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Characterization of telomeric defects and signal transduction pathways in Dyskeratosis Congenita cells

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CHARACTERIZATION OF TELOMERIC DEFECTS AND SIGNAL TRANSDUCTION PATHWAYS IN DYSKERATOSIS CONGENITA CELLS

by

Erik R. Westin

An Abstract

Of a thesis submitted in partial fulfillment
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Thesis Supervisor: Associate Professor Aloysius J. Klingelhutz

ABSTRACT

Telomere attrition is a natural process that occurs due to inadequate telomere maintenance. Once at a critically short threshold, telomeres signal the cell to cease division and enter a cell fate termed senescence. Telomeres can be elongated by the enzyme telomerase, which adds *de novo* telomere repeats to the ends of chromosomes. Mutations in the telomerase complex or telomere-related genes give rise to the premature aging disorder Dyskeratosis Congenita (DC). DC provides a unique model system to study human aging in relation to telomerase insufficiency and the subsequent accelerated telomere attrition. In this thesis, skin fibroblasts as well as keratinocytes and T-cells were analyzed from patients with Autosomal Dominant Dyskeratosis Congenita (AD DC) caused by a single allele mutation in the telomerase RNA component (TERC) that leads to telomerase haploinsufficiency. These cells were determined to have a severe proliferative defect and extremely short telomeres. It is demonstrated that the short telomeres in AD DC cells initiate a DNA damage response transduced by the p53/$p21^{WAF/CIP}$ pathway which mediate an elevation in steady-state levels of mitochondrially-derived superoxide and oxidative stress. Exogenous expression of the catalytic reverse transcriptase component of telomerase (TERT) activated telomerase in DC fibroblasts but resulted in reduced activity (~50% compared to control fibroblasts); however telomeres were successfully maintained, albeit at a short length. Simultaneous expression of both TERT and TERC led to robust telomerase activity and elongation of telomeres, indicating that TERC haploinsufficiency is a rate-limiting step in telomere maintenance in DC cells. Reconstitution of telomerase activity in AD DC cells ameliorated the proliferative defects, reduced the p53/$p21^{WAF/CIP}$ response and decreased oxidative stress. Increased superoxide and slow proliferation found in DC cells could also be mitigated by inhibiting $p21^{WAF/CIP}$ or by decreasing the oxygen tension to which the cells are exposed. Together, these results support the hypothesis that the insufficient

telomerase leads to critically short telomeres which signal the activation of $p21^{WAF/CIP}$, leading to increased steady-state levels of mitochondrial superoxide and metabolic oxidative stress as a means to engage senescence. These studies provide insight into mechanisms whereby shortened telomeres lead to premature aging in a humans and point to potential strategies to reduce the effects of tissue dysfunction in DC patients.

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PH.D. THESIS

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This is dedicated to the shoulders of giants that today's scientists and I are currently perched and to Kelly, my kids and the rest of my family who have shouldered the burden encountered when taking the road less traveled. And to SPW whose inspiration has led me on an unexpected but remarkable journey.

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LIST OF ABBREVIATIONS

AD DC: Autosomal Dominant Dyskeratosis Congenita

BMF: Bone Marrow Failure

CAST/EiJ: *Mus musculus castaneus* strain of mice

DC: Dyskeratosis Congenita

DDR: DNA Damage Response

DHE: Dihydroethidium

FACS: Fluorescence Activated Cell Sorting

GSH: Reduced Glutathione

GSSG: Oxidized Glutathione

HSC: Hematopoietic Stem Cell

HSF: Human Skin Fibroblasts

iPS: Induced Pluripotent Stem Cell

NHEJ: Non-Homologous End Joining

$O_2^{\bullet -}$: Superoxide

OIS: Oncogene-Induced Senescence

PF: Pulmonary Fibrosis

Q-FISH: Quantitative Fluorescence In Situ Hybridization

ROS: Reactive Oxygen Species

SA-β-Gal: Senescence-Associated β-Galatosidase (Assay)

SAHF: Senescence-Associated Heterochromatic Foci

SCID: Severe Combined Immunodeficiency Syndrome

SDF: Senescence-Associated DNA Damage Foci

SIPS: Stress Induced Premature Senescence

snoRNA: Small Nucleolar RNA

SOD: Superoxide Dismutase

T-Loop: Telomere-Loop

TRAP: Telomere Repeat Amplification Protocol

CHAPTER 1
REVIEW OF TELOMERES, TELOMERASE AND SENESCENCE-ASSOCIATED CELL FUNCTIONS

Introduction

Humans and all species are predisposed and preprogrammed to age due to a multiplicity of pro-aging factors. Organisms encounter immeasurable amounts of environmental toxins that result in cellular and DNA damage which promote aging. Mechanisms within biological programming ensure that a commitment to aging transpires. The developmental years of a species are necessary to generate and differentiate tissues that compose an organism. This is a period of expansive proliferation that allows an organism to progress from embryonic to adult stages. Once in the adult stage, proliferation slows and the effects of aging begin to appear. Organisms like flat worms and fruit flies are composed of terminally differentiated cells that cease to divide in adults and reside in this postmitotic state for the duration of the organism's life[1]. This postmitotic state restrains cells from re-entering the cell cycle, restricting self renewal and wound healing. During evolution, as organisms diversified and gained complexity, the evolution of the post-mitotic cell led to terminally differentiated cells that could re-enter the cell cycle if stimulated to do so (i.e. wound healing)[2]. The capacity to divide and undergo repair ensured that if this tissue became compromised then the fate of the organism is not inexorably linked to the tissue in question. To maximize proliferation, the cell has evolved to permit re-entry into the cell cycle but with limitations and safeguards. This ensures that older cells, which have presumably accumulated more mutations and damage than younger cells (thus more susceptible to tumorigenesis), are removed from the pool of proliferative cells[3]. This process guides the

cell's fate and leads it down a path resulting in senescence. A senescent cell, akin to a post-mitotic cell, is inhibited from further proliferation but remains metabolically active, thus providing support to the local cellular compartment[2].

Ultimately, complex species like mammals rely on terminally differentiated cells to be replenished over time to circumvent the accumulation of aged cells. Most tissues consist of many terminally differentiated cells that rely on a small pool of stem cells to slowly replenish damaged or aged tissues over time as a means to maintain cellular compartments and organ integrity[2]. Unlike terminally differentiated cells, adult stem cells, such as those involved in hematopoiesis, persist through most of an organism's life due to a combination of maintaining a quiescent state and access to housekeeping measures that prevent these cells from becoming susceptible to the rigors of long-term cell growth[4; 5]. However, during the lifespan of a stem cell, a point arises whereupon the replicative capacity of the cell diminishes thereby altering the composition and age of the compartment[6]. It is believed that it is at this crucial time point in the life of the organism the effects of aging begin to accumulate. The result is an inability of the stem cell population to efficiently replace aged/damaged cells leading to compromised tissues.

Although aging can be attributed to a wide spectrum of factors, two well characterized mechanisms that facilitate senescence and thus thought to contribute to aging are reactive oxygen species (ROS) and telomere attrition.

Senescence

Cell proliferation is essential to an organism as it facilitates organ development, aids in wound healing and an assortment of vital processes responsible for building and maintaining an organism. It is vital to the survival of the organism that a cell is responsive to pro-proliferative and anti-proliferative signals[7]. An appropriate response by cells to these stimuli prevents the organism from becoming susceptible to

precancerous lesions or deteriorating tissues. Along these lines, the cell has also devised a means to arrest proliferation if it has existed within the proliferative pool for an extensive period or has encountered damage. Senescence and apoptosis are cell fate determinants and are activated on a cell and/or stress-intensity specific basis[8].

The first general example in the scientific literature eluding to senescence dates back to the late 1800's[9]. However, this process is best characterized by Hayflick in the 1960's as a part of a seminal study assembled by Hayflick regarding the finite growth of cells called replicative senescence[10]. This report found that normal fibroblasts will proliferate in cell culture for what appeared to be a predetermined, experimentally reproducible amount of time before they entered an irreversible state of growth arrest. This phenomenon, now commonly known as the Hayflick limit, was not reproduced in cancer cells cultured under identical conditions suggesting a difference between the cell types could be informative regarding the ominous nature of cancer cells (Figure 1). Interestingly, this research sparked interest regarding mechanistic control over anti- and pro-proliferative states. Retrospectively, it is now understood that telomere attrition enforced the entry into senescence within Hayflick's cells and circumvention of this limitation in cancer cells was due to, in part, the ability of the cancer to activate telomerase[11] (discussed later).

Senescent cells acquire a multitude of characteristics that define this state[16]. For instance, as cells begin to enter senescence they slow their rate cell division leading up to a permanent growth arrest. Morphologically, senescent cells begin to flatten in cell culture and increase their cytoplasm-to-nuclear ratio indicative of an increased cell size. This change in cell morphology is thought to occur due to increased lysosomal biogenesis and excretion of matrix metalloproteinases[12; 13]. The discovery of the first biological marker that positively stains senescent cells [senescence-associated β-galactosidase histochemical stain (SA-β-Gal)] offered a means to better analyze this cell fate[13]. Using this assay, it was discovered that the fraction of positively stained cells increases in aged

cells *in vitro* and *in vivo*[13]. Other senescence-related diagnostic correlates include the increased prevalence of senescence-associated heterochromatic foci (SAHF)[14] and senescence-associated DNA damage foci (SDF)[15-17]. SDF are commonly found at the telomeres in senescent cells but can also be induced away from the telomeres under alternative senescent conditions (OIS, see below)[18].

A striking difference exists between the molecular profiles of normal and senescent cells. In conjunction with previously stated changes, senescent cells also undergo chromatin alterations[14] and modifications to their gene expression profile[19-23] as a means to attain and persist in this permanent growth arrest. As cells enter senescence, the cell cycle is arrested in G1[15; 24; 25] and reentry into the cell cycle is not unobtainable unless artificially induced in the lab. *In vitro* induction of senescence can be performed by several different methods. In cells that activate little to no telomerase, a net loss in telomere length will occur as cells are expanded *in vitro* and will eventually induce senescence (commonly referred to as replicative senescence)[12]. Telomere dysfunction and concomitant senescence can also be induced by expression of mutant telomere binding proteins such as dominant negative TRF2[26]. Stress inducing DNA damaging agents such as peroxide, UV, and γ-irradiation can cause what has been referred to as stress-induced premature senescence (SIPS)[27]. Finally, overexpression of an activated oncogene such as mutant Ha-Ras can also cause senescence (referred to as oncogene-induced senescence; OIS)[25]. In regards to OIS, senescent cells are often found within tumors demonstrating this safeguard is actively suppressing the escape of cancerous cells[28]. Alternatively, senescent cells are often found in aged organisms suggesting that aging-related pathologies may have an underlying increase in senescent cells[13; 29; 30]. The entry into senescence by passaged cells suggests that the accumulated wear-and-tear that accompanies older cells may be pro-tumorigenic and requires the cell to wind down its proliferation as a preventative measure. Senescence is not singly driven by prolonged

cell proliferation but in conjunction with aberrant proliferation that accompanies the expression of an oncogene[31] displaying the versatility of this cell fate.

To enter senescence, it appears that two distinct but functionally similar pathways may be activated either in concert or individually: the p53-$p21^{WAF/CIP}$ and $p16^{INK4A}$-RB pathways (hereafter $p21^{WAF/CIP}$ and $p16^{INK4A}$ will be referred to as p21 and p16) (Figure 2). p21 and p16 are cyclin-dependent kinase inhibitors that suppress cell proliferation but are also commonly found elevated in senescent cells[32]. Although senescence is thought to be irreversible, cells that senesce due to activation of the p53-p21 pathway can experimentally exit senescence by inactivating p53 that in turn extends their proliferative capacity[16; 18; 33; 34]. This does not appear to be the case after the sustained induction of the p16-RB pathway. Upon p16 activation, cells gain no proliferative advantage once p53, p16 or RB are inactivated indicating a difference in the degree of permanency between activating p53 vs p16[33]. This parallels information gleaned from studies investigating the kinetics of p16 and p21 during DNA damage. p21 appears to be transiently activated during DNA damage allowing a window of opportunity for the cell to repair damaged DNA and the subsequent reentry into the cell cycle[35]. Alternatively, once p16 is expressed it appears that this expression is sustained and accumulates over time. Together, p21 and p16 may work synergistically to enforce either a transient cell cycle arrest or a permanent arrest characteristic of senescence.

Telomeres and Senescence

Telomeres are found on the ends of each chromosome in every cell type of many organisms yet their maintenance differs among the different cell types. For instance, in a small fraction of cells (i.e. embryonic[36], germ[37], and hematopoietic[38] stem cells as well as activated T-cells[39]) telomerase is activated presumably to avoid entry into replicative senescence. In nearly every other cell type, telomere attrition takes place as a result from

the numerous restrictions placed on telomerase activity. Suppressing TERT expression is a typical means to regulate telomerase (or less frequently TERC); however, this restriction can also result by restricting access to telomeres[40-42]. Another factor that results in telomere diminution is the 'end-replication problem' whereby an inherent DNA replication restriction causes incomplete replication of chromosomal termini thereby leading to continuous attrition[43-45] (Figure 3). This leads to a net loss in telomere length yet it is presumed that other factors are involved in attrition[46]. Although telomerase activity is required in stem cell compartments to avoid replicative senescence, these cells will ultimately senesce albeit at a much slower rate than other cells[47; 48]. This loss is substantiated in progressively aged individuals that display a natural decrease in telomere length as they age[49-51]. Typically as a cell differentiates from an embryonic stem cell towards the more specialized adult stem cells and further towards a terminally differentiated cell, telomerase activity correspondingly decreases[52]. Similarly, the restrictions placed on telomerase during cellular development translate into a transition between stably maintained/elongated telomeres to a phase at which telomere attrition outpaces elongation. When considered over the lifespan of the organism, it appears that telomere attrition rates differ among age groups and can be categorized as tri-phasic[51]. Using blood cells collected from infants and young children to assess early attrition rates, this first phase appears to consist of a very rapid decline in telomere length samples. However this rate dramatically slows with unremarkable telomere loss into the mid-20's marking the end of the second phase. A final phase begins at which point the attrition rate increases and holds steady until death. An important question arises from these findings: if telomeres steadily decrease during organismal aging, are telomeres simply an inert biological marker or do they assume a more active role to help facilitate aging? To this end, many groups have closely examined this question[53]. In clinical studies examining telomere length among a variety of age groups, length was found to have some prognostic value towards predicting aging-related morbidity and mortality[54; 55].

Although advancing our understanding regarding the relationship between telomeres and aging, this evidence is highly correlative and must be substantiated by studies better characterizing the effects short telomeres have on cells.

Telomeres and Telomerase

Telomeres are a highly evolutionarily conserved mechanism utilized across many species to cap the ends of linear chromosomes[56; 57]. In the late 1970's, it was discovered that the unicellular eukaryote Tetrahymena has repetitive terminal DNA sequences ($TTGGGG^n$; in humans: $TTAGGG^n$) at the ends of its chromosomes[58] which were discovered later in other organisms suggesting that evolution devised a common solution towards maintaining chromosome termini. The fact that Tetrahymena telomere sequences could be utilized in yeast also supported a common evolutionary origin in chromosome termini maintenance[59] and began a search for a common terminal transferase that could prevent the inherent attrition problems associated with DNA replication. Maintaining telomeres is done by the ribonucleotide reverse transcriptase complex telomerase that is minimally composed of the catalytic component TERT and the RNA template, TERC[60-62] (Figure 4). The catalysis by this terminal transferase was characterized in Tetrahymena in 1985[60] and found dependent on an untranslated RNA component when cloned in 1987[61]. Continued studies of this RNA provided evidence as to the location of the telomere template sequence within this gene in 1989[62]. Subsequent studies manipulating telomerase indicated that telomerase is indeed required for telomere maintenance[63; 64] although alternative mechanisms of telomere elongation (referred to as ALT) have been found[65]. In humans, this complex was purified from HeLa cells in 1989[66]. Later, TERC and TERT were cloned in humans (and other species) supporting the commonality among these diverse organisms[67-70]. Together TERC and TERT assemble the minimal components required to reconstitute telomerase activity[71].

Research generated in the late 1970's and early 1980's that culminated in the elucidation and characterization of telomeres and telomerase activity in Tetrahymena and yeast won Liz Blackburn, her then graduate student Carol Greider and Jack Szostak the Nobel Prize in Medicine in 2009[72].

Telomeres tracts provide a platform for a complex of proteins called shelterin to bind with sequence specificity. This complex helps to distinguish telomeres from double-strand DNA breaks as well as a means to regulate telomere length/maintenance. In the 1970's James Watson and Alexey Olovnikov recognized for the first time that DNA polymerase is inherently flawed and cannot fully replicate linear DNA chromosomes[43; 44], today recognized as an inherent telomere attrition. Olovnikov postulated that this 'end-replication problem' may be problematic for the organism and may have implications relating to aging.

Telomeres and Shelterin

To help the cell discriminate between true double-stranded DNA breaks and chromosomal termini, the protein complex shelterin has been found *in vitro* to bind the telomere and creates an evolutionarily conserved structure called a T-loop[73] (Figure 5). Shelterin is composed of proteins that specifically bind double-stranded telomere repeats (TRF1 and TRF2) or the single-stranded 3' overhang (POT1). The mechanism by which a T-loop is formed is thought to occur by the invasion of the single stranded 3' overhang into an upstream region of telomere-tracts, displacing the G-strand and thus Watson-Crick basepairing with the C-strand. Within the T-loop, TRF1/TRF2/POT1 individually interact with telomere sequence and are tethered/stabilized together through TIN2 (POT1 requires an additional intermediary protein, TPP1)[74]. The presence of shelterin at the telomere provides a regulatory measure by preventing access to telomerase[75], as well as interactions with damage response proteins that would provoke a DNA damage response

(DDR; i.e. ATM/ATR)[76]. If this structure is disrupted or telomeres become critically short, the T-loop is thought to become unhinged[75]. This alteration to the secondary structure engages DNA damage responders, mobilizing p53, p21 and others to arrest the cell cycle and allow repairs or engage cell-fate decisions like senescence or apoptosis[16].

Telomerase-mediated telomere maintenance is restricted to late S-phase of the cycle and otherwise telomerase is prevented from gaining access to the telomere due to its sequestration away from telomeres[77]. However, shelterin provides protection from promiscuous telomerase activity in *cis* while also functioning to regulate telomere length and maintenance. Studies have shown that TRF1 or POT1 inhibition results in telomerase-dependent telomere elongation while elevated TRF1, TRF2 or POT1 decreases the equilibrated telomere length[26; 78-81]. Interestingly,TRF2 inhibition did not result in elongated telomeres but rather provoked end-to-end telomere fusions suggesting that TRF2 has an alternative function in regards to its function within shelterin[81]. These results support a mechanism whereby long telomeres acquire more shelterin and thus increase the likelihood that telomere will be inaccessible to telomerase.

In vitro, the T-loop can be minimally assembled by TRF2, independent of ATP (while TRF1 and TIN2 also assist to a lesser degree)[73; 82-85] suggesting an inherent propensity for this conformation[85]. This suggests that the procurement of the 3' overhang, thought to drive the formation of this structure, may not be dependent on POT1. The T-loop restricts telomerase activity but may also have a key role in relaying information regarding telomere dysfunction or length to the cell. Telomere dysfunction can be recreated *in vitro* by disrupting shelterin proteins which alters the T-loop secondary structure and mobilizing a response by the cell. For instance, excessive cell division[15] or the inhibition of TRF2 or TIN2 initiate a constitutive DNA damage response at the telomere[16]. This response can be abrogated by inhibiting single and double-stranded DNA damage responses (ATR and ATM, respectively)[17]. For example, p53 is simultaneously mobilized upon ATM activation which arrests the cell cycle via the p53-

regulated expression of p21[24]. TRF2 has been found partly responsible for preventing ATM activation via a direct interaction with ATM that conceals an ATM residue (serine 1981) which must be phosphorylated to activate ATM[76]. An interaction with ATM suggests that the presence of TRF2 may be instrumental in the suppression of a DNA damage response. This offers a potential mechanism whereby shelterin can activate/suppress a DDR through a direct interaction with DDR components[86]. Together, shelterin appears to undergo a conformational change as telomeres becomes shorter or damaged, enabling the cell to better recognize short or dysfunctional telomeres to mount an appropriate response.

Telomerase manipulation/knockout

Insight into the effects of telomere attrition on organismal aging and associated phenotypes has been investigated in model organisms by knocking out or inhibiting telomerase activity. Unlike telomerase deficiency in humans[87], these models take many generations to exert a phenotype (yeast:100 generations[88], Arabidopsis: five[89], the worm: five[90] and the mouse: three to five[64]). Some species like *Drosophila melanogaster* maintain telomeres in a unique manner compared to mammals relying on retrotransposons for telomere maintenance[91]. Mice harbor notoriously long telomeres and exert a telomere-related DNA damage response that is similar yet different compared to human responses[92-94]. This suggests that experimental procedures attempting to glean insight into the effects of telomere-related aging in humans, regardless of their evolutionary proximity, will not fully appreciate and replicate the mechanisms found in humans. However, mice have been extensively investigated and offer clues to telomere-related aging that may have implications for humans. Although mice fail to present an initial phenotype within the first generation after knocking out telomerase, subsequent generations exert an aging-related phenotype as telomeres continue to shorten. In one of

the first studies to characterize the effects of short telomeres and aging in telomerase knockout mice (mTerc$^{-/-}$), key pathophysiological evidence was found strengthening the association between aging and short telomeres[93]. Each successive generation of knockout mice acquired telomeres shorter than the previous, correlating with the onset of aging symptoms. For instance, graying hair, alopecia, ulcerated tissues (defects in wound healing), diminished hematopoietic progenitors and an increased cancer predisposition were among the age-related defects found in later generation telomerase knockout mice. However, these mice were surprisingly devoid of typical aging characteristics common to humans like cataracts, osteoporosis and vascular disease. This would suggest that telomeres are only one causative factor leading to aging and may not specifically contribute to certain aging phenotypes. Table 1 compiles information related to telomere and telomerase-related knock mice and display a spectrum of symptoms specifically related to telomere attrition in mice. Recently, other groups have attempted to assess Terc$^{-/-}$ in different strains of mice that have shorter telomeres (CAST/EiJ, thus more like humans; Table 1) and have demonstrated telomere shortening and aging-like phenotypes within one or two generations[95; 96].

Telomerase knockout mice that have been bred into p53$^{-/-}$[97; 98] or p21$^{-/-}$[99] knockout backgrounds lose some symptomology associated with aging suggesting that transduction of a short telomere signal by these two DNA damage responders is required to exert an aging phenotype. However, these mice have dramatically different lifespans as p53$^{-/-}$ Terc$^{-/-}$ mice acquire numerous tumors attenuating their lifespan compared to p53$^{+/+}$ Terc$^{-/-}$ mice. p21$^{-/-}$ Terc$^{-/-}$ mice gain an extension in lifespan without a concomitant increase in tumorigenesis indicating that p21 may be mobilized for only entry into senescence without secondary functions that could compromise genomic integrity[99]. The ability to generate short telomere mice also affords a unique opportunity to evaluate the tumor suppressive activity of short telomeres. For instance, mice can be generated that have an increased risk for tumorigenesis by knocking out APC[100] or p16[101]. However, crossing

these mice into a genetic background consisting of short telomeres partially rescues these mice from tumorigenesis. For this rescue to be successful, it appears that p53 must remain intact as $p53^{-/-}$ mice crossed into a short telomere background are highly susceptible to tumor formation[98; 100]. Overexpression of Tert in mice also supports the hypothesis that telomeres act as tumor suppressors: increased telomerase activity in mice leads to an increased incidence in cancer[102-107]. Despite some key differences, mice provide a relevant animal model, in evolutionary terms, in regards to telomere attrition, aging and associated functions.

Telomeres and Dyskeratosis Congenita

Human diseases that fail to maintain telomeres make an exceptional model to study the human-related effects of telomere attrition. Examples of aberrant telomere maintenance have been found in human disease providing evidence for the biological repercussions that transpire without sufficient telomerase. Telomeres provide a potent tumor suppressor that prevents cells from excessive proliferation. However, when telomeres become prematurely short they engage symptomology consistent with accelerated aging likely due to an early entry into senescence. There are a number of diseases whereby mutated proteins that interact with telomeres are believed to contribute to an aging phenotype. For instance the diseases Nijmegan breakage disorder (NBS1 gene) and Ataxia-telengiectasia-like disorder (MRE11) are caused by mutation in the MRN complex that facilitates DNA repair and involved in recognition of short telomeres. Another set of repair proteins, WRN, BLM, XPF/ERCC1 and ATM are also known to interact with telomeres and found to cause Werner, Bloom, xeroderma pigmentosum syndromes and ataxia telengiectasia, respectively[108]. However, mutations in telomerase and telomere-related genes offer unique insight into the pathogenesis of accelerated telomere attrition without directly compromising other/auxiliary pathways. Diseases that

fall into this category include those where bone marrow failure is prominent (aplastic anemia, Hoyeraal-Hreidarsson syndrome, paroxysmal nocturnal hemoglobinurea, myelodysplastic syndrome and Revesz syndrome) as well as pulmonary disorders. The diseases that arise from these mutations are quite dissimilar until considered within the context of Dyskeratosis Congenita. DC patients often present with a rare triad of leukoplakia, skin pigmentation and nail dystrophy however commonly acquire aplastic anemia and pulmonary fibrosis and harbor the symptomology similar to these other disorders (Figure 6; Table 1). A number of less common traits also arise in these patients including learning difficulties, epiphora and pulmonary disease. Traits symbolic of aging in DC patients include premature graying, osteoporosis, dental loss as well as the underlying telomere defect.

Although first characterized in 1910[109] the genetic underpinnings of DC were only recently discovered in 1998 upon the recovery of mutations within the pseudouridine synthase, dyskerin (DKC1, NAP57) gene[110] (Figure 4; Table 2). Dyskerin is a 57 KD protein that belongs to a complex of proteins that have a putative function in the post-transcriptional processing of pseudouridylating RNAs. In association with other components (H/ACA motif RNAs, NOP10, NHP2 and GAR1/NOLA1) DKC1 can isomerize uridine to pseudouridine in targeted RNAs[111]. Although telomerase does not have a known function in pseudouridylation, DKC1 binds the H/ACA motif within the TERC gene as a means to traffick and accumulate telomerase to Cajal bodies within the nucleus[112-114]. Due to dyskerin's role in pseudouridylation the DC phenotype was initially believed to arise due faulty pseudouridylation. In the following year experiments provided evidence for an interaction between dyskerin and TERC this H/ACA structural motif[115]. These patients' cells were found to have decreased levels of TERC thus in turn leading to decreased telomere length and telomerase activity. In 2001, the first DC telomerase mutation was uncovered in *TERC*, which segregated within affected members of a three generation Iowa kindred[116]. This was consistent with a disease model based on

telomere and telomerase dysfunction. Table 2 highlights these previous discoveries and the subsequent future mutations found in three more telomerase-associated genes (*TERT*[117], *NOP10*[118], *NHP2*[119]) and one constituent of the shelterin complex (*TINF2*[120; 121]). Some of these genes (i.e. *DKC1*) have limited disease presentation while others (i.e. *TERT*) display a wide variety of different disease manifestations. Each of these mutations leads to severely shortened telomeres and clinically-relevant repercussions. Interestingly, DKC1 functionality is not restricted to telomerase assembly or pseudouridylation as internal ribosomal entry sequence expression and microRNAs also appear to be processed by DKC1[122-124]. The fact that DC mutations appear to decrease total DKC1within the cell underscores the hypothesis that these alternative functions may also be impaired in X-linked DC patients[125]. This evidence emphasizes key differences between telomerase and DKC1 mutations[126].

