192)

VAS2870 (Figure 3.1) is a novel NADPH oxidase inhibitor created using rational drug design approaches to be more specific than classical inhibitors.(193) VAS2870 does not inhibit superoxide producing xanthine oxidase nor does it have any superoxide scavenging activity. The IC_{50} for NADPH oxidase inhibition by VAS2670 was determined to be 10.6 μM.(194) VAS2870 is also a reversible NADPH oxidase inhibitor. Interestingly, VAS2870 lessens oxidative stress with no effects on basal ROS production.(175) VAS2870 has also been shown to lessen PDGF-dependent smooth muscle cell chemotaxis without affecting cellular proliferation.(195) Although, more specific than its predecessors – the classical

NADPH oxidase inhibitors, off-target activity of VAS2870 has been identified. VAS2870 directly modifies cysteine residues of the ryanodine receptor–Ca2+ channel.(196)

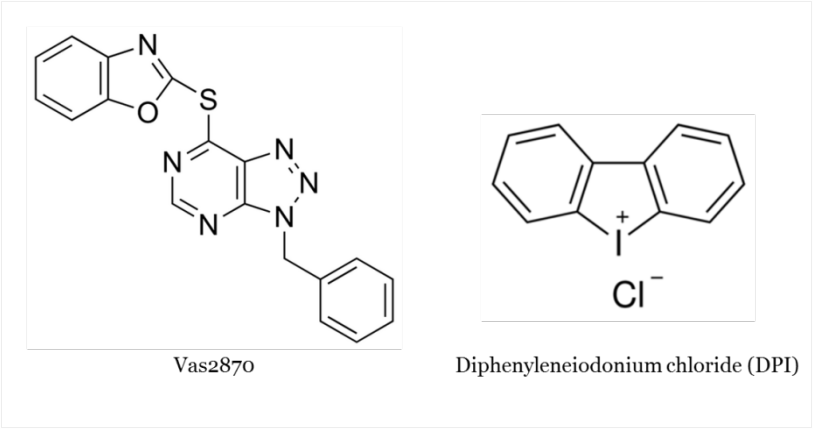

Figure 3.1. The structure of two NADPH oxidase inhibitors: VAS2870 and diphenyl iodonium (DPI) chloride.

There are also some naturally occurring compounds which appear to be protective against atherosclerosis. Estrogen, a class of hormones important in many biological processes, is believed to have vascular protective effects although the underlying mechanisms are poorly understood. Estrogen is believed to be responsible for the lower incidence of CVD in premenopausal women, a benefit which disappears post-menopause.(197) Estrogen inhibits vascular smooth muscle cellular proliferation(198) and inhibits oxLDL-induced inflammation in macrophages(199). Additionally, estrogen has been shown to induce vasodilation by stimulating the release of hydrogen sulfide from endothelial cells.(200) The anti-atherosclerotic effects of estrogen could also be partially mediated by telomerase. Estrogen activates TERT through estrogen-responsive element (ERE) in the *TERT* promoter (57) and could play a role in decreasing telomere dysfunction.

In our study of the effect on anti-atherosclerotic agents on telomerase, we used ASMC isolated from 4 month old atherosclerotic model of mitochondrial oxidative stress, SOD2+/- mice. SOD2+/- mouse has one copy of the gene coding for mitochondrial

superoxide dismutase, an important cellular anti-oxidant which scavenges superoxide. The activity level of SOD2 in SOD2+/- ASMC is about 42% that which is in wild type, resulting in increased levels of superoxide.(143) In accordance with a causative relationship of superoxide with atherosclerosis, the SOD2+/- mouse model suffers aortic stiffening, a precursor to atherosclerosis.(146) In Chapter II, we reported our observation of telomere erosion and surprisingly, elevated telomerase activity in SOD2+/- ASMC. Based on our observation that increased mitochondrial superoxide modulated telomerase activity in the atherosclerotic SOD2+/- model, we hypothesized that the anti-atherosclerosis properties of NADPH oxidase inhibitors may partially be the result of action on telomere maintenance. NADPH oxidase inhibitors should decrease the generation of superoxide resulting in decreased the effect of superoxide has on telomere biology. Furthermore, estrogen is already an established regulator of telomerase.(57, 201) Thus, for this study, we investigated if NADPH oxidase inhibitors and estrogen had any effect on telomerase in SOD2+/- ASMC.

B. Results

1. NADPH oxidase inhibitors have no effect on telomerase activity in SOD2+/- ASMC

Superoxide plays an integral role in the onset of many disease phenotypes such as atherogenesis. The SOD2+/- mouse model, which lacks an important mitochondrial scavenger for superoxide, exhibits pro-atherosclerotic features. The ASMC isolated from the SOD2+/- mice exhibited increased telomere attrition characteristic of the observed reduction of telomere length in CVD despite increased telomerase activity. Inhibitors of NADPH oxidase, the superoxide generating enzyme in cells, have been identified as possible therapeutic agents for atherosclerosis. In an effort to determine the effects these compounds have on telomerase, we treated cells with DPI or VAS2870

prior to assaying telomerase activity. We treated WT ASMC with 10 μM of DPI or VAS2870 for periods of time ranging from 1 – 12 hours. Using the TRAP assay to evaluate telomerase activity, we observed that telomerase activity increased with both compounds in a time dependent manner (Figure 3.2).Telomerase had a greater response to treatment with the more specific inhibitor, VAS2870 than DPI. However the effect VAS2870 had on telomerase lessened after 6 hours.

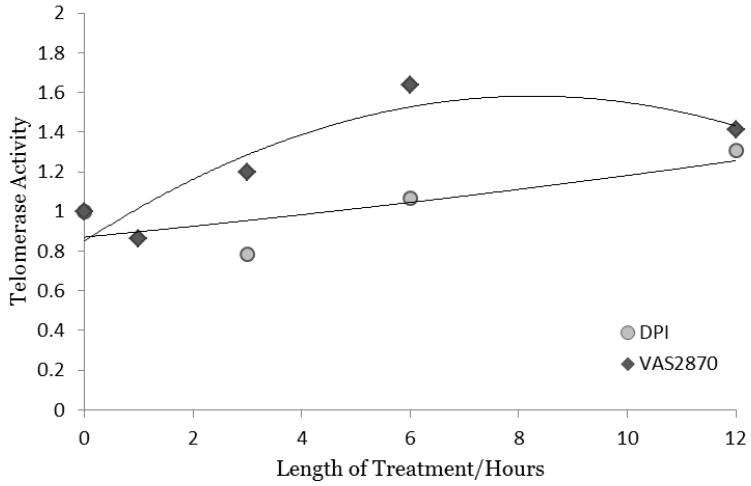

Figure 3.2. The effect of NADPH oxidase inhibitors on telomerase in WT ASMC. W4 ASMCs were treated with 10 μM of either Diphenyleneiodonium chloride (DPI) or VAS2870. WT ASMC was treated with DPI for time intervals of 3 hr, 6 hr and 12 hr. WT ASMC was treated with VAS2870 for time intervals of 1 hr, 3 hr, 6hr and 12 hr. Telomerase activity increased in a time-dependent manner for both compounds, however telomerase response to VAS2870 was greater than the response to DPI treatment. Telomerase response to VAS2870 began to diminish after 6 hours.

