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TELOMERE LENGTHENING TO ANTAGONIZE MYOCARDIAL AGING

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Biology

by

Christopher Travis Cottage

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2012
The Dissertation of Christopher Travis Cottage is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego
San Diego State University
2012
DEDICATION

This dissertation is dedicated to my friends and family, I could not have done this without you.
EPIGRAPH

“I try to think but nothing happens.”
Curly
The Three Stooges
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Chapter 1, in full has been accepted for the publication of the material as it appears in Stem Cells, 2012. Cottage CT, Neidig L, Sandararaman B, Din S, Joyo A, Bailey B, Gude N, Hariharan N, Sussman MA. The dissertation author was the primary investigator and author of this paper.

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Pim-1 Stimulates CPC Proliferation, San Diego State University Student Research Symposium – 2008

PUBLICATIONS


*All authors contributed equally.

is an early response to myocardial damage involving nucleolar proteins nucleostemin and nucleophosmin. PNAS, 2011 Apr 12;108(15):6145-50.


aspects of myocardial hypertrophy and compensation to pathological pressure overload. PNAS, September 16, 2008; 105(37): 13889 – 13894


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POSTER PRESENTATIONS


ABSTRACT OF THE DISSERTATION

Telomere Lengthening to Antagonize Myocardial Aging

by

Christopher Travis Cottage

Doctor of Philosophy in Biology

University of California, San Diego, 2012
San Diego State University, 2012

Professor Mark A. Sussman, Chair

Aging is associated with onset and progression of cardiovascular disease resulting from changes at both cellular and molecular levels. Accumulation of senescent cells in the myocardium has been implicated in deterioration of hemodynamic performance and impaired reparative processes. Cellular senescence can be linked to oxidative stress and repeated cellular divisions, both of which lead to telomeric shortening. Telomeres are the distal ends of chromosomes that consist of nucleotide repeats that function to protect important DNA from damage. Cells with critically short telomeres become senescent and are no longer able to divide, making them more likely to undergo apoptosis.
Telomeres are protected from shortening by multiple factors that protect the structure as well as regulate access to the telomere by telomerase (TERT), the enzyme responsible for telomere elongation. Maintenance of telomeres depends upon coordination of multi-molecular complexes that can be thought of as regulators of the “cellular clock” defined by telomeric length and tied to the aging phenotype. Myocyte replacement occurs throughout the mammalian lifespan and is thought to be dependent in part upon a cardiac progenitor cell (CPC) pool, the onset of senescence in the CPC population has been postulated to play a role in progressive structural and functional deterioration of the aged myocardium. Our lab has recently identified a serine/threonine kinase named Pim-1 to be protective in the heart downstream of the nodal kinase Akt. While investigating the functions of this newly found cardiac kinase we found that cardiomyocytes (CMs) as well as CPCs overexpressing Pim-1 had the ability to repeatedly divide in vitro and in vivo. The increased mitotic activity did not result in genetic instability, the cells maintained the ability to cycle several passages and still differentiate into the appropriate cardiac cell types. The goal of this study was to demonstrate that preservation of telomeres and a more youthful phenotype in CPCs is mediated by the cardioprotective kinase Pim-1. Herein we demonstrate that telomeres are rapidly lengthened with Pim-1 overexpression in CPCs and CMs, which coincides with increased proliferation in CPCs. Mechanistically, we find that Pim-1 dependent telomere lengthening results from interactions with TERT and is also dependent upon c-Myc activity. In vivo telomere length is preserved by Pim-1 activity in response to cardiomyopathic
stimuli and is coincident with enhanced TERT activity. Mechanisms maintaining or lengthening telomeres can serve as a “fountain of youth,” allowing cells to sustain a young phenotype and fight against the diseases commonly associated with cardiovascular dysfunction.
INTRODUCTION OF THE DISSERTATION
AGING AND CARDIOVASCULAR DISEASE