Although humans only have one *POT1* gene, mice carry two (*Pot1a* and *Pot1b*). $Terc^{+/-}$; $Pot1b^{-/-}$ are perhaps the best representative of the DC phenotype when taking into consideration whether the knockout displays the rare triad and bone marrow failure. The prerequisite Pot1b knockout in mice to generate a DC phenotype further suggests differences among human and mice related telomere maintenance. Table 1 also highlights whether knockout mouse models are able to recapitulate the effects related to DC mutations in other genes and indicates that no single mouse model is fully representative of the DC phenotype supporting a need for stronger human-related models.

While many tissues are affected in DC patients, it appears as though the most susceptible cells and cellular compartments are those with high cell turnover that would require heightened telomere maintenance. Bone marrow failure, for example, likely results from inadequate telomere maintenance in the hematopoietic stem cells which increases cell turnover and thus requires terminally differentiated cells to be replenished at a faster rate compared normal individuals[127]. This suggests that the adult stem cell population within these patients may be especially susceptible to the effects related to

insufficient telomerase offering insight into effects of short telomeres on stem cells, self-renewal and aging. Importantly, patients with AD DC experience the inter-generational phenomenon of disease anticipation[128; 129]. Disease presentation in affected DC patients from early generations appears to be less severe than those in later generations indicating that telomerase activity within the germline is compromised. This suggests that a patient's offspring are even more susceptible to the effects of telomere attrition than their parents.

Recognizing DNA Damage: the Double Strand Break

Telomere shortening and dysfunction set forth a signaling cascade to communicate a telomere-related dysfunction using the DNA damage response pathway[75]. Linearized telomere ends and double-strand breaks bear no significant differences to a cell surveilling for DNA damage and thus elicit nearly identical responses. Like double-stranded DNA breaks, short or dysfunctional telomeres accumulate signals within the telomere consistent with mounting a DNA damage response. One mark of DNA damage is the phosphorylation of the histone variant H2A, H2AX (γ-H2AX). The phosphorylation of H2AX serine-139 by ATM sets forth a DDR signaling cascade. This phosphorylation takes place after the MRN complex (MRE11/RAD50/NBS1) recognizes and becomes tethered to the double-strand break (DSB) whereupon MRE11 utilizes its nuclease activity to modify the break[130] and RAD50 bridges the divide between the two broken strands of DNA. Regarding the telomeres, TRF2 and MRN are thought to work in concert to achieve a similar signaling cascade[131]. The presence of MRN at the DSB (more specifically NBS1) plays a key role in the recruitment and activation of ATM[132; 133]. ATM (and to some degree ATR or DNA-PK; all in the PI3K-like kinase family)[134-136] phosphorylates H2AX thus initiating a robust DNA damage response by the cell. ATM is largely responsible for the phosphorylation taking place after double-stranded

DNA breaks while ATR performs this function after recognizing stalled replication forks and single-stranded DNA breaks. DNA-PK, on the other hand, appears to redundantly phosphorylate the DSB however this function appears to mediate functions related to non-homologous end joining (NHEJ)[137]. ATM phosphorylates two megabases[138] flanking the damage site and sets forth a spatio-temporal cascade that coordinates a series of protein interactions to repair the site of interest[139]. This recruitment tool aggregates MDC1, BRCA1, NBS1 (as part of the MRN complex), 53BP1 and others to the damaged site to begin repair[135; 140]. An essential step in mounting this defense is the subsequent recognition of gamma-H2AX by the C-terminal BRCT domains of MDC1[141; 142]. In experiments attenuating MDC1 activity, gamma-H2AX failed to recruit downstream components of the DDR (53BP1 and NBS1) to the damage site[142; 143]. However MDC1-independent DDR exceptions have been uncovered[144-146]. While the C-terminus of MDC1 is responsible for binding γ-H2AX, the N-terminus is phosphorylated by casein kinase 2 (CK2)[147-149] to facilitate the binding of NBS1. These interactions increase the recruitment of the MRN complex thereby heightening ATM recruitment and extending the phosphorylation of H2AX about the flanking DSB locus. This timeline is supported by studies investigating the temporal kinetics of DDR factors with local damage. MDC1 and NBS1 are the first to arrive at the damaged site while 53BP1 and BRCA1 begin aggregation shortly thereafter[150]. Although 53BP1 is trafficked to the damaged site, little is known about its apparent responsibility here. Potential 53BP1 functions during DDR include the maintenance of G2/M[151; 152] and intra-S checkpoint integrity[153] as well as a supportive role in NHEJ[154].

Transduction of DNA Damage Signal: Mediators of DDR

These local DNA damage events lay the groundwork to mobilize factors that provide a measured response to the damage. ATM and ATR (PI3K-like kinases) are

early mediators required to advance the DNA damage signal to appropriate DDR intermediates. Depending on the type of stress present, ATM and ATR have a specific set of proteins that they will activate. One such mechanism whereby ATM relays a DSB signal is by phosphorylating a key sensor, CHK2 while ATR phosphorylates CHK1 related to single-stranded DNA damage (UV damage, stalled replication forks)[155]. p53 is phosphorylated and activated by CHK1/2 and ATM. p53 is often considered the most important damage regulator within the cell. Without p53, DNA damage responses are severely compromised leaving unreliable repair mechanisms to carry the burden of maintaining the genome.

Transduction of the DNA Damage Signal: p53

p53 is perhaps one of the most fascinating and studied genes to date due to its ubiquitous functions in many distinct processes. p53 is known to have prominent roles in DNA damage, cell stress, metabolism, differentiation and development to name a few[156]. p53 was an attractive candidate gene to be mapped in the late 1970's and early 1980's due to an interest in its elevation in a number of cancer models[157-164] and is now known to be mutated in approximately 50% of cancers[165]. Multiple domains reside within p53 conferring DNA binding activity, an oligomerization domain, and a central domain that binds zinc for stability[166-168]. Many mutations that lead to tumorigenesis have been found in or around the DNA binding domain[168]. The ability to bind DNA confers some of p53's ability to regulate the cell cycle via transcriptional regulation.

p53 arrests the cell cycle during stress and cell damage. During dormant periods, p53 is regulated by MDM2, an E3 ligase that ubiquitinates p53 for nuclear export and subsequent proteasomal degradation[169; 170]. Interestingly, MDM2 is a transcriptional target of p53 creating a negative feedback loop wherein elevated p53 activity eventually leads to its own diminution[171]. In order to properly engage p53 during stress, p53 can be

protected from ubiquitination by the phosphorylation of key p53 residues or alternatively MDM2 can be sequestered away from p53[172; 173]. Once activated it is believed that p53 has the capacity to dictate the stress response (apoptosis vs senescence, for example) based upon the stimulant[8]. Once activated, a key gene targeted by p53's transcription activity is p21. This cyclin-dependent kinase inhibitor can arrest the cell cycle, however, other genes involved in a p53-measured response have also been elucidated[174]. The p53-dependent increase in p21 can pause the cell cycle to permit DNA repair or alternatively facilitate entry into senescence[175].

Transduction of the DNA Damage Signal: p21

p21$^{(CIP1/WAF)}$ (CDKN1A; p21) was independently cloned by two separate groups: one searching for proteins interacting with CDK2 (CIP1: CDK2 interacting protein) using the yeast-two hybrid assay[176] and another looking for proteins mediating p53's ability to act as a tumor suppressor (WAF1: wildtype p53-activated fragment)[175]. p21 is a cyclin-dependent kinase inhibitor that has the capacity to restrict progression through the cell cycle when activated. This inhibition relies on an interaction with Cyclin E and CDK2 however other cell cycle-related interactions have been found[177]. p21 arrests the cell cycle in at least two distinct manners: one via an interaction with PCNA (G1, S and G2 arrest)[178] and the other via p53-dependent activation during stress and DNA damage (G1, G1/S)[179; 180]. p53 acts as a p21 transcriptional activator upon cell damage and stress[175]. In response to irradiation for example, p21 appears to be activated by p53 to transiently arrest the cell cycle during repair while telomere dysfunction and senescence sustain p21 expression to fully engage a block in the cell cycle[15; 181].

Transduction of the DNA Damage Signal: p16

p16^{INK4A} (CDKN2A; p16) is another cyclin-dependent kinase inhibitor that arrests the cell cycle in G1 by interacting with Cyclin D and CDK's 4 and 6[182]. This activation

is thought to be a key step in the retention of a hypophosphorylated RB and thus prevent entry into the cell cycle. Using a number of cell lines believed to have underlying genetic defects on chromosome 9, positional cloning was used to identify the underlying genetic defect leading to these cancers. This locus shares exons with another gene, ARF (alternative reading frame), that is a distinct gene due to a frameshift in the use of p16's exons creating a novel peptide sequence[183]. In human cells, ARF expression facilitates the extranuclear export of MDM2 to assist in p53 stabilization[172]. p16 expression is negatively regulated by BMI1 and ID1 while positively regulated by ETS[184-186] that together differentially regulate p16 over time to permit elevated p16 levels in aged cells[7]. However, p16 expression is also induced during oncogene-induced senescence initiated by RAS[25]. This RAS pathway has been well characterized and is thought to be involved in the linear activation pathway of RAF, MEK and ERK which may lead to the phosphorylation of ETS although the p38 MAPK family has also been suggested to activate p16 in response to RAS[186; 187].

Transduction of DDR: p53, p21, p16 and ROS

p53, p21 and p16 each play significant roles in senescence and aging[188]. For instance, activation of p53 and p21 strongly correlate with the presence of telomere dysfunction and senescence while p16 has also been found to associate senescent cells and a number of aged tissues. Regarding telomere dysfunction, there appears to be a collective agreement that p53 and p21 are upregulated in response to short telomeres[15; 92; 189; 190] yet some controversy exists regarding p16's role[92; 191; 192]. Interestingly, aged and senescent cells have been found to harbor elevated levels of reactive oxygen species (ROS)[193]. Maintaining steady-state levels of ROS is important towards preserving cellular and tissue integrity[194]. A new association between p53/p21 and oxidative stress is becoming better understood while p16 may have an indirect or peripheral

association[195]. Elevated p53 and p21 levels have been reported in the face of ROS exposure as a result of cell stress and DNA damage[196-202] suggesting p53/p21 are a downstream component to this stress. However, a relationship between p53 and/or p21 activation have uncovered a dependency on this pathway to elevate ROS[8; 174; 203-205]. For instance, p53 or p21 (but not p16) overexpression have been found to elevate endogenous ROS[174; 205]. ROS can be elevated corresponding to the level of p53 activation thereby facilitating and directing entry into either apoptosis or senescence [8]. Similarly, p21 has also been found to elevate ROS during simulated telomere dysfunction[204]. Antioxidant intervention in each of these instances decreased ROS and improved proliferation suggesting a causal relationship between p21/p53 and ROS. In other studies, p21 and p16 were both closely studied for mechanisms whereby their expression led to senescence. These studies came to an agreement that p16 facilitates entry into senescence in an ROS-independent manner whereas p21-dependent senescence utilizes ROS[205; 206] However, p16 has been found activated by the presence of ROS in the p38 MAPK pathway[207] supporting a mechanism whereby p53/p21 elevate ROS to which p16 is responsive.

ROS and Disease

Although oxygen is commonly ascribed beneficial properties, life in an oxidative environment also carries with it the perils of a certain toxicity termed oxidative stress. This stress must be tightly regulated by cellular measures for the cell to operate optimally[194]. The onset of oxidative stress can originate either intra- or extracellularly and arises from the inability of the cell to mount an effective response to oxidative molecules that is cumulatively called reactive oxygen species (ROS). In excess, ROS needs to be detoxified via a number of enzymatic reactions tailored to diminish specific entities to achieve redox homeostasis consistent with optimal growth for the cell. These

oxidative molecules include free radicals like superoxide and hydroxyl radicals as well as stable species like hydrogen peroxide. ROS is typically generated from four sources: 1) the mitochondrial electron transport chain (superoxide, peroxide), 2) peroxisomal fatty acid metabolism (peroxide), 3) cytochrome P-450 reactions (superoxide) and 4) the respiratory burst generated by phagocytic and other cells (superoxide, peroxide, enzymatic nitric oxide, hyperchlorite)[3]. Perhaps one of the best characterized ROS reactions is the superoxide detoxification reaction. This employs one of three superoxide dismutase (*MnSOD*, *Cu(Zn)SOD* and *EcSOD*) genes in a reaction that generates hydrogen peroxide that in turn is detoxified by catalase or glutathione peroxidase[193]. Each of the SOD enzymes, in addition to catalase and glutathione peroxidases, are compartmentalized in the cell. Superoxide, or more broadly anions, are not thought to be able to cross lipid membranes[208] indicating that superoxide-derived stress has the potential to be compartmentalized rather than radiating away from the source across the cell.

Elevated ROS has the capacity to erode steady-state pathways and components (Figure 7). This deleterious activity is due to interactions between ROS and proteins, lipids and DNA. For instance, the interaction between ROS and proteins generates carbonyl derivatives which are used as a marker of oxidative stress[209; 210]. These oxidized peptide chains may assume sub-optimal secondary structures, altering protein function or cause aberrant protein cleavage. ROS interactions with lipids leads to lipid peroxidation which decreases membrane fluidity and disrupts proper interactions with membrane-bound proteins[211]. Finally, experiments assessing the interaction between DNA and ROS have found DNA is especially susceptible to ROS. Due to its proximity to mitochondrial superoxide production, mitochondrial DNA acquires a number of mutations over time[212; 213]. This provides evidence that elevated mitochondrial superoxide may erode mitochondrial function and provide a means that could slow cell growth[212-214]. Finally, oxidized DNA has been closely investigated as a possible avenue by which oxidative

stress could prime the cell for oncogenesis. Research supports this assertion as free radicals and oxidative damage carry out much of the damage and DNA mutagenesis caused by cigarette smoke[215]. Specifically, exposing DNA to ROS leads to numerous lesions, one of which is the well-characterized lesion 8-OxodG (8-Oxo-7,8-dihydro-deoxyguanine)[216]. This oxidized form of guanine is prone to erroneous DNA basepairing with its purine counterpart, adenine. It is interesting to note that telomeres are guanine rich and may be particularly susceptible to damage by ROS[216]. Together, it appears as though oxidative stress erodes cell function and mutates DNA providing a suboptimal cellular environment.

Oxidative stress is believed to have a hand in the pathology related to a number of diseases. Cardiovascular disease, diabetes, rheumatoid arthritis, atherosclerosis and neurological disorders like Parkinson's and Alzheimer's are all believed to have an underlying oxidative stress component. The presence of oxidative stress has been found in diabetic patients as a byproduct of hyperglycemia. It is known that hyperglycemia alters the manner in which superoxide is produced in the mitochondria culminating in ROS that exacerbates this disease[217-219]. In models of diabetes, antioxidant therapy has shown some promise supporting a causal role for ROS[220]. Another example of a disease thought to have an underlying oxidative stress-related effect is found in amyotrophic lateral sclerosis (ALS) patients. 10% of ALS patients have a mutation in the superoxide detoxifying gene Cu(Zn)SOD[221]. These patients appear to have elevated oxidative stress, however the genetic mutations uncovered to date lead to proteins that display a high degree of variability in regards to their superoxide dismutase activity[221]. This makes it difficult to reconcile the exact correlation among specific mutations, oxidative stress and resulting phenotype[221]. However mislocalization of this SOD, for example, would presumably prevent proper superoxide dismutation offering an explanation that may resolve questions about the spectrum of mutations[222]. Alternatively, pulmonary disease may arise if EcSOD is not properly regulated[223]. EcSOD expression is found in blood,

lung, heart, kidney and other tissues and has important functions in protecting against pulmonary fibrosis, inflammation and other oxidative stress related injury[224-236]. Interestingly, EcSOD is capable of decreasing telomere attrition rates in the face of normal and increased levels of oxidative stress[223] suggesting that EcSOD could regulate entry into senescence. Together these studies suggest that oxidative injury and oxidative stress play a significant role in human-related disease.

Oxidative damage-related diseases are the result of acute or prolonged exposure to this stress. Examples regarding the effects of presumably low doses of ROS (oxidative damage) and the subsequent phenotypic effects can be found in aging-related research. Nearly every model organism currently utilized for research has been interrogated to better understand the oxidative stress-related aging process and have to some common conclusions. A common finding among these studies is the degree to which heightened ROS exposure and the accelerated effects of aging. For example, mitochondria are a major source of superoxide during oxidative phosphorylation[237]. Superoxide is generated at two key loci along the electron transport chain, namely complex I (NADPH dehydrogenase) and complex III (ubiquinone–cytochrome c reductase)[237]. Even at steady-state levels, mitochondria are not capable of fully dismutating superoxide and estimates suggest that 1-2% of superoxide escape detoxification[238]. This source of superoxide has been analyzed and is considered a potential contributing factor to aging[239].

Generally, research supports a causal relationship between oxidative stress and aging. For instance, yeast knockouts of the MnSOD and Cu(Zn)SOD genes decrease lifespan suggesting a potential aging-related role for elevated superoxide[240; 241]. In support of this assertion, inhibition of superoxide production in these yeast SOD knockouts can rescue these effects[242]. ROS and superoxide have been proposed to have requisite functions for proper cell maintenance[243] however suppressing superoxide in this context rescues yeast from purported superoxide toxicity. Given the vast amount of time

that has passed since the last common shared ancestor between mammals and yeast, an argument could be made that unicellular yeast and multicellular organisms may differ in regards to oxidative stress-related aging mechanisms. However, similar studies have been carried out in *Caenorhabditis elegans* and *Drosophila melanogaster* that offer comparable conclusions. In *C. elegans*, the lifespan in wildtype and aged worms could be extended by 44% and 67%, respectively, when fed a steady diet of SOD and catalase mimetics[244]. Increased or decreased oxygen exposure in *C. elegans* has an inverse relationship between aging and oxygen exposure[245; 246]. In *Drosophila*, antioxidant intervention studies attempting to manipulate oxidative stress have not come to a consensus regarding the effects of oxidative stress on aging[247], perhaps due to the increasing organismal complexity in relation to *C. elegans*. In agreement with *C. elegans* findings, increasing oxygen tension in *D. melanogaster* decreases lifespan[248] and generates signs of premature aging[249]. Evidence from reverse genetics whereby artificially selecting for alleles that predispose flies to longevity found oxidative stress-related genetic alterations[250-254]. Heightened SOD and catalase enzymatic activity variants within these studies were discovered suggesting that increased ROS surveillance affords an extension in lifespan. Perhaps most revealing are Drosophila experiments enhancing or ablating SOD gene expression. These experiments confirmed the importance of these genes as null mutations[255; 256] and gene knockout studies[257; 258] severely attenuated lifespan. Genetic models constitutively activating SOD was found to be lethal in some studies[259] while other groups have found the opposite and were capable of extending lifespan[260-262] confounding the effects of superoxide within flies. However, experiments expressing SOD in neurons determined this targeted expression renders the flies viable, resistant to heightened stress and affords an extension in lifespan[263; 264]. This experiment suggests that multi-tissue expression could be detrimental to the organism while certain tissues may benefit from heightened superoxide regulation.

Due to its evolutionarily proximity to humans, mouse research has uncovered intricacies pertinent to the aging-oxidative stress relationship that yeast/worm/fly models would not have been able to divulge. However, these findings reveal that oxidative stress and aging appear to be related, but much more complicated than realized from studies in 'lower' organisms. Knockouts have been generated for each of the three SOD genes found in mammals. Since superoxide is compartmentalized and does not easily cross membranes[265], each SOD knockout offers insight related to the importance of superoxide regulation in their respective compartment. Surprisingly, the redundancy in enzymatic function among SOD genes does not translate to equivalent knockout phenotypes in the respective mice as a striking difference exists. Cu(Zn)SOD and EcSOD knockout mice are both viable but acquire distinct phenotypes that contrast with their wild-type counterparts[266-268] while MnSOD knockout mice are lethal just after birth[269; 270]. Cu(Zn)SOD$^{-/-}$ mice have a reduced lifespan and acquire hyperplastic nodules on the liver consistent with hepatocellular carcinoma[266]. EcSOD$^{-/-}$, on the other hand, are phenotypically normal under low-stress conditions but reveal a decreased lifespan if exposed to increased oxygen[268]. MnSOD heterozygotes have a normal lifespan yet acquire significantly more DNA damage and have a predisposition to tumorigenesis but lack other key oxidative stress markers confounding the superoxide-related effects on aging[271]. The reason for this dissimilarity between EcSOD/Cu(Zn)SOD and MnSOD may be traced back to the evolutionary history of these genes. EcSOD and Cu(Zn)SOD are evolutionary paralogs (EcSOD is also conjugated with copper and zinc carrying dismutase) believed to have arisen due to a gene duplication early in eukaryotic history[272] providing a type of evolutionary redundancy that is not shared with MnSOD. Alternatively, superoxide generated in the mitochondrial matrix may carry a magnitude of severity that is not found in cytosolic nor extracellular superoxide.

Interestingly, the presence of oxidative stress is not always detrimental to an organism. For instance, reduction of ROS can also have repercussions as some ROS are

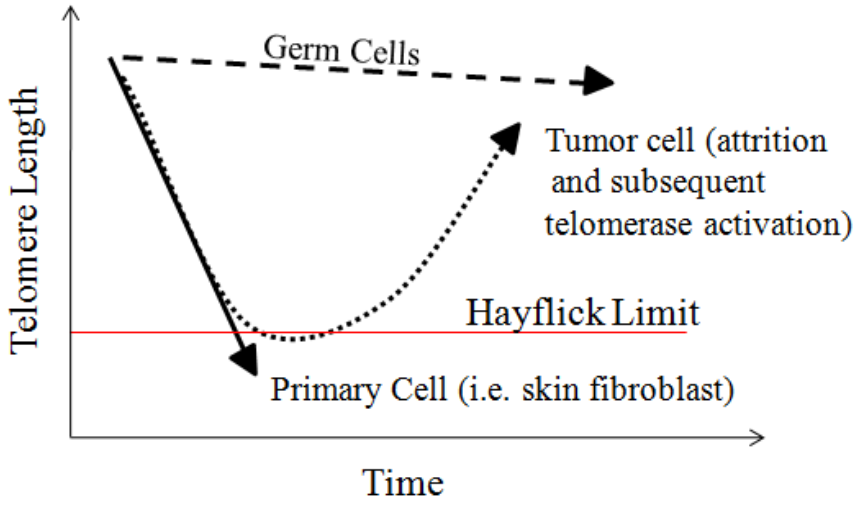
required for routine cellular processes (i.e. proliferation)[273]. A disease has been found that arises due to the inability of patients to mount an appropriate oxidative response to pathogens. Chronic granulatomous disease (CGD), is caused by defective phagocytosis in response to pathogens[274]. Normally, pathogens would be engulfed by phagocytes like neutrophils via phagocytosis. Once taken up, oxygen consumption is redirected to the NADPH oxidase complex generating an oxidative burst consisting of superoxide, hydrogen peroxide and other toxic ROS. However patients with this disease cannot mount an appropriate response which requires them to take prophylactic doses of antibiotics and antimycotics. This as well as other examples[243; 273] indicate that superoxide and ROS toxicity are context dependent and under some circumstances is beneficial. A balance must be struck between antioxidant measures and oxidative stress in order for the cell to optimally function (Figure 8).

Summary

At first glance, the heterogeneous pool of factors that facilitate aging may appear to unrelated however this review has provided a number of examples whereby senescence, oxidative stress, telomeres and DNA damage are interrelated and share common pathways. For instance, an increased DDR can elevate oxidative stress and vice versa. Senescence can be induced by DNA damage and oxidative stress. Telomere attrition is accelerated by oxidative stress and telomere attrition leads to senescence that is correlated with oxidative stress. Aging is an inevitable fate encountered by every species so it is not surprising that there is a level of redundancy found in aging mechanisms. Interestingly DDR, telomeres, senescence and to some extent oxidative stress each have primary functions responsible for suppressing tumorigenesis. Together, it appears that aging may have evolved to prevent excessive proliferation. In the following two chapters, I have investigated the effects of short telomeres in cells from

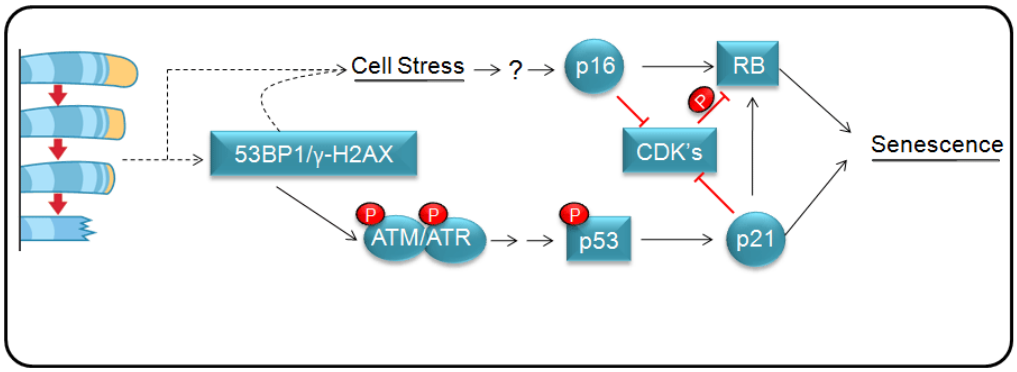
patients with the disease Dyskeratosis Congenita. These cells provide an opportunity to better characterize the effects of aging related to the short telomeres that these cells harbor. Of interest is the extent to which DDR, oxidative stress and senescence are intertwined in order to carry out a phenotype representative of the short telomeres. This research provides a model by which short telomeres engage a DNA damage response that is required to elevate ROS. This ROS, more specifically superoxide, is a candidate for carrying out the effects of aging in cell culture and may have broader implications towards understanding organismal aging.