Since we observed the greatest telomerase response in W4 ASMC following 6 hour treatment with 10 μM VAS2870, we decided to examine telomerase activity in SOD2+/- with VAS2870. We treated S2-4 with 1 μM and 10 μM VAS2870 for 6 hours. We also treated S2-4 with 10μM VAS2870 for 1 hour and 6 hours. We found that VAS2870 has very little effect on telomerase activity, regardless of concentration or time treated (Figure 3.3). Telomerase activity showed slight increase after 6 hours of treatment. Our efforts to increase VAS2870 concentration to 20 μM were not tolerated by S2-4; 6 hour treatment at 20 μM VAS2870 resulted in detachment of cells from cell culture surface and cell death. WT ASMC appeared seemingly healthy at 20 μM for 6 hours. So the cytotoxic effects of VAS2870 at high concentration maybe heightened in the already 'stressed' S2-4.

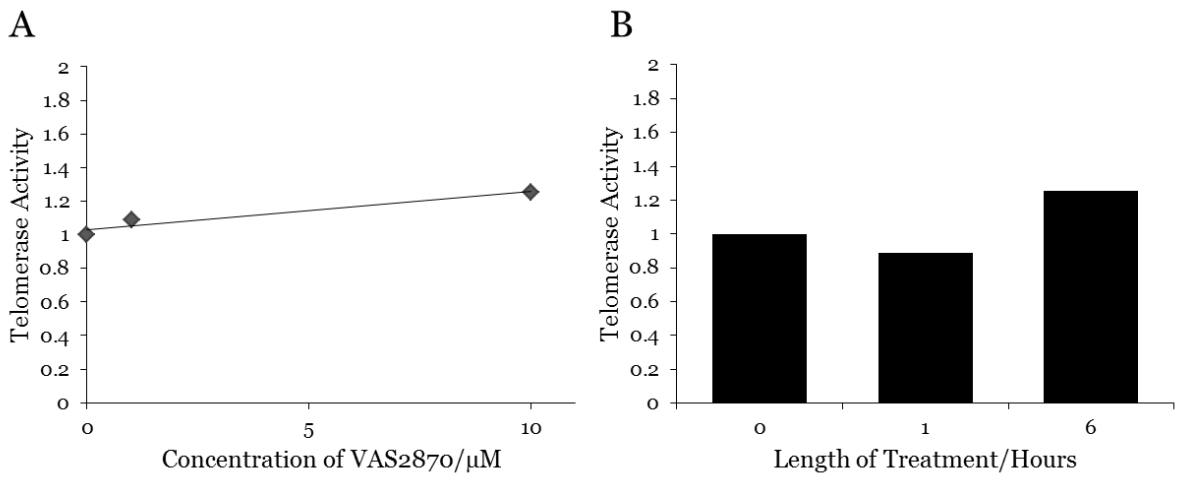

Figure 3.3. The effect of VAS2870 on telomerase activity of SOD2+/- ASMC. A)S2-4 ASMC was treated with VAS2870 in a dose dependent manner. Telomerase activity was measured after 6 hour treatment at 1 μM and 10 μM concentrations resulting in an insignificant change in telomerase activity. B) S2-4 ASMC was treated with VAS2870 in a time dependent manner. Treatment, with 10 μM VAS2870 for 1 hour and 6 hour time points, yielded little response in telomerase activity.

Since VAS2870 has negligible effect on telomerase activity in SOD2+/- ASMC, we considered if telomerase would respond differently to DPI treatment. VAS2870 is a more specific NADPH oxidase inhibitor than DPI. DPI has numerous targets in addition to NADPH oxidase including NADH dehydrogenase, xanthine oxidase and nitric oxide synthase. Nitric oxide, a product of nitric oxide synthase, is a highly reactive unstable radical. It appeared plausible that DPI inhibition of its other targets may have an effect on telomerase activity. We treated S2-4 with 10μM of DPI for 6 hours and observed no significant change in telomerase activity (Figure 3.4). Our results suggest NADPH oxidase inhibition has no effect on telomerase activity in high oxidative stress environment of S2-4.

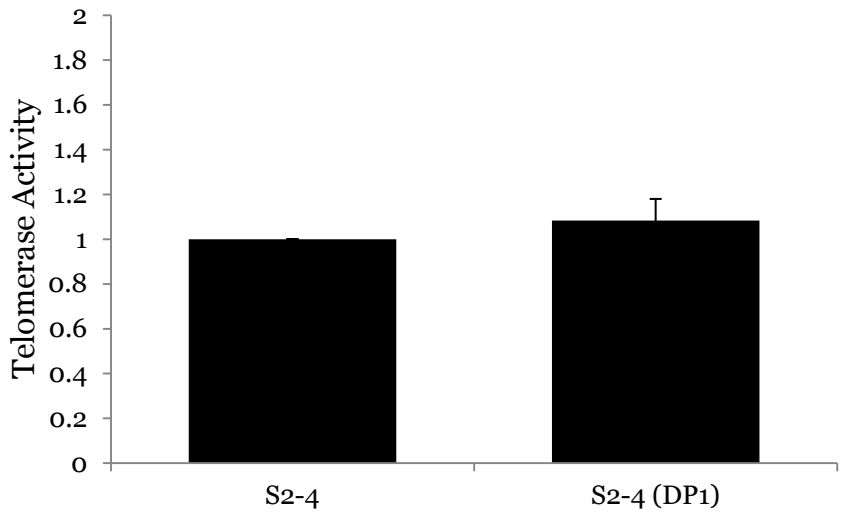

Figure 3.4. Diphenyleneiodonium chloride treatment of SOD2+/- ASMC. S2-4 and W4 were treated with DPI at 10μM concentration for 6 hours. Telomerase activity was measured after 6 hour treatment. DPI treatment did not cause any insignificant change in telomerase activity.

2. β-Estradiol Treatment yields telomerase response in SOD2+/- ASMC.

Estradiol is an established regulator of the catalytic subunit TERT, and consequently of telomerase activity. Estrogen modulation of telomerase activity may contribute to the progression of hormone sensitive cancers such as prostate and breast cancers.(54, 201, 202) Estrogen appears to be preventative against cardiovascular disease and the protective properties maybe related to increased telomerase activity. In this experiment, we treated WT and SOD2+/- ASMC for 52 hours at a concentration of 10 ng/ml prior to conducting a TRAP assay. Fifty-two hours allowed for sufficient time for estrogen transcriptional as well as post-translational regulation of TERT regulation of TERT. We observed no significant change in telomerase activity in WT ASMC. However, we observed a significant increase of 56% in telomerase activity (Figure 3.5). The response in S2-4 and not WT suggests that telomerase in SOD2+/- ASMC maybe more sensitive to estrogen than WT.

