TELOMERE LENGTH AND DYNAMICS IN HUTCHINSON-GILFORD PROGERIA SYNDROME

by

MICHELLE LEANNA DECKER

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Abstract

Hutchinson-Gilford Progeria Syndrome (HGPS) is a premature aging disorder caused by mutations in the gene LMNA, which encodes the nuclear matrix protein, Lamin A. Lamin A is found predominantly at the nuclear periphery but also throughout the nucleus in a 'nucleoplasmic veil'. The majority of HGPS patients have a single nucleotide mutation (1824 C→T) which results in the activation of a cryptic donor splice site causing a 150 nucleotide deletion in the mRNA and consequently a 50 amino acid in-frame deletion in the protein. The mutation results in aberrant processing and nuclear localization of the Lamin A protein. HGPS cells are characterized by misshapen nuclei, chromatin disorganization, accumulation of mutant Lamin A, short telomeres, DNA damage recruitment defect and early senescence.

To measure the telomere length of individual chromosomes, Quantitative Fluorescence in-situ Hybridization was used. The average telomere length in HGPS fibroblasts was greatly decreased compared to controls as well as highly variable. In contrast, the telomere length in hematopoietic cells which do not express LMNA was within the normal range for three out of four HGPS patient samples. These results suggest that mutant Lamin A decreases telomere length via a direct effect and that expression of mutant LMNA is necessary for telomere loss in HGPS.

Three different aspects of telomere biology were investigated: localization, mobility and attachment to the matrix. Telomeres were more localized to the nuclear periphery in HGPS fibroblasts than in wild type fibroblasts as well as having abnormal localization in regards to euchromatin/heterochromatin. To examine mobility, fluorescently tagged proteins were constructed to examine interactions between wild type and mutant Lamin A and telomeres during live cell imaging. Long telomeres in cells with the mutant protein did not
move the same distance as those in wild type cells. Mutant Lamin A did not bind DNA with the same affinity as the wild type Lamin A did.

These investigations show that telomeres and telomere dynamics are altered in HGPS cells. This is likely contributing to aspects of the pathology of the disease and would need to be taken into consideration in any therapeutic approach.
Preface

The work presented in Chapter 2 of this thesis has been previously published: Decker, M. L., et al. (2009). "Telomere length in Hutchinson-Gilford progeria syndrome." Mech Ageing Dev 130(6): 377-383. I contributed to the design of the hematopoietic cell section of the study, performed the RT-PCR, analyzed the data and wrote the manuscript. The Q-FISH and Flow-FISH were performed by Elizabeth Chavez and Irma Vulto, respectively. The remainder of the information presented in Chapter 3 has not been published and the experiments were designed, performed and analyzed by myself.

The UBC Ethics Board’s Certificates of Approval that pertain to the research presented in this thesis are B06-0117 and B10-0083.
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<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>Ch</td>
<td>Cherry</td>
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CWB</td>
<td>Cell wash buffer</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
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<tr>
<td>FTI</td>
<td>Farnesyl transferase inhibitor</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>HGPS</td>
<td>Hutchinson-Gilford progeria syndrome</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>iPS cells</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases</td>
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<tr>
<td>MCR</td>
<td>Multiple cloning region</td>
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<tr>
<td>MWB</td>
<td>Matrix wash buffer</td>
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<td>Nucleotide</td>
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<td>Orange</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PD</td>
<td>Population doubling</td>
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<tr>
<td>POT1</td>
<td>Protection of telomeres 1</td>
</tr>
<tr>
<td>Q-FISH</td>
<td>Quantitative-fluorescent in situ hybridization</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse-transcriptase polymerase chain reaction</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>STELA</td>
<td>Single telomere length analysis</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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</tr>
<tr>
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<td>Venus</td>
</tr>
<tr>
<td>WS</td>
<td>Werner syndrome</td>
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<tr>
<td>wt</td>
<td>Wild type</td>
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To my family
Chapter 1 – Introduction

1.1. Aging

Aging is a complex process which is influenced by both genetics and environment. Linkage and association studies on long-lived individuals have determined a long list of candidate genes involved in the aging process\(^1\,^2\). These genes have a wide variety of roles including DNA repair, tumour suppression, mitochondrial function and growth regulation (insulin and growth hormone pathways)\(^3\,^4\). The role of environment is supported by twin studies where even monozygotic twins had significant differences in lifespan and how they aged\(^5\).

Aging has been correlated with the onset of many degenerative diseases including osteoporosis, cancer, type II diabetes and dementia, as well as physical changes such as baldness, graying hair, and reduction in fitness. There are many hypotheses about the factors that contribute to aging including: the somatic mutation theory (increase in DNA mutations with age)\(^6\), the telomere loss theory (decline in replicative capacity is due to the loss of telomere length upon division)\(^7\,^8\), the mitochondrial theory (accumulation of mitochondrial DNA mutations with age)\(^9\), and the altered protein/waste accumulation theory (damaged proteins accumulate with time and interfere with normal cellular functions)\(^10\).

Recently, there has been a push to unify these theories as it is unlikely that any one mechanism is the cause of aging.

1.1.1. Progerias

In a few rare genetic diseases called ‘progerias’ (pro – early, geria – old), patients display an accelerated rate of aging\(^11\). Examples of these diseases include Hutchinson-
Gilford Progeria Syndrome (HGPS), Werner Syndrome (WS), Cockayne Syndrome and Restrictive Dermapathy\textsuperscript{11}. It is important to note that none of the progeroid syndromes have all the features of normal aging, so they are referred to as segmental premature aging diseases\textsuperscript{11}. The progeroid diseases fall into two broad categories based on causative mutation\textsuperscript{11}. Mutations are either in DNA damage repair pathway proteins (WS and Cockayne Syndrome) or in \textit{LMNA}, a nuclear filament protein and Lamin A processing proteins (HGPS and Restrictive Dermapathy)\textsuperscript{11,12}. The premature aging syndromes highlight the importance of the genetic factors in aging since long term environmental effects are unlikely to have as great an impact on the aging process in these patients.

\subsection{Hutchinson-Gilford Progeria Syndrome}

HGPS is a segmental premature aging disease first described by Hutchinson in 1886 and Gilford in 1904\textsuperscript{13}. The reported incidence of HGPS is 1 in 8 million, though the true figure might be closer to 1 in 4 million, taking into consideration unreported or misdiagnosed cases\textsuperscript{13}.

\subsubsection{Symptoms}

HGPS patients appear normal at birth, but within one to two years, begin to display the effects of the disease\textsuperscript{13}. Usually diagnosed in the first two years, initial symptoms include postnatal growth restriction, hair loss, and failure to thrive\textsuperscript{13,14}. The postnatal growth restriction leads to short stature and extremely low weight for height\textsuperscript{13}. Other symptoms consist of micrognathia, craniofacial disproportion, alopecia, prominent eyes and scalp veins, delayed dentition, thin high voice, pyriform thorac, and horse riding stance\textsuperscript{13,15}. Loss of subcutaneous fat causes skin to become lax and wrinkled, which along with a large head,
prominent scalp veins and a small jaw, give the children with this disease the aged appearance\textsuperscript{14,16,17}. Around an average age of 10, the children develop respiratory, cardiovascular and arthritic conditions similar to those commonly observed in an elderly individual\textsuperscript{15}. As the patients age, other symptoms develop including decreased joint mobility, and osteolysis\textsuperscript{14}. Death occurs at a mean age of 12.6 years (ranges from 1.5-27 years), most often due to heart attack or stroke caused by atherosclerosis\textsuperscript{14}.

As HGPS is a segmental aging disease, not all classic aging phenotypes are observed. Most notable is the lack of increase in cancer incidence or any immune system problems in HGPS\textsuperscript{15}. There is also no cognitive phenotype in the disease such as senile personality changes or Alzheimer’s disease\textsuperscript{15}. Patients exhibit no increase in sight problems over that expected for their age\textsuperscript{15}. Also, while patients often die of atherosclerosis, they have no increase in serum cholesterol or triglyceride levels compared to typical elderly\textsuperscript{18}.

1.2.2. Mutation

In 2003, two groups discovered a \textit{de novo} heterozygous mutation in the gene that codes for Lamin A (\textit{LMNA}) in patients with HGPS\textsuperscript{12,19}. In the study by Eriksson \textit{et al}, 2003, a region on chr1q was suspected due to two cases of uniparental isodisomy on Chromosome 1q and another with a 6Mb deletion in the same area\textsuperscript{12}. Candidate genes that fell in the affected region of all three cases were determined, including \textit{LMNA} which with its previous implications in other heritable diseases seemed a likely choice\textsuperscript{12}. Lamin A was also chosen as a candidate gene in de Sandre-Giovannoli \textit{et al}, 2003, based on other diseases with known Lamin A mutations that had similar symptoms\textsuperscript{19}. Sequencing of the gene in multiple patient samples determined that in approximately 90\% of cases, the causative mutation is a C$\rightarrow$T change at nucleotide (nt) 1824 in exon 11 of \textit{LMNA}\textsuperscript{12,19}. This is a silent mutation therefore does not code for an amino acid change but instead activates a cryptic donor
splice site which causes incorrect splicing between exon 11 and exon 12\textsuperscript{12} (Figure 1). This leads to an in frame 150 nt deletion in the mRNA and a subsequent loss of 50 amino acids in the protein\textsuperscript{12}. The deleted region includes a cleavage site that is important in the processing of pre-Lamin A to mature Lamin A\textsuperscript{11,15} (see detailed description of Lamin A processing later in Figure 2). The C-terminus is left modified with a farnesyl tail that is normally cleaved off\textsuperscript{20}. Thus HGPS is caused by the failure to properly process a precursor protein as a result of a splicing error in the mRNA illustrating how a single nucleotide mutation (from C to T) at the wrong place can result in a dramatic phenotype.

1.2.3. Cellular Phenotype

At the cellular level, the most characteristic feature of HGPS fibroblasts is the irregularly shaped nuclear membrane\textsuperscript{21}. Abnormalities of the nuclear membrane include lobulations, blebbing, micronuclei and invaginations, which increase in frequency and severity with time in culture\textsuperscript{21}. The replicative capacity of HGPS fibroblasts is diminished compared to those from unaffected individuals and growth is characterized by a period of hyperproliferation and an increase in the rate of apoptosis\textsuperscript{21,22}.