The aging process often coincides with a decrease in cellular function, which contributes to the increased likelihood of disease\(^3\). Cardiovascular disease (CVD) is the leading cause of death among the elderly, which is why understanding the molecular basis for the pathologies associated with aging is necessary\(^4\). One mechanism that contributes to the diseased pathology is a consequence of somatic cell senescence. Senescent cells are less viable and accumulation of these impaired cells leads to cardiac remodeling and cardiovascular diseases like hypertension and myocardial infarction (MI)\(^5\). Over the course of a lifetime cells divide to maintain homeostasis and allow for the removal of the older less functioning cells. As we age, the ability of the cell to proliferate and maintain the integrity of its DNA decreases. This inability to properly pass on genetic information to daughter progeny is a common contributor to the diseases associated with aging. CVD is the leading cause of death in the western world and accounts for more than $297.7 billion in related health care costs\(^4\). By comparison the predicted costs of all cancers combined is $228 billion, demonstrating the severity of CVD in terms of both mortality and economics\(^4\). The leading cause of death from CVD is a MI, MIs are coronary artery occlusions resulting in loss of blood flow to the distal regions of the clot. At the cellular basis, MI causes cardiomyocyte death and scar formation, diminishing cardiac contractility and results in dilation of the myocardium and heart failure. Treatments for MI are currently only capable of supporting the
heart with its reduced function, necessitating new strategies to protect or regenerate the damage.

**CARDIAC PROGENITOR CELLS**

Recent evidence has demonstrated that the heart is a regenerative organ unveiling new hope for antagonizing aging of the myocardium through enhanced repair\(^5\). Cardiac progenitor cells (CPCs) have been identified and characterized, reversing a long held precedent that the heart is a terminally differentiated organ\(^6\). CPCs are adult multi-potent stem cell that reside in niches throughout the heart and are responsible for maintaining cardiac cell homeostasis and minimal regeneration following injury\(^7\). CPCs, like all stem cells, self renew and are capable of differentiating into all forms of the cardiac lineage, myocyte, endothelial, and vascular cells\(^8\). Phenotypically, CPCs express c-kit a transmembrane receptor tyrosine kinase in addition to cardiac specific transcription factors GATA4, Mef2C, and Nkx 2.5\(^9\). Overtime CPCs age and become senescent, when this occurs they are less functional\(^10\). Diabetic cardiomyopathy, doxorubicin associated cardiomyopathy, and age associated CVD are thought to be a direct consequence of CPC dysfunction\(^11\)-\(^14\). CVD interventions utilizing CPCs are currently in clinical trials with promising results\(^15,\)\(^16\), however, autologous CPC treatment will involve CPCs from aged and debilitated individuals. Thus to fully combat the devastating effects of CVD, CPCs will need to be engineered to reverse the aging phenotype and enhance myocardial regeneration.
Herein we identify Pim-1, a cardioprotective kinase as a molecular intervention capable of delaying the aging phenotype in vivo and acutely reversing aging aspects while stimulating proliferation in vitro.

\textit{Pim-1}

Pim-1 is a serine-threonine kinase belonging to the Calmodulin-dependent protein kinase family together with two other highly conserved family members (Pim-2 and Pim-3). Pim-1 was discovered in mice as a preferential site of integration for the Moloney murine leukemia virus and named Pim-1 (Proviral Integration for Moloney Virus) over 25 years ago. Pim-1 plays pivotal roles in cellular proliferation, differentiation, metabolism, and survival by phosphorylating and interacting with multiple targets. A literature search reveals how dynamic the expression and activity of Pim-1 is depending upon cell type and response to stimuli, either pathologic or homeostatic. Specifically, Pim-1 is expressed in various hematopoietic sites including thymus, spleen, bone marrow, and fetal liver, but can also be found in the heart, oral epithelia, prostate, hippocampus, vascular smooth muscle, and many tumorigenic cell types (reviewed in). Pim-1 overexpression coincides with increased cell cycling during fetal development and in the hematopoietic system. Upon maturation, Pim-1 is down regulated in most organs until induced by pathologic stimuli promoting survival-signaling. Presence of Pim-1 in neoplastic cell types can mean a poor prognosis depending on the type and place of the malignancy. For example, the presence of Pim-1 with c-Myc; a synergistic partner, support a positive prognosis in prostate
adenocarcinoma, yet the opposite is true in the case of mantle cell lymphomas (reviewed in\textsuperscript{18}). Ironically, Pim-1 promotes survival and regeneration in tissues considered to be resistant to neoplastic transformation such as the heart. Interestingly, the same molecular mechanisms that produce relatively bleak outcomes in cancer patients also produce possible therapeutic interventions in patients with cardiomyopathy.