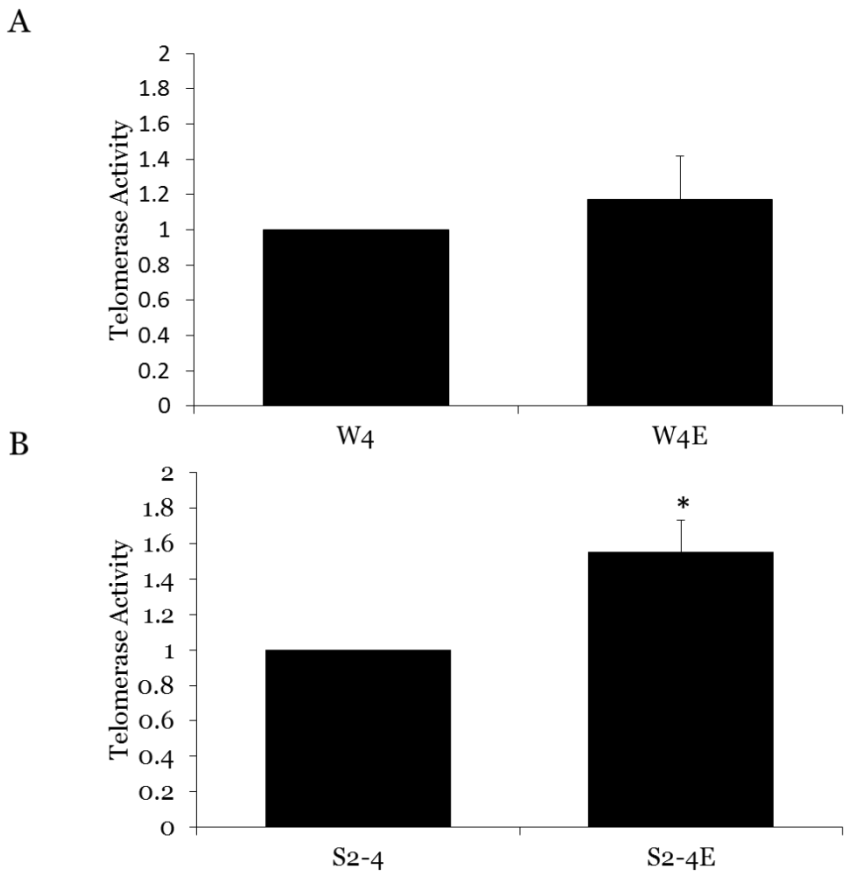

Figure 3.5. Estradiol treatment of WT and SOD2+/- ASMC. WT and SOD2+/- ASMC were treated with estradiol at a concentration of 10 ng/ml for 52 hours. A) Estradiol treatment did not alter telomerase activity of W4 SMC. B) Telomerase activity was approximately 56% greater in S2-4 after estradiol treatment. (*P=0.0357 vs S2-4)

C. Discussion

In Chapter II, we characterized telomere biology in SOD2+/- ASMC and found a phenotype of telomere erosion and increased telomerase activity. Furthermore, the telomere dysfunction in SOD2+/- ASMC was present at 4 month before disease progress and therefore might have contributed to the cardiac dysfunction and aortic stiffening observed in the SOD2+/- mouse model. Notably, telomere attrition is a phenotype seen in humans suffering from forms of CVD including atherosclerosis. In an effort to further study the relationship telomere dysfunction has on disease development, in this chapter we investigated the effects

two types of anti-atherosclerotic agents had on telomerase activity in cultured ASMC. We chose to assay for telomerase activity and not telomere length because changes in telomere length requires long term culturing to observe and primary ASMC cannot be cultured extensively. Telomeric DNA is lost at a rate of 40-200bp per cell division while appreciable changes in telomerase activity can be seen within hours. The types of anti-atherosclerotic agents we examined were the hormone: estradiol and the NADPH oxidase inhibitors: VAS2870 and DPI. Although not completely understood, the therapeutic aspects of NADPH oxidase inhibitors and estradiol are attributed to different pathways. NADPH oxidase inhibitors decrease superoxide, a form of ROS suggested to be causative in atherogenesis. Estrogen, on the other hand, affects multiple pathways as well as increases telomerase expression, and consequently can offset telomere erosion.

In our experiments with DPI and VAS2870, we observed increased telomerase activity in WT but not in SOD2+/- ASMC. The results observed in WT are not surprising. In Chapter II, we observed a small but significant decrease in telomerase activity with acute increase in superoxide by mitochondrial destabilizer rotenone. It follows that if the NADPH oxidase inhibitors caused a decrease in superoxide, an increase in telomerase activity would be expected. VAS2870, having a greater response on telomerase activity in WT than DPI, maybe due to VAS2870 being a more specific inhibitor and DPI being involved in other interactions which are also known to increase other forms of oxidative stress. With the SOD2+/- ASMC, we expected NADPH oxidase inhibitors to decrease superoxide production and reverse the increase in telomerase activity observed in the cell type compared to WT. Instead we found VAS2870 and DPI had negligible effects on telomerase activity. The non-response of telomerase to the NADPH oxidase inhibitors in SOD2+/- ASMC could be a

consequence of compounds inhibiting superoxide production, but not decreasing the superoxide that had already accumulated in the cell or the cellular response to chronic increased oxidative stress resulted in changes that rendered the cells inert to acute changes in superoxide levels. Furthermore, DPI and VAS2780 do not act as superoxide scavengers and are unable to eliminate superoxide in the cell. Additionally, SOD2+/- ASMC has 42% of SOD2 superoxide scavenging ability present in WT, thus ridding the cell of superoxide is much less efficient than in WT. Perhaps, treating SOD2+/- ASMC with a superoxide scavenger to lower superoxide levels before DPI or VAS2870 treatment would have produced different results. Longer treatments with the compounds may also prove informative but the restriction imposed by the number of passages primary cell culture should undergo, limits the duration of treatment. Treating the mouse model with NADPH oxidase inhibitors, would allow for longer term treatment that could possibly rescue the telomere attrition in treated SOD2+/- mice.

Estradiol is thought to be responsible for the observation that pre-menopausal women having low incidence of CVD. The mechanisms by which estradiol is preventative is poorly understood; the hormone regulates many pathways including telomerase activation. In our experiments, we examined if estradiol had any effect on telomerase activity and found, the hormone had no effect in WT but had a significant effect in SOD2+/- ASMC. Our results suggest that telomerase, which was already up regulated in SOD2+/- ASMC, was more sensitive to estradiol regulation than WT. Estradiol may have a greater effect on telomerase of the mitochondrial dysfunctional SOD2+/- ASMC because telomerase may be more epigenetically open to activation and estradiol has mitochondrial protective function.(203) It is important to note, that although estradiol further increases telomerase activity in SOD2+/-

ASMC, it is likely that the enzyme would not be able to counteract the telomere attrition detected. This conclusion is based on the observation that telomeres continued to shorten despite elevated telomerase in SOD2+/- ASMC. As well as the fact, estradiol treatment only increased telomerase activity 56% while telomerase activity in S2-16 was 3- times greater than in S2-4 and was unable to elongate the telomeres. Reversal of telomere erosion may necessitate the reduction of oxidative damage at the telomeres.