In HGPS cells there is always a loss of peripheral heterochromatin and sometimes a loss of internal heterochromatin\textsuperscript{21}. Epigenetic alterations in HGPS include genome wide down-regulation of histone H3 trimethylation on lysine 9 and loss of histone H3 trimethylation on lysine 27 on the inactive X chromosome, which suggests that chromatin organization in HGPS cells could be severely compromised\textsuperscript{23}. It has been reported that telomeres from HGPS fibroblasts are significantly shorter than aged matched and parental controls however; a mechanism was not determined\textsuperscript{24}. Recruitment of DNA damage signaling proteins including 53BP1 and RAD51 is compromised in HGPS cells and general genomic instability has been observed\textsuperscript{24,25}. 
Figure 1: Cryptic splice site causes abnormal splicing of *LMNA* on Chr 1q in HGPS cells.

(A) Normal splicing of LMNA between exon 11 and 12. (B) Aberrant splicing between the activated cryptic splice site of exon 11 and the beginning of exon 12 due to activation of a cryptic splice site by the mutation found in HGPS. Red arrow indicates the splice site that is activated in HGPS.
Figure 2: Processing of Lamin A in with and without the HGPS causing mutation.

*Left:* The normal processing pathway of pre-Lamin A to mature Lamin A. *Right:* Processing pathway of Lamin A with the HGPS mutation. The deletion of the second cleavage site prevents the removal of the farnesylated tail. This research was originally published in the Journal of Lipid Research. Young, S.G., Fong, L.G. and Michaelis, S. Prelamin A, Zmpste24, misshapen cell nuclei, and progeria--new evidence suggesting that protein farnesylation could be important for disease pathogenesis. *J Lipid Res.* 2005; 46:2531-58. © the American Society for Biochemistry and Molecular Biology.
1.3. Lamin Proteins

The lamin proteins (A and B), are involved in many important functions including DNA replication, transcription, chromatin organization, nuclear positioning/shape, and assembly/disassembly of the nucleus during cell division\textsuperscript{26,27}. All nuclear lamins are classified as Type V intermediate filament proteins and confined to the nucleus for most of the cell cycle\textsuperscript{26}. There are four A-type lamins (A, A\textDelta10, C and C2) encoded by the *LMNA* gene via alternate splicing\textsuperscript{26}. Lamin A and C are expressed in almost all differentiated tissues except for cells of the hematopoietic lineage. Lamin C2 is only expressed in the testis while A\textDelta10 is found in cell lines derived from colon, lung and breast carcinomas, respectively\textsuperscript{27}. The three B-type lamins are expressed from two genes, *LMNB1* and *LMNB2*\textsuperscript{27}. Expression of at least one B-type lamin is required for cell viability\textsuperscript{28}.

1.3.1. Structure of Lamin

All lamin proteins have three domains; a N-terminal head domain, a coiled-coil rod domain and a large globular tail domain with an immunoglobulin fold\textsuperscript{26}. B-type lamins and Lamin A have a CaaX motif (where ‘a’ is aliphatic, and X is any amino acid) at the C-terminus. This motif is targeted for post-translational modifications (Figure 2, *Left*)\textsuperscript{29}. The cysteine is farnesylated by a farnesyl transferase, followed by cleavage of the final three amino acids by ZMPSTE24 and RCE1\textsuperscript{29}. The farnesylated cysteine is methylated by ICMT\textsuperscript{29}. Then in Lamin A, the final 15 amino acids including the farnesylated tail are cleaved by ZMPSTE24. There is no second cleavage event in the B-type lamins so the C-terminus is left farnesylated, localizing the proteins to the nuclear membrane\textsuperscript{29}. 
1.3.2. Binding Partners

Lamin A has many different types of binding partners including other nuclear lamina and matrix proteins such as Lamin C, Lamin B, LAP2α and actin\(^{26}\). Lamin A binds directly and indirectly to DNA and histones\(^{26}\). This is thought to allow lamins to act as scaffolding networks for multiprotein complexes\(^{26}\). This is important for the function of RNA PolII and splicing factor compartments\(^{30}\). Lamin A also binds gene regulatory factors such as the cell cycle regulator, pRb, and the transcription factors SREPB, MOK2 and BAF\(^{26}\). Signaling proteins such as PKCα and 12(S)-lipoxydase have been reported to interact directly with Lamin A and the lamin scaffold structure is required for PKCα signal transduction\(^{31,32}\). Lamin A has also been found in complexes with DNA repair proteins\(^{33}\).

1.3.3. Laminopathies

Lamin A mutations, as well as mutations in other lamina proteins, have been found to cause a wide variety of diseases termed laminopathies, which include cardiomyopathies, muscular dystrophies, lipodystrophies, neuropathies, dermopathies, and premature aging syndromes\(^{34}\). Some of the diseases are limited to specific tissue types while others have a broad tissue specificity\(^{35}\). One of the major questions in the study of laminopathies is how Lamin A mutations can cause such a variety of diseases in different tissue types\(^{34}\). Since multiple mutations in different proteins are able to cause overlapping phenotypes, there appears to be a lot of interplay between the different nuclear lamina and matrix proteins.
1.3.4. Mechanism of Lamin A Pathology

The two main hypotheses for the pathology of Lamin A mutations include the mechanical stress hypothesis and the gene regulation hypothesis, however these are not necessarily mutually exclusive\textsuperscript{36}. The mechanical stress hypothesis states that lamin mutations cause abnormalities and fragility of the nuclear structure resulting in increased susceptibility to cellular damage from physical/environmental stress\textsuperscript{36}. Tissues under high torsional stress such as skeletal and cardiac muscle and adipose tissue are often affected in laminopathies\textsuperscript{34}. These mutations may disrupt the associations between Lamin A and the cytoplasmic intermediate filament proteins\textsuperscript{34}.

The gene expression hypothesis states that Lamin A mutations affect transcription factor function and consequently alter the gene expression profile. The nuclear envelope and matrix play a role in tissue specific gene expression through interactions with chromatin components\textsuperscript{36}. As mentioned above, several transcription factors bind directly and indirectly to Lamin A\textsuperscript{34}. Some of these are involved in important cell fate decisions and regulation of apoptosis\textsuperscript{34}. It has been shown that Lamin A mutations can cause problems in cell cycle control and other aspects of gene regulation\textsuperscript{37,38}.

1.4. Telomeres

Eukaryotic DNA has unique challenges due to its linear nature as the ends of chromosomes, if not protected, would be prone to breakdown and degradation by nucleases. As well, the ends of chromosomes could resemble double strand breaks and if recognized as such would be subject to processing and repair. Protection from these processes is accomplished by nucleoprotein structures called telomeres\textsuperscript{39}.
1.4.1. Telomeric Structure

Telomeres consist of repetitive, G-rich DNA sequences and associated telomere binding proteins\textsuperscript{40}. In vertebrates, the telomere sequence is composed of (TTAGGG)\textsubscript{n} repeats, varying from 2-10 kb in humans\textsuperscript{41}. Most telomeric DNA is double stranded. However, the very end of the chromosome forms a 3’ single strand overhang between 50 and 300 nt long. The telomeres and associated proteins create a protective structure called a T-loop where the 3’ overhang inserts into the upstream double stranded telomeric DNA\textsuperscript{39,40} (Figure 3A and B). The T-loop structure was first identified by electron microscopy of telomeric restriction fragments\textsuperscript{39}. It was proposed that by sequestering chromosome ends into T-loops, telomeres cannot be recognized as double strand DNA breaks and prevents degradation by nucleases\textsuperscript{39}.

The T-loop and telomere binding proteins are referred to as a telosome or shelterin complex\textsuperscript{40}. The first protein in the shelterin complex to be discovered was Telomere Repeat Factor 1 (TRF1) due to its specificity for double stranded telomere repeats\textsuperscript{42}. TRF1 is a negative regulator of telomere length; over-expression leads to a decrease in telomere length while deletion of the protein causes abnormally long telomeres\textsuperscript{43}. Telomere Repeat Factor 2 (TRF2), found through sequence homology, binds to the telomere repeats and protects the 3’ overhang from nucleases and DNA damage detection\textsuperscript{43,44}. TRF1 can induce bending and looping of the DNA while TRF2 is thought to facilitate the insertion of the single stranded tail into the double stranded telomeric DNA\textsuperscript{43,44}. TRF1-interacting nuclear protien 2 (TIN2) functions to tether TRF1 and TRF2\textsuperscript{45}. As well TIN2 binds TPP1 which serves to recruit Protection of Telomeress 1 (POT1) to the complex\textsuperscript{45,46}. POT1 binds the G-rich single stranded overhang, preventing DNA damage signaling\textsuperscript{47}. The final shelterin complex protein is Rap1 which is recruited to telomeres via TRF2 and binds directly to telomeric repeats\textsuperscript{48}. 
Figure 3: Schematic of shelterin on telomeric DNA.

The role of Rap1 is not well understood but when Rap1 is deleted telomeres will continuously lengthen\(^49\). Rap1 also has a role in telomere recombination and fragility and in transcriptional gene regulation\(^49\). Other proteins found at telomeres include DNA damage response proteins such as Ku70/80, RAD50, MRE11 and NBS1\(^50\).

1.4.2. Mechanisms of Telomere Loss

Telomeres are required to protect the ends of linear chromosomes against genomic instability caused by terminal fusions, recognition as double strand breaks and prevention of degradation of genetic information due to DNA replication\(^40\). There are two main mechanisms of telomere loss, the end replication problem and sporadic telomere loss\(^51\). During each cell division, there is a loss of a small amount telomeric DNA at every chromosome end in a mechanism termed the ‘end replication problem’\(^52\). Due to the nature of lagging strand synthesis, there will always be a stretch of DNA at the end of the chromosome, where the RNA primer was located, that will not be able to be replicated\(^51\). A loss of about 50-200 nt per division has been reported in humans\(^50,52,53\). Sporadic telomere loss is due to stalled replication forks and failure to repair DNA damage at the telomere\(^51\). It can be of a variable amount, from a few nucleotides to the entire telomere\(^54\).