Pim-1 was first discovered in mice and subsequently cloned into human cell lines, genomic mapping identified Pim-1 on chromosome 17 in mice and chromosome 6 in humans. Six exons make up the 313 amino acid protein, which has two isoforms stemming from alternative start sites, the larger 44kD and smaller 34kD isoforms contain a kinase domain, a proton acceptor site, a glycine loop motif, and a phosphate-binding site\textsuperscript{19}. Both isoforms of Pim-1 are short-lived (~5 minutes to 6 hours depending on cell type) and constitutively active. Paradoxically, Pim-1 is known to phosphorylate itself despite the fact that it does not contain the consensus sequence needed for phosphorylation. Auto-phosphorylation of Pim-1 results in constitutive activation\textsuperscript{20}. Selective peptide mapping identified the Pim-1 consensus sequence as either (K/R)$_3$-X-S/T-X or R/K-R/K-R/R/K-X-S/T-X where X is an amino acid with a small side chain but neither basic nor acidic\textsuperscript{21}. Pim-1 has a variety of targets throughout the cell, explaining the ubiquitous expression of Pim-1. It is thought that subcellular localization defines targets, in this way nuclear Pim-1 stimulates proliferation and mitochondrial/cytoplasmic localization promotes survival.
**CONTROLLING PIM-1**

The constitutive activity of Pim-1 allows for function independent of post-translational modification, such that regulation of Pim-1 expression must occur at the transcriptional and translational level. *Pim-1* gene expression can be induced by a large array of cytokines: most interleukins, granulocyte macrophage-colony stimulating factor (GM-CSF), epidermal growth factor (EGF), leukemia inhibitory factor (LIF), and interferon-alpha. Cytokine stimulation results in activation of multiple pathways such as the Janus kinase-signal transducer and activator of transcription (Jak-STAT) and nuclear factor κB (NFκB) growth factor signaling pathways. Interleukin 5 and 7 (IL-5 and IL-7) activate STAT5, whereas GM-CSF and EGF activate STAT3. STAT3/STAT5 are both able to bind to the *Pim-1* promoter and induce transcription\(^\text{19}\). Platelet derived growth factor (PDGF) also increases *Pim-1* transcription in vascular smooth muscle cells resulting in enhanced proliferation which can be blunted with Jak-STAT pathway inhibitors\(^\text{22}\).

The hormone Prolactin induces *Pim-1* transcription independent of STAT transcription factors, instead through PI3K/AKT signaling pathways establishing Akt as an upstream regulator of Pim-1 activity\(^\text{23}\). In addition, pathological stimuli such as DNA damaging agents like peroxide or 5-fluorouracil are known to stimulate *Pim-1* through kruppel-like factor 5 binding to the *Pim-1* promoter activating transcription and subsequently preventing apoptosis\(^\text{24}\). Other DNA damaging treatments such as hypoxia elevate levels of Pim-1 mRNA and protein by inhibiting its ubiquitin-mediated proteasomal degradation and causes Pim-1 translocation from the cytoplasm to the nucleus\(^\text{25}\).
*Pim*-1 mRNA has a short half-life due to the presence of five copies of the AUUU(A) destabilization motif in the 3’ untranslated region (UTR)\(^ {17}\). The 5’ UTR of Pim-1 contains multiple secondary structures due to repetitive GC rich regions, thereby complicating translation. In order for *Pim*-1 translation to be more efficient the transcript relies on cap dependent translation that requires a 7-methylguanosine cap at the 5’ end of the message to promote ribosomal assembly supported by abundant increase in Pim-1 protein upon removal of the 5’ UTR or overexpression of translation initiation factor E4 (eIF4E)\(^ {26}\).

In addition to Pim-1 transcriptional control, Pim-1 is also regulated through protein stabilization, specifically interactions with several molecules and complexes that inhibit ubiquitination and dephosphorylation. Several facets of Pim-1 activity rely on synergistic partners that enhance and sustain kinase activity by preventing degradation. One such partner is heat shock binding partner 90 (Hsp90) upon association of Pim-1, Hsp90 prevents ubiquitin-mediated degradation, whereas inhibition or silencing of Hsp90 promotes rapid Pim-1 proteolysis\(^ {27}\). Conversely, Hsp70 binds ubiquitinated Pim-1 and directs Pim-1 to the proteasome for degradation\(^ {27}\). Dephosphorylation of Pim-1 ceases activity and promotes degradation. Protein phosphatase 2A (PP2A) acts upon both Pim-1 and Pim-3, once dephosphorylated, Pim-1 is quickly ubiquitinated and shuttled to the proteasome\(^ {28, 29}\).
Studies utilizing transgenic mice to overexpress Pim-1 and knockdown techniques to eliminate expression concluded that Pim-1 contributes to cell cycle progression in several cell types. Subsequent studies revealed that this is achieved by modulating multiple targets throughout the progression of the cell cycle. During G1-S progression Pim-1 binds and phosphorylates CDC25A increasing phosphatase activity which in turn activates CDK2 and CDK4. In addition, Pim-1 increases CDK2 activity by phosphorylating cell cycle inhibitors p21/Cip1 and p27/Kip1, targeting them for nuclear export and degradation. In a similar fashion Pim-1 enhances progression through G2/M by phosphorylating the N-terminus of CDC25C enhancing phosphatase activity. To further stimulate transition into M phase Pim-1 phosphorylates C-TAK-1 an inhibitor of CDC25C at multiple sites reducing its kinase activity allowing CDC25C to promote G2/M transition (Figure 1.1).