D. Conclusion and Future Directions

We found that NADPH oxidase inhibitors can regulate telomerase activity, as seen in WT ASMC, but may not be able to modulate telomerase in an environment with high oxidative stress, as seen in SOD2+/- ASMC. Our experiments concerning the effects of NADPH oxidase inhibitors on telomerase are particularly significant as there is very little in the literature investigating superoxide, NADPH oxidase and telomerase. We also confirmed telomerase activation by estradiol as well as showing that telomerase maybe more sensitive to estradiol regulation when experiencing high oxidative stress. An improvement on our study would be to include a superoxide scavenging compound in our cell treatment procedure to eliminate superoxide levels while VAS2870 or DPI reduces superoxide production. Another option to reduce superoxide already produced would be to increase the time between treatment and cell collection to allow the SOD2+/- ASMC more time to naturally clear superoxide since its superoxide scavenging ability is reduced significantly. While, VAS2870 and DPI are two of the more common NADPH oxidase inhibitors used in scientific studies, it may be beneficial to use a more specific inhibitor such as the novel Nox4 inhibitor, GKT137831. The more specific inhibitor may be able to attenuate the superoxide production

more efficiently. Another interesting follow-up experiment would be to investigate the reasons for this increased sensitivity of telomerase in SOD2+/- ASMC to estradiol. One possible mechanism for increased sensitivity to estradiol is up regulation of estrogen receptors.

This study also had limitations. Due to the risks of the primary cells undergoing transformation, the cells are used between passage #3 and #10 and this doesn't provide enough time to observe the long term effects of estradiol and the other test compounds on telomere length. Follow up studies in mice, would allow for longer treatment regimens and time for telomeres to response. A major goal is to find a treatment which decreases superoxide levels as well as modulate telomerase and use this treatment in SOD2+/- mice and see if treatment is able to offset the telomere erosion as well as reduce the development of CVD phenotypes.

E. Methods

1. Materials

VAS2870 and Diphenyleneiodonium chloride (DPI) were purchased from Sigma. Cell culture grade β-estradiol was purchased from MP Biomedicals

2. Cell Culture.

Mouse aortic smooth muscle cells (AMSC) isolated were from 4- month C57BL/J6 wild-type (WT) and superoxide dismutase 2 haploid deficient (SOD2+/-) mice. SOD2+/- mice were backcrossed at least 8 times into C57BL/J6 background. ASMC were derived from several mice. ASMC were cultured in Gibco® Dulbecco's Modified Eagle's Medium

(DMEM) supplemented with 10% Fetal Bovine Serum (FBS). AMSCs were a gift from Marschall Runge, PhD, MD (McAllister Heart Institute, UNC-CH).

3. Estradiol Treatment.

Twenty μg/ml estradiol stock solution was prepared by dissolving x mg β-estradiol to x ml absolute ethanol and then diluting ethanol solution 1:50 in phenol red free DMEM. Stock solutions were stored at -20 ˚C until needed. Approximately 50, 000 ASMC were plated in each well of a 6-well plate and grown in Gibco® phenol red free DMEM supplemented with 10 % charcoal-stripped FBS, sodium pyruvate and L-glutamine for 48 hours. Cells were then treated with estradiol at 10 ng/ml concentration in Gibco® phenol red free DMEM supplemented with 0.1 % charcoal-stripped FBS, sodium pyruvate and L-glutamine.

4. VAS2870 and DPI treatment.

Twenty mM DPI and 25 mM VAS2870 stock solutions were prepared in DMSO and stored at -20 ˚C until time of use. ASMC at 50-70 % confluence in 6-well plate were serum starved in Gibco® DMEM supplemented with 0.1 % FBS for at least 12-16 hours. Cells were then treated with varying concentrations of VAS2870 or DPI diluted in media supplemented with 0.1 % FBS.

5. Telomere repeat amplification protocol (TRAP).

Primer extension reactions contained 0.5 μg whole cell lysates, 0.1 μg TS primer (5′-AATCCGTCGAGCAGAGTT), TRAP buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 63

mM KCl, 0.05% Tween20, and 1 mM EGTA) and 50 μM of each dNTP. Reactions were incubated at 30 ºC for 30 min. Telomerase extension reactions were then amplified with 0.1 μg ACX primer (5′-GCGCGG(CTTACC)$_3$CTAACC), 0.1 μg NT primer (5′-ATCGGCTTCTCGGCCTTTT-3'), 0.5 units Taq DNA polymerase, 0.5 μl Taq buffer (Invitrogen), dNTPs to a final concentration of 50 μM and 0.001 amol TSNT template (5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3') used as a loading control. The reactions were heated to 95 ºC for 5 minutes and then subjected to 33 PCR cycles at 95 ºC for 30 s, 60 ºC for 30 s, 72 ºC for 60 s. Reaction products were separated on a 12.5 % nondenaturing polyacrylamide gel, stained with SYBR Green I (Molecular Probes), and imaged on a phosphorimager (Storm 860). Reactions were quantified using ImageQuant and the product intensity for each reaction was normalized to the TSNT internal standard

CHAPTER IV: TELOMERASE AND OXIDATELY MODIFIED NUCLEOTIDES

A. Introduction

Reactive oxygen species (ROS) has been shown to regulate telomerase activity indirectly through transcription and post translational modifications.(69, 76, 204) Other mechanisms by which ROS could affect telomerase activity include oxidative damage of nucleotide precursors in nucleotide pools, and direct oxidation of telomerase RNA and the G-rich telomere. Because nucleobases are susceptible to oxidation, damaged nucleotides can be used as substrates by polymerases resulting in a variety of mutations. Many studies have reported that the incorporation of oxidized deoxynucleotides into DNA during replication or DNA repair is an important source of mutagenesis.(205, 206) Accordingly, the presence of oxidatively damaged deoxynucleotides increased the occurrences of error in leading and lagging strand synthesis.(207) The purines, adenine and guanine are the most vulnerable nucleobases to redox modification, and increased oxidation of purines in the dNTP pool increases mutagenesis.(208) Deoxyadenine triphosphate is oxidized to generate 2-hydroxy-2'deoxyadenosine 5'triphosphate (2-oxo-dATP). Incorporation of 2-oxo-dATP leads to G:C→T:A transversion as 2-oxo-dATP preferably pairs with guanine.(206, 209) Since telomerase does not contain a guanine in its template region, the enzyme is not likely to

utilize 2-oxo-dATP even if present in the dNTP pool. However, 2-oxo-dATP could be incorporated into telomeric DNA during lagging strand synthesis.