Eventually telomere length will reach a critical point where the telomere can no longer form the protective T-loop structure\(^40\). At this point most cells undergo senescence or apoptosis\(^55\). Failure to do this could lead to genomic instability, malignant transformation and cancer\(^55\). The addition of the telomere elongation enzyme, telomerase, will increase the lifespan of cells\(^55,56\).
1.4.3. Telomerase

First discovered in the ciliate *Tetrahymena*, telomerase, a ribonucleoprotein reverse transcriptase, has the ability to elongate critically short telomeres\textsuperscript{56,57}. Telomerase is active in embryonic tissues and in small amounts in certain adult cell lineages. Telomerase is composed of two subunits; TERT, the catalytic subunit and TERC, the RNA template\textsuperscript{56}. Other proteins are required for telomerase function including dyskerin, NOP10, NHP2 and GAR1\textsuperscript{56}. Telomerase activity is regulated by either modulating enzyme activity or by controlling access to the telomere. Ninety percent of cancers reactivate *TERT* in order to prolong their ability to divide\textsuperscript{55}. The identification of human diseases, such as Dyskeratosis, where telomerase activity is dysfunctional highlights the importance of telomere elongation throughout the lifespan of the organism.

1.5. DNA Damage

1.5.1. Genomic Instability

DNA damage and genomic instability, often associated with cancers, also has an important role in normal and premature aging. Over time, DNA accumulates mutations from a variety of sources including chemical, environmental, and metabolic as well as errors obtained during replication\textsuperscript{59}. In order to combat this, the cell has multiple DNA damage repair mechanisms as well as cellular processes such as apoptosis and senescence to take a cell out of the replicative pool\textsuperscript{59}.
1.5.2. Senescence

Cells derived from human tissue have a finite replicative potential called a ‘Hayflick number’\(^6\). After the Hayflick number has been reached, the cell undergoes ‘cellular senescence’. One of the key contributing factors that determine when a cell will undergo senescence is telomere length. When telomeres reach a critical minimum length the protective t-loop structure can no longer be formed which triggers a DNA damage response. Senescence can also be activated early by cellular stress, oncogene activation, loss of tumor-suppressor function and unrepairable DNA damage\(^{61-63}\).

The hallmark of senescence is arrest of the cell cycle. Other cellular alterations include morphological changes, activation of tumor-suppressor networks, β-galactosidase activity, altered chromatin structure and changes in secreted factors. The last of these characteristics can alter the microenvironment around the senescent cell and affect non-senescent cells. Cells must have multiple features in order to be classified as senescent\(^6\).

1.6. Objectives and Hypothesis

The overall objective of the project was to determine how telomere function is perturbed due to the Lamin A mutation found in HGPS. Different aspects of telomeres were examined including length, localization, mobility, attachment to the matrix and the DNA damage response. The global aim of this work was to gain a better understanding of the role of telomeres in normal aging and of the interactions between telomeres and the nuclear matrix.

The working hypothesis for this thesis was that Lamin A has an important role in the organization of chromosomes in the nucleus and that disruption has detrimental effects on telomere function, chromatin organization and the DNA damage response.
Chapter 2 – Telomere Length in Hutchinson-Gilford Progeria Syndrome

2.1. Synopsis

Hutchinson-Gilford Progeria Syndrome (HGPS) is a rare premature aging disorder caused by mutations in the gene \textit{LMNA}, which encodes the nuclear matrix protein Lamin A\textsuperscript{12,19}. Previous research has shown that the average telomere length in fibroblasts from HGPS patients are shorter than age matched controls\textsuperscript{64}. How mutations in Lamin A lead to shortened telomere length is not known nor is the contribution of individual chromosome ends to the low average length understood.

To measure the telomere length of individual chromosomes, Quantitative Fluorescence \textit{in situ} Hybridization (Q-FISH) was used. In agreement with previous studies, the average telomere length in HGPS fibroblasts was greatly reduced; however, the telomere length at specific chromosome ends was extremely variable. In contrast, the telomere length in hematopoietic cells which do not express Lamin A at detectible levels, was within the normal range for three out of four HGPS patient samples. These results demonstrate that mutant Lamin A decreases telomere length via a direct effect and that expression of mutant \textit{LMNA} is necessary for telomere loss in HGPS.

2.2. Material and Methods

2.2.1. Cell Lines and Patient Samples

The HGPS fibroblast cell lines AG03513, AG06297 and AG11498 were obtained from the NIA Aging Cell Repository (Coriell Cell Repository, Camden, NJ). Cells were
cultured in Dulbecco’s modified eagle medium (DMEM) containing 15% fetal calf serum (FCS), 200µM glutamine, 100 U/ml penicillin and 100 g/ml streptomycin, at 37°C in a 5% CO₂ atmosphere incubator. When the experiments were performed, the HGPS cell lines were at the following population doublings: AG03513 – PD 17, AG06297 – PD 35 and AG11498 – PD 7. The six healthy control fibroblast cell lines that were used in the telomere length studies have been previously reported.

Blood samples were drawn from four patients diagnosed with classical HGPS after informed consent from patients and their parents. Blood was drawn in Heparin or EDTA tubes and shipped at room temperature. Upon arrival blood samples were frozen until analysis. Controls for Flow-FISH were 400 healthy persons ranging from birth to 100 years of age as previously reported.

2.2.2. Quantitative-Fluorescence in situ Hybridization

Q-FISH was performed as previously reported. Briefly, metaphase cells were harvested, fixed with methanol-acetic acid then dropped onto slides. Slides were fixed with formaldehyde, treated with pepsin, and dehydrated with ethanol. The hybridization mix containing the Cy3-labeled (CCCTAA)₃ peptide nucleic acid telomere probe was added to each slide which was then denatured at 80°C for 2 minutes prior to incubation at room temperature for 1 hour. Slides were washed, counterstained with DAPI then mounted using DABCO. Images were acquired and analyzed as described.

2.2.3. RNA Extraction and RT-PCR

RNA was extracted from BJ neonatal human foreskin fibroblasts, and T cells and granulocytes from control samples using the RNAeasy kit (Qiagen). A reverse-transcriptase
polymerase chain reaction (RT-PCR) assay was performed with the isolated RNA to make complementary DNA (cDNA). The cDNA was amplified with either primers for LMNA (forward: CAAGGCATCTGCCAGCGG and reverse: TTTCTTTGGCTTCAAGCCCC) or β-actin (forward: AGAGATGGCCACGGCTGCTTC and reverse: GCATTTCGCGTGGACGATGGAG). PCR products were visualized on a 1.5% agarose gel by ethidium bromide staining.

2.2.4. Flow-FISH

Details of the Flow-FISH method are as previously described. Briefly, red blood cells from thawed samples were lysed with NH₄Cl. Leukocytes were denatured in formamide at 87°C, hybridized with a fluorescein-conjugated (CCCTAA)₃ peptide nucleic acid probe, and counterstained with LDS751 DNA dye. Analysis of fluorescence was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The cell types analyzed included total leukocytes, granulocytes, total lymphocytes, CD45RA-positive/CD20-negative naive T cells, CD45RA-negative memory T cells, CD20-positive B cells, and CD57-positive NK/NKT cells. Bovine thymocytes were used as an internal standard in every sample. Cellquest Pro software (BD Biosciences, CA, USA) was used to quantify the flow cytometry results; median telomere lengths were calculated using an automated Microsoft Excel calculator.

2.2.5. Statistical Analysis

HGPS samples were compared to control samples using an independent t-test, assuming unequal variance. A p-value of <0.005 was considered significant. Telomere length will be demonstrated in box plot histograms.
2.3. Results

2.3.1. Telomere Length in HGPS Fibroblasts

To investigate the nature of telomere shortening in HGPS, Q-FISH was performed on metaphase chromosome spreads from three HGPS primary fibroblast cell lines derived from biopsies of three different patients (Figure 4). The fibroblast cell lines from HGPS patients were grown using standard culture conditions. Cells from early passage cultures were arrested in metaphase and used for telomere length analysis. At least twelve metaphases were analyzed for each cell line. No significant chromosomal abnormalities such as fusions, translocations or aneuploidy were detected in the metaphases from HGPS patients (data not shown).

The samples AG11498 and AG06297 have the common 1824 C→T mutation; however, the mutation status of AG03513 is unknown although the patient had classical HGPS symptoms. Telomere length in AG06297 and AG03513 were significantly shorter than the control samples. AG11498 was approximately the same length as the control samples and is short for the age of the patient based on data from Allsopp et al., 1992 (Table 1)\(^6\). While AG11498 was not shorter than control cells it should be noted that the average age of the donors for the control cell lines was 50 years which accounts for this. Telomere lengths were variable between different chromosome ends as well as in the same chromosomes between samples (Figure 5 and 6). Signal free ends, representing telomeres that are too short to detect by Q-FISH, were observed in the HGPS samples but not in the normal fibroblast metaphases (Table 1) (Figure 4, white asterisks).

No evidence was found for consistent biased shortening of any particular chromosome end between each HGPS samples, although all samples exhibited some
Figure 4: Quantitative-FISH analysis of individual telomeres in fibroblasts from 3 different patients with HGPS.

The PNA probe specific for telomeric DNA is in shown in yellow while DAPI staining for DNA is shown in blue. Telomere ends with undetectable telomere signals are indicated using a white asterisk. (A) AG03513, (B) AG06297 and (C) AG11498.
Table 1: Mean telomere length and frequency of signal free ends in HGPS fibroblasts measured using Q-FISH.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age (years)</th>
<th>p arm (kb ± SEM)</th>
<th>q arm (kb ± SEM)</th>
<th>Total (kb ± SEM)</th>
<th>Signal free ends</th>
<th>% signal free ends</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>49.7(^a)</td>
<td>–</td>
<td>–</td>
<td>6.17 ± 0.03</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AG03513</td>
<td>13</td>
<td>3.18 ± 0.05</td>
<td>3.46 ± 0.05</td>
<td>3.32 ± 0.05</td>
<td>43</td>
<td>1.83</td>
</tr>
<tr>
<td>AG06297</td>
<td>8</td>
<td>3.60 ± 0.04</td>
<td>3.40 ± 0.04</td>
<td>3.48 ± 0.04(^b)</td>
<td>88</td>
<td>2.84</td>
</tr>
<tr>
<td>AG11498</td>
<td>13</td>
<td>6.57 ± 0.06</td>
<td>6.34 ± 0.06</td>
<td>6.45 ± 0.06</td>
<td>3</td>
<td>0.13</td>
</tr>
</tbody>
</table>

\(^a\) The control is the average of 6 normal fibroblast cell lines. p and q arm data values were not collected separately for the normal controls.