Pim-1 is expressed ubiquitously throughout the cell, but during mitosis Pim-1 appears to be enriched in the nucleus facilitating cell division. Pim-1 stabilizes the spindle poles by interacting with Nuclear Mitotic Apparatus protein (NuMA) during mitosis, most likely phosphorylating NuMA as cells with kinase dead forms of Pim-1 do not co-localize with NuMA and have a higher frequency of apoptosis. It is thought that Pim-1 phosphorylates NuMA to promote complex formation at the microtubule (-) end and provide a docking site for dynein and dynactin to promote proper chromosome segregation. In addition to
NuMA, Pim-1 also phosphorylates heterochromatin protein-1 (HP-1) to further contribute to spindle fiber assembly during mitosis.

The transcription factor c-Myc is a well-characterized partner for Pim-1, known to promote cellular proliferation and differentiation when the two are co-expressed. Pim-1 can bind, phosphorylate, and stabilize c-Myc, facilitating cell cycle progression by promoting c-Myc dependent transcription of target genes\textsuperscript{34}. To accomplish this, c-Myc together with its heterodimer partner Max, bind to E-box promoter elements to regulate approximately 11-15% of all human genes. Interestingly, it is speculated that Pim-1 may contribute to the regulation of around 20% of the c-Myc regulated genes\textsuperscript{35}. Several c-Myc regulated molecules are necessary for cell cycle progression; including CDC25A, CDK2, and CDK4. During times of Pim-1 induced cell cycle amplifications, c-Myc is also found to be highly expressed\textsuperscript{36}. It is thought that Pim-1 regulates c-Myc dependent transcription by phosphorylating Histone 3 on Serine 10. This phosphorylation is required for c-Myc to bind to DNA and promote transcription\textsuperscript{35}.

\textit{Pim-1 and Cell Survival}

Mitochondrial membrane integrity regulates the release of pro-apoptotic cytochrome c and subsequent caspase cleavage eventually ending in DNA fragmentation and cell death (Figure 1.2)\textsuperscript{37}. Pim-1 acts as a survival kinase at the mitochondria via pro and anti-apoptotic Bcl-2 family members\textsuperscript{1}. During normal homeostatic conditions Bcl-2 family members Bcl-2 and Bcl-X\textsubscript{L} reside in the outer membrane regulating mitochondrial outer membrane
permeabilization\textsuperscript{38}. Upon apoptotic stimuli, pro-apoptotic family members Bax and Bad associate with Bcl-2 and Bcl-X\textsubscript{L}, permeabilizing the outer membrane resulting in cytochrome c release. Pro-survival kinase Pim-1 phosphorylates BAD on Serine 112 resulting in translocation from the mitochondria to the cytoplasm and binding to 14-3-3 scaffold proteins\textsuperscript{24}. Overexpression of Pim-1 increases levels of both Bcl-2 and Bcl-X\textsubscript{L} in mitochondrial fractions protecting the cells from hydrogen peroxide stress \textsuperscript{39}. In addition to forming heterodimers with Bcl-X\textsubscript{L}, Bcl-2 interacts with pro-apoptotic family members Bax and Bak to prevent membrane permeabilization and cytochrome c release (Figure 1.2). Many more cell survival related targets have been proposed using bioinformatic software. Elucidating the unknown is a topic of future research geared at both harnessing and inhibiting the role of Pim-1 and cell survival.