Guanine is more susceptible to oxidation than adenine due to its high electron density and low oxidation potential.(210) Oxidation of dGTP usually produces 8-oxo-7,8-dihydrodeoxyguanosine triphosphate (8-oxo-dGTP). Both DNA polymerases and reverse transcriptases have been shown to incorporate 8-oxo-dGTP with varying levels of discrimination.(207, 211) Interestingly, in cell culture, the presence of 2-oxo-dATP enhances the mutagenic properties of 8-oxo-dGTP, and increases the occurrence of A:T→ C:G mutations.(209) It is unclear the mechanisms which 2-oxo-dATP in dNTP pool enhances 8-oxo-dGTP but it is believed 2-oxo-dATP prevents hydrolysis of 8-oxo-dGTP. Kamath-Loeb et al. reported that both low and high fidelity reverse transcriptases were more efficient in utilizing 8-oxo-dGTP when using a RNA template than a DNA template. Telomerase, a reverse transcriptase with its own RNA template, has been shown to use modified nucleotide analogues as substrates.(212, 213) It is then plausible that telomerase may use 8-oxo-dGTP base as a substrate and increase oxidative damage lesions in telomeric DNA. Moreover, the accumulation of oxidative damage in telomeric DNA can result in telomere dysfunction.(148, 149)

Additionally, oxidatively modified nucleotides may be able to alter telomerase activity. Evidence suggests that oxidatively modified DNA precursors can reduce polymerase activity as the rate of incorporation of oxidatively damaged nucleotides is slower than with unmodified nucleotides. The insertion efficiencies for 8-oxodGTP incorporation by a variety of polymerases was $>10^4$-fold lower than for dGTP incorporation.(214) Subsequently, incorporation inefficiency is decreased when incorporating a nucleotide opposite a

oxidatively modified base.(215) In studies of telomerase, telomerase activity was inhibited by use of nucleotide analogues. Nucleoside analogues such as dideoxyguanosine (ddGTP)(216), azidothymidine 3'-azido-2',3'-dideoxythymidine (AZT)(216, 217), arabinofuranyl-guanosine triphosphate (Ara-GTP)(218), dideoxyinosine triphosphate (ddITP)(218), 7-Deaza-2'-deoxyguanosine 5'-triphosphate (7-deaza-dGTP) (219, 220) and 6-thio-7-deaza-2'-deoxyguanosine 5'-triphosphate (TDG-TP)(220) were all shown to inhibit telomerase activity, with varying degrees of potency. TDG-TP was found to be a particularly potent (IC_{50} = 0.06 μM) and specific telomerase inhibitor. Fletcher el al. also found TDG-TP could be used as a substitute substrate for dGTP, but only at relatively high concentrations.(220) Notably, chain terminating nucleotiside AZT has clinical relevance. AZT was the first nucleoside reverse transcriptase inhibitor approved for the treatment of HIV-1.(221) In addition to the inhibition of telomerase, many of these nucleotide analog were able to produce telomere attrition (216, 218)and decreased proliferation in cancer cells.(217) Furthermore, nucleotide analogues were also found to decrease telomerase processity.(213) Once incorporated, the presence of oxidatively damaged nucleobases in telomeric product may inhibit telomerase activity depending on the location of the lesion in the telomeric sequence.(152) In this chapter, we examined the possibility that telomerase may use oxidatively modified nucleotides as a substrate and these nucleotide analogs may inhibit telomerase activity.

B. Results

1. Telomerase distinguishes between dGTP and 8-oxo-dGTP

Since telomerase has been shown to use nucleotide analogues as substrates,(212, 213) we hypothesized that telomerase would utilize 8-oxo-dGTP as a substrate. To determine if 8-oxo-dGTP can be used as a telomerase substrate and compete with dGTP incorporation, we incubated recombinant telomerase with increasing concentrations of 8-oxo-dGTP in a direct telomerase assay containing 0.33 μM [α-^{32}P]- dGTP. If 8-oxo-dGTP could compete with dGTP either as a substrate or inhibitor, we would detect reduced radioactivity in the telomerase products and perhaps a change in the characteristic banding pattern of telomerase extension product. Surprisingly, we found that telomerase activity was not affected by 8-oxo-dGTP (Figure 4.1B), and the enzyme exhibited at least a 700 fold preference for dGTP. Direct telomerase assay with increasing concentrations of dGTP containing 0.33 μM [α-^{32}P]-dGTP was a control. As expected, dGTP was able to compete with [α-^{32}P]- dGTP (Figure 4.1A). Additionally, nucleotide analogues have been shown to be able to reduce telomerase processivity,(213) we found the presence of 8-oxo-dGTP had negligible effect on processivity of the enzyme (Figure 4.1C).