\(^b\) \(p < 0.005\) of mean telomere length between controls and HGPS samples calculated using an independent t-test assuming unequal variance.

\(^c\) \(p < 0.0001\) of mean telomere length between controls and HGPS samples calculated using an independent t-test assuming unequal variance.
extremely short chromosomes including chromosome 18q in AG06297 (Figure 5). The telomeres of chromosome 17p, which have been previously reported to be among the shortest\textsuperscript{65,70}, were below the mean telomere length in all HGPS samples however in AG11498 it was not statistically different from the mean telomere length for that sample (Figure 6). In addition, the telomeres of chromosome 4q, often one of the longest telomeres and always above the mean in normal individuals\textsuperscript{65}, was only significantly different than the mean telomere length in AG03513.

In the control fibroblast samples, the average telomere length of chromosome 18, which is gene poor, were above the total mean telomere length. However, the average telomere length of the gene-rich chromosome 19 was always found to be below the mean telomere length in the control samples. In the HGPS samples, the telomere length of chromosomes 18 and 19 was more similar to each other than they were in control samples. In some cases the telomere length of chromosome arms did not follow the pattern of the control samples (i.e. Chr18q was below the mean telomere length in AG06297 while it was always found above the mean in controls) (Figure 5 and 6). In summary, chromosome-specific differences in telomere length observed in cells from normal control individuals were not preserved in all HGPS cell lines. This suggests that the more random chromatin arrangement in the HGPS cells affects telomere length.

2.3.2. Cell Type Specific Expression of \textit{LMNA}

To investigate if the expression of mutant Lamin A is directly involved in the generation of short telomeres, we examined telomere lengths in hematopoietic cells, which reportedly either do not express \textit{LMNA} or express it at much lower levels\textsuperscript{71,72}. To confirm expression levels, RT-PCR was performed on granulocyte and T cell mRNA samples and a fibroblast cell line (BJ) as a control (Figure 7). The only sample that expressed \textit{LMNA} at
Figure 5: Telomere length is short in HGPS cell lines and highly variable between chromosomes.

Box plot histograms of telomere fluorescence values in the HGPS fibroblast cell line AG06297. Telomere lengths of chromosomes from HGPS cells are highly variable. The horizontal line in the large box represents the 50th percentile; the upper and lower margins of the box are the 25th and 75th percentile; the small box inside represents the mean; the vertical lines above and below the box are the 5th and 95th percentile respectively; the ‘×’ represents the 1st and 99th percentile; the ‘–’ represents the minimum and maximum values. (A) The p arm and (B) the q arm of the indicated chromosomes.
Figure 6: The pattern of telomere length in HGPS cell lines does not always follow that observed in normal cell lines.

Average telomere length of individual chromosomes in three normal fibroblast samples (control 1, control 2, and control 3) and three HGPS fibroblast samples (AG03513, AG06297 and AG11498) measured by Q-FISH. Highlighted are chromosomes 17p (red), 4q (blue), 18p and 18q (green) and 19p and 19q (orange)
Figure 7: *LMNA* is not expressed in granulocytes (G), or T cells (T) but is expressed in the fibroblast cell line (BJ).

β-actin was used as an internal control for the amount of cDNA in each PCR reaction.
detectable levels was the BJ control fibroblast cell line sample while the granulocyte and T cell samples had no band confirming the lack of *LMNA* expression in these hematopoietic cells (Figure 7).

2.3.3. Telomere Length in HGPS Hematopoietic Cells

Blood samples were obtained from four HGPS patients, all of whom have the common C→T mutation at nt 1824 of *LMNA* and presented clinically with classical HGPS symptoms (personal communications, Dr. William A. Gahl). The median telomere lengths of subsets of hematopoietic cells, including T cells, B cells, NK cells, and granulocytes were measured using flow-FISH. The average telomere length in all subsets of hematopoietic cells was consistent with the expected length for age-matched controls in three out of the four patient samples examined (Figure 8). One sample, HGPS 3, had very low telomere length in all subsets of cells. This patient did not exhibit a more severe form of the disease compared to the other patients at this stage. The normal telomere lengths in three out of four patients suggest that the expression of Lamin AΔ50 leads to the short telomeres observed in the fibroblast samples rather than a systemic affect of the disease.

2.4. Discussion

HGPS is a segmental aging disorder which resembles aging in specific tissues both physiologically and at the cellular level. The phenotype of short telomeres has been seen in other premature aging diseases including Werner Syndrome where telomere dysfunction has been proposed to cause genomic instability73. Our results from telomere length analysis of fibroblasts from HGPS patients are consistent with the results described by Allsopp et al, 199264 (Table 1). By combining Q-FISH with karyotyping, the telomere length of individual
Figure 8: Telomere length in hematopoietic cells according to age in patients with HGPS and normal donors.

Telomere length is normal in three of the HGPS patient samples measured. The vertical axis represents telomere length in kilobases. Lines in the figures indicate the 1st, 10th, 50th, 90th, and 99th percentiles determined from 400 normal control subjects. Symbols represent subjects: four patients with HGPS (coloured solid square), and 18 normal donors (yellow solid circles). (A) Lymphocytes and (B) granulocytes.
chromosome ends was studied in the hope to gain insight into the mechanism of telomere shortening in HGPS.

The length of individual chromosomes from HGPS patients did not always follow the established patterns of the control samples (Figure 5 and 6). Chromosome 17p has been previously reported to have the shortest telomeres, and in the HGPS samples it was also below the mean telomere length but not the absolute shortest\textsuperscript{66,70}. Chromosomes 18 and 19, which are relatively gene-poor and gene-rich respectively, have defined nuclear territories\textsuperscript{74,75}. Chromosome 18, typically located at the periphery of the nucleus in proliferating cells, is more internally localized in HGPS cells\textsuperscript{74}. Loss of peripheral heterochromatin may lead to this change in nuclear position and this alteration in chromatin organization could affect telomeres structure. In the HGPS fibroblasts, the telomere length in chromosomes 18 and 19 generally followed the pattern of the wild type cells, however, the telomere lengths of these two chromosomes are more similar (Figure 6). This taken with the chromosome 17p differences suggests that there may be differences in telomeric chromatin in HGPS.

The median telomere length was measured in hematopoietic cells by flow-FISH to determine if cells with reduced or no expression of \textit{LMNA} also had short telomeres. In three out of the four samples, telomere length was within the normal \textit{LMNA} expected range for the age of patient (Figure 8). This implies that the expression of mutant Lamin A is, in general, required for the generation of short telomeres. This puts into question previous results that found problems in hematopoietic cells from HGPS patients\textsuperscript{19}.

The lack of obvious telomere shortening in cells that do not normally express Lamin A argues that the effect on telomere length in cells is direct rather than indirect. However one sample, HGPS 3, had very low telomere length in all subsets of hematopoietic cells (Figure 8). This difference could be due to an indirect effect of the Lamin A mutation (e.g. loss of cells supporting the hematopoietic stem cells) or due to normal variation in telomere
length in the population. Parental blood samples were not available for analysis so the possibility that this particular patient inherited short telomeres cannot be excluded.

Many mechanisms have been proposed to explain the cellular defects in HGPS. The simplest mechanism for decreased telomere length in HGPS would be a larger amount of telomeric DNA lost with each cell division (e.g. due to increased exonuclease processing due to increased access to the DNA). Increase loss at each division was not observed by Allsopp et al., 1992 when telomere length was measured by TRF analysis. However, it is possible that the sensitivity of this technique was insufficient to detect a small increase in telomere loss with each replication round. It would also not account for the variation observed. Similar to a previously proposed mechanism, an increase in apoptosis and/or senescence in HGPS cells could cause a compensatory increase in cell division in remaining cells. This likely has a partial role in the short telomere phenotype; however it does not explain the observed variability in telomere length, especially the ultra short telomeres (Table 1). A third mechanism is failure to repair DNA damage at telomeres, causing losses of variable amounts of DNA. This would explain both the high variability in telomere length between chromosomes and the increased frequency signal free ends (Table 1 and Figure 4). Unrepaired DNA damage could also prevent the cells from completing cell division leading to apoptosis or senescence. The generation of short telomeres in HGPS is likely not caused by one simple mechanism but a complex interplay of all these.
Chapter 3 – Telomere Dynamics in Hutchinson-Gilford Progeria Syndrome

3.1. Synopsis

Hutchinson-Gilford Progeria Syndrome (HGPS) is a premature aging syndrome caused by mutations in the gene encoding the nuclear matrix protein, Lamin A. Lamin A is found predominantly at the nuclear periphery but also located throughout the nucleoplasm. A single nucleotide mutation (1824 C→T), which causes a silent amino acid change in Lamin A has been found in the majority of cases\textsuperscript{12,19}. HGPS cells are characterized by misshapen nuclei, chromatin disorganization, accumulation of the mutant protein and early senescence.

Three aspects of telomere biology were examined: localization, mobility and attachment to the nuclear matrix. Using FISH, it was determined that telomeres were mislocalized in HGPS cells compared to controls. Telomeres were not found at the euchromatin/heterochromatin border and they appeared to have a more peripheral localization in the HGPS cells. To study the interactions between wt or mutant Lamin A and telomeres, cell lines were created that expressed a Venus-tagged TRF1 to distinguish telomeres and Cherry-Lamin A Δ50. Live cell imaging of these cells to examine mobility of telomeres as well as the interaction between Lamin A and TRF1 demonstrated that the mobility of the larger telomeres, as determined by the fluorescence intensity, was reduced in the cells expressing the mutant Lamin A while it was unchanged in cells expressing wt Lamin A. Using nuclear matrix extraction, it was determined that DNA did not bind the mutant Lamin A as well as it did wt Lamin A. The DNA damage protein, 53BP1, was also found to have an altered pattern compared to wt cells. In HGPS cells, there were many very small foci of 53BP1 compared to wt cells where there were only 1-2 large foci.
Taken together, these results suggest that telomere dynamics are altered in HGPS. Whether this is a consequence or a cause of the short telomere length also observed in the disease is unknown. However, it is possible that it accounts for some of the cellular pathology observed in the disease including premature senescence.

3.2. Materials and Methods

3.2.1. Cell Lines and Cell Culture

The HGPS cell lines AG06297 and AG11498 were obtained from the NIA Aging Cell Repository (Coriell Cell Repository, Camden, NJ). BJ foreskin fibroblasts, HeLa cancer cell line and the PG13 cells were obtained from the ATCC.