Figure 1: The Hayflick Limit



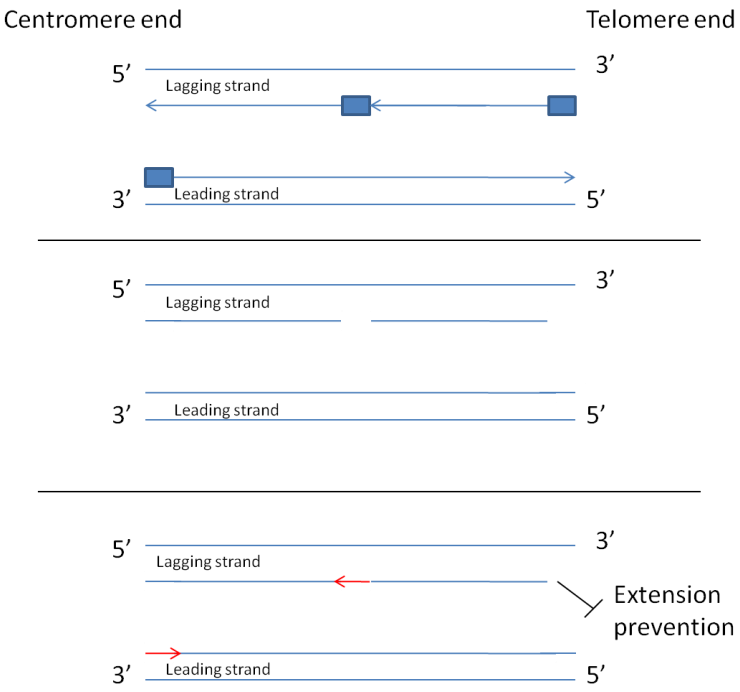
A generalization of the Hayflick limit is demonstrated as telomeres decrease (Y-axis) over time (X-axis) limiting replicative capacity. Primary fibroblasts lack telomerase activity and thus have decreasing telomere length over time. Germ cells mobilize telomerase and thus circumvent replicative senescence over long periods of time. Cancer and tumor cells can evolve from primary cells that lack telomerase activity but aberrant telomerase activation can lead to maintained telomeres thereby preventing replicative senescence.

Figure 2: The Telomere-Related Senescence Pathway



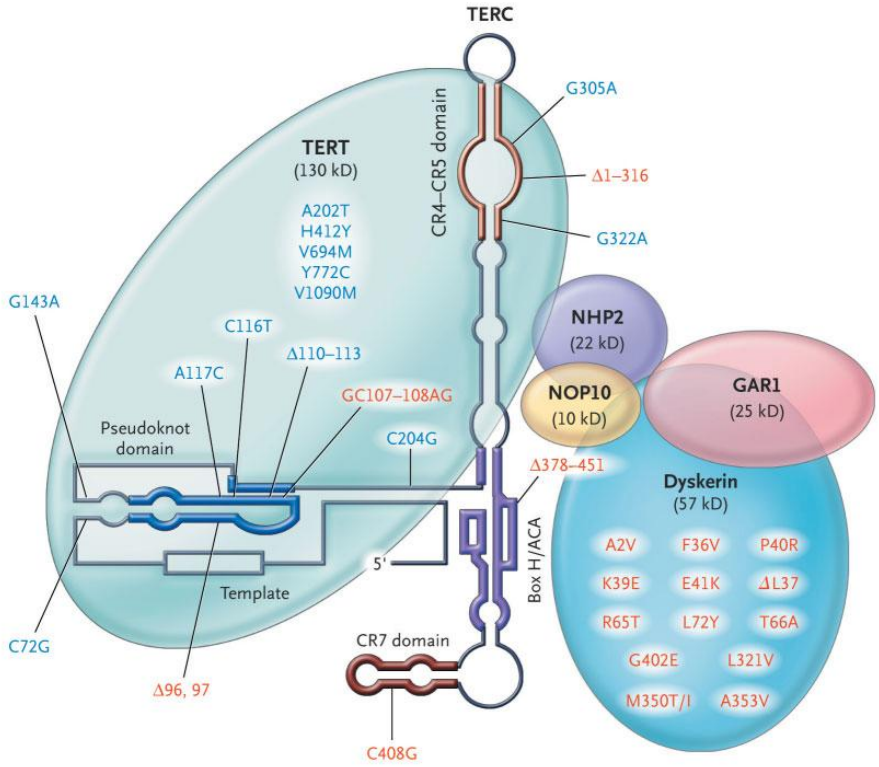
During telomeres attrition, telomeres (yellow; representative image an end of a chromosome) will reach a critical telomere length and begin to accumulate marks of DNA damage that include phosphorylated H2AX (γ-H2AX) and 53BP1. This DNA damage accumulation and pathway will also be activated at non-telomeric DNA damage foci. γ-H2AX and 53BP1 will activate ATM/ATR and eventually p53 and its transcription target p21. Cell cycle arrest will ensue in G1 thereby facilitating a senescent state. p16 can also be upregulate during telomere attrition but is not well understood. Telomere attrition imagery derived from: http://nobelprize.org/nobel_prizes/medicine/laureates/2009/press.pdf

Figure 3: The End Replication Problem



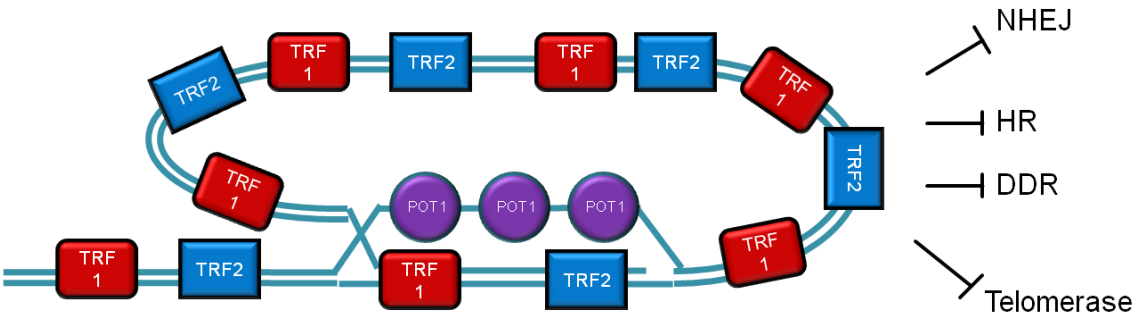
An inherent problem exists when DNA replication is performed when comparing internal DNA (noted on left by 'Centromere end' and the chromosome terminus (noted on right as 'Telomere end'. RNA primers are seeded (blue squares) on to the DNA (blue lines) and extended in a 5' to 3' direction. Once extended, the primers are digested and filled with DNA by DNA polymerase and DNA ligase (red arrows). At the terminus of the 3' parental strand at the teloemere end there is insufficient clearance for gap filling nor an additional RNA primer to extend this lagging strand.

Figure 4: The Telomerase Complex



The telomerase complex is composed of a catalytic reverse transcriptase (TERT), an RNA template strand (TERC) and accessory proteins (shown here as NHP2, NOP10, GAR1 and Dyskerin). Mutations within this complex cause DC (red), bone marrow failure (blue) and other diseases. Current research has also found mutations in NHP2 and NOP10 that cause DC. The terminal 3' hairpin structure (Δ378-451; Box H/ACA motif) is deleted in cells studied in this thesis. Image from Yamaguchi *et al.*[275]

Figure 5: The Telomere End Forms a Secondary Structure (T-Loop)



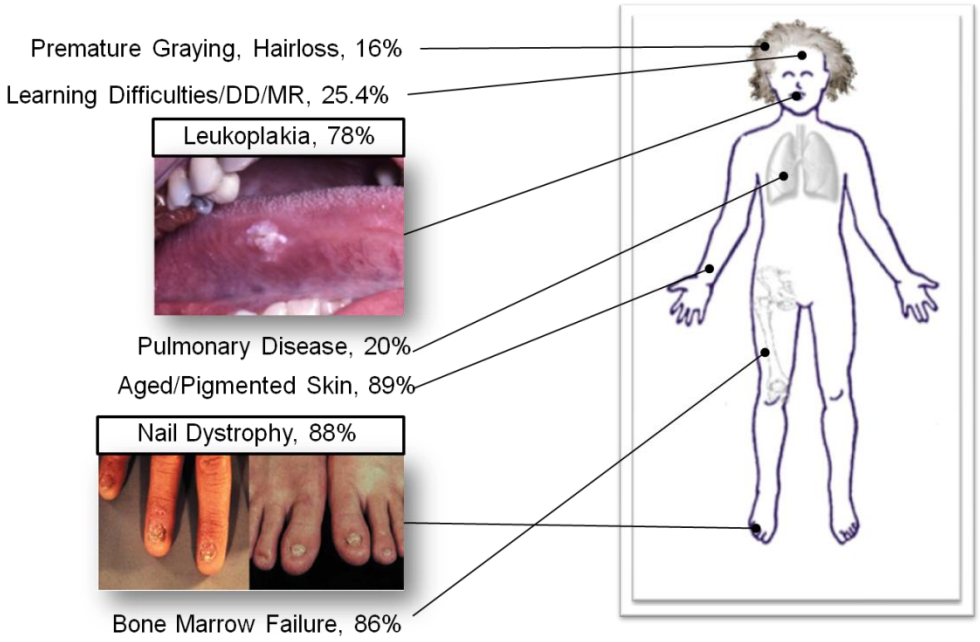
The telomeric 3' single-strand DNA invades an upstream region of the telomere to form a T-loop (and smaller D-loop). This conformation requires a protein complex called shelterin that is composed of TRF1, TRF2, POT1 and other. Within this conformation the telomere distinguishes itself from a double-strand DNA break to prevent a DNA damage break response from the cell. In this conformation, the telomere is also able to prevent aberrant telomerase elongation and pathways resulting in modifying the telomere end (homologous recombination, non-homologous end joining).

Table 1: Comparison of DC and Telomerase- and Telomere-Related Knockout Mice

	DC Phenotype	Terc -/- (Multiple Strains)	Terc -/- CastEiJ Strain	Terc +/+; Short telomeres CAST/EiJ	Terc -/-; Pot1b -/- (single mouse; died P13)	Terc +/-; Pot1b -/-	Tert -/-	Tert +/+/+	Dkc1 -/- or -/Y	Dkc1 hypomorph +/- or -/Y	Trf1 -/-
Skin Pigmentation	**89%**	no	no	no	no	**yes**	no	increased wound healing/growth	embryonic lethal	**skin dyskeratosis**	**yes**
Nail Dystrophy	**88%**	no	no	no	no	**yes**	no	no		no	no
Bone Marrow Failure	**86%**	**prolif. capacity compromised**	**yes**	**yes**	**yes (severe)**	**yes**	no	no		**yes**	no
Leukoplakia	**78%**	no	**intestinal epithelia similarity?**	**intestinal epithelia similarity**	no	no	no	no		no	**yes**
Epiphora	30%	no	no	no	no	no	no	no		no	no
Learning Difficulties	25%	late generation: hypoactivity	no	no	no	no	no	no		no	no
Pulmonary Disease	20%	no	no	no	no	no	no	no		lung abnormalities; yes	no
Short Stature	19%	late generation: decreased body weight	no	no	no	decreased body weight	vertebrae issues	no		no	yes
Dental Caries/Loss	17%	no	no	no	no	no	no	no		no	no
Esophageal Stricture	17%	no	no	no	no	no	no	no		no	yes
Hair Loss/Graying	16%	late generation	no	no	no	no	no	no		no	absent hair follicles
Hyperhiderosis	15%	no	no	no	no	no	no	no		no	absent sebaceous glands
Malignancy	10%	decreased incidence	no	no	no	no	decreased incidence	increased incidence		increased incidence	no
Intrauterine growth retardation	7%	late gen., decrease fetal size	no	no	no	no	no	no		no	no
Liver disease/peptic ulceration/enteropathy	7%	intestine: late generation	intestine: yes	no	yes	intestine	late gen: incr. GI cell apopt.	no		no	no
Ataxia	7%	no	no	no	no	no	no	no		no	no
Hypogonadism/undescended testes	6%	late generation	yes	no	lack of germ cells	yes	later generation	no		no	no

Table 1 continued.
This table incorporates data compiled regarding prevalent DC symptomology[276], mouse knockout models related to telomerase and telomeres (Mouse Genetics Informatics [MGI, http://www.informatics.jax.org/]) and research highlighting specific knockout strains[95; 96]. Multiple different genetic backgrounds were used among mouse models however CAST/EiJ mouse strains were highlighted as these mice have a reduced telomere length reminiscent and comparable to humans which predispose these mice to a phenotype carrying DC symptomology and thus a better model for telomere-related studies. The category 'DC Phenotype' lists the percentage of patients displaying the respective symptom. Skin pigmentation, nail dystrophy, bone marrow failure and leukoplakia are among the most prevalent symptoms and mouse models recapitulating these symptoms are highlighted in red. Terc +/-; Pot1b -/- mice regarded as the best model to recapitulate DC symptomology.

Figure 6: The Dyskeratosis Congenita Phenotype



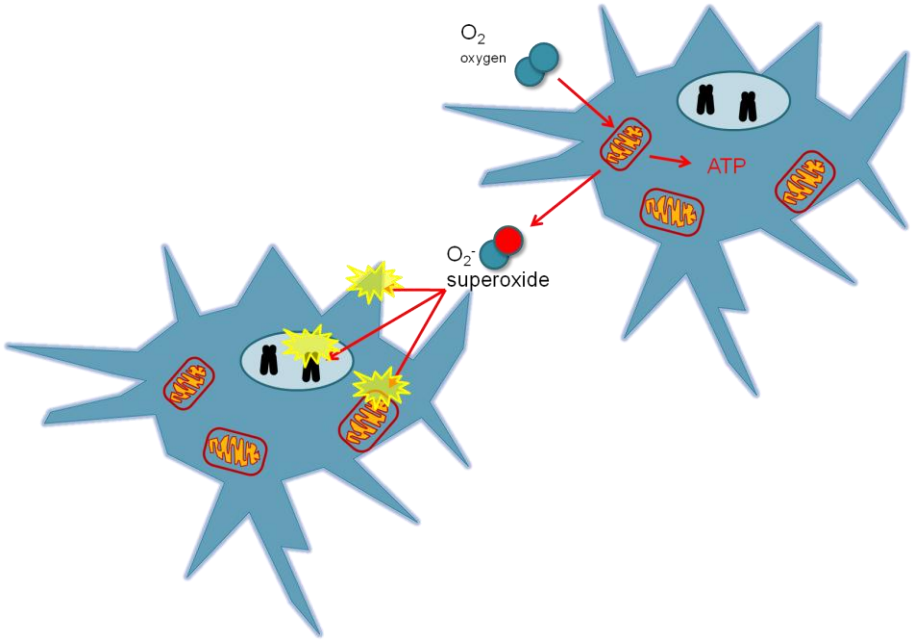
DC is accompanied by a triad of uncommon symptoms that include leukoplakia, pigmented skin and nail dystrophy. These patients also are severely predisposed to bone marrow failure and increased risk for cancer and pulmonary disease. Many other symptoms are common in this disease[276].

Table 2: Location of DC Mutations and Associated Protein Functions

Gene	Alias	Wildtype Gene Function	Total # of Unique Mutations Uncovered (All Diseases, Percentage of Total)	# of Mutations Unique to DC (% of total mutations at gene)	Mutation Comments	Diseases
TERT	*TP2, TRT, EST2*	Catalytic component of telomerase	42 (28%)	7 (17%)	Located throughout gene	Aplastic Anemia, Coronary Artery Disease, Autosomal Dominant & Autosomal Recessive DC, Idiopathic Pulmonary Fibrosis, Hoyeraal-Hreidarsson Syndrome, Cri-du-chat (?)
TERC	*TER, TR*	RNA template strand of telomerase	40 (27%)	11 (28%)	Located throughout gene, No DC Mutations in N-Terminus	Aplastic Anemia, Autosomal Dominant DC, Idiopathic Pulmonary Fibrosis
DKC1	*NAP57, CBF5, NOLA4*	Binds, trafficks, accumulates telomerase via the scaRNA TERC domain; putative pseudouridine synthase; part of snoRNP complex	44 (30%)	44 (100%)	Conc. of mutations in N-term, many mutations result in exon 15 loss	X-linked recessive DC, Hoyeraal-Hreidarsson Syndrome
NHP2	*NOLA2*	Part of snoRNP complex responsible for rRNA processing	3 (2%)	3 (100%)	All mutations concentrated in exon 4	Autosomal Recessive DC
NOP10	*NOLA3*	Part of snoRNP complex responsible for rRNA processing	1 (1%)	1 (100%)	Located in exon 2	Autosomal Recessive DC
TINF2	*TIN2*	Intermediate binding protein among shelterin components (binds TPP1, TRF1, TRF2)	18 (12%)	15 (83%)	All mutations concentrated in exon 6a	Autosomal Dominant DC, Revesz Syndrome, Aplastic Anemia
			148 (100%)	81 (55%)		

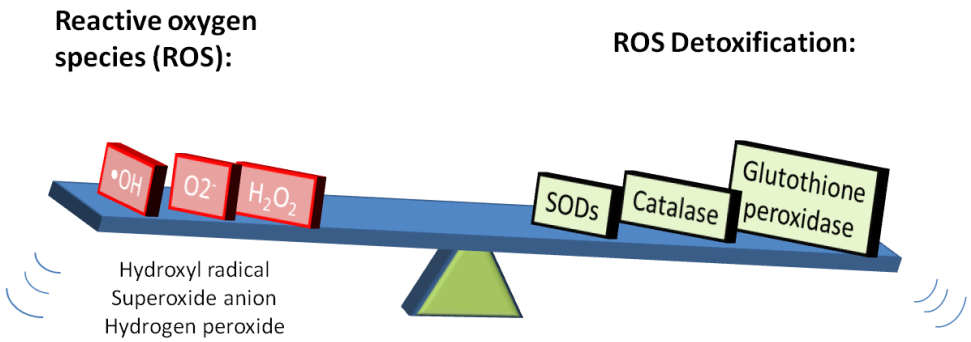
DC and other diseases have underlying genetic mutations in telomere and telomerase genes that lead to severely shortened telomeres that presumably lead to disease presentation. These mutations are found in the minimal telomerase complex (*TERT* and *TERC*), a snoRNA protein complex responsible for post-transcriptional rRNA modifications (*DKC1*, *NHP2*, *NOP10*) and a telomere-binding protein that tethers the protein complex shelterin (*TIN2*). Data acquired from: http://telomerase.asu.edu/diseases.html[57] and updated as of March 2010.

Figure 7: Simplified View of Superoxide



This schematic is a simplified view of one ROS, superoxide. Although created in multiple loci within the cell, a predominant souce is the mitochondria. During oxidative phosphorylation, oxygen is consumed and a small percentage (~1%) is reduced to superoxide. For illustrative purposes, superoxide is shown crossing the cell membrane although this rarely happens due to strict compartmentalization within the cells. As in the figure, superoxide can react negatively with DNA, proteins and lipids thereby altering their structure and function. Superoxide is believed to be an important component of the Free Radical theory of aging related to heightened oxidative stress.

Figure 8: Balancing Oxidative Stress



Oxidative stressors like peroxides, superoxide and others can erode cell function but have important roles in proliferation and immune responses. Antioxidant measures can be detrimental if interupting steady-state processes essential for cellular maintenance. Alternatively, excessive oxidative stress is deleterious and needs to be ameliorated before excessive damage ensues

CHAPTER 2

TELOMERE RESTORATION AND EXTENSION OF PROLIFERATIVE LIFESPAN IN DYSKERATOSIS CONGENITA FIBROBLASTS

Introduction

Telomeres consist of hexameric tandem repeats (TTAGGG) of DNA located at the chromosome ends and are necessary for maintaining chromosome integrity, function, and replication [277; 278]. Telomeres normally shorten with each cell division and it is thought that this eventually results in cellular dysfunction, aging, and genetic instability [108; 279; 280]. Shortening of telomeres is primarily due to what has been termed "the end replication problem", which simply means that DNA polymerase cannot completely replicate the 5' end of newly synthesized linear DNA strands because it requires a 3' primer [281]. In addition, a number of other mechanisms for telomere loss have been described [282]. Telomeres can be maintained by telomerase, an enzyme complex consisting of an RNA component called TERC that acts as a template for a reverse transcriptase component TERT to catalyze the addition of telomere repeats to the telomere ends [70; 283-287]. Telomerase is active in human germline cells and most cancers [37; 288]. Most normal human somatic cells, such as fibroblasts, express TERC but have low to undetectable levels of TERT and almost undetectable levels of telomerase activity. Some highly proliferative cells such as activated B and T lymphocytes also have active telomerase, although in these cells it is thought to be tightly regulated [289-291]. Definitive proof that telomere shortening is involved in cellular senescence was provided in a seminal study in which it was demonstrated that expression of TERT could activate telomerase, maintain telomeres and extend the lifespan of human cells without any other apparent changes [292; 293].

Dyskeratosis Congenita (DC), inherited in both an X-linked and an autosomal dominant (AD) manner, is a premature aging syndrome characterized by bone marrow failure, leukoplakia, abnormal skin pigmentation, and nail dystrophy [294-296]. A variety of somatic abnormalities normally seen in aged individuals have also been reported in DC, including aplastic anemia, hair loss, gray hair, osteoporosis, cancer, and pulmonary and hepatic fibrosis. DC patients also display an increased risk for malignancy, though bone marrow failure is the main cause of early mortality. The X-linked version of the disease is caused by mutations in dyskerin, a protein that interacts with TERC and is required for TERC accumulation [110; 115; 294; 297]. Measurement of telomeres from the somatic cells from patients of the X-linked form of the disease revealed that they were shorter than normal [115; 298]. The autosomal dominant form of DC (AD DC), a more rare form of DC, is generally caused by mutations in TERC [116; 299], although a recent paper reported a 3-generation AD DC kindred that was associated with a mutation in TERT [300]. Other studies have shown that certain forms of aplastic anemia, without overt signs of DC, are also associated with mutations in TERC and TERT [275; 299; 301-303]. These latter results suggest that certain hematologic disorders besides DC may also be associated with defects in telomere length maintenance.

We identified a large three-generation kindred of AD DC individuals with an TERC mutation that display all of the typical features associated with DC [116]. The mutation in this family creates a 3' truncation of the last 74 bases of TERC. Interestingly, this region has been shown to be important for binding to dyskerin, thus potentially linking the X-linked and autosomal forms of the disease [304]. It has been speculated that some TERC mutations may act in a dominant negative fashion, interfering with the normal TERC expressed from the other allele [305; 306]. This does not appear to be the case for the TERC mutant found in our family as it expressed at low to undetectable levels and reconstitution experiments indicate that has no dominant negative effect [307]. A recent study suggests that some naturally derived TERC mutants can act in a

dominant negative fashion, although none of these mutants had a large scale deletion [301]. Average telomere lengths in lymphocytes from AD DC patients are shorter than normal [116; 128; 298; 308] and AD DC lymphocytes exhibit proliferative and functional defects [309]. Telomere length analysis of individual chromosomes in AD DC cells failed to detect a bimodal distribution of telomere lengths, but instead demonstrated that cells had shortened telomeres overall, suggesting that shortened telomeres inherited from the affected parent may be preferentially elongated during development at the expense of longer telomeres from the unaffected parent [128].

Reconstitution of telomerase components and telomere length in DC cells could be considered as a potential means to prevent premature senescence and cell dysfunction *in vitro* and *in vivo*. A recent report demonstrated that exogenous expression of TERT in X-linked DC fibroblasts was able to extend lifespan but was insufficient to elongate telomeres without co-expression of TERC [310]. To our knowledge, no definitive experiments have been performed to determine whether telomerase activation in cells from patients with autosomal dominant DC can restore telomere length. We hypothesize that the short telomere defect in these cells is not irreversible and that rescue can be attained upon mobilization of telomerase the subsequent maintenance of telomeres thereafter. In the present study, we have transduced AD DC fibroblasts with different combinations of TERT and TERC vectors and demonstrate that telomerase activation can restore telomere length and rescue cells from senescence. Our studies indicate that co-expression of TERT and TERC together may provide a more efficient means to restore and elongate telomere length than expression of TERT alone.

Results

AD DC fibroblasts proliferated at rates similar to normal cells at early passage. However, the proliferative lifespan of the AD DC cells was about half that of their

normal counterparts (figure 9a). To quantitatively assess telomere signal between the AD DC and normal cells and in the cells as they were passaged in culture, we utilized an established real time PCR methodology that measures relative levels of telomere repeats as compared to a single gene copy control (36B4)[311] which allows . Using this method, we found that telomere signal in the early passage AD DC cells was significantly less than that observed in early passage normal cells (~3 fold less) and even less than that observed in normal cells at the point of senescence (figure 9b). This is similar to data obtained previously on lymphocytes from 3rd generation patients from this family [309]. Thus, somatic cells from AD DC patients start out with extremely short telomeres and a proliferative lifespan *in vitro* that essentially mirrors proliferative defects *in vivo*.

To determine whether telomerase and telomere length could be reconstituted in AD DC cells, we generated a replication defective feline immunodeficiency virus (FIV) vector that co-expresses TERC and eGFP from separate promoters (figure 10a). In this construct, the TERC gene contains a large portion (515 bp) of the endogenous 3' end, which we reasoned would allow for proper processing of the RNA into the mature form [297; 312]. As has been described previously [297], a snoRNA U3 promoter was utilized to drive TERC expression, whereas eGFP was expressed from a CMV promoter. This vector, designated FIV TERC/eGFP, was used to generate VSV-G pseudotyped virus and tested for functionality in telomerase negative VA13 cells. VA13, which utilizes an ALT mechanism of telomere elongation, is negative for expression of both TERC and TERT, but a variant was generated that expresses TERT but still remains telomerase negative [297]. Infection of TERT VA13 cells with our vector resulted in high telomerase activity, demonstrating that the vector was efficient and functional at expressing TERC (figure 10b). Northern analysis also verified that cells transduced with the TERC/eGFP construct expressed a mature TERC RNA of approximately 450 bases in length (figure 10c).