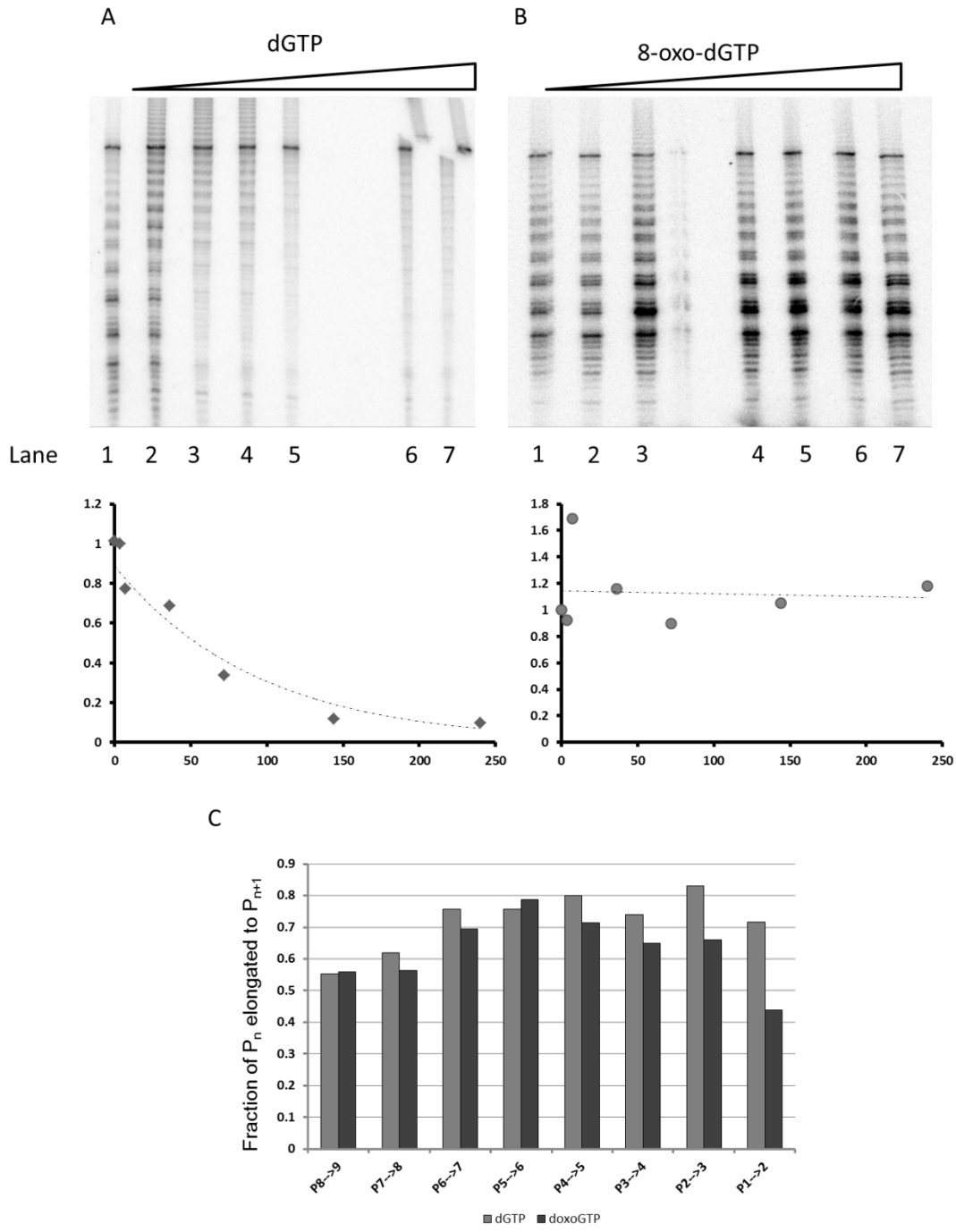

Figure 4.1. Telomerase can distinguish between dGTP and 8-oxo-dGTP. Direct human telomerase reactions contained 0.33 µM radiolabeled [α-^{32}P]-dGTP titrated with increasing concentrations of dGTP and 8-oxo-dGTP. (A) Telomerase direct assay with increasing concentration of unlabeled dGTP: 0µM (lane 1), 3.6 µM (lane 2), 7.2 µM (lane 3), 36 µM (lane 4), 72 µM(lane 5), 144 µM (lane 6) and 240 µM (lane 7). (B) Telomerase activity assay with increasing concentration of cold 8-oxo-dGTP: 0µM (lane 1), 3.6 µM (lane 2), 7.2 µM (lane 3), 36 µM (lane 4), 72 µM(lane 5), 144 µM (lane 6) and 240 µM (lane 7). (C) Chart depicting the fraction, Pn that has been elongated by telomerase to Pn+1 for both unlabeled 8-oxo-dGTP and dGTP both at 3.6 µM concentration.

2. Telomerase distinguishes between dTTP and 8-oxo-dGTP

While, 8-oxo-dGTP has the ability to base pair with all 4 deoxyribonucleotides(222), the nucleotide has been shown to be preferentially incorporated opposite cytosine or adenine. (223) In the non-mutagenic base pairing with cytosine, 8-oxodGTP forms Watson-Crick hydrogen bonds. However, when 8-oxo-dGTP is base paired with adenine, both bases assume Hoogsteen geometry.(222) Adenine base pairs with thymidine, so 8-oxo-dG:dA pairing is mutagenic, and the incorporation of 8-oxo-dGTP often leads to A:T to C:G (A→C) transversions in mammalian cells.(209) Therefore, we hypothesized that telomerase can incorporate 8-oxo-dGTP opposite adenosine, and 8-oxo-dGTP can compete with dTTP. To test this hypothesis, we conducted direct telomerase assays containing 0.33 μM [α-^{32}P]-dTTP and increased unlabeled 8-oxoGTP concentrations gradually. Increasing unlabeled dTTP and dGTP were used as controls. Not surprisingly, unlabeled dTTP competed with 0.33 μM [α-32P]- dTTP (Figure 4.2A), however, we found that 8-oxo-dGTP blocked [α-32P]- dTTP incorporation and increasing concentration had little effect on activity (Figure 4.2B). Additionally, the telomeric products were shorter in our [α-^{32}P]- dTTP telomerase assay containing 8-oxo-dGTP versus an assay containing dGTP, most likely due to telomerase not recognizing 8-oxo-dGTP as a substrate. Accordingly, we observed smaller products in our [α-^{32}P]- dTTP telomerase assay containing 3.6 μM 8-oxo-dGTP (Figure 2B, lane 2) than our assay containing 3.6 μM dGTP (Figure 4.2B, lane 7). As expected, increasing concentrations of dGTP had little effect on dTTP incorporation also (Figure 4.2C).