HGPS cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 15% fetal calf serum (FCS), 200 µM glutamine, 100 U/mL penicillin and 100 g/mL streptomycin at 37°C in a 5% CO₂ atmosphere incubator. When the experiments were performed, the HGPS fibroblasts were at the following PDs: AG06297 – PD 37 and AG11498 – PD 9. BJ fetal foreskin fibroblasts were cultured in F10 medium containing 15% FCS, 200 µM glutamine, 100 U/ml penicillin and 100 g/ml streptomycin at 37°C in a 5% CO₂ atmosphere incubator. BJ fibroblasts were between 30 and 40 PDs when experiments were performed. HeLa cells and PG13 cells were cultured in the same conditions as the HGPS cells except 10% FCS was used.

To calculate population doublings between platings, the following formula was used:

$$ PD = \frac{\log N - \log N_0}{\log 2} $$

Where $N_0$ is the starting number of cells and $N$ is the final number of cells.
3.2.2. Viral Construct Construction

Retroviral constructs were derived from the MIG plasmid, which contains both a MSCV promoter and an Internal Ribosome Entry Site (IRES)-Green Fluorescent Protein (GFP) cassette downstream of the multiple cloning region (MCR). The LMNA and LMNA\(\Delta 150\) genes were cloned into the MCR after PCR amplification from cDNA generated from RNA extracted from the HGPS fibroblasts line, AG06297. RNA was extracted from AG06297 sample using the RNAeasy kit (Qiagen). A RT-PCR assay was performed with the isolated RNA to make cDNA. Primers were used that added restriction enzyme sites to the 3’ and 5’ end of the gene to facilitate cloning. Correct plasmids were selected based on restriction enzyme digest and sequenced to confirm there were no point mutations. One correct plasmid of each type was selected and used from then on.

A similar procedure was used to make the different fluorescent fusion proteins. The gene for a fluorescent protein (Venus, Orange or Cherry) was cloned upstream of the gene of interest. Possible clones were screened via restriction enzyme digestion and a correct plasmid selected for use.

3.2.3. Retroviral Production and Infection

The day previous to transfection, PG13 cells were plated in 4 mL of growth medium in a 6 cm tissue culture dish at a density of ~1.5-2 million cells and allowed to attach overnight. Cells were approximately 75% confluent and in plateau growth phase for optimum transfection efficiency.

Five minutes prior to transfection, chloroquine was added to the cells to a final concentration of 25 μM. The DNA was prepared by adding, to a 15 mL Falcon tube, 10 μg of the plasmid DNA and dH₂O to bring the final volume to 450 μL. 50 μL of 2.5 M CaCl₂ was
added and thoroughly mixed. To the DNA/CaCl\textsubscript{2}, 0.5 mL of 2X HBS (50mM HEPES, pH 7.05, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, 1.5 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.0) was added and bubbled vigorously for 10 seconds with an automatic pipettor, left to sit for 30 seconds and added dropwise to the cells. Plates were gently mixed to evenly distribute the DNA and placed in a 37°C incubator. The media was replaced with fresh media 8 and 24 hours post transfection. The plates were moved to a 32°C incubator 24 hours post transfection. At 48 hours post transfection, the virus containing media was removed and centrifuged at 1200 rpm for five minutes to pellet any debris. Fresh media was added to the cells and virus was harvested the same way at 72 hours post transfection. The virus containing media was filtered through a 0.45 µm filter and, if not to be used right away, frozen at -80°C.

To infect target cells, 1 µL of 10 M polybrene was added to the cells. 1.5 mL of viral supernatant was added to target cells and incubated at 37°C. After 24-48 hours, cells were washed with fresh media and allowed to grow for approximately two weeks. Using flow cytometry, viral expression was determined based on the fluorescent protein expressed (GFP, Venus, Orange or Cherry fluorescent protein) from the transfected plasmid.

3.2.4. RT-PCR and PCR

RNA was extracted from BJ + Lamin A and BJ + Lamin A ∆50 cells using the RNAeasy kit (Qiagen). RT-PCR was performed with the isolated RNA to make complementary DNA (cDNA). The cDNA was amplified with either primers for endogenous *LMNA* (forward: CAAGGCATCTGCCAGCGG and reverse: TTTCTTTGGCTTCAAGCC) or the transgene *LMNA/LMNA∆150* (forward: CAAGGCATCTGCCAGCGG and reverse: CGTACGGTAGTAACTG). PCR products were visualized on a 1.5% agarose gel by ethidium bromide staining.
3.2.5. Western Blot

Cells were harvested, centrifuged and washed in phosphate-buffered saline (PBS; 3.2 mM Na$_2$HPO$_4$, 0.5 mM KH$_2$PO$_4$, 1.3 mM KCl, and 135 mM NaCl) before resuspension in protein loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.003% bromophenol blue, 6 M urea, 5% β-mercaptoethanol) and mixed by tapping. To extract proteins, the cells were sonicated twice for 15 seconds while on ice. Protein extracts were stored at -20°C until used.

SDS-PAGE gels were prepared as standard; 8% acrylamide gels were used in all cases. Before loading onto the gel, the protein extracts were heat denatured at 95°C for three minutes. Gels were run at 150V until the dye marker was at the bottom of the gel, approximately one hour. The proteins were transferred to a nitrocellulose membrane at 100V for 1.5 hours, and then washed in PBS. The membrane was then blocked with PBS-Milk/Tween (PBS-MT; PBS with 5% milk powder and 1% Tween-10%) for one hour at room temperature. The primary antibody was incubated overnight at 4°C on a rocking platform. The membrane was then washing one for 10 minutes with PBS-MT and five times for 10 minutes with PBS-T, followed by incubation with a secondary antibody for 30 minutes at room temperature on a rocking platform. The membrane was washed once with PBS-MT for ten minutes then five times with PBS-T for 10 minutes and finally in PBS three times. The chemiluminescence reagent (SuperSignal West Pico Chemiluminescent Substrate, Pierce) was added and after five minutes the signal was detected.

3.2.6. Immunofluorescence

To perform immunofluorescence (IF), ~2 x 10$^4$ cells were grown on coverslips over night. The following day, the coverslips were washed with Tris-buffered saline (TBS) and
fixed with 2-4% formaldehyde (diluted in TBS) for 20 minutes at room temperature. Cells were rinsed twice for 5 minutes with TBS to remove any remaining fixation solution. Cells were permeabilized and blocked with 0.2% Triton X-100 and 1% bovine serum albumin (BSA) and incubated for 20 minutes before the primary antibody was added for between one hour and overnight, depending on the antibody. Following incubation, cells received two 5 minute washes with TBS before the secondary antibody was added for a 1 hour incubation at room temperature. The coverslips were again rinsed with two 5 minute TBS washes. Nuclei were counterstained with DAPI for 5 minutes then rinsed with TBS. Vectashield was added to the coverslips which were then mounted on slides, sealed and stored in the dark until used.

For the nuclear shape investigations using BJ + Lamin A and BJ + Lamin A ∆50, ten random images were taken using the DeltaVision microscope at a 40x objective. Cells were visually assessed as normal, lobular or containing micronuclei. For BJ + Lamin A, n=149 and for BJ + Lamin A ∆50, n=80.

For DNA damage studies, BJ fibroblasts and the HGPS cell line AG06297 were used. Random images were taken using the DeltaVision system at a 40x objective.

3.2.7. PNA-FISH

For FISH, coverslips were prepared as for immunofluorescence including the washing, fixing and permeabilization until the primary antibody step. Cells were treated with RNA:T1 at 37°C for 10 minutes then washed in twice for two minutes in TBS. Hybridization mixture (70% formamide, 0.25% NEN in maleic acid, 10 mM Tris-HCl, pH 7.4, 0.5 µg/mL PNA Tel Cy-3, 5% MgCl₂ Buffer) was added to coverslips which were covered with a slide and placed in an 80°C oven for 3 minutes to denature DNA and incubated for 2 hours at room temperature. Coverslips were removed from slides with wash solution I (70%
formamide, 10 mM Tris-HCl, pH 7.4, 0.1% BSA) and washed twice for 15 minutes with wash solution I. Coverslips were rinsed with wash solution II (0.1 M Tris, pH 7.4, 0.15 M NaCl, 0.08% Tween 20) three times for five minutes then counterstained with DAPI. Vectashield was added to the coverslips before being mounted on a slide, sealed and stored in the dark until used.

3.2.8. Live Cell Imaging

A modified model system was utilized to examine telomere mobility. Cells were infected with virus carrying a Venus-TRF1 (V-TRF1) fusion gene and either a Cherry (Ch)-Lamin A or a Ch-Lamin A Δ50 gene. As with the other model system described, cells were flow sorted based on the expression of fluorescent transgene. Using live cell imaging with the DeltaVision system, time-lapse movies were made of the sorted cells. All cells were imaged the same way to remove any bias. Image stacks of cells co-expressing V-TRF1 and Ch-Lamin A or Ch-Lamin A Δ50 were taken every 45 seconds for 450 seconds. Using the program Volocity, the telomeres could be tracked between each image to determine characteristics such as total distance moved, velocity and area covered.

3.2.9. Nuclear Halo Extraction

Isolation of nuclear matrices was based on a modification of a previously described procedure. Cells used in these experiments included: HeLa, HeLa-Lamin A, and HeLa-Lamin A Δ50. Plateau phase cells were trypsinized and 10⁸ cells were used for the experiments. Cells were washed in cold PBS, pH 7.4 then with 25 mL of cold cell wash buffer (CWB; 50 mM KCl, 0.5 mM EDTA, 0.05 mM spermidine, 0.05 mM spermine, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM Tris-HCl (pH 7.4) and 0.5% thiodiglycol) prior to
centrifugation at 1000 rpm for 5 minutes to pellet cells. The cell pellet was resuspended in CWB plus 0.1% digitonin (at 5 mL per 2 x 10^7 cells) and passed through a 20-gauge needle to lyse the cell membrane and release nuclei. Lysis was confirmed via microscopy. The suspension was loaded onto a 10% glycerol-CWB cushion at a ratio of 10 mL of suspension to 5 mL of cushion and centrifuged at 2400 rpm for 10 minutes at 4°C. The nuclei were washed with CWB with 0.1% digitonin and then suspended in 5 mL of modified CWB (with 0.1% digitonin and 0.5 mM CuSO_4 but without EDTA) and incubated at 37°C for 20 minutes. Nineteen volumes of LIS solution (10 mM Lithium diiodosalicylate, 100 mM lithium acetate, 0.1% digitonin, 0.05 mM spermine, 0.125 mM spermidine, 0.25 mM PMSF, 20 mM Heps-KOH (pH 7.4)) was added to nuclei and incubated for an additional 10 minutes at room temperature to generate nuclear halos. The nuclear halos were collected by centrifugation at 1000 rpm for 10 minutes at room temperature then washed twice with matrix wash buffer (MWB; 20 mM KCl, 70 mM NaCl, 10 mM MgCl_2, 10 mM Tris-HCl (pH 7.4)) with 0.1% digitonin and then twice with MWB. Finally, the halos were washed with the restriction enzyme buffer (NEB Buffer 2; New England BioLabs). 1 x 10^6 halos were digested with RsaI and HinfI (2U/µg of DNA) at 37°C overnight. The nuclear halos were pelleted via centrifugation at 3000 rpm for 10 minutes and both the supernatant and the pellet (resuspended in NEB Buffer 2) were saved.