We then performed experiments to determine whether the FIV TERC/eGFP vector could reconstitute TERC expression and telomerase activity in AD DC cells. As it is known that normal fibroblasts express barely detectable levels of TERT, we reasoned that activation of telomerase in AD DC and normal fibroblasts would require exogenous expression of TERT. AD DC and normal fibroblasts were therefore transduced with different combinations of vectors to express GFP alone, TERC alone, TERT alone, or TERC and TERT together. Approximately 90% transduction efficiency was obtained using the FIV constructs at an MOI of 5, as assessed by GFP expression (data not shown). Cells that had been successfully transduced with the TERT retroviral vectors were selected in G418, whereas cells that were transduced with the lentiviral vector were followed for eGFP expression. Cells were passaged as pools or individual colonies were ring-cloned and subcultured. Analysis of TERC levels in the vector only cells by quantitative RT-PCR demonstrated that AD DC cells had approximately half as much TERC transcript as normal cells, as would be expected due to one defective copy of TERC in AD DC cells (figure 11a). Based on this result, it was not surprising that expression of TERT alone activated telomerase in AD DC fibroblasts but to a lower level (~1/2) than what was observed when TERT alone was expressed in normal fibroblasts (figure 11b). Interestingly, expression of TERT alone, in both AD DC and normal fibroblasts, resulted in higher levels of TERC, suggesting that TERT can stabilize TERC as has been previously proposed (figure 11a) [310]. As expected, both AD DC and normal fibroblasts transduced with the TERC vector alone had higher levels of TERC than untransduced cells, but TERC expression alone did not activate telomerase in either cell type. Expression of TERC and TERT together in AD DC fibroblasts, on the other hand, brought the level of telomerase to approximately the same as what was observed in normal fibroblasts that expressed TERT alone. Co-expression of TERC and TERT in normal fibroblasts caused telomerase activity that was higher than that observed with

TERT alone, indicating that TERC levels were a limiting component in cells that overexpressed TERT.

We next assessed telomere signal, as a measure of telomere repeats, in the cells by quantitative PCR. Telomere signal corresponded well with telomerase activity (figure 11b and 3c). Transduction of vector or TERC alone did not activate telomerase or elongate telomeres in either AD DC or normal cells, and TERT AD DC cells had approximately half the telomere signal of TERT normal cells (figure 11c). Telomere signal was clearly much greater in AD DC or normal cells that expressed TERC and TERT together. Thus, our results indicate that AD DC cells were partially deficient for telomere elongation upon TERT expression alone but that co-expression of TERT and TERC together resulted in robust telomerase activity and telomere elongation in both AD DC and normal cells.

Experiments were next undertaken to determine the fate of transduced cells over long term culture. To do so, we cultured three clones each of TERT and TERT/TERC expressing cells for an additional 20 passages (~60 pd). Comparison of cells at approximately 15 pd post-cloning (E) to cells at approximately 75 pd post-cloning (L) demonstrated that AD DC fibroblasts clones expressing TERT alone maintained shorter telomeres than normal fibroblasts expressing TERT alone, again reflecting haploinsufficiency of TERC in the AD DC cells (figure 12a). None of the TERT DC clones exhibited elongation of telomeres beyond that observed at early passage and at least one of these clones exhibited telomere shortening over time. Both the normal and AD DC clones expressing TERT and TERC together, however, generally had longer telomeres at later passage, with some clones exhibiting extremely long telomeres. Measurement of telomerase activity in early and later passage transduced DC cells indicated heterogeneity among different clones, and several of the TERT and TERT/TERC clones exhibited a decrease in telomerase activity over time (figure 12b). The reason for this loss is unknown. Not surprisingly, the one TERT/TERC DC clone,

DC-TERT/TERC-C, with the longest telomeres at later passage also maintained the highest levels of telomerase at later passage (figures 12a and 12b).

Despite differences in telomerase and telomere signal, all AD DC normal clones expressing TERT alone or TERT and TERC together had a significantly extended lifespan which was greater than 3 times that of vector alone (>75 pd versus ~20pd post subcloning), and at this time these cells appear to have been immortalized. Visual examination of the transduced AD DC fibroblasts demonstrated that expression of TERT alone or TERT and TERC together caused a "rejuvenated" phenotype in that they exhibited a morphology that was similar to early passage normal fibroblasts (figure 13). No consistent differences in cell growth or morphology were observed between cells that expressed TERT alone and cells that expressed TERT and TERC together. In contrast, cells expressing TERC alone did not appear to be phenotypically different than vector alone, indicating that expression of TERC alone has no profound consequences in AD DC or normal fibroblasts.

To further characterize how TERC and TERT expression affected telomere length in AD DC and normal fibroblasts, telomere specific Q-FISH was performed on a subset of transduced cells. Using Q-FISH, it was demonstrated that vector AD DC fibroblasts had telomere lengths that were approximately half that of normal (6.7 kb versus 11.2 kb) (figure 14a and 14b). Expression of TERT alone resulted in telomere lengths that were intermediate in length (11.2 kb for TERT DC and 24.4 kb for TERT normal) whereas co-expression of TERT and TERC together resulted in long telomere lengths (32 kb for TERT/TERC DC and 51 kb for TERT/TERC normal). Interestingly, the TERT/TERC DC clone at early passage exhibited two subpopulations that resulted in an apparent bimodal distribution of telomere length (figure 14b). This pattern was not observed in DC cells transduced with vector or TERT alone, indicating that DC cells do not start with a bimodal distribution. Furthermore, two distinct subpopulations of cells with different telomere lengths were apparent in the early passage DC-TERT/TERC-C clone (data not

shown). Assessment of a later passage of this particular clone demonstrated even more extensive elongation (average telomere length of ~114 kb) and a more normal distribution of length (figure 14c), suggesting that the subpopulation with longer telomeres outgrew the population with shorter telomeres. Interestingly, all the transduced cells at early and later passage retained an apparently normal diploid karyotype (data not shown). Overall, these results indicate that TERT alone can activate telomerase and, for the most part, maintain telomeres in AD DC cells, but that TERT and TERC together leads to higher telomerase activity and longer telomeres.

To examine the effects of telomerase reactivation in AD DC fibroblasts, we measured telomere length on individual chromosomes through Q-FISH and karyotyping. Most chromosomes in AD DC cells that expressed vector alone or TERC alone had short telomeres with some chromosomes, such as chromosome 2, having extremely short telomeres (figure 15). Upon TERT expression, overall telomere length on all the chromosomes increased and, interestingly, even very short telomeres were restored to a length that was similar to other chromosomes. Our results indicate that telomerase acts on the shortest telomeres, preferentially, and that all telomeres are brought to a new baseline length. These results nicely validate studies on lymphocytes from AD DC individuals and unaffected children of an AD DC parent which suggested that telomerase acts on the shortest telomeres during development [128].

Discussion

In this study, we have demonstrated that telomerase reconstitution in AD DC cells restores telomere length and significantly extends cellular lifespan. Expression of TERT alone, although sufficient to extend the lifespan of AD DC cells, did not result in telomeres that were as long as TERT expressing normal cells. However, our results indicate that even limited telomerase activity acts on the shortest telomeres in the AD DC

cells. Co-expression of TERT and TERC together caused high levels of telomerase activity and greatly extended telomere length in both AD DC and normal fibroblasts. Our results provide important insights into how TERT and TERC synergize in human cells to restore telomeres and point to possible strategies to extend telomere length in hematopoietic stem cells or other cells that are refractory to telomere elongation by expression of TERT alone.

Clearly, telomerase is tightly regulated in normal cells and levels are critical for determining whether telomeres are maintained or not. In AD DC families with mutations in TERC, it is the level of TERC that is rate limiting for telomerase activity whereas in cell culture skin fibroblasts telomerase is limited by the suppression of TERT expression. Telomerase haploinsufficiency during development is thought to result in telomere shortening and telomeres become even shorter in dividing cells from tissues of highly proliferative organs such as the hematopoietic system and the skin. Mouse knockout studies support this conclusion in that TERC or TERT heterozygotes exhibit telomere shortening over time and with increasing generations [95; 300; 313]. In our experiments, overexpression of TERT resulted in telomerase levels that were approximately half that of TERT expressing normal cells, reflecting the expected haploinsufficiency for TERC in AD DC cells. Our finding that TERT expression alone in AD DC cells led to an overall increase in average telomere length as compared to cells with vector alone indicates that limiting levels of telomerase act on the shortest telomeres. This result is in line with mouse studies demonstrating that crosses between telomerase knockout mice with short telomeres and mice with wildtype telomerase and long telomeres results in progeny with an intermediate telomere length equilibrium characterized by elongation of the shortest telomeres and preferential maintenance of short telomeres [313; 314]. Our findings also complement results showing that unaffected homozygous wild type children from affected AD DC patients have shorter average

telomere length overall but no specific set of chromosomes that have extremely short length [128; 300].

Recent studies indicate that co-expression of TERC and TERT together results in higher telomerase and longer telomeres than TERT expression alone, providing further evidence that TERC levels can limit telomere maintenance [310; 315]. In our studies, exogenous expression of TERC and TERT together also resulted in robust telomerase activity and greatly extended telomeres in some clones. Although our experiments indicated that TERT alone resulted in extended lifespan and longer telomeres than cells transduced with vector alone, we did not observe extensive telomere elongation over time in these cells and, in at least one case, we observed telomere shortening. This latter result is similar to a recent study by Wong and Collins showing that TERT expression alone in fibroblasts from X-linked DC patients resulted in higher telomerase and significantly extended lifespan but not telomere elongation [310]. Any differences in telomere length maintenance upon expression of TERT alone between X-linked and autosomal dominant DC cells could be due to a variety of factors including expression levels of dyskerin in the different cells.

Whether transduction of TERC alone could activate telomerase in other somatic cells from AD DC patients is unknown, but may in part be dependent on the level of endogenous TERT, which is nearly undetectable in fibroblasts. Some cells such as hematopoietic stem cells appear to be somewhat refractory to immortalization by TERT, and TERT expression alone did little in terms of maintaining telomeres [316]. Co-expression of TERT and TERC together might provide a more efficient means to activate telomerase, restore telomeres, and extend proliferative lifespan in these cell types. In our studies, co-expression of TERT and TERC sometimes led to telomeres that were incredibly long. Such long telomeres are not generally observed in diploid human cells and it is possible that these cells have lost mechanisms to regulate normal telomere length. In this regard, it will be of interest to ascertain levels of proteins known to be

involved in telomere length regulation (e.g. TRF1, TRF2, and POT1) in cells with such long telomeres [74].

Since there is the potential of upregulating telomerase to ameliorate problems associated with telomere shortening and poor cell proliferation in DC patients (and even the elderly), it will be important to determine whether AD DC cells with restored telomeres retain normal cellular function. TERT has been shown to have functions that go beyond its ability to elongate telomeres and these functions could interfere with normal cell physiology [317]. Extensive telomere elongation may also disrupt normal cell responses. For example, there is evidence that telomere length plays a significant role in regulating response to DNA damaging agents and oxidative stress [318-320]. The cells that we have generated should be of significant value in determining whether restored telomere length, particularly extremely long telomeres, alters responses to these agents. Exogenous expression of TERT may also result in cellular transformation and the consequences of telomerase activation and telomere elongation with regard to the carcinogenic process in AD DC cells is unknown. It should be noted that telomere length is not the only factor that regulates senescence in human cells. Several studies, including our own, indicate that telomere-independent stress-related pathways such as $p16^{INK4a}$ upregulation are important for inducing a senescence response even in the presence of high telomerase activity and long telomeres [321-325]. It is interesting to note that in our experiments, normal fibroblasts senesced with a telomere length that was longer than that observed in AD DC fibroblasts, indicating that other factors may come into play in determining when senescence in culture occurs.

Although still speculative, it is possible that problems associated with telomerase mutations and telomere shortening could be "fixed" in adult stem cell populations by transduction of telomerase component genes. One could envision that such a strategy might be useful for treating certain patients with aplastic anemia, including DC patients, where there is a demonstrated association between telomere shortening and disease.

Even if one could safely extend telomeres in hematopoietic stem cells to extend their lifespan, thus potentially alleviating bone marrow failure, other tissue in the affected individuals could still fail due to premature telomere shortening and senescence in the cells of these tissues. Nevertheless, our studies are proof of principle that it is possible to restore telomeres and proliferative capacity in cells from AD DC patients and provide an entry point to develop strategies to alleviate telomere shortening *in vivo*.

Materials and Methods

Cell Culture

The University of Iowa Internal Review Board approved this study and all subjects gave informed consent. Fibroblasts were obtained from 4 mm skin punch biopsies of a third generation AD DC affected patient [309] and a normal age-matched control. Briefly, the epidermis was removed by overnight dispase treatment at 4^{o}C. The underlying dermis was incubated in collagenase overnight at 37^{o}C and dissociated by pipetting. Cells were spun down and replated in culture dishes in 10% FBS/DMEM with Fungizone, penicillin, and streptomycin for the first passages, with removal of Fungizone for subsequent passages. The cells were split 1:8 when they were 90% confluent. TERT expressing VA-13 cells were kindly provided by Kathy Collins (Berkeley) and grown in 10% FBS/DMEM with penicillin and streptomycin.

Retroviral Constructs, and Transduction of Cells

A feline immunodeficiency virus (FIV) vector that co-expresses TERC and eGFP from separate promoters was generated by inserting the TERC gene with a 5' upstream U3 promoter and the TERC endogenous 3' end (a gift from Kathy Collins, Berkeley) into the Mre11 site of eGFP pVETL (kindly provided by Beverly Davidson, University of Iowa). The TERC gene contains a large portion of the endogenous 3’ end (515 bp) which

allows processing of the RNA into the mature form, resulting in a normal 451 base product [297; 312]. FIV constructs were pseudotyped with VSV-G at the Gene Vector Core at the University of Iowa using previously described methods [326]. The TERT-pBABE-neo vector [324] was obtained from Robert Weinberg (Whitehead Institute, Cambridge) and the pLXSN vector from Denise Galloway (Fred Hutchinson Cancer Research Center, Seattle). These latter MLV based vectors were packaged in Phoenix Amphotropic packaging lines according to previously published protocols [322]. All retroviral infections were carried out overnight in the presence of polybrene at 8 ug/ml. All cells were dually infected with combinations of an FIV or MLV based vector for consistency. Infection with eGFP FIV or TERC eGFP FIV vectors were performed at ~2 MOI and infection was monitored by visualizing green fluorescence after 48 hours. Cells infected with TERT-pBABE-neo or pLXSN were selected in 1 mg/ml G418 for 10 days. Cells were passaged as pools or plated at high dilutions and ring cloned after approximately 10 days growth. Clones were selected on the basis of green fluorescent expression (presence of eGFP FIV or TERC eGFP FIV).

Real Time PCR to Quantify Telomere Signal

Real time quantitative PCR were performed based on the protocol from Cawthon *et al.*[55; 311; 327]. Genomic DNA was isolated from one 100 mm confluent plate by trypsinizing and pelleting the cells and using the Qiagen Blood/Tissue DNA kit according to manufacturer's instructions. DNA was eluted in 100 ul volumes. 35ng of DNA was amplified using two different sets of primers, one for telomere sequences, and the other for the house keeping gene 36B4. The composition of the master mixes was identical except for the primer pairs. PCR reactions contained 1X 6-ROX reference dye (Invitrogen), 0.2X SYBER Green (Roche), 15mM Tris-HCl pH 8.0, 50mM KCl, 2mM $MgCl_2$, 0.2mM each dNTP, 5mM DTT, 1% DMSO and 1.25U AmpliTaq Gold DNA

polymerase. Telomere primer concentrations were: tel1, 270nM (5'-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3') and tel2, 900nM (5'-TCCCGACTATCCCTATCCCTATCCCTATCCCCTATCCCTA-3'). The 36B4 primer concentrations were: 36B4u, 300nM (5'-CAGCAAGTGGGAAGGTGTAATCC-3') and 36B4d, 500nM (5'-CCCATTCTATCATCAACGGGTACAA-3'). The PCR profile for both primer sets was 95^{o}C for 10 min, followed by 30 cycles at 95^{o}C for 15 sec, and 54^{o}C for 2 min. Samples were run in triplicate in a 25 ul volume. The T/S ratio was calculated as $[2^{Ct(telomere)} / 2^{Ct(36B4)}]^{-1}$ or $2^{-\Delta Ct}$. Once the T/S ratio was calculated, it was made relative to one sample (usually normal fibroblasts or vector control) as indicated and replicate values were averaged. For error bars, standard error of the mean was calculated from the 3 independent replicates.

Q-FISH and Karyotyping

Quantitative fluorescent in situ hybridization (Q-FISH) with Cy-3-labeled $(CCCTAA)_3$ peptide-nucleic acid and subsequent analysis of digital images were performed as described [308; 328]. Briefly, we hybridized a peptide nucleic acid (PNA) probe $(CCCTAA)_3$ specific for mammalian telomeres (Applied Biosystems) for two hours at room temperature to methanol/acetic acid-fixed cells followed by heat denaturation at 72°C for 3 min. To remove nonhybridized PNA probes, slides were washed with 0.05% Tween 20 containing PBS at 56°C for 15 min and visualized by using a Nikon Eclipse E800 fluorescence microscope. For each cell line, at least 400 chromosomes were analyzed and the mean fluorescence intensity was correlated to telomere length as ascertained by utilizing plasmids with fixed numbers of telomere repeats [328]. Image acquisition and analysis software (TFL-Telo, developed in the Lansdorp laboratory and available from the flintbox.com network) was utilized for analysis and display of data.

For analysis of telomere length from individual chromosomes, both Q-FISH and karyotyping was performed as previously described [329].

Measurement of TERC Levels

Total RNA was collected from cultured primary fibroblast samples using Tri-Reagent followed by chloroform extraction and ethanol precipitation per the manufacturer's instructions followed by purification using RNeasy (Qiagen) according to manufacturer's protocol. cDNA was constructed from 1.8ug of isolated RNA using the RetroScript reagents and protocols (Ambion). TERC RNA expression levels were analyzed using Real-Time PCR (RT-PCR). Briefly, cDNA was amplified in triplicate using TERC primers[1] (Forward: 5'-TCTAACCCTAACTGAGAAGGGCGTAG-3'; Reverse: 5'-GTTTGCTCTAGAATGAACGGTGGAAG-3') in conjunction with SYBR-GREEN PCR Master Mix (ABI) and analyzed on an ABI PRISM 7000 Sequence Detection System. Primers amplifying 18s RNA (Forward: 5'-CCTTGGATGTGGTAGCCCGTTT-3'; Reverse: 5'-AACTTTCGATGGTAGTCGCCG-3') were run in parallel to normalize RNA levels among fibroblast samples and ΔCt was calculated for each sample by the equation: $\Delta Ct = Ct_{TER} - Ct_{18S}$. Fold differences in TERC mRNA levels were calculated relative to normal fibroblasts infected with empty vector (Vector N-HSF-1) by using the equation: $2^{(\Delta\Delta Ct)}$ where $\Delta\Delta Ct = \Delta Ct_{(\text{Vector N-HSF-1})} - \Delta Ct_{(\text{Cell Type})}$.

Quantitative Telomerase Assay

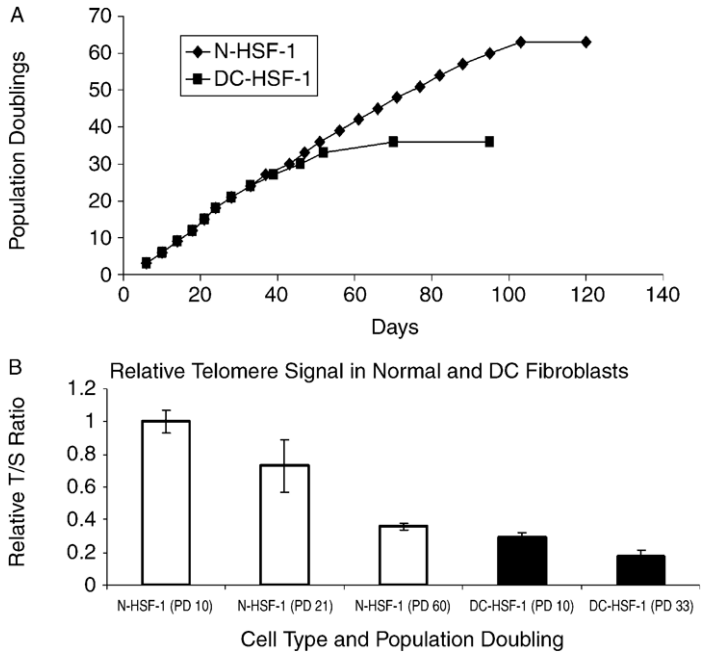
Real time quantitative TRAP assays were done based on a protocol from Hou, M. *et. al.* [330]. Briefly, a 50ul reaction mixture containing 1X SYBER Green buffer (PE Applied Biosystems), 2.5mM each dNTP, 1.5mM $MgCl_2$, 1mM EGTA, 1ug T4 Gene 32 protein (NEB), 0.1ug each of primers TS (5'-AATCCGTCGAGCAGAGTT-3') and

ACX [5'-GCGCGG$(CTTACC)_3$CTAACC-3'], 1 unit AmpliTaq Gold polymerase, and 1ul (1000 cell equivalent) of protein lysate. Reactions were performed in the ABI PRISM 7700 thermal cycler (Applied Biosystems). The PCR mixture was incubated at room temperature for 30 minutes followed by 40 cycles (95^oC for 10 min, 95^oC for 15 sec, and 60^oC for 1 min). Triplicate threshold values (C_t) were compared with standard curves derived from serial dilutions of telomerase-immortalized E6 LXSN E7 LXSH keratinocytes (5000, 1000, 200, 40 cells).

Northern Analysis

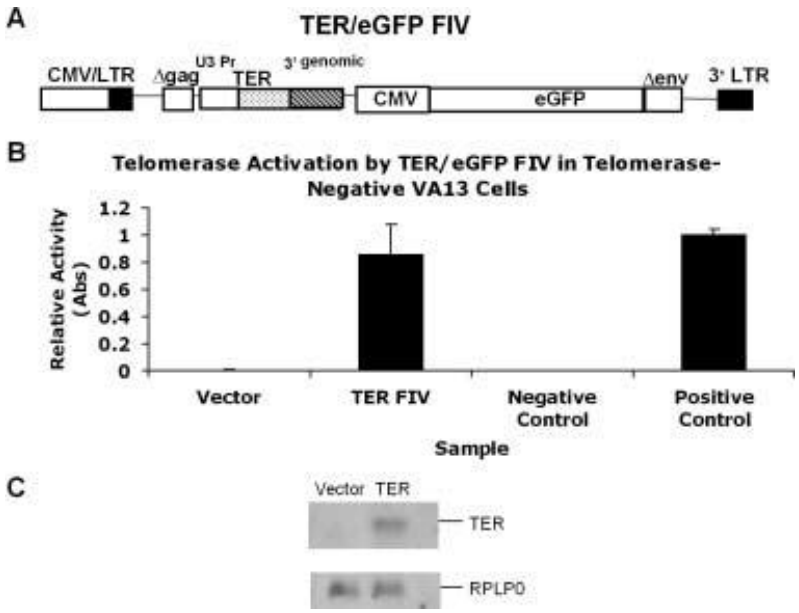
DIG Northern analysis was conducted using a DIG Northern Kit as described in the manufacturer's protocol (Roche Applied Science). Total RNA (2 ug) was separated on a 2% denaturing agarose gel and transferred to Hybond N+ membrane (Amersham). DIG labeled hTERC antisense RNA was synthesized using T7 polymerase and SfoI cut pBSV3hTR500 (hTERC plasmid from Kathy Collins) as a template. Antisense DIG-labeled RPLP0 (Ribosomal Protein Large P0, aka 36B4) from a SalI digested pGEM5 plasmid clone of RPLP0 was used as a loading control. After hybridization and washes, chemiluminescent detection was performed with anti-DIG-AP antibody and CDP-Star (Roche Applied Science) followed by exposure in a FujiImager.

Figure 9: Premature Senescence and Short Telomeres in AD DC (DC-HSF-1) as Compared to Normal (N-HSF-1) Fibroblasts.



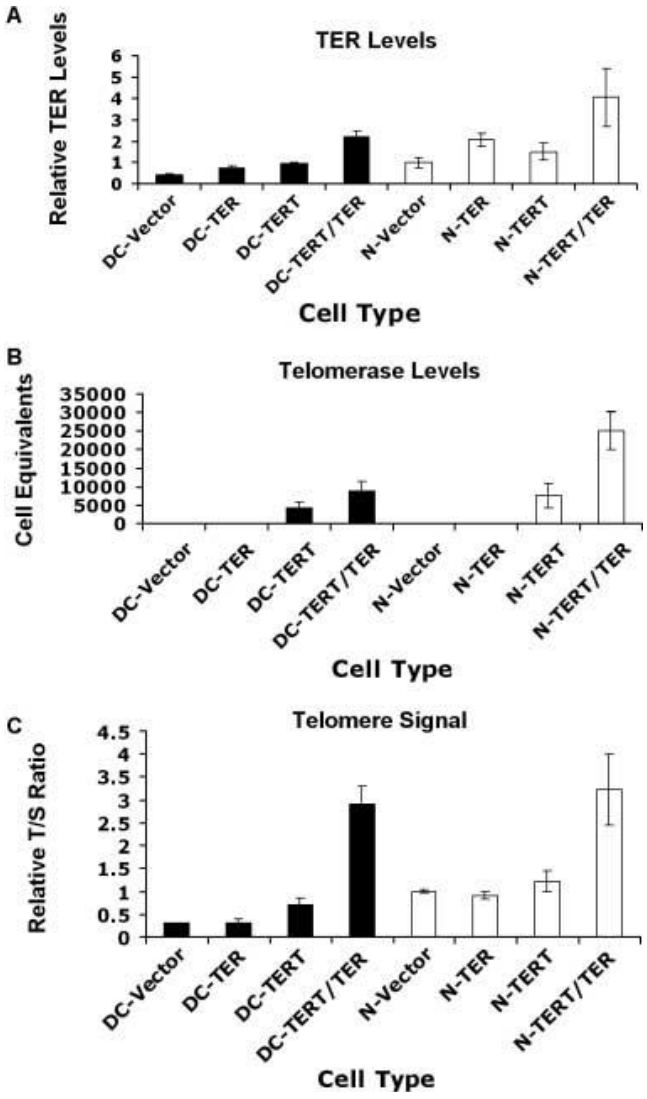
A. DC-HSF-1 cells proliferated for approximately half the lifespan of normal cells. **B.** Quantification of telomere signal in normal and AD DC fibroblasts at early passage and senescence. Telomere signal was ascertained by real-time PCR methodology as describe in the Materials and Methods. The T/S ratio represents the ratio of telomere signal over that of a single gene copy control (all relative to the T/S ratio of N-HSF-1 at early passage). All error bars represent standard error of the mean from three replicate assays.

Figure 10: Reconstitution of Telomerase in TERC Negative Cells.