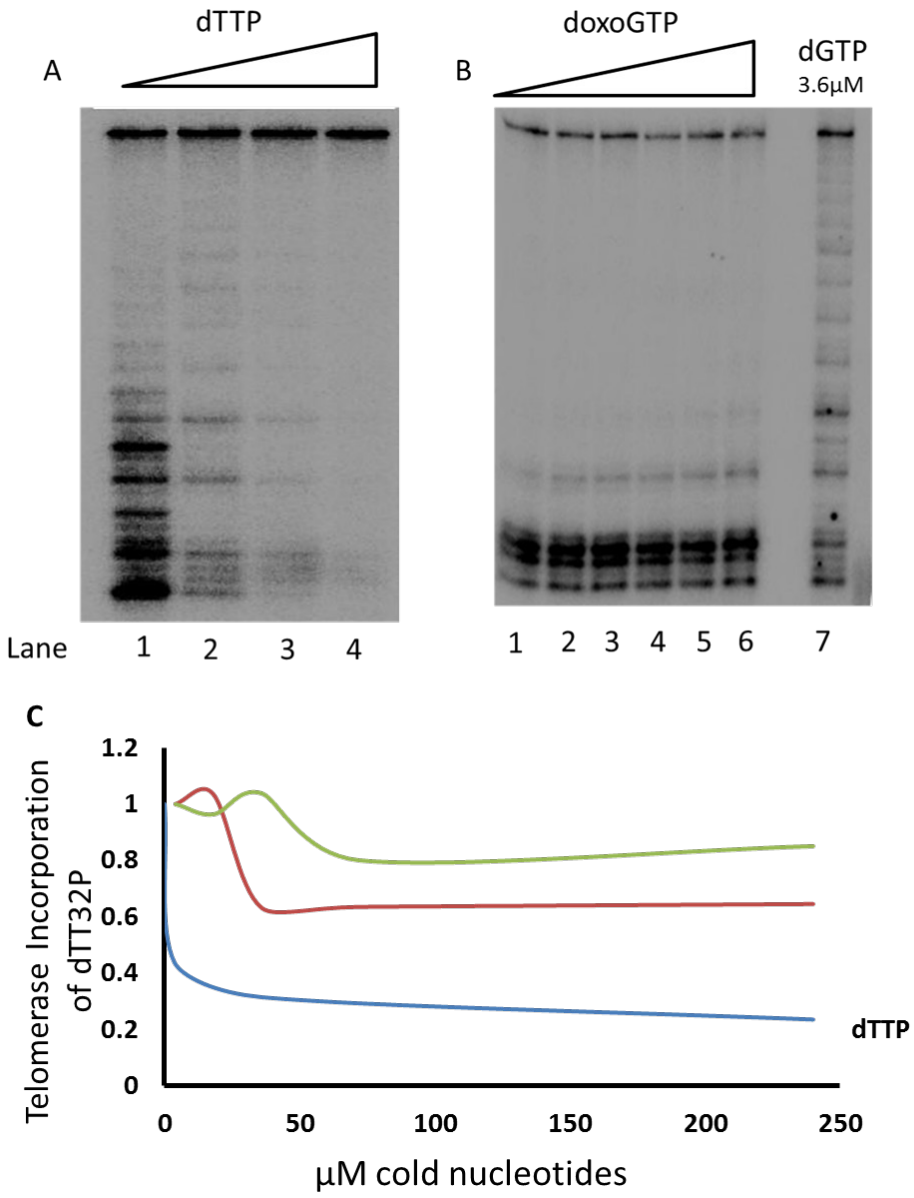

Figure 4.2. 8-oxo-dGTP has little effect on telomerase incorporation of dTTP. Telomerase direct activity assays all containing 0.33 µM [α-^{32}P]-dTTP had increasing concentration of unlabeled (A) dTTP and (B) 8-oxo-dGTP. Unlabeled dTTP concentrations were 0 µM (lane 1), 3.6 µM (lane 2), 36 µM(lane 3) and 240 µM (lane 4). Unlabeled 8-oxo-dGTP 3.6 µM (lane 1), 18 µM (lane 2), 36 µM (lane 3), 72 µM(lane 4), 144µM (lane 5), 240 µM (lane 6) and 0 µM (with 3.6 µM dGTP)(lane 7) (C) Plot of Telomerase incorporation of [α-^{32}P]-dTTP against increasing concentrations of cold/unlabeled 8-oxo-dGTP (green), dGTP (red) and dTTP (blue).

C. Discussion

These studies suggest that 8-oxo-dGTP has negligible effects on telomerase activity and processivity. This product form the oxidation of the dNTP pools is neither incorporated as a substitute for dGTP or dTTP. However, it is possible that in cellular conditions, oxidative damage to nucleotides have a greater effect on telomerase than what is observed these *in vitro* biochemical assays.

While our studies including 8-oxo-dGTP had negative results, there are other experiments we could do to explore other ways ROS may impact other redox sensitive entities involved in telomerase synthesis. This includes direct oxidation of the protein subunit. The thiol functional group of cysteine (Cys) is recognized as a molecular target of redox chemistry and oxidation of Cys within a polypeptide chain has been reported as a regulatory mechanism.(224) The two electron oxidation of Cys yields sulfenic acid (CysSOH), which further oxidizes to sulfinic acid ($CysSO_2H$) and sulfonic acid ($CysSO_3H$). Alternatively, oxidation of cysteine can result in the formation of disulfide bonds through a reaction with a second thiol either from glutathione (GSH), another Cys, or other cellular molecules containing thiol groups.(224-226) It is known that ROS inhibits the activity of protein tyrosine phosphatases via oxidation of an active site cysteine.(227, 228) In addition, there are examples of small molecule telomerase inhibitors that appear to target a free thiol in hTERT for alkylation.(229, 230) Therefore it is plausible that hTERT can be regulated by ROS through direct oxidation of one of its cysteine residues.

A previous student of the Jarstfer lab, Dr. Joana Soares, performed experiments exploring hydrogen peroxide effects on telomerase. She discovered that the activity of immuno-purified telomerase was reduced significantly with hydrogen peroxide treatment and

that catalase, a hydrogen peroxide scavenger, could protect telomerase inhibition by hydrogen peroxide (Figure 4.3A). Furthermore, she found that inhibition by hydrogen peroxide did not result from oxidation of hTERC or telomeric primer (data not shown) but was most likely due to action on hTERT. In an effort to identify the Cys residues that are oxidized, Dr. Soares hypothesized that if oxidation of TERT is evolutionarily conserved then cysteine residues that are susceptible to oxidation would also be conserved in TERT from organisms that are genetically distant from human such as *Tetrahymena thermophila*. It was determined that *T. thermophile* telomerase is redox sensitive (data not shown) and 4 conserved Cys residues that are likely responsible for the inhibition were identified based on sequence conservation (Figure 4.3B). Mutagenesis of the Cys to Ser yielded four hTERT mutants: C413S, C842S, C998S, and C1043S, and 3 of the hTERT mutants: C413S, C998S, and C1043S were inactive (Figure 4.3C). The hTERT C842S mutant which retained telomerase activity remained sensitive to ROS suggesting, it was not the residue whose oxidation resulted in telomerase inhibition (Figure 4.3D). Dr. Soares's results identified several cysteines crucial to telomerase activity, but did not directly identify a redox sensitive residue. Although, one could expect that a conserved cysteine residue, for example C413, which is essential catalytically could also be ROS sensitive.