To purify the DNA, both fractions (attached – pelleted matrices and soluble – supernatant) were incubated overnight with proteinase K (100 µg/mL) in a solution of 10 mM EDTA, 0.5% sodium dodecyl sulfate and 10mM Tris-HCl (pH 7.4) at 37°C. DNA was purified using phenol chloroform extraction and precipitated with isopropanol. DNA was resuspended and stored in 10 mM Tris-HCl (pH 7.4) at -20°C.
3.3. Results

3.3.1. Model System of HGPS in Human Fibroblasts

Due to the inherent nature of HGPS fibroblast cells to senesce prematurely in culture, and the rarity of the disease in the population and therefore patient samples, a model system was devised to eliminate the need to solely use patient derived fibroblast samples. Since the mutation found in the majority of HGPS patients is a dominant mutation, adding the gene that encodes the mutant protein should be sufficient to cause the same alternations found in patients. A caveat of this approach is that expression of the mutant allele should be at least similar to the two wild type (wt) alleles combined. BJ fibroblasts, a newborn foreskin line with a normal lifespan of at least 80 population doublings (PDs), were modified to simulate artificial HGPS cells. The wt Lamin A gene (LMNA) and the mutant Lamin A gene (LMNA∆150) were cloned into the viral vector ‘MIG’ under the control of the moderate strength promoter, MSCV. Using the PG13 viral system, virus containing the region of interest was generated and BJ fibroblasts infected.

Successful infection was determined by using the vector’s IRES-GFP sequence. After infection, cells were flow cytometry sorted based on GFP expression. Cells not expressing a transgene formed a distinct non-fluorescent population (black dots, Figure 9A) while cells expressing the transgene ranged from low to high expression likely based on the copy number and integration location (low expressing cells, green dots; high expressing cells, red dots, Figure 9A). To determine the actual expression level, cDNA was generated from sorted cells using RT-PCR. When PCR was performed, primers used spanned the deletion region of LMNA to determine levels of wt LMNA and LMNA∆150. Endogenous and exogenous LMNA were distinguishable since the PCR for the exogenously expressed gene used a primer in the IRES of the transgene. The expression level of the wt LMNA transgene
Figure 9: Expression of \( \text{LMNA}\Delta150 \)-IRES-GFP in BJ fibroblasts.

(A) FACS plot of BJ fibroblasts expressing \( \text{LMNA}\Delta150 \)-IRES-GFP. GFP and propidium iodine (PI) are measured in arbitrary units of fluorescence intensity. Black dots represent cells negative for GFP expression, green and red dots represent low and high GFP expressing cells respectively. (B) PCR of \( \text{LMNA} \) over the region that includes the cryptic splice site in HGPS to determine expression level of the transgene in comparison to the endogenously expressed wt gene. In the cells expressing wt \( \text{LMNA} \), the transgene is expressed at approximately the same level as the endogenous gene while in the \( \text{LMNA}\Delta150 \) cells, the transgene is expressed at a much higher level. (C) Western blot with a Lamin A antibody of BJ cells, BJ cells expressing \( \text{LMNA} \) or BJ cells expressing \( \text{LMNA}\Delta150 \)
was approximately the same as the endogenous *LMNA* mRNA, however the *LMNA*Δ150 transgene was expressed at a much higher level indicative of Lamin A over expression not being well tolerated at high levels (Figure 9B). The PCR product from the *LMNA*Δ150 transgene is 150 nt smaller than the *LMNA* which might account for some of the difference in the amount of product produced. However, a Western blot with an antibody specific to the head region of Lamin A (which is unaffected by the mutation) determined protein levels. Expression of the transgene proteins correlated with mRNA expression levels signifying that the mRNA is translated properly (Figure 9C).

The model system for HGPS was tested to determine if it resembled key characteristics found in patient fibroblast samples. When population doublings were measured over a 14 week period all three cell lines (BJ, BJ + Lamin A, and BJ + Lamin A Δ50) grew at the same rate until approximately 12 PDs post sorting (around day 50) when the growth rate of the cell lines expressing a transgene started to decrease (Figure 10A). The growth rate of the BJ + Lamin A Δ50 cells decreased and eventually stopped growing, possibly reflecting a ‘senescence-like’ state. The decrease in growth rate of the BJ + Lamin A fibroblasts was unexpected, however, the cells did not stop growing over the course of the experiment. The population doublings of cells expressing transgenes was only strictly followed once. However, it was observed with subsequent usages of the cells that cell growth of the BJ + Lamin A Δ50 slowed down compared to controls every time these cells were put into culture. When HeLa cells were infected with the virus containing either *LMNA* or *LMNA*Δ150, the cells also had a growth impediment compared to uninfected HeLa cells. Unlike with the BJ fibroblasts, the HeLa cells with LMNAΔ50 did not stop growing in the time-span of the experiment (data not shown).

From past studies, the easiest discernable cellular change observed in the HGPS cells is abnormalities of the nuclear membrane shape\(^1\). Immunofluorescence with a Lamin
Figure 10: BJ fibroblasts expressing mutant Lamin A resemble key aspects of patient HGPS fibroblasts.

(A) Population doublings of BJ fibroblasts (blue), BJ fibroblasts expressing exogenous wt Lamin A (red) and expressing exogenous Lamin A ∆50 (green). Time 0 indicates the date at which transgene expressing cells were sorted. Cells expressing mutant Lamin A decrease in growth rate around day 50 and stop doubling after about 75 days in this experiment. (B) Bar graph summarizing nuclear morphology of BJ fibroblasts expressing exogenous wt Lamin A (n=149) or Lamin A ∆50 (n=80). Multiple images were taken of fields of cells from both lines and cells were categorized by eye. Cells with lobulations or micronuclei increase in frequency with expression of mutant Lamin A. A representative example of what would have been classified as a normal cell (C) and a cell with lobulations (white asterisk) and micronuclei (white arrow) (D).
A antibody and DAPI confirmed that the nuclear membrane in the cells expressing exogenous Lamin A ∆50 had a higher incidence of cells with lobulations, invaginations, and micronuclei when compared to both BJ and BJ + Lamin A (Figure 10 B-D). The frequency of these changes increased with time in culture as observed in HGPS fibroblasts (data not shown). This coupled with the decrease in growth rate and eventual growth arrest of the BJ + Lamin A ∆ 50 cells compared to wt cells suggests that the model recapitulates the cellular abnormalities observed in fibroblasts from HGPS patients.

3.3.2. Telomere Organization in HGPS

In Chapter 2, it was found that telomeres in HPGS cells are much shorter than in normal fibroblasts.\textsuperscript{64,78} It is possible that this, as well as the chromatin defects in HGPS cells creates problems for telomere function. To determine if there were any differences in telomeres of HGPS cells and wt cells various aspects of telomere biology were examined.

Telomere location in the nucleus was investigated using FISH on interphase control and HGPS cells. One distinct difference between the control BJ fibroblasts and HGPS cells was the intensity of the telomere fluorescence (Figure 11). In both HGPS cell lines, the telomeres were less intensely stained in comparison to the BJ fibroblast telomeres. This is consistent with the short telomeres found in HGPS cells as discussed in Chapter 2. Furthermore, there appeared to be differences in chromatin appearance between the BJ and HGPS fibroblasts. The distinction between euchromatin (light blue DAPI staining) and heterochromatin (dark blue DAPI staining) in the BJ fibroblasts was distinct. In the HGPS cells, the borders are more blurred. In the BJ fibroblasts, the telomeres were located at the borders between the two chromatin types (Figure 11). However, in the HGPS cell lines AG11498 and AG06297, telomeres were randomly distributed in the euchromatin and heterochromatin and not always located at the border between them. The blurriness of the
Figure 11: Telomere fluorescence in HGPS cells is less intense and telomeres do not appear to be localized to the euchromatin-heterochromatin borders as in control cells

Fluorescent in situ hybridization (FISH) on BJ fibroblasts and AG11498 fibroblasts (HGPS) using a Cy3-PNA probe (red) to telomeric DNA. DNA is stained with DAPI (Blue). In the merged image, telomere fluorescence has been enhanced to allow telomeres to be visible.
chromatin types could account for some of the localization differences. Also, when three-dimensional images were observed using imaging software, it appeared that telomeres were more localized to the periphery in the HGPS fibroblasts. This was difficult to quantify with the imaging software available and is not readily observable in Figure 11, however, a peripheral localization of telomeres in HGPS fibroblasts was consistently observed. These experiments were repeated twice with consistent results. Also, the results for both HGPS cell lines tested were similar.

For the purpose of live cell imaging, a fluorescently tagged version of Lamin A and Lamin A Δ50 was created. The IRES-GFP was removed from the MIG plasmid since expressing cells could be sorted based on the fluorescence from the tagged protein. However all other steps for creation of the cells were the same as with the original model system. Both Orange (Og)-Lamin A and Og-Lamin A Δ50 were expressed at levels less than the endogenous Lamin A, however, Og-Lamin A Δ50 is expressed much more than Og-Lamin A (Figure 12A). Over-expression of Lamin A was not well tolerated as seen previously with the original model system.