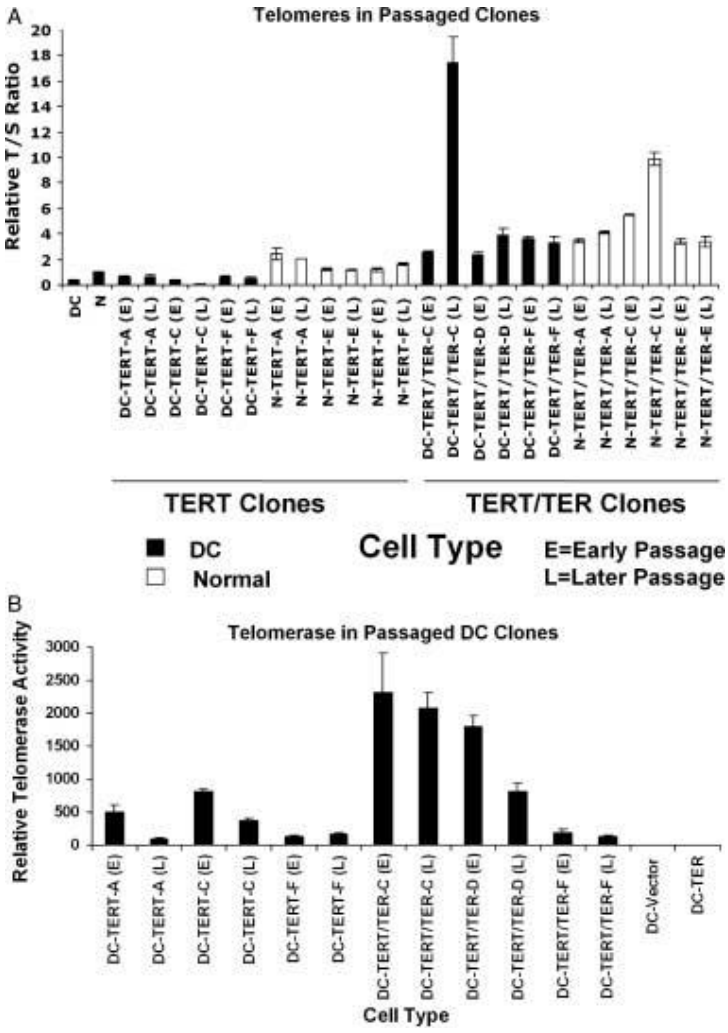
A. The TERC/eGFP FIV lentiviral construct**.** The TERC gene (also called TERC) along with a U3 small nucleolar (sno) RNA polymerase II promoter was inserted into the replication defective eGFP Feline Immunodeficiency Virus (FIV) construct. **B**. The TERC/eGFP FIV virus and eGFP vector alone were pseudotyped with VSV-G and transduced into TERT expressing, TERC negative VA13 cells. Transduction resulted in high telomerase activity in these cells, indicating functionality of the introduced TERC gene. **C**. Northern blot showing the accumulation of mature TERC of approximately 450 bases in cells transduced with the TERC/eGFP FIV virus as compared to eGFP FIV alone. RPLP0 is an internal control for RNA loading.

Figure 11: Reconstitution of TERC, Telomerase, and Telomere Length in AD DC Fibroblasts.



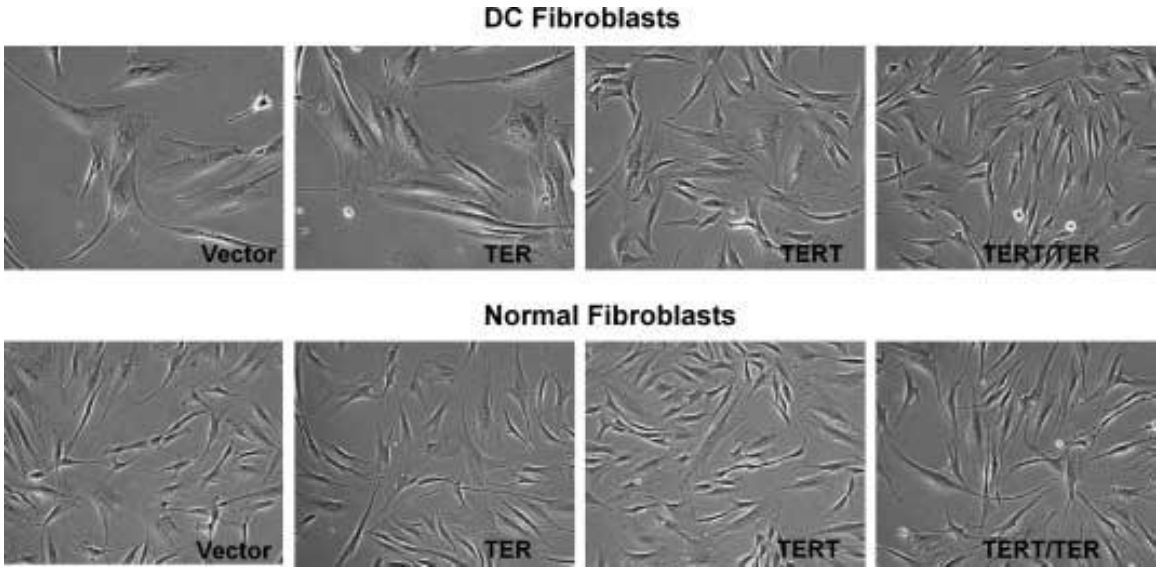
Cells were transduced with vector, TERC alone, TERT alone, or TERC and TERT together. AD DC cells are in blue and normal cells in red. **A**. TERC (also called TERC) levels in transduced cells as measured by quantitative RT-PCR. Values are relative to vector transduced normal cells (N-Vector). **B.** Telomerase activity as measured by a real-time TRAP assay (in cell equivalents as compared to the activity of a standard E6/E7 immortalized HSK cell line). **C.** Relative telomere length in transduced cells. Quantification of telomere signal was ascertained by real-time PCR methodology. The T/S ratio represents the ratio of telomere signal over that of a single gene copy control (all relative to the T/S ratio of N-Vector). All error bars represent standard error of the mean from 3 replicate assays.

Figure 12: Telomeres Lengths Among Different Cell Types



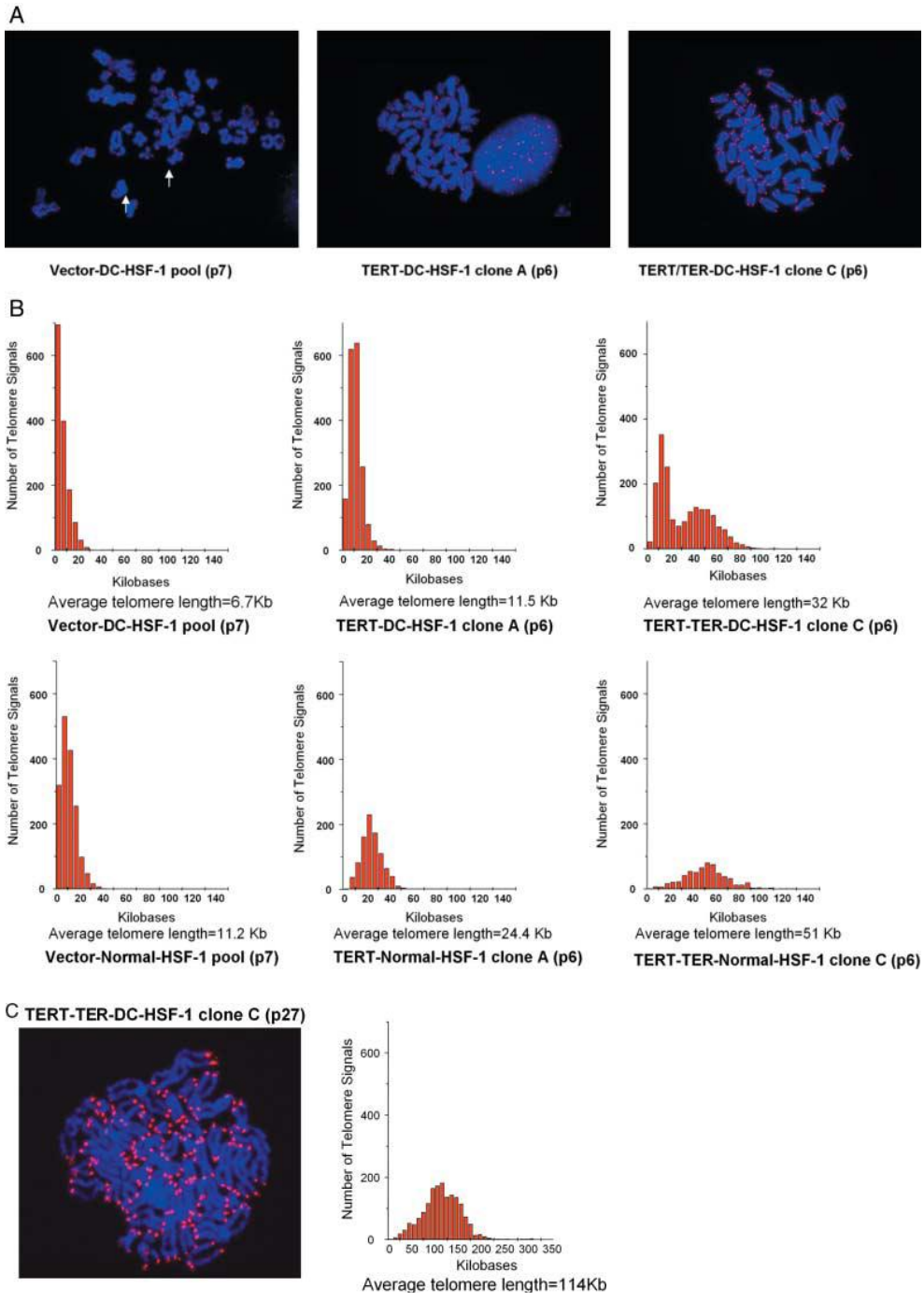
A. Telomere length over long-term passaging in TERT and TERT/TERC transduced AD DC (DC) and normal (N) fibroblast clones. E=Early Passage (P5, ~15 pd post cloning), L=Later Passage (P25, ~75 pd post cloning). Three clones of each cell type were analyzed. Telomere length was assessed as in figure 3 with a quantitative PCR assay. Relative T/S ratio is the ratio of telomere over single gene signal made relative to early passage normal (N) fibroblasts. All values represent the average or three replicate assays. Error bars are standard error of the mean. **B**. Telomerase activity in early and late passages of TERT and TERT/TERC (TERC also called TERC) transduced DC cells. "E" and "L" designations are similar to those described in 4A. Error bars represent standard error of the mean for three replicate assays.

Figure 13: Telomerase Reconstitution "Rejuvenates" AD DC Fibroblasts.



Normal and DC fibroblasts were transduced with vector, TERC, TERT, or TERT/TERC as described in the Materials and methods. TERC also called TERC as in this figure. Photographs were taken within 3 passages after selection (100X magnification).

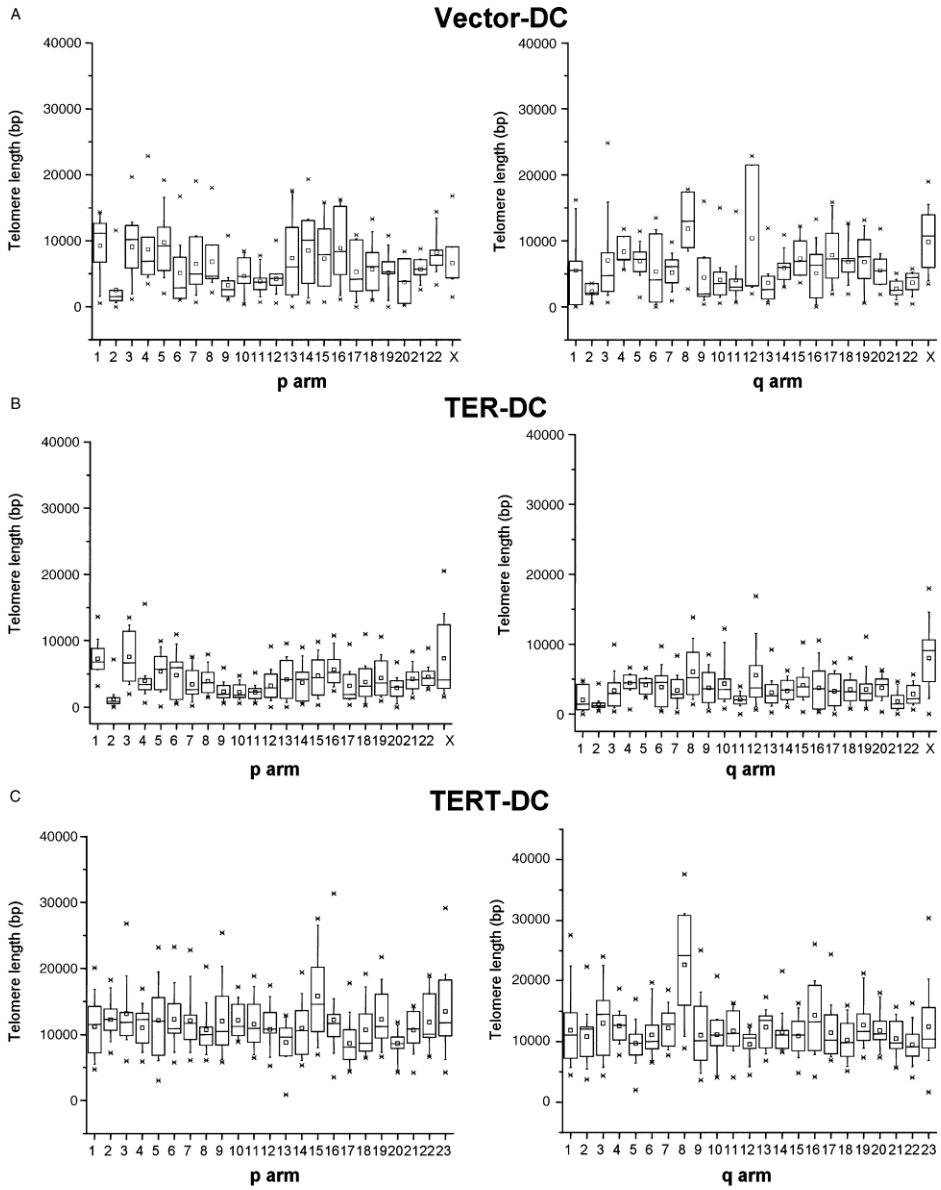
Figure 14: Telomere Length as Assessed by Q-FISH.



A. Representative metaphases of vector only AD DC fibroblasts (Vector-DC-HSF-1), TERT expressing AD DC fibroblast clone at early passage (TERT-DC-HSF-1 clone A), and TERT-TERC expressing AD DC fibroblast clone at early passage (TERT-TERC-

Figure 14 continued.
DC-HSF-1 clone C). TERC also called TER as in this figure. Q-FISH was performed using a PNA telomere specific probe (see Materials and Methods). **B**. Histogram representation of telomere signals from >400 telomeres per cell type of transduced early passage AD DC fibroblasts including Vector-DC-HSF-1, TERT-DC-HSF-1 clone A, and TERT-TERC-DC-HSF-1 clone C (top 3 panels) and transduced early passage normal (N-HSF-1) fibroblasts Vector-N-HSF-1, TERT-N-HSF-1 clone A, and TERT-TERC-N-HSF-1 clone C (bottom 3 panels). The Y-axis on each graph represents frequency of events whereas the X-axis represents telomere length (as ascertained by calibration with controls). Average telomere lengths are shown beneath each graph. **C**. Extensive elongation and a normal distribution of telomere length in later passage TERT-TERC DC cells.

Figure 15: Effects of Telomerase on Telomere Length at Individual Chromosome Arms.



Graphs represent telomere length on the p and q arms from individual chromosomes of early passage cells expressing eGFP only (A), TERC (B), or TERT (C). The distribution of telomere length at individual chromosome arms in 7 to 20 metaphase cells is expressed in box plots. In each box plot, the stars represent the first and 99th percentile of the telomere length values, the lines represent the 10th and 90th percentile, and the boxes represent the 25th and 75th percentile. Median values are given by the small box and the 50th percentile of the telomere length distribution by the horizontal bar in the box. TERC also called TER as in this figure.

CHAPTER 3

P21$^{WAF/CIP}$ MEDIATES OXIDATIVE STRESS AND SENESCENCE IN DYSKERATOSIS CONGENITA CELLS WITH TELOMERE-RELATED DYSFUNCTION

Introduction

Cellular aging is believed to involve the interaction between biological programming and numerous environmental factors that culminate in cells losing the ability to proliferate and becoming senescent. Senescence is a cell fate that many cells enter at the end of their lifespan that consists of existing in a metabolically active state without further cell division. Two well-characterized intracellular mechanisms that are believed to induce senescence are: telomere-associated[44; 127; 331] and oxidative stress-associated senescence[194; 332], both of which are thought to be causative factors in aging[333].

Reactive oxygen species (ROS) are a diverse set of reactive molecules such as hydrogen peroxide, organic hydroperoxides, superoxide ($O_2^{\bullet -}$), and hydroxyl radicals ($^{\bullet}OH$)[194] which, in excess, can cause oxidative stress and facilitate entry into senescence[334]. ROS have been found to be elevated in aged tissues[335] and can be manipulated *in vitro* to induce senescence[336; 337]. In agreement with the idea that reactions involving O_2 can contribute to senescence, increasing ambient O_2 tension in cell culture environments has also been found to hasten entry into senescence[338] while decreasing the O_2 tension from 21% to ~4% increases replicative lifespan[339]. In order to mitigate the potential toxicity associated with elevated ROS, cells express several redundant antioxidant enzyme systems including superoxide dismutases (SOD) [Cu(Zn)SOD, MnSOD and EcSOD], catalase, peroxiredoxins, and glutathione peroxidases[194]. When steady-state levels of ROS exceed antioxidant capacity, oxidative

stress ensues which can contribute to cell death or entry into senescence. Phylogenetic studies investigating the role of oxidative stress in aging have found that ROS production or detoxification are strong determinates of organismal lifespan[194; 340].

Telomere shortening is also believed to be involved senescence, acting as a mitotic clock to limit replicative lifespan[341]. Telomeres are a highly conserved evolutionary mechanism utilized across many species to cap the ends of linear chromosomes[56; 57]. In humans, telomeres are composed of tandem arrays of the hexameric DNA repeat, $TTAGGG^{n}$. Telomeres shorten with each successive cell division unless maintained by telomerase, a ribonucleoprotein reverse transcriptase capable of adding *de novo* repeats to chromosomal termini. One factor that results in telomere diminution is the 'end-replication problem' whereby DNA replication fails to faithfully replicate chromosome ends leading to continuous attrition[43-45]. Aside from protecting genes within the chromosome from undergoing erosion, telomeres also provide another function by forming a protein-containing secondary structure called a T-loop (telomere-loop) that prevents the cell machinery from recognizing the telomere as a double-stranded DNA break[73]. Once critically shortened, the telomere structure is disrupted, initiating a pathway that is believed to activate a DNA damage response (DDR). This telomere-associated DDR involves, in part, a sequence of events that lead to deposition of DDR 'marks' such as 53BP1 and γ–H2AX within the telomere that lead the subsequent activation of the kinases ATM/ATR, CHK1/CHK2, the tumor suppressor p53 and the p53-regulated cyclin-dependent kinase inhibitor (CDKi), $p21^{WAF/CIP}$[15; 92; 189; 190]. Evidence also supports a role for the $p16^{INK4A}$-RB pathway, in mediating telomere-associated cellular senescence[92; 191; 192].

In a small fraction of cells (i.e. embryonic[36], germ[37], and hematopoietic[38] stem cells as well as activated T-cells[39]) telomere maintenance is performed by a complex of proteins that together constitute the enzymatic activity of telomerase. Telomerase is minimally comprised of a reverse transcriptase catalytic moiety (TERT) and its RNA

template strand (TERC)[61; 62]. Telomere extension by telomerase can extend the proliferative capacity of some cells that would otherwise be susceptible to replicative senescence[342]. However, aberrant telomerase activity has potentially grave consequences as it has been found to be activated in approximately 90% of tumors/cancers[343].

A human disease caused by inadequate telomere maintenance, Dyskeratosis Congenita (DC), is primarily a bone marrow failure disorder but also displays a wide variety of other aging phenotypes that make DC a highly relevant model for understanding the relationship between telomeres and human aging[344]. Clinical features of DC patients include a rare triad of nail dystrophy, skin pigmentation and oral leukoplakia in addition to premature alopecia, graying of hair and osteoporosis[345]. These symptoms, coupled with a high incidence of bone marrow failure, liver dysfunction, pulmonary fibrosis and malignancy suggest that certain tissues may be more susceptible to telomere dysfunction *in vivo*. The first reported mutation to cause autosomal dominant Dyskeratosis Congenita (AD DC) was described in a three-generation family harboring a mutation in the 3' terminus of the TERC gene[116]. In this family, the disease is caused by genetic haploinsufficiency where affected individuals have one wildtype and one mutant copy of TERC[297]. In addition, mutations in at least five other telomerase or telomere-related genes have been found to cause DC[110; 117-121]. Mutations in TERT and TERC have also been reported in patients with pulmonary fibrosis and aplastic anemia (and other bone marrow failure syndromes) without clinical manifestations common to DC[299; 346], indicating that insufficient telomerase and critically short telomeres may play a key role in the pathogenesis of non-DC diseases that display symptoms related to DC patients.

Previous work by our lab identified short telomeres, decreased proliferative capacity, increased population doubling time, and a prematurely senescent phenotype in DC cells that were ameliorated by exogenous expression of telomerase components[87]. We hypothesize that the short telomere defect found in these patients' cells relies on the

transduction of a telomere-associated DNA damage response to mobilize causative factors to induce a premature senescent phenotype. In the current study, we have uncovered evidence of increased steady-state levels of superoxide and oxidative stress in DC cells that is initiated by insufficient telomerase (short telomeres) and mediated by the p53/p21$^{WAF/CIP}$ pathway. In addition, the proliferative defect in DC cells is 1) O_2 dependent, 2) corrected by introduction of exogenous telomerase and 3) partially ameliorated by inhibition of p21$^{WAF/CIP}$. Both exogenous telomerase and inhibition of p21$^{WAF/CIP}$ reduce steady-state levels of superoxide. These studies are the first to show that telomere dysfunction can lead to increased steady-state levels of superoxide by way of activating p53/p21$^{WAF/CIP}$ in cells from DC patients and suggests new mechanistic insights that could be exploited to provide some relief from the symptoms of this disease.

Results

We have reported proliferative defects caused by short telomeres in AD DC cells[87; 128]. In addition to this defect, AD DC fibroblasts also exhibit a three-fold increase in the percentage of cells positively stained by the senescence-associated β-galactosidase (β-gal) marker in comparison to control cells at the same passage (Figure 16A). In light of increased β-gal staining, DC cells were investigated for the presence of a heightened DDR, a finding that has been reported elsewhere among senescent cells[15]. In regards to a telomere-DDR, 53BP1 is trafficked relatively early to short or dysfunction telomeres and serves as an indicator for the steady-state levels of DDR within a population of cells. Immunofluorescence was employed to enumerate the number of 53BP1 foci per nuclei to ascertain whether DC cells harbor a heightened number of DDR foci. The majority of signals within control cells were in the lowest range (0-4 foci) while DC cells had more foci per nuclei in the higher bin ranges (5-9 foci and 10-14 foci; Figure 16B). Thus, DC cells have a significant increase in 53BP1 foci ($p<0.001$) which is indicative of a heightened DDR.

In skin fibroblasts, the mobilization of telomerase is restricted by low or absent TERT expression. We have shown previously that exogenous expression of TERT in AD DC cells is sufficient to activate telomerase and maintain telomeres, albeit at a short length[87]. Compared to untransduced AD DC cells, TERT expressing AD DC cells displayed a dramatic decrease in 53BP1 foci ($p<0.001$; Figure 16C). Thus, telomerase activation significantly reduced 53BP1 foci in DC cells suggesting that the increase in 53BP1 in DC cells can be attributed to foci formed at the telomeres.

Given the heightened number of 53BP1 foci in DC cells, we hypothesized that the p53 pathway was activated in these cells, as reported elsewhere in cells with dysfunctional telomeres[16]. For this purpose, we examined levels of phosphorylation at the serine-15 residue of p53 that is known to occur in the presence of DNA damage[347]. Levels of phosphorylated serine-15 were found to be significantly increased in DC fibroblasts compared to controls ($p<0.001$), whereas total p53 levels were not ($p<0.23$) (Figure 17A and Figure 18). Upon activation, p53 can transcriptionally regulate a number of genes, including $p21^{WAF/CIP}$ which was slightly elevated in DC cells as compared to control cells (Figure 17B). Exogenous expression of sh-*p53* or *TERT* significantly decreased $p21^{WAF/CIP}$ levels (Figure 17B), supporting the hypothesis that short telomeres in DC cells upregulated $p21^{WAF/CIP}$ via p53. We also observed an increase in levels of another CDKi, $p16^{INK4A}$, in DC cells as compared to controls; however, this increase was not attenuated by *TERT* expression (Figure 19). This indicates $p16^{INK4A}$ levels were sustained via a telomerase-independent mechanism. Overall, our data support the existence of a heightened p53/$p21^{WAF/CIP}$ mediated DDR in AD DC fibroblasts that can be repressed upon telomerase activation.

Recent reports have indicated that activation of the p53 pathway is associated with an increase in ROS levels[8; 174]. To investigate whether the p53 pathway found to be active in DC cells altered intracellular redox state, DC and control fibroblasts were analyzed for their ability to oxidize dihydroethidium (DHE) [348], which is believed to be a

surrogate marker for steady-state intracellular superoxide levels. Initial experiments using fluorescent microscopy indicated that AD DC fibroblasts exhibit increased DHE oxidation compared to controls (Figure 20A). To quantify this difference, cells acquired from five AD DC patients and two controls were incubated with DHE and analyzed by FACS (fluorescence-activated cell sorting). All AD DC skin fibroblast samples displayed a statistically significant increase in DHE oxidation compared to controls (Figure 20B).

To determine if the origin of superoxide production could be localized to mitochondria, MitoSOX oxidation (believed to be targeted to mitochondria) was compared between DC and control cells. In support of the hypothesis that mitochondria significantly contributed to superoxide levels in DC cells, all five sets of skin fibroblasts acquired from DC patients showed a significant increase in MitoSOX oxidation relative to control (Figure 20C). Univalent reduction of O_2 to form superoxide is believed to occur during normal O_2 metabolism with approximately one percent of total O_2 consumption resulting in the formation of superoxide[238], which has been suggested to contribute to the aging process[239]. Of importance, pegylated-CuZnSOD (polyethylene glycol conjugated-CuZnSOD; PEG-SOD) and pegylated-catalase (PEG-CAT) are cell permeable scavengers of superoxide and hydrogen peroxide, respectively, which can be used to determine the involvement of superoxide and hydrogen peroxide in oxidation of fluorescent probes[59]. When DC cells were supplemented with PEG-SOD or PEG-CAT and subjected to DHE-FACS analysis, the results shown in Figure 21A and 21B clearly demonstrate that the PEG-SOD inhibitable DHE oxidation (but not PEG-CAT) was significantly elevated in DC cells, clearly demonstrating that the increase in DHE oxidation seen in DC cells was attributable to increased steady-state levels of superoxide in DC cells relative to control. Further evidence that DC fibroblasts have increased levels of oxidative stress as compared to normal cells was demonstrated by the observation that DC cells had significantly higher percentages of glutathione disulfide (%GSSG) (a major

intracellular redox buffer) relative to normal cells (Figure 22). To extend these observations to other cell types from DC patients, DHE oxidation was measured in T-cells[349] and skin keratinocytes[350] from DC patients. Consistent with the findings in fibroblasts, DHE oxidation in T-cells[349] and skin keratinocytes[350] from DC patients was found to be significantly elevated (Figure 23A & B) relative to control, supporting the hypothesis that several different cell types from DC patients demonstrate increases in steady-state levels of intracellular superoxide. Overall, these results support the hypothesis that telomerase insufficiency and the short telomeres observed in cells from DC patients are accompanied by increased steady-state levels of intracellular superoxide.