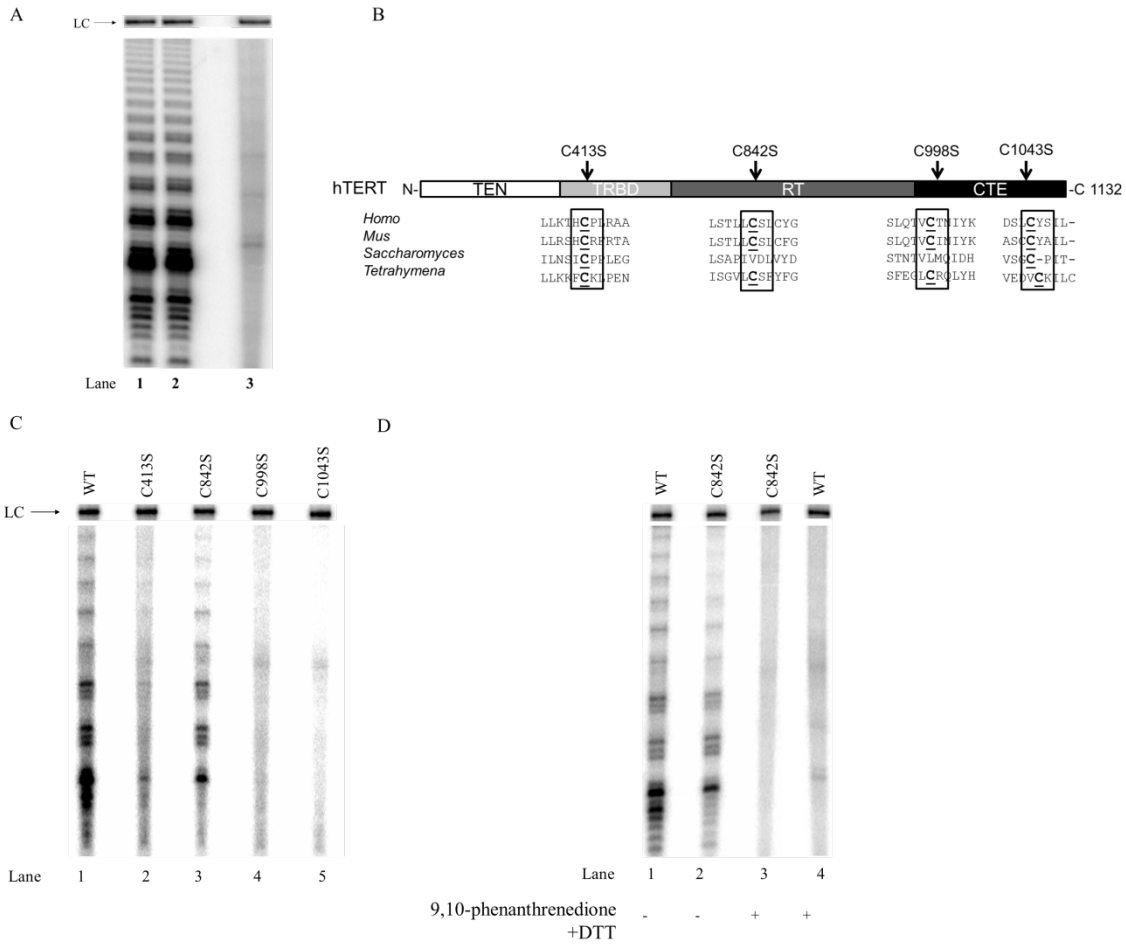

Figure 4.3. Hydrogen peroxide may inhibit recombinant telomerase through reaction with cysteine residue. (A) Direct telomerase assay using cell extracts from recombinant human telomerase, incubated with DMSO (lane 1), with hydrogen peroxide (50 mM) and catalase (10 U) (lane 2), or with hydrogen peroxide (50 mM) alone (lane 3). (B) Cysteine residues conserved between human and tetrahymena telomerase. (C) Telomerase activity of WT telomerase (lane 1), C413S mutant (lane 2), C842S mutant (lane 3), C998S mutant (lane 4), or C1043S mutant (lane 5). (D) Telomerase activity of WT telomerase (lane 1), C842S mutant (lane 2), C842S mutant treated with 9,10-phenanthrenedione (50 mM) and DTT (5 mM) for 1 hr prior to telomerase assay (lane 3), or WT telomerase treated with 9,10-phenanthrenedione (50 mM) and DTT (5 mM) for 1 hr prior to telomerase assay (lane 4).

D. Conclusion and Future Directions

Our exploratory studies with 8-oxo-dGTP coupled with previous results reveal new insights into the effect ROS has on telomerase activity. We found that 8-oxo-dGTP has little effect on telomerase activity. However, while 8-oxo-dGTP is the most abundant and

extensively studied product of guanine oxidation, numerous guanine oxidation products exist such as spiroiminohydantoin, guanidinohydantoin, imidazolone and oxazolone.(210) These products may be present in relatively small quantities in cells but could have biological significance. Exploratory studies on whether or not any of these compounds affect telomerase may yield more promising results. Additionally, we also know that hydrogen peroxide directly inhibits telomerase, most likely though action on Cys residue. However, we have not identified the Cys that maybe responsible. Alternative approaches, such as mass spectrometry, will be required to identify the hTERT lesions.(224, 225)

Furthermore, it might be of interest to determine the effects other forms of ROS have on telomerase directly. For example, in Chapter II, we showed that increased mitochondrial superoxide in cellular conditions resulted in increased telomerase activity, while cytoplasmic superoxide resulted in decreased telomerase activity. We don't however know if superoxide has any direct effect on telomerase or if the changes in telomerase activity observed was a downstream effect or consequence of superoxide action elsewhere.

E. Methods

1. Recombinant telomerase isolation.

HEK293T cells (2-6 x 10^5 per well in 6-well plates) were transiently transfected with plasmids expressing hTERT and hTR. The hTERT plasmid pVan107-FLAG (0.75 μg) and pBS-U1-hTR (3.75 μg) using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Forty-eight hours post-transfection, cells were collected and lysed in CHAPS lysis buffer (10 mM TrisHCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 % CHAPS, 10 % glycerol,

supplemented before use with protease inhibitor cocktail III (Calbiochem) and 5 mM β-mercaptoethanol) by incubating at 4 ºC for 30 min on a rotator. Lysed cells were clarified by centrifugation 13,000 × g at 4 ºC for 10 min. Protein concentration of extracts was determined using the Coomassie Plus Assay kit (Pierce). The extracts were flash frozen and stored without loss of activity for several months at -80 ºC. Flag-tagged telomerase was immunoprecipitated with anti-FLAG M2 affinity agarose beads (Sigma-Aldrich, St. Louis, MO) and used where noted.

2. Direct human telomerase Activity.

Human telomerase activity was measured as previously described.(231) Reactions contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM spermidine, 1 μM human telomerase primer 5'-$(TTAGGG)_3$-3', 0.5 mM dATP, 0.5 mM dTTP, 2.9 μM dGTP, 0.33 μM [α-^{32}P]-dGTP (3000 Ci/mmol; Perkin Elmer) and telomerase from crude cell extracts or affinity purified. Inhibition studies included varying concentrations of inhibitor. Primer extension was carried out at 30 ºC for 90 min. A radiolabelled 114 oligonucleotide was used as loading and recovery control and was added before products were isolated by phenol-chloroform extraction and ethanol precipitation. Products were resolved on a 10% denaturing urea/TBE gel. Gels were dried, imaged on a Storm 860 Phosphorimager, and quantified with ImageQuant. The intensities of each band in each sample were summed and normalized to the loading control. To determine the effects of 8-oxo-7,8-dihydrodeoxyguanosine triphosphate (8-oxo-dGTP) on telomerase activity, direct telomerase activity assays were performed as described with increasing concentrations of 8-oxo-dGTP.

For [α-^{32}P]-dTTP assay, reactions contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM spermidine, 1 μM human telomerase primer 5'-$(TTAGGG)_3$-3', 0.5 mM dATP, 3.6 μM dGTP, 0.33 μM [α-^{32}P]-dTTP (3000 Ci/mmol; Perkin Elmer) and immune-purified telomerase. 8-oxodGTP was added at concentrations between 3.6 and 240 μM, in an effort to determine the effects of 8-oxo-7,8-dihydrodeoxyguanosine triphosphate (8-oxo-dGTP) on dTTP incorporation. Increasing concentrations of dGTP and dTTP were used as control titrations.

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