The Orange protein did not seem to interfere with normal Lamin A function as the protein was properly located in the nucleus (Figure 12B) and disassembled and reassembled normally during cell division. In the case of Lamin A Δ50, the protein induced nuclear envelope/matrix abnormalities such as invaginations, lobulations, micronuclei and internal Lamin A structures. Via microscopy, it was observed that there was a very high amount of variability in the expression level of Og-Lamin A from barely visible to highly over-expressed. In cells highly over-expressing Og-Lamin A (based on fluorescence intensity when viewed through the microscope), aggregates could be seen to form in the nucleus suggesting that in cells where there was too much Lamin A, it could not properly
Figure 12: HeLa cells expressing either Og-Lamin A or Og-Lamin A ∆50

(A) Western blots of Lamin A ∆50 (LAΔ), and Lamin A (LA) using an antibody for Lamin A/C. (B) Images of HeLa cells expressing either Og-Lamin A or Og-Lamin A ∆50. The cells expressing wt Og-Lamin A had aggregates in some cells (white arrows). In the cells expressing the mutant Lamin A, the cells exhibit invaginations, folds in the nuclear membrane, and internal Lamin A structures (indicated by white asterisks). DNA is stained with DAPI (Blue).
incorporate into the nuclear lamina. Multiple cell lines were created with these constructs
and results were consistent each time.

To examine telomere mobility a fluorescently tagged version of TRF1, a telomere
specific binding protein, was used to visualize telomeres during live cell imaging. Venus (V)-
TRF1 was created for this purpose however it had to be highly over-expressed in these cells
for it to be visible via microscopy (Figure 13A). Nevertheless, this did not appear to have any
observable negative effects on cell growth or function. To examine telomere mobility in a
HGPS system, cells co-expressing V-TRF1 and Chy-Lamin A Δ50 were used (Figure 13B).
The Cherry fluorescent protein was selected over Orange fluorescent protein for these
experiments since the fluorescent spectrum of Cherry does not overlap at all with that of
Venus thereby reducing any imaging artifacts however the colour of the fluorescent protein
did not change the observed results in regards to the affect of expression on nuclear
morphology.

The program Volocity, which has the ability to track the movement of objects
between images, was used to track telomere movement over a time course. Velocity
calculates the most likely route the telomere took to get to the new position (Figure 13C).
With the assumption that the amount of TRF1 on a telomere correlates with the size of the
telomere and that the ratio of non-fluorescent:fluorescent TRF1 is approximately equal on all
telomeres in a given cell, the fluorescent value of the V-TRF1 spots can determine relative
telomere length. Based on the fluorescent value, telomeres were divided into three groups:
small, medium and large. Each cell was calculated individually and telomeres were split in
thirds to determine groups since the total fluorescence was variable between cells (n=10 for
each cell line). In HeLa cells expressing V-TRF1, all telomeres regardless of size moved
approximately the same distance over the time course of the experiment (Figure 13D). In
Figure 13: Mobility of fluorescently tagged telomeres in HeLa cells and HeLa cells expressing Cherry-Lamin A △50

(A) A Western blot of V-TRF1. (B) Merge of Venus-TRF1 and Cherry-Lamin A △50 in HeLa cells. (C) Velocity composite image of telomeres tracks through images taken every 45 seconds over 450 seconds. Telomere movement in (D) HeLa and (E) HeLa expressing Lamin A △50. Low, medium and high refer to the intensity of the fluorescence representing the telomere.
HeLa cells co-expressing V-TRF1 and Chy-Lamin A Δ50, small and medium telomeres moves the same distance while the longer telomeres moved a shorter distance and with less velocity (Figure 13E). The longer telomeres also moved in more confined area compared to small or medium telomeres. This suggests that they are somehow constricted in their ability to move. This experiment was repeated once with results being consistent between and within the experiments.

3.3.3. Telomere Attachment to the Nuclear Matrix

The function of the nuclear matrix is altered in HGPS fibroblasts. To better understand how this affects DNA in general and telomeres specifically, nuclear matrix attachment was examined. Using a modified nuclear halo extraction protocol, the percentage of telomeres attached to the matrix in normal cells was determined. As expected, there was much more DNA in the soluble fraction than in the attached (to the matrix) fraction (Figure 14A). However, the telomere distribution between the fractions did not mirror the total DNA. Telomeres were enriched in the attached fraction compared to total DNA (42% telomeric DNA vs. 10% total DNA) (Figure 14B and C). The telomeres in the attached fraction also had a slight increase in the mean telomere length over those in the soluble fraction (Figure 14B).

The same HeLa cell lines that were used in the live cell imaging studies were used to examine how Lamin A expression changes DNA-matrix attachment. The nuclear halo extractions were performed on HeLa, HeLa + Og-Lamin A and HeLa + Og-Lamin A Δ50. With the cells expressing HeLa + Og-Lamin A, there was a large increase in the amount of DNA in the attached fraction in comparison to HeLa cells, consistent with Lamin A binding DNA (Figure 14D). This also confirms that the exogenously expressed Lamin A integrates
Figure 14: Mean telomere length is increased in attached fraction and mutant Lamin A does not bind DNA as well as wild type Lamin A.

(A) Ethidium bromide staining of soluble (S) and attached (T) fractions from nuclear halo extraction of HeLa cells. (B) Hybridization of a TTAGGG repeat probe to a Southern transfer of the gel in pictured in A. (C) Bar graph representing percentage of DNA via intensity of ethidium bromide staining from A and B. (D) Ethidium bromide staining of T and S fractions from HeLa, HeLa expressing transgene lamin A del50 (HeLaΔ) and HeLa expressing transgene lamin A (HeLa-LA).
into the nuclear matrix and is able to function normally. However, the cells expressing Og-Lamin A ∆50 did not show an increase in the amount of DNA bound to the matrix (Figure 14D). This implies a defect of the Lamin A ∆50 in binding DNA which deserves to be studied further.

These nuclear matrix attachment studies were performed once therefore further experiments are needed to confirm the validity of these results however the results are interesting and deserve further inquiry.

3.3.4. DNA Damage in HGPS Cells

HGPS fibroblasts have a defect in DNA damage repair. Immunofluorescence staining of phosphorylated H2AX (γH2AX) and 53BP1, two important proteins in the early DNA damage response, were compared in BJ fibroblasts and HGPS cells. In non-irradiated BJ fibroblasts it was common to find one to two large 53BP1 foci (Figure 15). In the HGPS cells (AG06297) these large foci were not observed, however there were many small foci per cell suggesting many sites of DNA damage (Figure 15). This experiment was repeated once and a minimum of 40 cells were examined for both HGPS and BF fibroblast. Results were consistent between cells for each cell line.

3.4. Discussion

The intrinsic growth defect of HGPS cells make patient cells difficult to study. To avoid this problem, a model system was created that recapitulated key aspects found in HGPS fibroblasts including premature senescence in culture, persistent DNA damage and morphological changes in nuclear shape (Figures 9 and 10). This model system also allowed the addition of genes encoding fluorescently-tagged proteins for live cell imaging.
Figure 15: Immunofluorescence with 53BP1 on fibroblasts from control and HGPS cells.
Control fibroblasts have 1-2 large 53BP1 foci while HGPS fibroblasts had many small foci. The small 53BP1 foci in the AG06297 HGPS fibroblast line have been enhanced.
allowing a unique look at the dynamics of individual proteins as well as the interaction of different proteins with each other.

An unexpected discovery was that when the wt Lamin A protein was over-expressed, it caused defects in cellular replication (Figure 10A). Lamin A interacts with a wide variety of proteins, as well as chromatin and DNA directly. Any number of these interactions could be altered when it is over-expressed. It is also possible that, similar to what has been shown for the mutant Lamin A, too much wt Lamin A leads to a stiffening of the nuclear membrane. However, this did not lead to obvious defects in the morphology of the cells over expressing Lamin A (Figure 10B).

One of the shortcomings of the model system is the over-expression of transgenes. The cells still have the endogenously expressed Lamin A and on top of that are expressing extra wt Lamin A or Lamin A Δ50. However, since the mutant Lamin A works in a dominant negative fashion, effects of the mutant protein can in principle still be observed. Ideally, to get a better model, a partial knock-down of wt Lamin A combined with the addition of the mutant Lamin A could be used. Another limitation of this model system is that it has the premature senescence defect which limits the growth potential of individual batches of cells as seen in the HGPS fibroblasts. To alleviate this problem, an inducible promoter could be used to turn on the expression of the mutant gene once appropriate cell numbers had been reached.

A goal of these studies was to determine if telomere organization and function was altered in HGPS. Cells from HGPS patient samples had a different distribution pattern of telomeres than in wild-type cells. Telomeres in HGPS cells were not localized to the euchromatin/heterochromatin boundaries as in control cells (Figure 11). The altered chromatin state of the genome in HGPS cells, as evident by the differences in DAPI staining between control and HGPS cells, could bring about this aberrant localization.
It has been previously shown that telomeres in wt fibroblasts tend to have a more internal localization. However, in interphase HGPS fibroblasts, telomeres were found to have an increased localization to the nuclear periphery. There is an increase in Lamin A at the nuclear periphery due to the farnesyl tail that does not get cleaved off in the mutant Lamin A (Figure 2). It is possible that the increase in Lamin A at the nuclear membrane increases the percentage of telomeres that are found there. It was extremely difficult to quantify telomere localization due to the weak fluorescence of telomeres (short in HGPS) and limitations of the imaging software used on flat fibroblast cells. Efforts to solve this problem are continuing.

Another aspect of telomere dynamics, mobility, was studied using live cell imaging. The major difference found between control cells and those expressing mutant Lamin A was in the movement of larger telomeres. Large telomeres in the HGPS model system moved less than their counterparts in control cells and had a more confined area of movement (Figure 13 D and E). Taken together this suggests that the movement of larger telomeres is somehow constricted. Whether this is directly due to the mutant Lamin A or a secondary effect of the general state of the chromatin and nucleus is unknown. While it is known that Lamin A ∆50 itself is not as mobile this suggests that other components of the nucleus may also not be able to move around as freely in HGPS cells.