To determine if there was a causal relationship between diminished telomerase function and alterations in steady-state levels of superoxide, the effects of TERT expression on steady-state levels of DHE oxidation was determined in DC cells. DC and control cells were infected with vector alone or the same vector containing TERT, which mobilizes telomerase activity in skin fibroblasts. TERT-expressing DC cells exhibited a significant 50% decrease in both DHE and MitoSOX oxidation, relative to empty vector treated cells (Figures 24A and B), indicating overexpression of TERT in DC cells decreased steady-state levels of superoxide. This data, along with our previous work[45], indicates that TERT expression and telomerase activation in DC cells can significantly reduce steady-state levels of superoxide and DNA damage responses as well as improving cell proliferation[45].

Although the primary function of telomerase is to maintain and elongate telomeres, recent reports have uncovered extra-telomeric secondary functions of TERT that include putative functions within the mitochondria and regarding heightened DNA damage responses[351; 352]. To determine whether any of these other functions accounted for the effects of TERT expression on superoxide levels in DC cells, TERT variants with targeted mutations in key domains were exploited. These TERT modifications include mutations that 1) disrupt the ability of TERT to be trafficked to the mitochondria (i.e.

R3E/R6E)[353; 354], 2) mutations that disrupt telomere elongation[355] but retain the ability to assist in a putative DNA damage function [i.e. TERT-DAT mutants][356], and 3) a dominant negative TERT unable to elongate telomeres (i.e. DN-TERT)[357]. The TERT mutants were stably expressed in DC cells and selected for antibiotic resistance. These DN-TERT and TERT-DAT mutants failed to decrease DHE, while the R3E/R6E TERT mutant decreased ROS comparable to wildtype TERT (Figure 24C). These results indicate that the putative mitochondrial targeting signal in TERT and DNA damage-related function are dispensable for decreasing steady-state levels of superoxide in DC cells, suggesting that the erosion of telomeres, and not mitochondrial localization or damage-related responses, is critical to the to the decreased superoxide levels demonstrated by DC cells expressing TERT. Overall, these results indicate that the telomere-elongating function of TERT must remain intact in order to reduce steady-state levels of superoxide in DC cells.

To further test the hypothesis that short telomeres lead to increased steady-state levels of superoxide, dominant-negative TRF2 (DN-TRF2) was overexpressed in control cells because this manipulation has previously been found to disrupt telomeres[358]. Control cells expressing DN-TRF2 acquired a significant 38% increase in DHE oxidation relative to vector control ($p<0.005$; Figure 25), demonstrating that inducing telomere dysfunction in non-DC cells could also lead to increased steady-state levels of superoxide. Together, this data suggests that proliferative defects in DC cells have short/dysfunctional telomere origins which can be corrected by telomerase-dependent telomere maintenance.

To determine whether expression of p53 and $p21^{WAF/CIP}$ was casually related to the increased steady-state levels of superoxide seen in DC cells, cells were infected with retroviral vectors expressing short-hairpin RNA sequences targeting p53 or $p21^{WAF/CIP}$, selected with appropriate antibiotics, and then evaluated for DHE oxidation. Stable knockdown of *p53* or $p21^{WAF/CIP}$ transcripts caused significant reductions in DHE

oxidation to levels comparable to normal fibroblasts (Figure 26A) and this suppression of steady-state levels of superoxide was maintained with further passaging (data not shown). In contrast, experiments performed in parallel, knocking down $p16^{INK4A}$, failed to decrease ROS during cell passage suggesting a specific role for $p53/p21^{WAF/CIP}$ in regulating superoxide levels in DC cells (data not shown). Continued passaging of cells with knocked down $p21^{WAF/CIP}$ uncovered a remarkable extension of proliferative lifespan (≈70 day lifespan extension; Figure 26B). Knockdown of p53, on the other hand, resulted in a crisis early after transduction (data not shown) as these cells presumably due to mitotic distress[359]. These results indicate that p53 and $p21^{WAF/CIP}$ are both involved in mediating the increased ROS in DC cells, but that the effects of knocking down these proteins on cell proliferation are not equivalent. Indeed, previously reported studies have demonstrated differential effects of removing p53 or $p21^{WAF/CIP}$ in the context of telomerase deficiency on organism and cell survival[99; 360].

Cell culture is routinely performed under atmospheric O_2 tension (i.e. 21%). *In vivo*, however, most cells are exposed to significantly less O_2. Previous research has revealed that skin fibroblasts are afforded an extension of lifespan when grown in low O_2 (~4% vs. 21% atmospheric O_2) and conversely demonstrate diminished lifespan at levels greater than atmospheric O_2 [339; 361]. To determine the biological effects of manipulating O_2 tension, mid-passage DC and control fibroblasts were grown in sub-confluent conditions under atmospheric (21%) or low O_2 (4%) and passaged over a period of 14 days. DC demonstrated prolonged growth kinetics compared to control cells grown under ambient O_2 as previously described (population doubling time: 2.4 days vs. 1.7 days, respectively; solid gray vs. solid black lines)[87]. Control cells grown in 4% O_2 displayed similar growth kinetics compared to those grown in ambient O_2 (population doubling time: 1.7 days vs. 1.7 days; black lines). In contrast to normal cells, DC cells cultured in low O_2 acquired significantly improved growth kinetics compared to those in ambient O_2 (population doubling time: 2.4 days for ambient vs. 1.9 days for low O_2;

Figure 27A, gray lines). At the termination of this 14 day proliferation experiment, DC cells demonstrated a two-fold increase in cell number grown in low O_2 compared to DC cells grown in atmospheric O_2 tension. Interestingly, no significant changes in 53BP1 foci were found in DC cells grown in ambient O_2 vs. low O_2, indicating that alterations in the DNA damage response was not contributing to the improved proliferative response seen in the DC cells in low O_2 (Figure 27B). In addition, $p21^{WAF/CIP}$ levels were found to remain stable between cells grown in 4% O_2 and those grown in ambient O_2, suggesting that growth in low O_2 caused a dissociation between DDR and inhibition of proliferative potential (Figure 27C). This is in contrast to TERT expressing DC cells in which levels of $p21^{WAF/CIP}$ were found to also be decreased (Fig 7C).

To determine if DC cells grown in 4% O_2 demonstrated alterations in steady-state levels of mitochondrial superoxide, MitoSOX oxidation was determined under the different O_2 tensions. Interestingly, MitoSOX oxidation was significantly reduced in 4% O_2 but only for DC cells and not normal cells (Figure 27D). These results indicate that low O_2 conditions specifically improved DC growth kinetics as well as decreasing steady-state levels of mitochondrial derived superoxide. These data suggest that growth in low O_2 conditions can partially inhibit the telomere-related effects on proliferative potential in DC cells as well as suppressing steady-state levels of mitochondrial derived superoxide in the absence of causing changes in the DNA damage response or expression of $p21^{WAF/CIP}$.

Discussion

Telomere-related dysfunction and the ability to reverse this dysfunction by overexpressing TERT in DC cells affords a unique opportunity to isolate the effects of this dysfunction in otherwise ‘young’ cells that have not been extensively passaged in culture. DC cells have prematurely shortened telomeres as well as characteristics of

cellular aging which include increased β-Gal staining, a large cytoplasmic-to-nuclear ratio, increased population doubling time and decreased lifespan in culture. Given that DC patients display a unique clinical phenotype and acquire symptoms of premature aging, we set out to further characterize the molecular mechanisms whereby telomere dysfunction engages cellular aging in this novel and highly relevant human model system.

The DNA damage response (DDR) related to telomere attrition has been well-characterized in previous reports in senescent cells[17] or cells undergoing telomere dysfunction[16]. The increase in 53BP1 foci seen in DC cells extends and confirms these findings in the DC model system. Likewise, the current finding that p53/p21$^{WAF/CIP}$ is responsive to telomere dysfunction also confirms in the DC model system findings that were anticipated based on results in other model systems[17; 362]. Interestingly, p53 and p21$^{WAF/CIP}$ activation have also been shown to occur in response to increased steady-state levels of ROS[196-202], but no previous publications have demonstrated a direct causal relationship between telomere dysfunction, expression of p53 and p21$^{WAF/CIP}$ and increases in steady-state levels of ROS[8; 174; 203-205] in human DC cells.

In the current study, cells from DC patients were utilized to demonstrate that p53/p21$^{WAF/CIP}$ expression is causally related with telomere dysfunction-induced activation of a DNA damage response, reduced proliferative capacity, and increased steady-state levels of superoxide. The ability of p53 to modulate ROS has been previously characterized[8; 174; 363] and depending on the magnitude of activation in response to stress, p53 induced increases in ROS can result in either senescence or apoptosis[8, 78]. Furthermore, two papers characterizing the role of p21$^{WAF/CIP}$ in causing increases in steady-state levels of ROS have found that both conditional endogenous induction of p21$^{WAF/CIP}$ as well as enforced p21$^{WAF/CIP}$ expression using viral vectors result in increased steady-state levels of ROS[78, 79]. In the first study[204] the authors found when p53 was intact, cells expressing exogenous p21$^{WAF/CIP}$ would become apoptotic

while cells that had lost or inactivated p53 underwent senescence. Both these biological outcomes could be inhibited by the thiol antioxidant N-acetyl cysteine (NAC) supporting the conclusion that oxidative stress was involved in the mechanism. In the second study[79] the authors also found that enforced expression of $p21^{WAF/CIP}$ increased β-Gal staining that was suppressed by NAC, again supporting the conclusion that ROS caused entry into senescence. These previous results, in conjunction with our data demonstrating the involvement of $p21^{WAF/CIP}$ in regulating growth inhibition and steady-state levels of superoxide in DC cells, supports a significant role for telomere-related dysfunction in regulating cellular processes that impact steady-state levels of superoxide through $p21^{WAF/CIP}$ in human diseases.

ROS have been demonstrated to play a role in causing irreversible senescence in previously characterized model systems[337], supporting a potential role for ROS in the senescence phenotype seen in the DC cells with dysfunctional telomeres. Recent evidence has led to the speculation that $p21^{WAF/CIP}$ may be a key intermediate between dysfunctional telomeres and clinical aging phenotypes. Proof of principal support was obtained for this hypothesis when it was demonstrated that crossing $p21^{WAF/CIP}$ knockout mice into a genetic background consisting of dysfunctional telomeres rescues the related phenotype without increasing risk for tumorigenesis[99]. The current findings with cells from DC patients showed that knocking down $p21^{WAF/CIP}$ using shRNA resulted in a remarkable increase in lifespan and maintenance of proliferative capacity while also decreasing steady-state levels of superoxide to levels comparable to those seen in cells from normal humans. Interestingly, DC cells expressing $p21^{WAF/CIP}$ shRNA did eventually undergo senescence, suggesting that factors other than $p21^{WAF/CIP}$ also govern senescence. Most importantly, the results in this report demonstrate using cells from patients with a human disease of aging (Dyskeratosis Congenita) that telomere-related dysfunction is associated with significant increases in steady-state levels of superoxide as well as reduced cell proliferation that can be inhibited by expressing $p21^{WAF/CIP}$ shRNA.

The mechanism by which insufficient telomerase and shortened telomeres lead to increased steady-state levels of superoxide is currently unclear. Previously a study by Pérez-Rivero *et al.*[364] examined mouse fibroblast aging and found cells with short telomeres have decreased catalase (CAT) expression (which is expected to lead to increased steady-state levels of hydrogen peroxide) as well as increased TGF-β1 and Collagen IV. In another study by Lee *et al.* T-oligos were utilized to mimic telomere dysfunction[365], and this was found to activate p53 causing an apparent increase NADPH oxidase-dependent ROS levels based on the finding that ROS levels could be attenuated by treatment with a non-specific inhibitor of flavin containing oxidases (diphenyliodonium). The data in the current study does not clearly identify the source of increased steady-state levels of superoxide in the DC cells, but the results with MitoSOX oxidation being altered, coupled with the differential effects seen with manipulations of O_2 tension, support the hypothesis that mitochondria may represent a significant source of superoxide in DC cells containing dysfunctional telomeres.

The findings of this current work support the hypothesis that oxidative stress may have a previously unappreciated function in the constellation of symptoms and pathologies found in DC patients. Given previous reports in other disease pathologies, it is logical to hypothesize that oxidative stress may contribute to bone marrow failure and pulmonary fibrosis seen in DC patients[299; 346; 366-370]. Bone marrow failure has been attributed to the failure of the hematopoietic stem cell niche and stroma to support the most immature bone marrow cells[371-373] and this niche may be especially susceptible to dysfunctional telomeres[374; 375] as well as oxidative stress[376-382] associated with increased steady-state levels of superoxide. The oxidative stress mechanism is also likely to contribute to pulmonary fibrosis as ROS mediated signaling has been hypothesized to contribute to cell damage and profibrogenic stimuli in many other disease states involving fibrosis[234-236]. Given the widespread investigation of antioxidant therapies to mitigate these types of tissue injury processes, it is possible that DC patients could

benefit from adjuvant antioxidant therapies[383] which could represent relatively non-invasive and potentially effective strategies for this disease.

In conclusion, the current study supports the hypothesis that that insufficient telomerase that leads to telomere attrition activates a DNA damage response that is accompanied by increased steady-state levels of superoxide in a p53/p21$^{WAF/CIP}$ dependent fashion in cells from DC patients. This represents a significant finding because it suggests in a very relevant human model of aging that telomere dysfunction and oxidative stress may not be mutually exclusive mechanisms promoting organismal aging but may represent an integrated process amenable to both genetic as well as pharmacological manipulation.

Materials and Methods

Cells

Cells from DC subjects and healthy controls were obtained with consent and approval from the University of Iowa Internal Review board. These patients are part of a multi-generational kindred with a deletion that encompasses the terminal 74 base pairs of the processed/expressed hTERC gene giving rise to a haploinsufficient, autosomal dominant form of DC[116]. Skin fibroblasts were acquired via punch biopsies and were grown in DMEM supplemented with 10% fetal bovine serum as previously published[87]. Lymphocytes were isolated following Ficoll-Hypaque (GE Healthcare) gradient separation of whole blood obtained following venipuncture and grown in RPMI and 10%FCS with antibiotics as previously described[349]. $CD3^+$ lymphocytes were isolated by positive selection using recommended protocols (Stem Cell Technology). Keratinocytes were isolated and cultured as previously described[350]. All experiments were performed using age and sex-matched Normal-2 and DC-1 unless indicated otherwise.

Growth Kinetics

DC and control skin fibroblasts were plated at 15,000 cells per 60mm tissue culture plate in triplicate sets. Cells were in media conditions previously mentioned under either normoxia (21%, ambient O_2) or reduced O_2 (4%). Cells were passaged at 50-70% confluency to maintain log phase growth. At intermittent periods, triplicate sets were washed, trypsinized and counted using a Coulter Counter. Error bars represent the standard deviation of the triplicate sets.

Retroviral constructs and transduction of cells

The mutant mito-TERT R3E/R6E construct was generated by removing the mitochondrial localization signal of TERT[353; 354]) by site directed mutagenesis (QuikChange, Stratagene) and inserting this into pLXSN-TERT. LXSN-TERT and FIV-U3-hTERC have been published elsewhere[87]. In order to produce virus carrying the genes of interest, retroviral plasmids were incubated with GenePORTER (Genlantis) according to the manufacture's protocol. This cocktail was then added to amphotropic-expressing Phoenix cells[384] and supernatants were prepared as previously published[385]. Fibroblast infections took place in the presence of 8ug/mL polybrene overnight. Cells were selected with a respective antibiotic (puromycin (1ug/mL), neomycin (1 mg/mL) or hygromycin (200ug/mL)). All cells were analyzed prior to senescence or crisis.

Immunofluorescence

Skin fibroblasts were grown under normal conditions and treated as previously published[15]. Briefly, cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS and subsequently blocked in serum. The 53BP1 antibody (Novus) was diluted 1:500 and incubated overnight. The cells were washed in TBST, incubated

with secondary antibody, washed and stained with ToPro-3 (Invitrogen). Cells were visualized with a Zeiss 510 multiphoton confocal microscope, captured and randomized for blinded analysis. Statistical analysis was applied using Student's t-test.

DHE, GSSG/GSH, MitoSOX

DHE (Molecular Probes, Eugene OR) assays were performed as previously described[386; 387]. Briefly, triplicate samples of skin fibroblasts, keratinocytes or T-cells were analyzed during log phase growth and incubated in 10 uM DHE for 40 minutes. The cells were washed, trypsinized and centrifugated. The cells were brought up in PBS and subjected to FACS analysis to quantify relative DHE oxidation levels by comparing the mean fluorescent intensity averages of the triplicate sets. Assays of MitoSOX (Molecular Probes) oxidation were performed similarly however incubated for a total of 20 minutes and at a 2 uM concentration. Antimycin A (10 uM) was used as a positive control to stimulate DHE and MitoSOX oxidation. Graphs include error bars that represent triplicate sets.

For inhibition of DHE oxidation experiments using PEG-SOD and PEG-CAT (Sigma) cells were incubated with 100 U/mL PEG-SOD or PEG-CAT for one hour and then labeled with 10 uM DHE and incubated for another 40 minutes following the standard DHE-FACS protocol. The amount of SOD inhibitable (or catalase inhibitable) DHE oxidation was calculated based on the equation: $DHE_{Control} - DHE_{PEG\text{-}SOD}$ = the amount of the mean fluorescent intensity attributable to $O_2^{\bullet -}$ or H_2O_2.

Glutathione analysis

Glutathione levels were measured as described previously[388]. Briefly, fibroblasts were scrape harvested, spun down and homogenized in 50 mmol/L potassium phosphate buffer (pH 7.8) containing 1.34 mmol/L diethylenetriaminepentaacetic acid buffer. Total

glutathione was determined as described[389]. GSH and GSSG were distinguished by addition of 2 uL of a 1:1 mixture of 2-vinylpyridine and ethanol per 30 uL of sample followed by incubation for 1 h prior to assay[390]. Levels of glutathione were normalized to cellular protein levels.

Quantitative Real-Time PCR

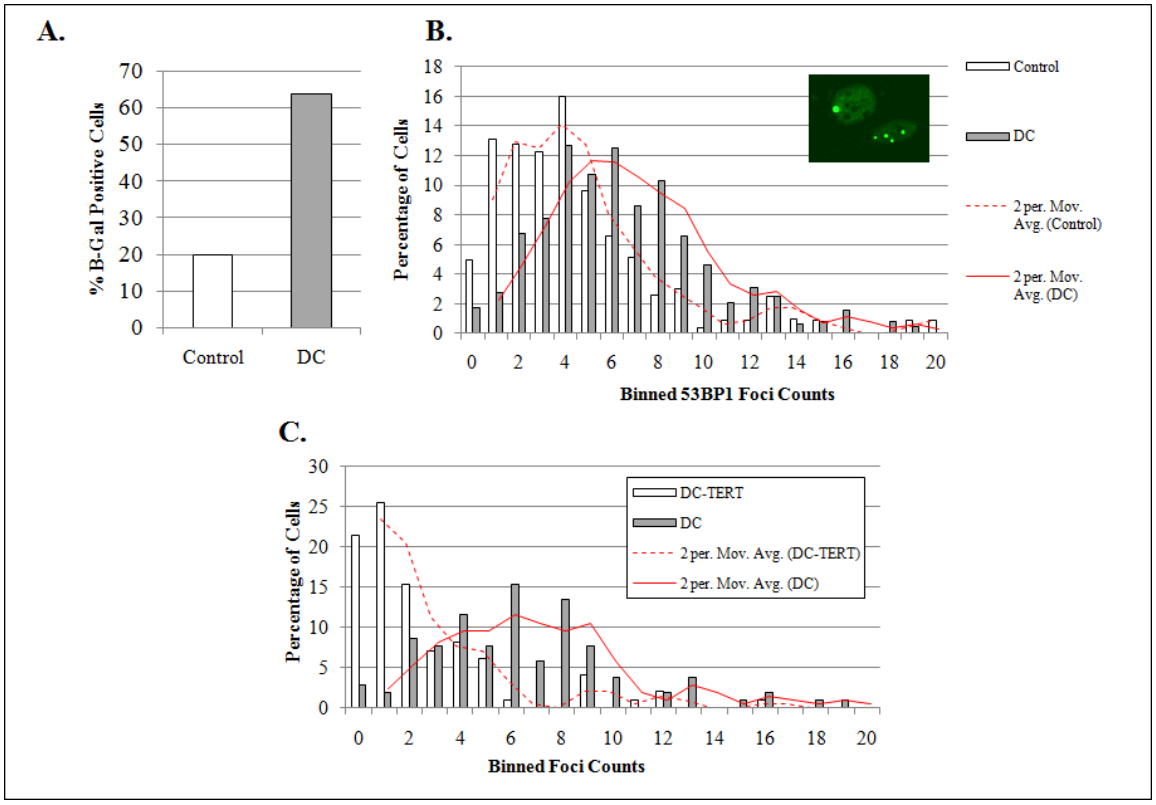
Quantitative real-time PCR (Q-RT-PCR) was utilized to verify diminution of transcript levels when utilizing RNAi technology or for elevated levels of $p16^{INK4A}$. Real-time PCR primers were generated using transcript sequences acquired from NCBI or the UCSC genome browser and analyzed using IDTDNA PrimerQuest analysis (Coralville, IA). Primer sequences are available upon request. RNA was acquired from fibroblasts by scraping skin fibroblast cultures with 1mL of TRIzol (Invitrogen). RNA was extracted using RNeasy technology (Qiagen) and utilized for cDNA synthesis (RetroScript, Ambion). The Q-RT-PCR reaction consisted of synthesized cDNA, oligonucleotide primers (Integrated DNA Technologies) and 2X SYBR-green master-mix (Applied Biosystems) and was run on an ABI PRISM Sequence Detection System (model 7900HT or 7700). The equation $2^{-(\Delta Ct-\Delta RPLP0)}$ was utilized to quantify overall transcript changes between the cell types and normalized to a single gene control, RPLP0.

Western Analysis

Standard Western techniques were utilized as previously described[385; 391]. Briefly, whole cell extracts were acquired from cell scrapings containing WE16 lysis buffer supplemented with protease and phosphatase inhibitors. Protein were separated by SDS-PAGE, transferred to a PVDF membrane and stained with the following antibodies: $p21^{CDKN1A}$ (Pharminogen) or Actin (Santa Cruz). Secondary antibodies conjugated with

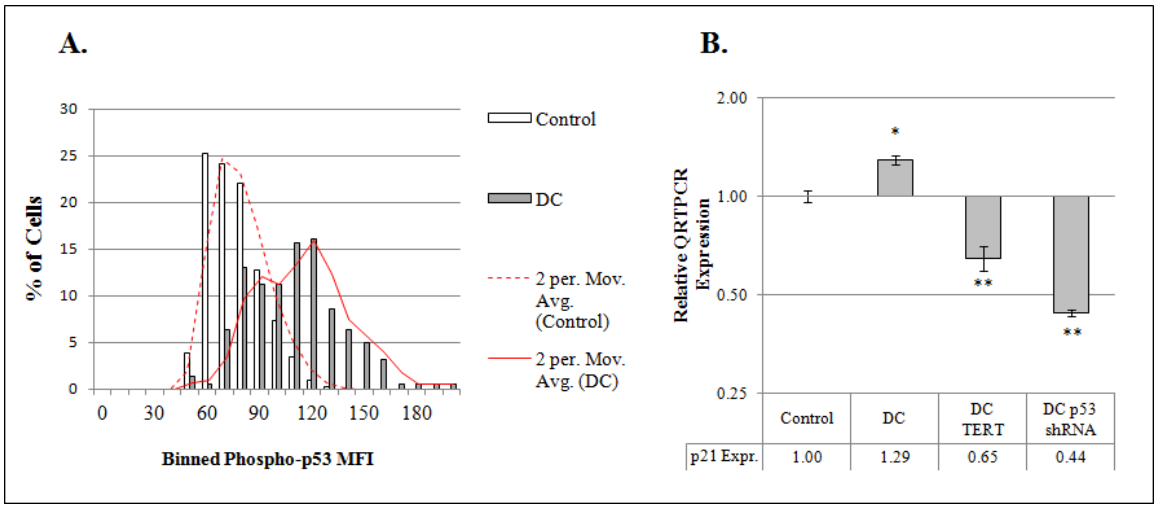
HRP were used to illuminate blots with Western Lightning Chemiluminescence Reagent (Perkin-Elmer) and visualized on a Fugi LAS 3000 chemiluminescent imager.

Figure 16: Heightened Telomere DNA Damage Response in DC Cells is Mitigated by Telomerase Activation.



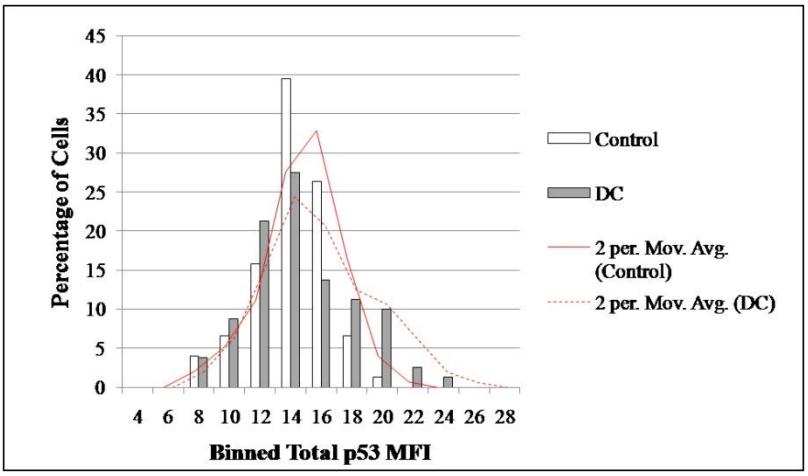
A. Mid-passage age/sex matched control and DC (patient: DC1) cells were fixed and stained with β-galactosidase to ascertain senescent levels in DC and control cell skin fibroblasts. **B.** 53BP1 antibody was incubated with fixed mid-passage DC and control skin fibroblasts, visualized with a Zeiss 510 multiphoton confocal microscope and imaged for subsequent analysis. Blinded images of 53BP1 focal points were quantified and binned based on counts-per-nucleus. The Y-axis represents the average number of signals per cell acquired per 100 cells over two separate experiments. A moving average (red line) was calculated to highlight the dissimilarity between the two data sets. The data was subjected to the Student's t-test (one-tailed, unequal variance; $p<0.001$) indicating a significant increase in the total 53BP1 foci per nucleus in DC cells. **B Inset.** Typical example of 53BP1 stained foci of two nuclei. **C.** DC cells stably infected with a retrovirus expressing TERT were analyzed for changes in 53BP1 foci compared to telomerase negative DC cells. This data was subjected to the Student's t-test (one-tailed, unequal variance; $p<0.001$).