Biologists struggle to understand the mechanisms that regulate cellular proliferation and survival in order to create beneficial clinical treatments. The serine threonine kinase Pim-1 is capable of manipulating these pathways in a variety of ways and in a variety of cell types. Oncologists view Pim-1 as an oncogene capable of driving tumorigenesis with other oncogenes like c-Myc. In most hematopoietic malignancies, Pim-1 propels cell proliferation while simultaneously promoting survival, creating a “recipe for disaster” and poor prognosis. It is because of this that pharmacologists are actively synthesizing new Pim-1 inhibitors in hopes of manipulating the pathway enough to halt metastasis. In contrast, cardiovascular biologists treat persistent survival and proliferation as a breakthrough capable of resuscitating cardiac cells during times
of myocardial damage. Current studies engineering cardiac stem cells from both mice and humans to overexpress Pim-1, then adoptively transferring them into damaged myocardium. By overexpressing Pim-1, CPCs are able to regenerate damage and increase cardiac output\textsuperscript{20, 40, 41}. Others utilize cardiotropic adenoviruses overexpressing Pim-1 to promote cellular proliferation and survival in cardiac cells specifically\textsuperscript{42}. When administered to mice by tail vein injection this virus was capable of reversing the effects of diabetic cardiomyopathy without surgery\textsuperscript{42}. Whether Pim-1 is a target for therapeutic inhibition or is a method of empowering stem cells, Pim-1 remains a signaling molecule under intense investigation.

**TELOMERES**

New revelations in the field of cardiac cell aging have linked malfunction of cardiac cells to the shrinking of telomeres\textsuperscript{43}. Telomeres are the distal ends of chromosomes comprised of several kilobases of specific DNA repeats and numerous telomere-binding proteins. The limited mitotic activities associated with cellular senescence are a direct consequence of telomere shortening, as telomeres act as a “cellular clock” winding down with each division until the telomeres become critically short\textsuperscript{44}.

James Watson termed the phenomenon of progressive telomere shortening during mitosis as the “end replication problem\textsuperscript{45}.” This “problem” describes the loss of up to 150 bases of telomeric repeats at the end of replication. This occurs as a result of RNA primers needed for DNA polymerase
to replicate single stranded complimentary strands from 5’ to 3’. Once replication is complete, the RNA primer falls off, revealing a single stranded terminal end that is subsequently removed by endogenous nucleases, shortening the telomere\textsuperscript{46}. If a telomere becomes too short a few things may occur; the cell reaches what is termed the cells Hayflick limit and becomes senescent, the cell undergoes apoptosis, or telomerase, the enzyme that extends telomeres may become activated.

**TELOMERASE**

Telomerase (TERT) is a reverse transcriptase that antagonizes senescence by lengthening telomeres\textsuperscript{47}. TERT consists of a catalytic subunit and a RNA template referred to as Telomerase RNA component (TR)\textsuperscript{48}. TERT-deficient mice possess phenotypes consistent with advanced aging after four breeding generations, demonstrating how mouse telomeres are inherently long\textsuperscript{48}. In addition, TERT knockout mice acquire dilated cardiomyopathy due to decreased proliferation, increased apoptosis, and elevation of p53\textsuperscript{49}. In murine myocytes, TERT is highly expressed and active during development and is progressively downregulated as the animal ages, but still persists throughout life with varying levels of activity\textsuperscript{50, 51}. When TERT was transgenically overexpressed in myocytes following the alpha myosin heavy chain promoter, increases in myocyte proliferation and survival following infarction were demonstrated\textsuperscript{52}. In the absence of pathology, these mice are hyperplastic but do not live longer than control. TERT overexpression beyond physiological levels
results in undesirable hypertrophic remodeling, suggesting that ideal TERT levels are difficult to maintain and TERT regulation is most likely a more viable therapeutic. CPCs contain TERT at significantly elevated levels, similar to other undifferentiated stem cells and neoplastic cells known to be proliferative. Activation of TERT in the heart is a consequence of damage and most likely aids in the repair process. Thus TERT expression and activity are required for normal homeostasis in order to maintain cardiac structure and function. TERT is subject to regulation by phosphorylation and protein-protein interactions by chaperones that stimulate its assembly and activity. One noteworthy activator of TERT is Akt, which is known to phosphorylate TERT, leading to enhanced activity and longer telomeres. TERT phosphorylation by Akt is promoted and dependent upon interactions with the chaperone HSP90. Transcription of TERT is tightly regulated as well, transcription can be promoted by many canonical proliferative transcription factors most notably c-Myc. When c-Myc is overexpressed exogenously or in transformed neoplastic cells TERT message is significantly upregulated. Inhibitory signaling influences TERT as well, Protein Phosphatase 2A (PP2A) inhibits TERT activity and soluble factors like Transforming Growth Factor 1β (TGF1β) promote canonical signaling leading to inhibition of TERT transcription. Increased TERT activity is commonly associated with oncogenic transformation, however promoting TERT activity in the heart where transformation is notoriously resistant can be utilized to enhance myocardial survival and repair.
Collectively the pieces of information from this introduction indicate that the molecular trends associated with age involve telomere shortening and a reduction of TERT expression and activity. The presence of functional CPCs within the myocardium allow for replenishment of senescent and apoptotic myocytes. Dysfunctional or senescent populations of CPCs are unable to differentiate into functional cardiomyocytes, which leads to an accumulation of poorly functioning senescent cardiomyocytes. Cardiomyopathy occurs when senescent myocytes amass, signaling pathologic eccentric hypertrophy resulting in myocardial dilation, decreases in hemodynamic function, and heart failure. The possibility of Pim-1 interceding at the beginning by inhibiting telomere erosion or promoting telomere lengthening represents an exciting molecular intervention to reverse the adverse effects of aging.
Figure 1.1. Pim-1 and the Cell Cycle.