The ability of the nuclear matrix to bind DNA was tested using nuclear matrix extractions. It was found that the HeLa cells expressing mutant Lamin A had no increase in the amount of DNA in the attached fraction while there was a significant increase in the amount of attached DNA in HeLa cells over expressing wt Lamin A. This indicates that mutant Lamin A does not bind DNA as well as the wt Lamin A. In HGPS cells, where there would only be half the amount of normal Lamin A, this lack of DNA binding capacity could contribute to the pathologies observed.
When 53BP1 was examined in unirradiated control and HGPS fibroblasts the difference was striking. The HGPS cells did not possess the large foci found in wt cells but instead had many small foci. Persistent DNA damage has been observed in HGPS cells before\textsuperscript{24,25}. One possibility is they are (or some of them are) reflective of telomeres that are so short they are being recognized as DNA damage. From the telomere length measurements performed on the HGPS fibroblasts, it is known that there are telomere-free ends as well as extremely short telomeres. In a study by Yamauchi \textit{et al.} 2008, they noted that persistent foci in normal cells are larger than the initial foci formed upon DNA damage. However they did find that in cells lines from AT and NBS patients, persistent foci were small and attributed it to a defect in DNA damage repair\textsuperscript{79}. Previous studies have shown that certain aspects of DNA damage repair are deficient in HGPS cells\textsuperscript{24,25}. It is possible that related defects to those found in AT and NBS are causing the same persistent small foci in HGPS cells whether or not the initial DNA damage is caused by short telomeres or other sources.
Chapter 4 – Discussion

4.1. Overall Analysis

Human aging is a complex process with many different contributing factors, including genetics and environment, which is incompletely understood. The premature aging syndromes such as HGPS and WS may provide insight into the pathology of specific aspects of the aging process. While it is not a perfect representation of aging, HGPS has many tissue specific symptoms that are frequently observed over the course of normal human aging. The results obtained from the experiments described in this thesis have revealed that key aspects of telomere biology in HGPS fibroblasts are perturbed; telomeres are short in cells expressing mutant Lamin A and telomere dynamics are abnormal in these cells.

A striking aspect of the short telomere length in HGPS fibroblasts was that the length of individual chromosome ends did not always follow the limited number of patterns established for control samples (Figure 6). This possibly reflects changes in heterochromatin status in HGPS cells as well as the changes in nuclear position of chromosomes. It is known that HGPS cells have an altered chromatin state and it is possible that this could lead to changes in the specific chromatin pattern at telomeres. This is supported by a recent study that performed ChIP on telomeric DNA from HGPS cells showing that the histone pattern at telomeres is changed. This alteration in chromatin state could affect access of the telomeres to proteins such as DNA repair enzymes.

When hematopoietic cells, which have reduced or no expression of LMNA were examined (Figure 7), three out of the four samples had telomere lengths within the normal expected range compared to age-matched controls (Figure 8). The lack of obvious telomere shortening in cells that do not normally express Lamin A argues that the effect on telomere
length in cells is direct rather than some systemic consequence of the disease. The lack of expression of \textit{LMNA} in hematopoietic cells likely accounts for the normal immune system function that is observed in HGPS patients\textsuperscript{15}.

Three mechanisms were proposed earlier for the decreased telomere length in HGPS cells\textsuperscript{76,78}. They were:

1. A larger amount of telomeric DNA lost with each cell division.
2. An increase in apoptosis and/or senescence in HGPS cells leading to an increase in division in remaining cells\textsuperscript{76}.
3. A failure to repair DNA damage at telomeres, causing losses of variable amounts of DNA.

These three mechanisms are not mutually exclusive. Failure to repair DNA would not only lead to variable telomere length but also to persistent damage that could cause apoptosis/senescence.

Telomeres may have a critical role in the persistent DNA damage and early senescence in HGPS fibroblast cells observed in culture\textsuperscript{81}. It has been shown that there is a deficiency in the DNA damage response in HGPS cells\textsuperscript{24,25}. We have also observed that there are many very small, persistent 53BP1 foci in HGPS cells. These persistent foci could be short telomeres that are being recognized as sites of DNA damage which cannot be repaired since there is no telomerase expressed in fibroblasts. Such DNA damage, if left unrepaired, could eventually cause the cells to senesce or undergo apoptosis and causing remaining cells to divide more in order to make up the deficit. Cellular senescence in tissues leads to changes in gene expression in the affected cells and has been demonstrated to alter the microenvironment around the cell\textsuperscript{82}. Short telomeres have also previously been correlated with an increased risk of heart disease\textsuperscript{83}. Senescence due to telomere length could contribute to, or exacerbate, the pathological changes in arteries that lead to heart attacks, strokes and eventually death in the patients.
In a study by Huang et al, 2008, it was demonstrated that over-expression of either Lamin A ∆50 or wt Lamin A caused a decrease in telomere length\(^8\). This suggests a very active role for Lamin A in the maintenance of telomere length. As this was an over-expression study, it is difficult to say if this observation is just merely due to cellular changes from too much Lamin A in general rather than a specific effect. Also, the over-expression studies described in this thesis show that a growth defect, similar, but to a lesser extent, to expression of the mutant Lamin A protein. It is possible that the decrease in telomere length seen in the Lamin A over-expressed cells is due to a higher cell turnover. Research by Han et al, 2008, showed a role for the tumour-suppressor ING1 in HGPS\(^8\). With its role in both apoptosis and chromatin structure it is possible that this also has a secondary effect leading to the short telomere phenotype observed. Lamin A might have many effects on cell turnover as well as roles in adult stem cells through chromatin remodeling\(^8\), nuclear organization\(^8\) and other factors.

The genome is divided into discrete domains of high or low Lamin B binding\(^7\). The lamina-associated domains have specific characteristics including low gene density, low gene expression and enrichment of repressive chromatin marks. It has been demonstrated that in HGPS cells, Lamin B localization is perturbed. As the lamina is important as a scaffold for multi-protein and chromatin containing complexes\(^6\), mislocalization of Lamin B due to the mutation found in HGPS could lead to disruption of normal function of these complexes. Since telomeres attach to the nuclear matrix, the nuclear architecture dysfunction caused by the Lamin A mutation may lead to dysfunction in the structure and function of telomeres\(^8\). These complexes could have a role in the maintenance, repair and replication of telomeric DNA which has been established to be disrupted in HGPS which could lead to problems including an increase in replication errors, failure to repair, and accessibility of enzymes at telomeres in particular and the genome in general. This could
cause the generation of short telomeres in HGPS fibroblasts and symptoms of the disease in the patients.

Multiple aspects of telomere biology are perturbed in HGPS cells. Since the expression of telomerase can prolong the life-span of HGPS cells in culture and suppress the proliferation defect, this implies an important role for telomeres in the pathology of HGPS\textsuperscript{89}. The localization of telomeres to the nuclear membrane could affect the access of proteins including DNA damage repair complexes or histone modifying enzymes. The impaired mobility of telomeres suggests that the binding of telomeres to the scaffold of the nucleus is altered in HGPS. Taken together, the studies described in this thesis imply that telomere biology is altered due to the Lamin A $\Delta$50 mutation and that this likely goes on to influence the pathology of the disease.

4.2. Applications

This research, especially in regards to the telomere length data, could be important in the evaluation of any therapy for HGPS including the current trial on farnesyl transferase inhibitors (FTIs). Many of the other premature aging syndromes as well as other diseases that have a short telomere phenotype have a marked increase in cancer incidence\textsuperscript{90}. This is not observed in HGPS, possibly due to the very early death of patients with the disease. However, if clinical trials for FTIs are successful, or if other therapies are discovered in the future and the lifespan of patients is extended, then the extreme loss of telomere length could eventually lead to an increased likelihood of cancer occurrence. It is currently unknown whether the FTIs halt the loss of telomere length that was observed in fibroblasts. Even if it does, in children that receive the therapy later in their lives, it is possible they might still be at risk.
4.3. Future Directions

As a part of the work for this thesis, it was confirmed that telomeres in HGPS, while of variable length, are overall short in comparison to control cells. As part of the future work for this project, a better elucidation of the mechanism of telomere shortening should be undertaken. Three possible mechanisms have been proposed in the discussion for how this could be occurring. To determine the rate of loss per cell division, HGPS cells and control cells could be compared at each cell passage by STELA (Single Telomere Length Analysis). STELA is a PCR based technique to amplify the telomeres from specific chromosomes. This technique would allow a more accurate measure of the loss of telomeric DNA between passages than terminal restriction fragment length analysis which was used in Allsopp et al., 1992. STELA would also determine if there is an increased number of outliers, which represent very short telomeres, in HGPS cells compared to controls. Outliers have a statistically significant difference in telomere length compared to the median length of all telomeres. This would determine if large amounts of telomeric DNA are lost from HGPS cells more commonly than from controls. These could also be examined in the inducible HGPS model system discussed in Chapter 3 where the starting point of the cells would be the same if a clonal population was used.

Due to the rarity of HGPS, patient samples are limited which has led to a lot of the research being done on over-expression models (like those described in this thesis), and the mouse models that have been developed. Recently, induced pluripotent stem cells (iPSCs) have been derived from patient samples of human diseases including HGPS. These cells can then be differentiated into the cell types that are specifically afflicted in the particular disease of interest, with the hope that early events in the pathology of the disease could be observed. The iPSCs derived from HGPS fibroblasts are at a developmental stage prior to LMNA expression and lose the disease phenotype including nuclear envelope
shape and epigenetic alterations\textsuperscript{92}. However, when differentiated, \textit{LMNA} is re-expressed and the expected disease phenotypes return\textsuperscript{92}. One advantage to using iPSCs would be the ability to study cell types that would be normally hard to obtain from biopsy samples (such as vascular or muscular stem cells)\textsuperscript{93}. Also, unlike the over-expression models these are derived from actual patient samples and have the correct ratios of wt Lamin A to mutant Lamin A and more cells can be grown than when using fibroblast cell lines. HGPS fibroblasts do not reprogram into iPSCs as readily as normal fibroblasts however the ones that did were karyotypically normal\textsuperscript{93}. A concern with using iPSCs is that they may harbor some of the epigenetic profile of the adult cells they were derived from\textsuperscript{94}. However, these cells would be an excellent way to determine early steps in the pathological process of HGPS and would be of great use to further studies into the role of telomeres in HGPS.

Experiments on the DNA damage response and the role of telomeric DNA in persistent DNA damage observed in HGPS cells should be continued. It is currently unknown what the source of the persistent damage is. Telomere-FISH could be combined with γ-H2AX or 53BP1 IF to determine if this all or some of this damage is occurring at telomeres. These experiments, as well as the experiments on telomere length outlined above should be combined with the use of FTIs to elucidate their effect of telomere length. As mentioned before, this could have important clinical implications.
References