Figure 17: Activation of the p53/p21$^{WAF/CIP}$ Pathway in DC Cells.



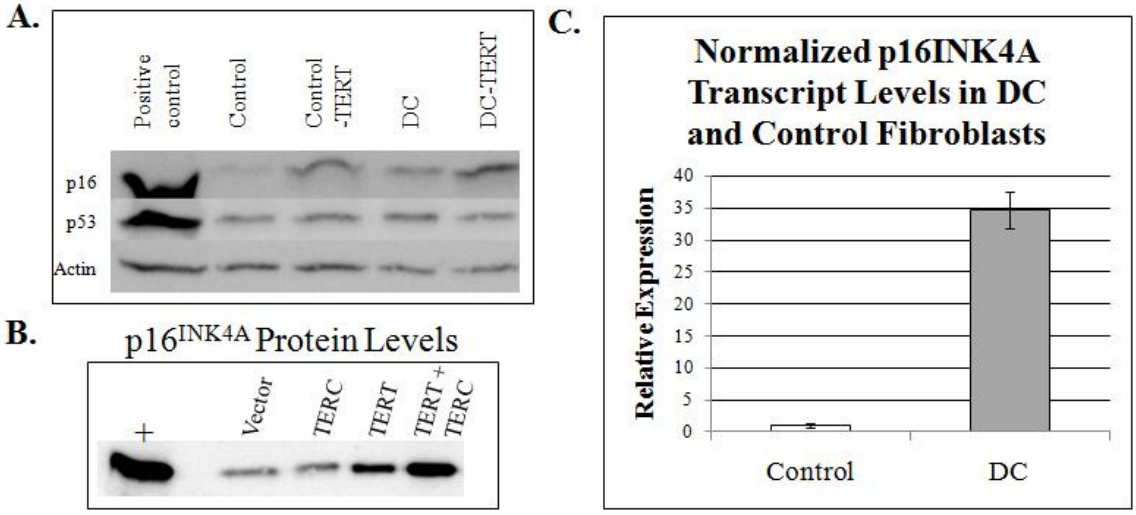
A. Mid-passage DC (DC1) and control cells were grown under routine conditions, fixed and stained with p53 serine-15 antibody, a residue phosphorylated during DNA damage. Images were captured using Zeiss 510 multiphoton confocal microscope and the intensity of nuclear signals were quantified using ImageJ and binned. P-values were generated based on Students t-test ($p<0.001$). A moving average (red line) was calculated to highlight the dissimilarity between the two data sets. **B.** RNA was obtained from control cells, DC cells (DC1), DC cells expressing exogenous TERT, or p53 shRNA grown under routine conditions. cDNA was generated from this RNA to evaluate the relative amount of p21$^{WAF/CIP}$ expressed in these cells. Values were generated based on triplicate QRTPCR values, graphed (log_2) and data analyzed for statistical significance (*: Control . DC, $p<0.001$; **: DC. DC-TERT and DC vs. DC p53 shRNA, $p<0.001$). Error bars represent standard deviation of triplicates.

Figure 18: Similar Total p53 levels in DC and Control Cells.



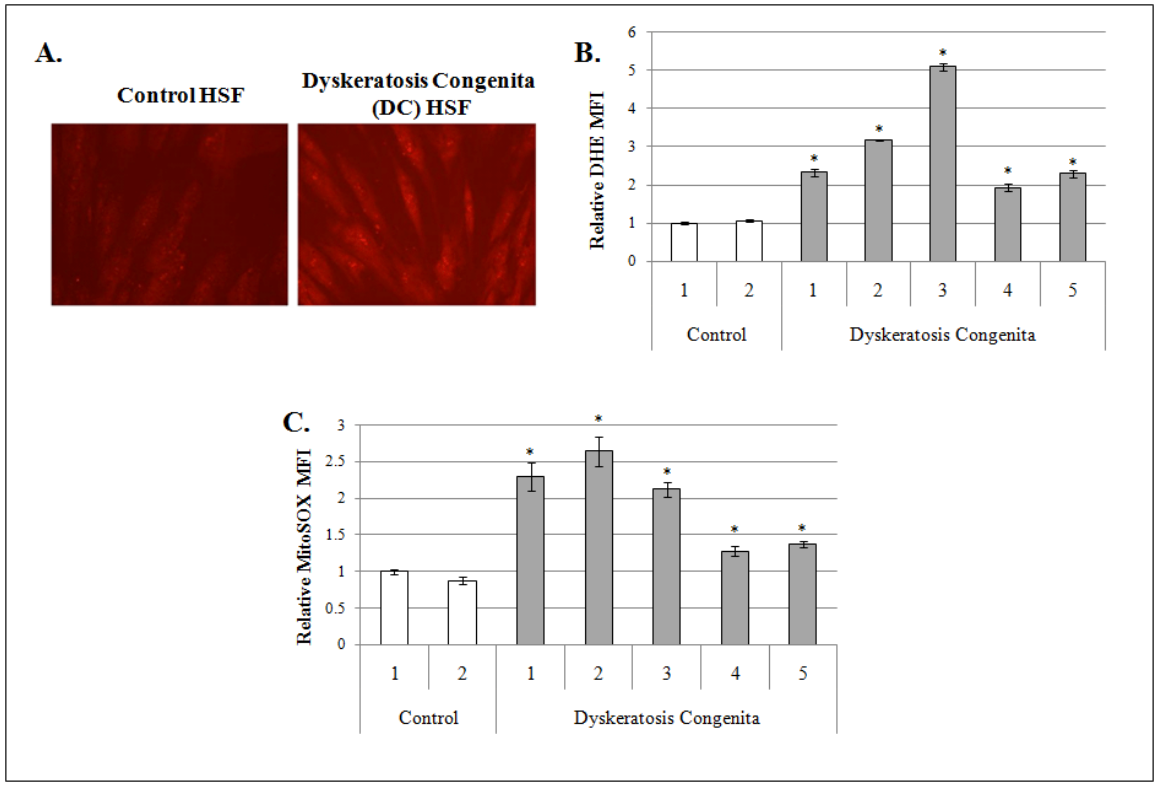
Mid-passage DC (DC1) and control cells were grown under routine conditions, fixed and stained with an antibody assessing total p53 levels. Images were captured using Zeiss 510 multiphoton confocal microscope and the intensity of nuclear signals were quantified using ImageJ and binned. P-values were generated based on datasets generated between DC and control cell nuclear fluorescence indicating similar levels of total p53 between DC and control cells (Students t-test, $p<0.23$). A moving average (red line) was calculated to highlight the similarity between the two data sets.

Figure 19: Increased transcript and protein levels of p16INK4A in passaged DC fibroblasts.



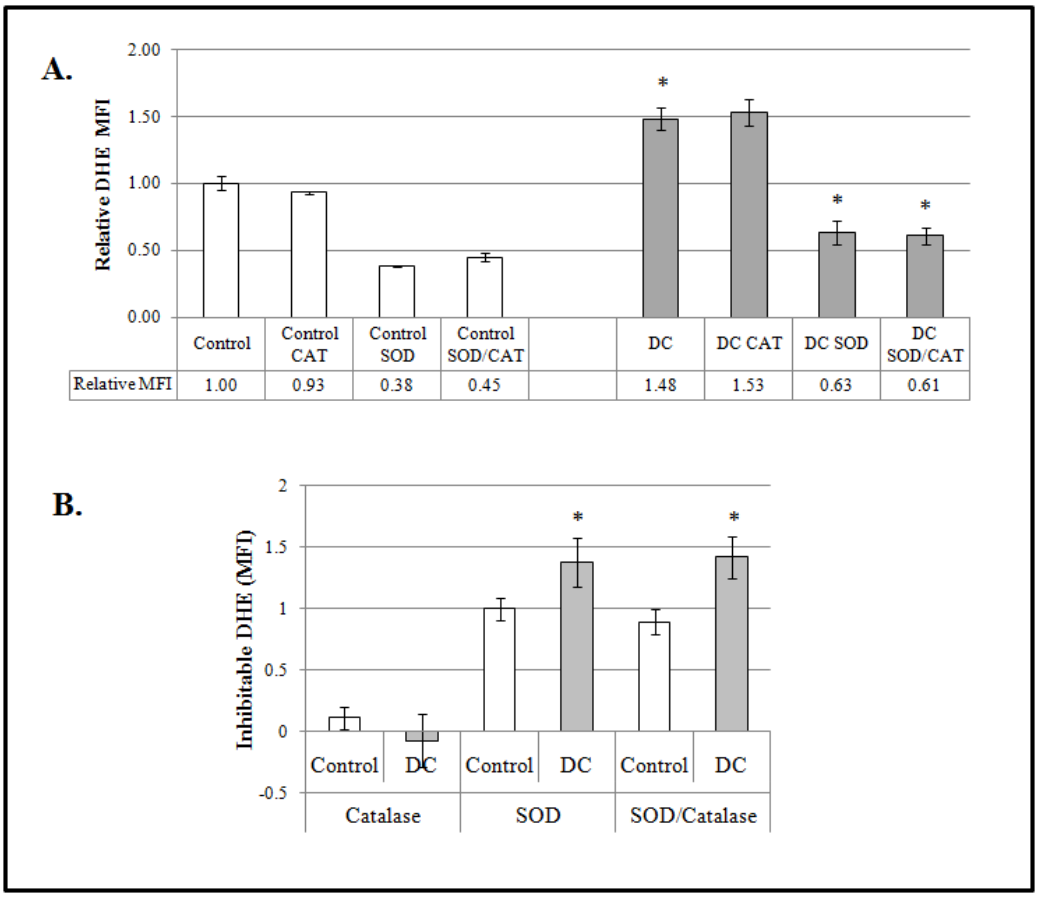
A. p16INK4A and total p53 protein levels were assessed in DC and control fibroblasts by SDS-PAGE at equivalent passage and grown in parallel. **B**. DC cells were stably infected to express one or both telomerase components and analyzed by SDS-PAGE. **C**. p16INK4A transcript levels of DC and control fibroblasts at equivalent passage using real-time PCR quantification. Samples were normalized to an internal control and values made relative to 'Normal' (value = 1).

Figure 20: Measurement of Elevated Steady-State Levels of Superoxide in DC Fibroblasts.



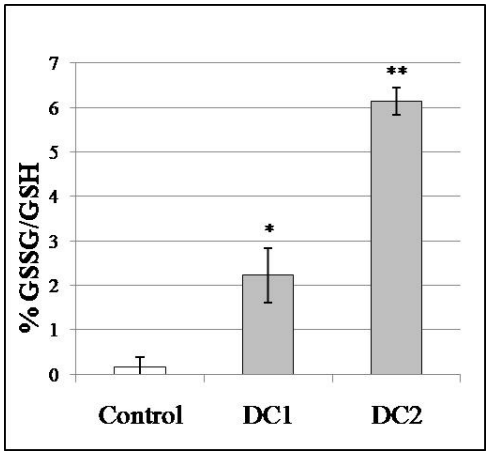
Qualitative assessment of control and DC (DC1) (**A**) human skin fibroblasts (HSF) incubated with dihydroethidium (DHE) visualized at identical exposures with a Zeiss 510 multiphoton confocal microscope. **B**. Samples from two controls and five DC family members were quantified for DHE oxidation using FACS analysis. DHE oxidation values represent the mean fluorescent intensity (MFI) from the measurement of 10,000 cells. The error bars are reflective of standard deviation in triplicate analyses of cells from each individual. Data is normalized to the MFI value from control fibroblasts. * indicates statistically significant increase in DHE oxidation compared to controls, $p<0.001$. **C.** MitoSOX oxidation determined in mid-passage DC and control cells with FACS analysis. Values are relative to controls and error bars indicate standard deviation of triplicate sets of MFI determined on triplicate sets of 10,000 cells. Statistical significance based on Student's t-test ($p<0.01$).

Figure 21: An Increase in Inhibitable Superoxide Levels in DC cells



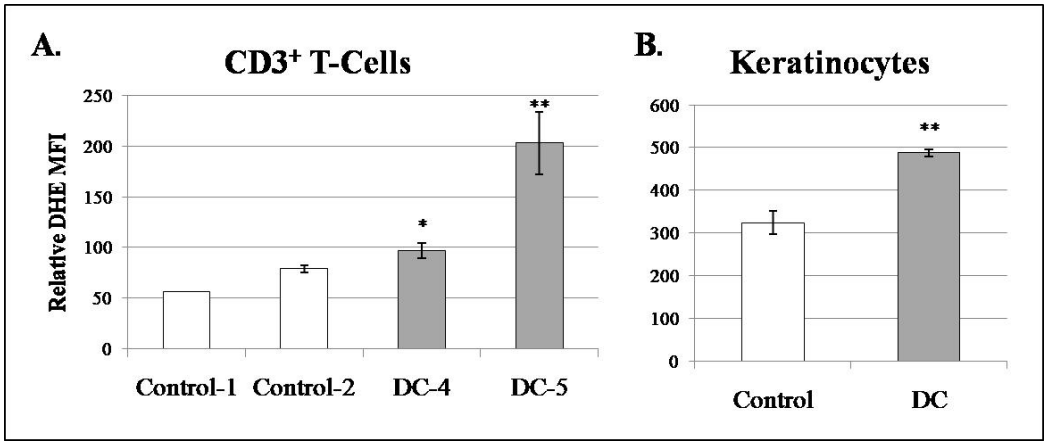
DC (DC1) and control fibroblasts were grown under routine conditions in the presence of DHE or DHE + pegylated SOD and/or CAT (CAT or Catalase = DHE oxidation attributable to hydrogen peroxide, SOD = DHE oxidation attributable to superoxide, SOD/Catalase = DHE oxidation attributable to both superoxide and hydrogen peroxide). **A.** DC cells displayed a significant elevation in DHE staining compared to controls ($p < 0.002$) while cells treated with PEG-Catalase did not display a significant difference in the DHE decrease compared to a decrease in controls (DC DHE – DC PEG-CAT DHE vs Control DHE – Control PEG-CAT DHE; $p < 0.19$). However, the difference in between DC cells and those treated with PEG-SOD and both PEG-SOD/PEG-CAT displayed a significant DHE difference compared to controls ($p < 0.04$ and $p < 0.004$, respectively). All values normalized to untreated Control. **B.** The values are an alternative view of **A.** and represent the difference between cells incubated in DHE and cells incubated with their respective antioxidant. The MFI average of triplicate sets of cells incubated with DHE + SOD or CAT was subtracted from the MFI average of cells incubated with DHE alone. All values are normalized to control cells incubated with PEG-SOD. P-values are the same as in **A.** (SOD: $p < 0.04$; CAT: $p < 0.002$).

Figure 22: Oxidative Stress in DC Cells as Determined GSSG/GSH and MitoSOX Measurements.



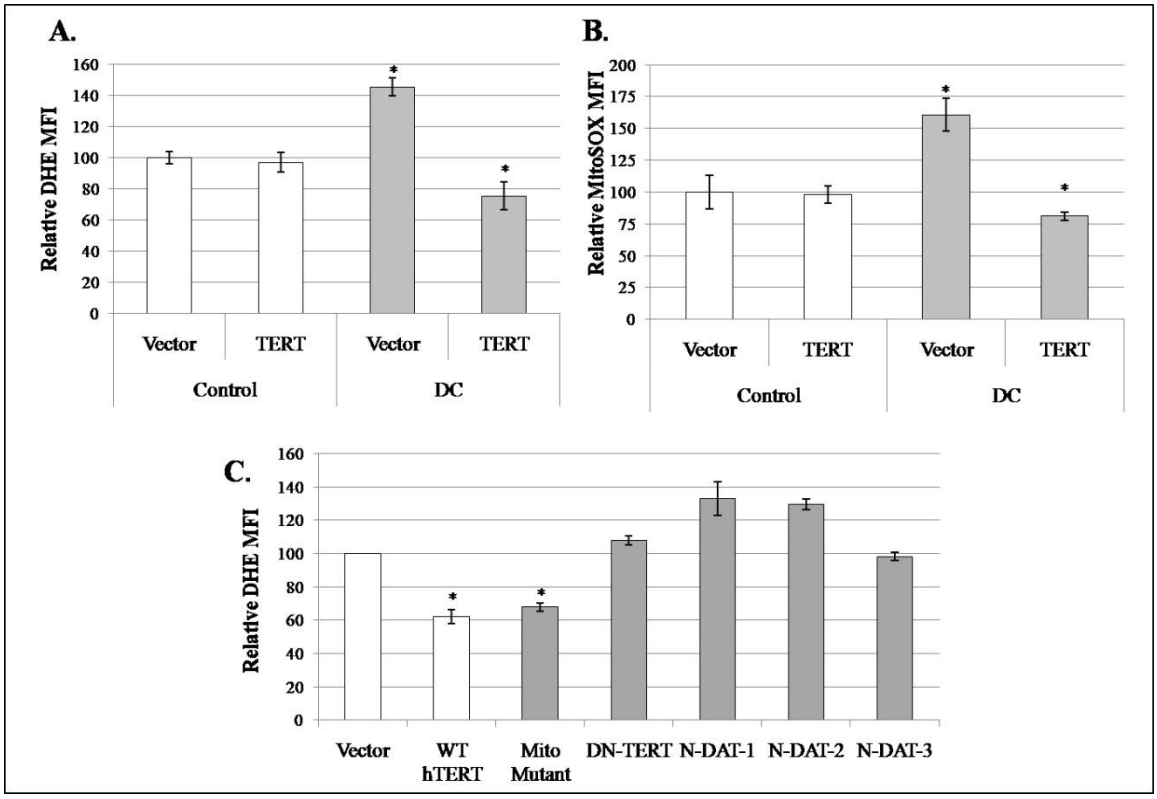
Mid-passage DC and control fibroblast samples were analyzed for glutathione disulfide and glutathione to determine the percent of total glutathione that was in the disulfide state (%GSSG) N.D. indicates levels that were not detectable by the assay. Statistical significance based on Student's t-test analysis of duplicate cell culture sets (*= $p<0.05$; ** = $p<0.001$).

Figure 23: Increased Steady-State Levels of DHE Oxidation in T-Cells and Keratinocytes from DC Patients.



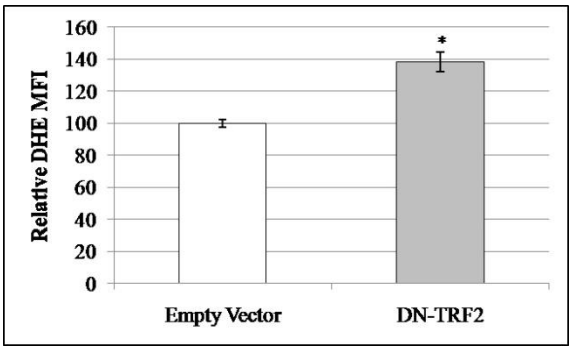
Triplicate sets of freshly acquired and positively selected $CD3^+$ T-cells (**A**) and skin keratinocytes (**B**) were acquired from DC subjects and were subjected to DHE-FACS analysis. Error bars indicated standard deviation of triplicate sets. * indicates statistically significant increase in compared to age-matched controls (Student's t-test): (T-cells: Control-1 vs. DC-5; Control-2 vs. DC-4); *: $p<0.05$; **: $p<0.001$.

Figure 24: Decreased Levels of DHE Oxidation Seen in DC Cells Over Expressing TERT.



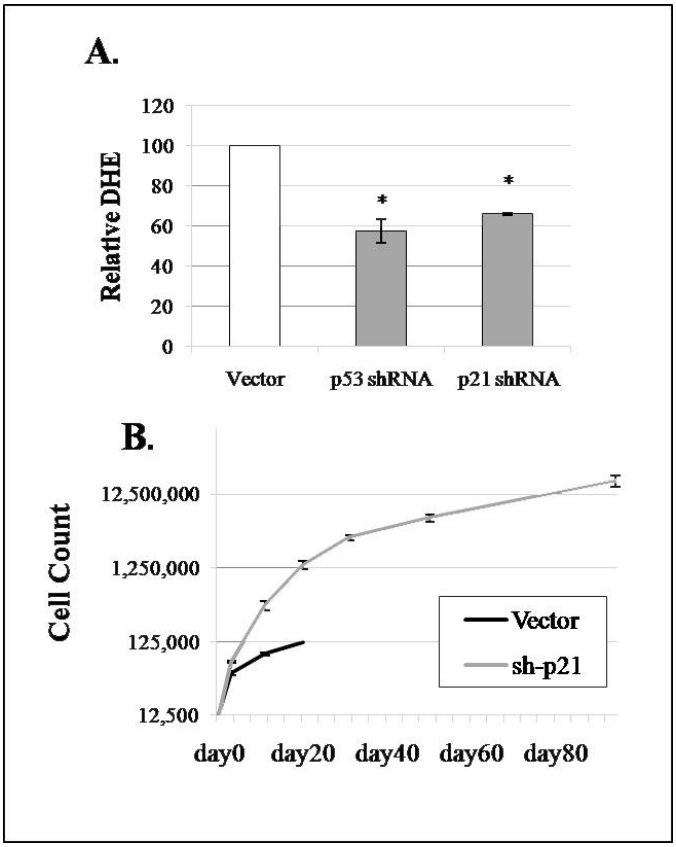
A & B. DC skin fibroblasts (DC1) were infected with retroviral vector alone or with a TERT expressing vector and selected for a pure population. These cells were then subjected to DHE-FACS (**A**) or MitoSOX-FACS (**B**) analysis. Error bars indicate standard deviation of triplicate sets (* indicates a significant increase in DC cells compared to normal controls or significant decrease in DC-TERT expressing cells compared the empty vector DC control (Student's t-test: $p<0.005$). Cells were normalized to empty vector controls. **C.** DC cells were stably infected with the indicated retroviral vector, selected via antibiotic resistance and analyzed using the DHE-FACS assay. DHE oxidation values are relative to an empty vector control. Mito-Mutant (R3E/R6E), dominant-negative TERT, and N-DAT mutants 1-3 have been reported elsewhere. Error bars indicate standard deviation of triplicate sets of 10,000 cell cohorts analyzed individually. * indicates statistical significance decrease in DHE compared to controls, $p<0.001$.

Figure 25: Dysfunctional Telomeres via Expression of Dominant Negative TRF2 Leads to Increased DHE Oxidation.



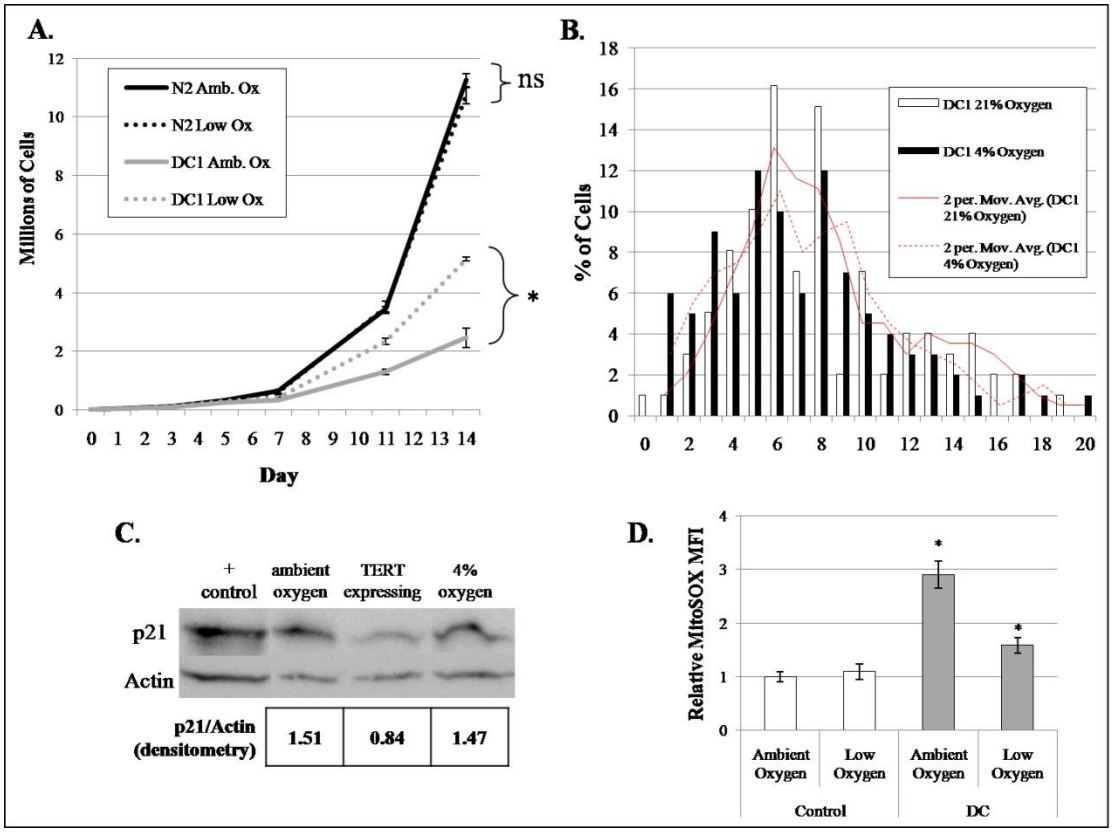
Mid-passage normal cells were infected with a retroviral vector expressing a dominant-negative form of TRF2 (DN-TRF) as well as an antibiotic resistance gene. Post-infection, the cells were selected for a pure population and analyzed via DHE-FACS. Statistical significance was assessed via the Student's t-test. * indicates $p<0.005$.

Figure 26: Consequence of p53 or p21$^{WAF/CIP}$ shRNA Expression on DHE Oxidation, MitoSOX Oxidation, and Proliferation in DC Cells.



A. Mid passage DC cells (DC1) were stably infected with shRNA knocking down the expression of p53 and p21. Cells were analyzed using DHE-FACS analysis at regular intervals to assess DHE staining and late passage vector cells and their shRNA counterparts are presented here. Vector controls and shRNA cells were performed in parallel (triplicate) to evaluate relative DHE oxidation. Values are relative to an empty vector control analyzed with the cells of interest. Error bars indicated standard deviation of triplicate sets. Symbol indicates statistically significant decrease in DHE oxidation compared to controls: * $p<0.001$. **B.** Mid-passage DC fibroblasts expressing a vector control or p21 shRNA were plated at equivalent cell numbers and passaged in subconfluent conditions until senescence. Triplicate plates of cells were counted using a Coulter counter.

Figure 27: Reducing O_2 Tension Suppresses Inhibition of Proliferative Potential in DC Fibroblasts.



A. DC (DC1) and control fibroblasts were grown at either ambient oxygen (21%) or low oxygen (4%) over a two week period. Triplicate sets of plates were passed at ~50-70% confluency and quantified by a Coulter counter. Error bars represent the standard deviation of each triplicate set counted. * indicates significant difference (Student's t-test) in total cell counts at Day 14: $p<0.001$; ns indicates no statistically significant difference in cell counts. **B.** DC and Control cells were grown in low and ambient oxygen for a week, fixed and assessed with immunofluorescence to quantify 53BP1 foci. The foci of each nucleus were counted and binned accordingly. Comparisons were drawn using Student's t-test indicating no statistical difference ($p<0.25$). **C.** $p21^{WAF/CIP}$ levels were evaluated by Western blotting in DC cells grown under routine cell culture conditions (ambient oxygen), transduced with TERT (also ambient oxygen) as well as grown in low oxygen (4%). $p21^{WAF/CIP}$ levels were quantified by evaluating band intensities via Fuji Multi Gauge densitometry software (version 2.3). These values were then normalized by dividing the quantified $p21^{WAF/CIP}$ band intensity by the intensity of their respective loading control (actin). A moving average (red line) was calculated to highlight the similarity between the two data sets. **D.** DC and control cells were grown in low and ambient oxygen for one week and then incubated in MitoSOX and subjected to FACS analysis. The data generated was subjected to the Student's t-test for significance. Significance was found when analyzing control cells grown in ambient

Figure 27 continued.
oxygen vs. DC cells grown in ambient as well as DC cells grown in ambient vs. DC cells grown in low oxygen ($p<0.005$ for both).

CHAPTER 4

CONCLUSIONS AND PERSPECTIVES

The correlative relationship between telomere attrition and aging is well-founded and has been investigated in numerous aging models[331]. Telomere attrition acts as a mitotic clock believed to have general tumor suppressive mechanisms preventing excessive proliferation and facilitating entry into replicative senescence[341]. However, telomeres are typically considered a signal while other cellular components downstream of this signal are thought to act as the causal mechanism toward generating a senescent phenotype. The role of telomere shortening in human aging and the mechanism by which shortened telomeres facilitate senescence are not entirely clear, but research presented within this thesis offers new insight into these issues. In Chapter 2, studies were presented characterizing the relationship between telomere shortening and proliferative defects in skin fibroblasts acquired from DC patients. The activation of telomerase in DC cells was an important finding uncovered in Chapter 2 that indicated that the DC phenotype at the cellular level was not irreversible and telomere maintenance could rescue these proliferative defects. This rescue was particularly insightful as it appeared that the short-telomere biology causing these proliferative defects hinged on one of two possible explanations (that may not be mutually exclusive): Either this rescue transpired due to the heightened telomere maintenance/elongation upon mobilization of telomerase or, the presence of the telomerase enzyme had extra-telomeric functions enabling this rescue. However, Chapter 2 did not necessarily resolve this issue. The research presented in Chapter 3 is direct result of the questions that arose from Chapter 2 and focused on three key issues: 1) the importance of telomere maintenance versus telomerase mobilization in rescuing proliferative defects in DC cells, 2) the means by which the cell communicates the presence of short telomeres to the cell and 3) the causative factors that are signaled by short telomeres to direct a senescent phenotype.

Together Chapters 2 and 3 offer data supporting a model integrating multiple variables that facilitate telomere-related aging/senescence. Briefly, in Chapters 2 and 3, I have presented data supporting a model whereby DC cells harboring short telomeres initiate a signal to the cell activating p53 and p21. This activation is necessary for the cell to elevate reactive oxygen species, a known and putative causative factor utilized by the cell to recapitulate short-telomere biology and enforce an aged phenotype.

The causative agents that lead to aging are numerous; however, to understand the magnitude by which a single element leads to aging, experiments must isolate an element independent of other contributing factors. Dyskeratosis Congenita provides a unique model system to study the role of telomerase insufficiency and telomere shortening related to aging and offers insight that may otherwise be unobtainable. First, the disease symptomology is surprisingly specific in its presentation, signifying that certain cellular compartments may be acutely susceptible to the effects of short telomeres. Second, a number of these compartments are compromised during routine aging underscoring commonalities between routine aging and accelerated telomere attrition observed in DC. Finally, an aging phenotype arises in cells acquired from relatively youthful DC patients suggesting these aging indices are predominantly telomere-related. Taken together, DC patients offer an opportunity to study the effect of telomere-related aging by minimizing other aging-related variables.

The free radical theory of aging suggests that free radicals erode cellular factors, thereby creating a suboptimal cellular environment which promotes aging[193]. This theory also encompasses ROS as a facilitative mechanism to promote aging[3]. Interactions between ROS and cellular components (DNA, proteins, lipids) can be deleterious that support the inclusion of ROS into the free radical theory of aging. ROS arise as byproducts of reactions that take place *in vitro* and *in vivo* however the effects of ROS *in vivo* are difficult to study[193; 392]. The correlation between ROS and aging has spurred research to determine whether ROS has a causal role in the aging process. Perhaps one

of the best *in vitro* methods to study this relationship is investigating the aging-related effects of cells as they reach maximal cell division and enter senescence. Senescent cells have been found to accumulate in both extensively passaged cells and in aged organisms supporting this as an appropriate model[13; 29; 30]. In fact, ROS have been demonstrated to play a role in the irreversible nature of senescence[337] suggesting that the presence of elevated ROS may facilitate entry into senescence. In data presented in this thesis, DC cells acquire elevated levels of ROS and senesce prematurely due to short telomeres. However, induction of senescence by alternative means (i.e. DNA damage) has also been found to elevate ROS[393-397]. This suggests that increasing cellular ROS is not a telomere length-dependent mechanism but likely a necessary component toward suppressing proliferation and entering senescence. It is possible that an activated DNA damage response, regardless of a telomere or DNA origination, may have a significant role in ROS regulation.

Data generated in this thesis support the utilization of an intact DNA damage response triggered by short telomeres to engage senescence (Figure 28). In the context of DC cells and telomere dysfunction based on this thesis and other studies[16], the DDR is believed to involve the following sequence of events: deposition of 53BP1/γ-H2AX at the damaged site which in turn activate ATM/ATR, CHK1/CHK2, p53 and p21[16]. The subsequent decrease in 53BP1 foci and p53 phosphorylation in TERT expressing DC cells suggests that at least part of the DDR is signaled by telomere dysfunction. The fact that low oxygen exposure did not decrease the DDR suggests that the proliferative gains made by DC cells exposed to low oxygen is DDR-independent. I hypothesize that these gains are due to an uncoupling of the DDR and responsive downstream factors related to ROS. This could be tested in DC sh-p21 expressing cells grown in low oxygen to determine whether the same proliferative gains are indeed acquired. This uncoupling interrupts the signal flow from short telomeres to causative factors, one of which may include mitochondrially-derived superoxide. This is supported by data presented here

demonstrating diminished mitochondrial superoxide when exposed to a low oxygen environment; however this can only be demonstrated as a correlative relationship at this time. An experiment optimized to investigate the effects of superoxide on DC cells would be to express adeno- or retroviral vectors of each of the SOD genes, similar to previous studies[273]. The use of a viral vector would permit the targeted expression of a SOD gene to specific cellular compartments. If superoxide has a deleterious effect in these cells then it's suppression during this period is likely to have beneficial effects on the cell's ability to enter and sustain a period of cellular division compared to controls.

Disruption of the DDR by p21 increases proliferative potential and decreases ROS in DC cells. Although previous research has found that ROS activate p53 and p21[196-202] while others find p53 and p21 can induce ROS[8; 174; 203-205], the decrease in ROS upon expression of p21 or p53 shRNA clearly indicates that the upregulation of ROS lies downstream of the DDR pathway in DC cells. In a mouse model of telomere dysfunction ($Terc^{-/-}$), mice acquire several aging indices similar to those found in DC patients[64; 93; 99]. When these mice are crossed into a $p21^{-/-}$ or $p53^{-/-}$ background these deficits are rescued at the expense of severe tumorigenesis in $p53^{-/-}$ mice[97; 98] but interesting without increasing tumor formation in $p21^{-/-}$ mice[99]. Research investigating $Terc^{-/-}$ mice have been performed and have also found an elevated ROS supporting our conclusions regarding short telomere cells[397]. The fact that proliferative defects in the $Terc^{-/-}$ mouse cells can be ameliorated upon removal of p21 support that ROS may have similar origins in mouse and human individuals with short telomeres using an evolutionarily conserved mechanism to carry out short-telomere cellular programming.

It would be reasonable to consider that DC senescence is mechanistically similar to other models of senescence. Cellular alterations related to other senescence models should be considered as potential accessory means to engage senescence in DC cells. For instance, microarray studies evaluating differential gene expression between senescent and controls have leant insight into the transcriptional profile of a senescent cell[20; 397].

These studies have found that senescence programming is cell type specific and, in fibroblasts, are related to mitochondrial biogenesis/function, stress responses and other processes. These studies may have a bearing on the understanding of senescence in DC cells. Alternatively, proinflammatory genes and hydrogen peroxide may also be increasing senescence rates in DC cells[398]. An interesting model that has arisen from these experiments is the 'bystander effect'[399]. The bystander effect postulates that non-senescent cells residing within a cellular pool consisting of senescent cells may be susceptible by proximity via a paracrine secretory phenotype. This suggests that as a small number of cells begin to enter senescence they may begin to excrete factor that accelerate the entry into non-senescent cells thereby initiating a process that erodes a cellular niche. However, some groups have found that a senescent niche may also prime certain cell types for tumorigenesis[400] so careful research must be performed to characterize the effects of senescent cells on their neighbors.

The correlation between ROS and senescence has recently been evaluated in regards to models of telomere dysfunction. Two studies have provided evidence that telomere dysfunction may alter ROS in cells, yet p21 was not implicated in this process. Pérez-Rivero *et al.*[364] examined mouse fibroblast aging and found cells with short telomeres have decreased catalase (CAT) expression (increasing hydrogen peroxide) as well as increased TGF-β1 and Collagen IV. A second study by Lee *et al.* employed T-oligos to mimic telomere dysfunction[365]. T-oligos activated p53 and a NADPH oxidase-dependent rise in ROS which could be attenuated via a NADPH oxidase inhibitor (DPI). Attempts to evaluate changes in gene expression and subsequent enzymatic activity (CuZnSOD, MnSOD, catalase), other potential origins (NADPH oxidase; nitric oxide synthase), locations (extracellular) and constituents (peroxide) of this stress failed to offer any further insight into this stress (data not shown), in contrast to previous publications using other model systems[364; 365]. In the studies presented in this thesis, the increased ROS appears to originate from the mitochondria and is largely comprised of superoxide,

but not peroxide. More specifically, it appears that low oxygen decreases mitochondrial superoxide and improves proliferative kinetics. Although correlative, this suggests that superoxide may potentiate the cell for senescence.

This thesis provides compelling evidence that p21 is activated by a telomere-associated DDR leading to increased superoxide and potentially other related ROS. Evidence presented here corroborates a number of investigations regarding a telomere-related DNA damage response that activates p53 and p21 via 53BP1 and presumably the ATM/ATR and CHK1/CHK2 pathways. Other studies have indicated a causal role for p21 towards elevating ROS as a means to facilitate entry into senescence as well. Overexpression of p21 in normal fibroblasts hastens entry into senescence while simultaneously elevating ROS[205]. These cells can be rescued by antioxidant treatment (NAC) supporting the hypothesis that p21 mediates this early senescence by mobilizing ROS. Genetic manipulation of p21 has also attempted to better understand its role in ROS/senescence. Distinct domains within the p21 protein mediate many protein-protein interactions within the nucleus and the cytoplasm[401; 402]. These interactions include Cyclins, CDKs, PCNA, GADD45 and a host of other proteins. The interaction between PCNA and p21 is believed to assist in mediating a cell-cycle arrest by inhibiting the progression of the DNA replication fork during periods of stress or damage[403]. However, two independent research groups performed systematic deletions of p21 domains and found that the Cyclin/CDK domains were required for elevating ROS while the PCNA domain is dispensible[204; 205].

In two recent papers, another group has attempted to elucidate the origins of ROS in senescent cells. In their 2007 paper[404], a correlation was found between senescent cells and mitochondrial dysfunction. Specifically, they found numerous indices related to mitochondrial dysfunction: decreased mitochondrial membrane potential, increased mitochondrial biogenesis and increased flux of Ca^{2+}. Elevated ROS activated the mitochondrial uncoupling protein UCP2 to facilitate mitochondrial dysfunction.

Together this dysfunction was found to generate a mitochondrial-derived signaling pattern called a retrograde response which engages nuclear reprogramming and general cellular alterations[405]. However, blocking ROS using antioxidant measures (decreasing oxygen tension, antioxidants) or disrupting UCP2 expression, mitochondria were rescued from dysfunction. Interestingly it was postulated that this dysfunction becomes part of a positive-feedback loop where elevated mitochondria superoxide increases senescence markers and decreases telomere length. As damage accumulates, ROS increases damaging DNA which thus increases the DDR in turn elevating ROS further. In support of this, it has been demonstrated that the guanine-rich telomeres are inherently more susceptible to damage caused by ROS than other DNA sequences[46; 404]. Antioxidant intervention extended cellular lifespan and slowed telomere attrition however the frequency of DNA damage foci remained stable. The number of 53BP1 foci was not altered in DC cells exposed to low oxygen in agreement with these findings and suggests that telomere length and telomere-related DNA damage foci may have unequal contributions regarding entry into senescence. Further analyses will be required to understand this discrepancy. In their 2010 paper[393], *in silico*, *in vitro* and *in vivo* evidence was presented attempting to explain the relationship between p21 and mitochondrial dysfunction. Using data generated by control and senescent cells analyzed by expression arrays, the group assembled a potential pathway that supports p21 as a key intermediary between a DDR and mitochondrial dysfunction. This proposed *in silico* pathway suggests that p21 interacts with GADD45 to activate p38MAPK, GRB2 and TGFβ. The conclusion of this pathway is thought to contribute to mitochondrial dysfunction and elevated ROS.

Research presented in Chapter 2 and 3 suggest that telomere maintenance or diminishing ROS are potential avenues towards ameliorating the effects of DC in these patients. A number of recent studies have shed light onto genetic and pharmacological interventions that may be worth pursuing in the near future. Dyskeratosis Congenita is a

heterogeneous disorder that is difficult to treat. The symptomology spectrum extends from minor defects like dyskeratotic nails and skin pigmentation to the more ominous bone marrow failure (BMF) and pulmonary fibrosis (PF). Clinically, if fortunate to find a matching donor, these patients could receive bone marrow or lung transplant potentially alleviating these life-threatening ailments. Even with a matched donor, conditioning DC patients for bone marrow transplant by myeloablative treatment, for example, has its own set of DC-related problems[406]. If successful, these treatments would circumvent BMF and PF yet extending the patient's lifespan may unmask other symptomology hidden by early mortality. Of course, the clinical panacea is to correct the underlying genetic defect using the patient's own cells. One immediate benefit to performing an autologous transplant is the patient would not reject their own MHC-compatible cells. By acquiring one's cells, genetic therapy may be performed *ex vivo*. However, gene therapy trials have had a poor track record. The first gene therapy trials were attempted from 2000-2003 in patients with diagnosed forms of severe combined immunodeficiency syndrome (SCID)[407-410]. Researchers acquired hematopoietic stem cells (HSCs) from SCID patients and infected them *ex vivo* with viral vectors carrying a wild-type copy of the mutated gene. Once infected and expanded, these progenitor cells were transplanted back into the patients in a manner similar to a bone marrow transplant. Although this therapy rescued almost all of these patients from this immune disorder (9 of 10), a number of these patients (4 of the 9 rescued) developed T-cell leukemia due to insertional mutagenesis indicating the treatment had deregulated tumorigenic safeguards. Although the likelihood that a single viral infection would potentiate a cell for oncogenesis is very small, the likelihood increases several fold when millions of viral particles infect millions of cells as a means to increase the odds of a successful infection and recover sufficient cells for transplant. With the advent of updated, safer vectors, gene therapy to treat Dyskeratosis Congenita patients may be possible. *Ex vivo* viral infection of DC hematopoietic stems cells with a stably or transient TERC expressing vector could

potentially elongate telomeres *in vitro.* A single infection of TERC without TERT should not be limiting as HSC's express both[411]. Expanded cells could be transplanted in the patient, potentially staving off BMF. However, telomerase is thought to prime cells for tumorigenesis as it is activated in a high percentage of tumors[343]. Although telomere correction may have taken place to prevent bone marrow failure, this treatment could expose the patient to an unacceptable risk for cancer. Perhaps a more attractive candidate for gene therapy may be to target p21. Mice with short telomeres deficient for p21 are rescued for many aging-related phenotypes without a concomitant increase in tumorigenesis[99]. Given the extension of lifespan found in DC cells expressing p21 shRNA, p21 disruption may provide an avenue to alleviate DC symptomology.

An alternative therapeutic approach could take advantage of skin cells harvested from Dyskeratosis patients to generate iPS cells (induced pluripotent stem cells)[412]. iPS are typically generated by stably overexpressing four transcription factors (OCT4, KLF4, SOX2 and MYC) that have been found to maintain embryonic stem cells and are believed to manipulate the genetic expression profile by altering the epigenetic landscape[413]. These transformed cells acquire embryonic stem cell-like characteristics but have some differences[414]. Only a small percentage of cells are recovered through this methodology and these odds are decreased in DC cells as telomerase deficiency[415], senescence[416; 417] and possibly oxidative stress[418] are particular strong barriers in the formation of iPS cells. Nevertheless, iPS cells have been generated from cells acquired from the same DC fibroblasts that were utilized in this thesis[419]. The short telomere and senescence defects were averted during the iPS transformation process as TERC, TERT and DKC1 were upregulated mobilizing telomerase and preventing short telomeres from driving entry into replicative senescence. These studies generally make use of retroviral vectors that stably express iPS transcription factors, which would be an unviable avenue for clinical use given the integration issues found in SCID patients. Recently, iPS have been generated using episomal expression and protein-based means supporting alternative avenues to

generate cells that are free from oncogenic potential found from retroviral integration[420; 421]. The formation of iPS cells currently has no clinical bearing for a DC patient as these cells cannot be directly transplanted. For DC patients to take advantage of this technology, iPS reprogramming must be taken a step further to generate HSCs and HSC-derived lineages.

Sickle cell anemia has been investigated in a humanized-version of a mouse model as a proof-of-principle investigation into the potential use of iPS for clinical intervention in humans[422]. iPS will not generate blood cells if transplanted and thus require further manipulation. To do so, this group overexpressed HOXB4, a key transcription factor in the derivation of blood cells from progenitors[423]. This procedure was coupled with homologous recombination to correct the mutated gene (a step that could prove beneficial in DC cells during this procedure) which prevented aberrant sickling in daughter cells produced by HSCs. The group found that both myeloid and erythroid cells were generated in the rescued mice however engrafted progenitor cells produced predominantly myeloid cells. This proof-of -principle research proved effective however better mechanisms must be developed to become a viable clinical approach in the future. For DC patients to take advantage of this technology all cells differentiated from hematopoietic stem cells must be generated (which to date has not been successfully performed) to circumvent bone marrow failure.

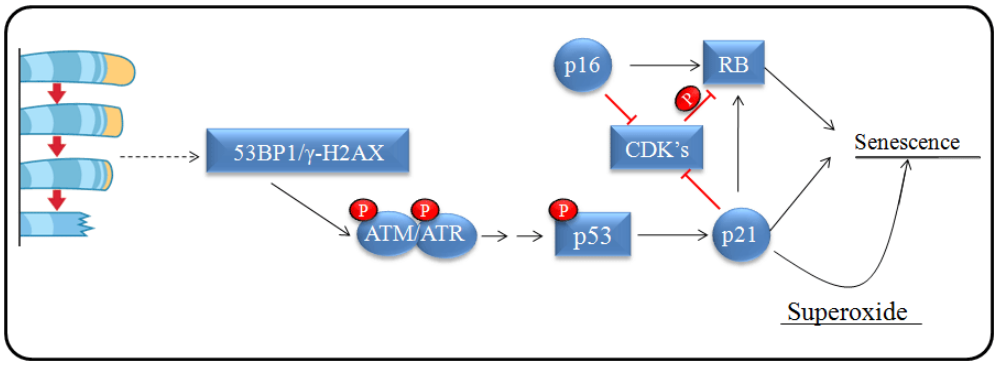
Given the findings within this thesis, oxidative stress may have a previously unappreciated function in the constellation of symptoms found in DC patients. In fact, the 50% of DC patients have no underlying genetic mutations in the known DC genes[424]. With the knowledge that DC cells have an underlying increase in ROS and the p21/p53 pathway modulate this signal, it may be prudent to expand the search for causative DC mutations that lead to hyperactive p53 or p21 activity as well as mutations in redox-related genes. Oxidative stress may also have a significant role in the pathogenesis of bone marrow failure and pulmonary fibrosis given that telomerase mutations have also

been found in these patients[299; 346; 366-370]. Bone marrow failure has been attributed to the failure of the hematopoietic stem cell niche and stroma to support the most immature bone marrow cells[371-373]. This stem cell niche may be especially susceptible to either dysfunctional telomeres[374; 375], aberrant generation of oxidative stress[376-382] or a combination of the two. These circumstances are also likely to apply to pulmonary fibrosis as ROS misregulation is likely to increase cell damage and the prevalence of fibrotic lesions[234-236]. If this holds true, one component of diseases caused by short telomeres will be elevated ROS levels. Given that decreasing oxygen tension in DC cells led to improved growth kinetics and decreased mitochondrial superoxide, it may be prudent to investigate whether DC patients could benefit from adjuvant antioxidant therapies like those attempted in patients with HIV, acetaminophen overdoses or pulmonary fibrosis which diminish ROS via n-acetyl cysteine supplementation[383; 425; 426]. For therapeutic purposes, adjusting steady-state ROS levels may be more feasible than more invasive means involved in bone marrow and lung transplantation.

Chapter 2 of this thesis quantifies telomere lengths in DC cells and reveals that the short telomere effects in these cells were capable of being rescued upon introduction of telomerase activity. This suggests that the senescence-related phenomena found in these cells have origins related to their short telomere status. Chapter 3 assesses the downstream signaling pattern stemming from these short telomeres and has found a p53/p21-dependent increase in mitochondrial superoxide and total ROS. Together, these chapters generate the framework for a means whereby short telomeres lead to an aged phenotype. This work supports combining two independent aging-related models (telomere and free radical related aging) that operate in tandem to enforce a short telomere-related cellular phenotype (Figure 29). Future work will require direct manipulation of specific ROS to fully appreciate the extent to which individual ROS facilitate entry into ROS. p21 may be carrying out a subset of telomere-related signaling in response to an activated p53 yet p53 is known for a spectrum of unrelated functions

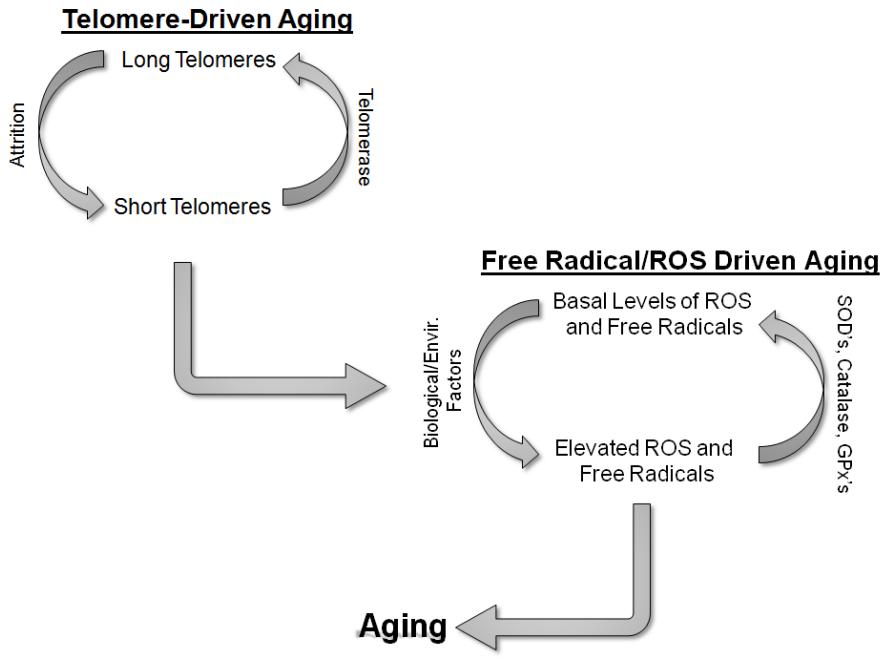
that could be operated in parallel. Further experiments will need to be carried out where p53 and p21 are individually assessed regarding their ROS function independent of their counterpart as well as assessing the effects of attenuating both p53 and p21 activity. In sum, DC cells offer a direct mechanism to study a human-related aging model in the context of telomere dysfunction. The significance of this research spans aging and cancer-related research and may provide insight into suitable interventions on behalf of DC and DC-related clinical disorders.

Figure 28: A Model of the p53, p21 and Superoxide Response to Short Telomeres



DC cells have short telomeres that acquire elevated marks of DNA damage (53BP1), which lead to an elevated p53 response (Ser15 phosphorylation) and the activation of p21. DC cells expressing p21 shRNA decreases mitochondrial superoxide and gain an extension in lifespan. Exposure of DC cells to low oxygen appears to uncouple the short telomere effects from an ROS response as these cells simultaneously decrease mitochondrial superoxide and are afforded proliferative gains. p16 is another attractive CDKi that is elevated in aged tissues that warrants future investigation into its properties related to DC cells. Telomere attrition imagery derived from: http://nobelprize.org/nobel_prizes/medicine/laureates/2009/press.pdf

Figure 29: Biological Circuitry: From Telomeres to ROS to Aging



Telomere attrition and the Free Radical Theory of Aging both offer mutually exclusive evidence towards understanding the molecular effects of aging. Telomere attrition is one factor that leads to aging while this can be countered by the activation of telomerase. Alternatively, free radicals and reactive oxygen species can be elevated via environmental stimuli or intracellular mechanisms. Once ROS have been elevated, these highly reactive molecules can be detoxified via superoxide dismutase, catalase or glutathione peroxidases, for example. However, data within in this thesis supports a model whereby short telomeres are a biological factor that elevates ROS and thus promotes aging via an entry into premature senescence. DC cells appear to require both pathways in order to carry out the effects of short telomeres by way of elevating a potential causative ROS, mitochondrially derived superoxide.

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