Pim-1 together with c-Myc work synergistically to drive G1/S transition by stabilizing CDK2/Cyclin D and CDK/Cyclin E complexes by phosphorylating CDC25A and cell cycle inhibitors p21/Cip1 and p27/Kip1. Pim-1 phosphorylates c-Tak1, inhibiting c-Tak1 kinase activity, promoting CDC25C driving G2/M transition. During mitosis, Pim-1 interacts with NuMa at the spindle poles, which is thought to promote the segregation of chromosomes. The exact role of Pim-1 during mitosis is currently under investigation but it is known that Pim-1 interacts with and phosphorylates HP-1.
Pim-1 inhibits apoptosis by phosphorylating many substrates, involving up-regulation of anti-apoptotic proteins, Bcl-2 and Bcl-Xₐ (in the cytosol and mitochondria respectively) while directly phosphorylating and inhibiting pro-apoptotic protein, Bad. Bcl-2 in turn is known to inhibit pro-apoptotic proteins, Bax and Bak. Bax translocates into the mitochondria upon stress and induces the release of cytochrome c, which initiates the caspase cascade and apoptosis. Pim-1 inhibits tBid-induced cytochrome c release in hearts, thereby inhibiting apoptosis. Pim-1 also maintains mitochondrial integrity by protecting against oxidative stress-induced mitochondrial inner membrane depolarization and calcium-induced matrix swelling.
CHAPTER 1:

INCREASED RATE OF MITOSIS AND TRANSIENT
TELOMERE LENGTHENING IN CARDIAC PROGENITOR
CELLS OVEREXPRESSING PIM-1
INTRODUCTION

Regenerative medicine to combat the devastating loss of myocardial contractility resulting from MI has demonstrated promising initial results\textsuperscript{16}. Increased left ventricular ejection fraction, decreased infarct size, and increased hemodynamic function following adoptive transfer of autologous c-kit+ CPCs suggests that infusion of CPCs play a pivotal role in cardiac regeneration\textsuperscript{16, 40, 41}. However, CPCs isolated from patients are heterogeneous in nature and suffer from consequential impairment rooted in the underlying cardiomyopathic disease of the donor. Specifically, MI survivors typically are elderly, suffer from chronic cardiomyopathies or diabetes and thus possess CPCs with compromised regenerative potential\textsuperscript{10, 63}. Aged CPCs exhibit short telomeres, inactive TERT, and impaired proliferation, thereby limiting replicative capacity and generation of the population required for effective cellular cardiomyogenic treatment. Strategies aimed at accelerating proliferation and extending replicative lifespan of CPCs will be essential to overcome inherent limitations of harvested patient CPC populations derived from weak, aged, or damaged myocardium.

Telomeres are a nucleoprotein complex at the ends of linear chromosomes consisting of several kilobases of 5'-TTAGGG-3' DNA repeats and telomere associated proteins collectively known as the Shelterin complex\textsuperscript{64, 65}. Shelterin assembles tightly on the telomere to prevent chromosomal instability and inhibit DNA damage machinery\textsuperscript{66, 67}. Repeated rounds of mitosis or endogenous oxidative stress successively diminishes telomeric repeats resulting
in critically short telomeres\textsuperscript{68}. Short telomeres can lead to chromosomal fusions, cellular senescence, apoptosis, or transformation\textsuperscript{69-71}. Telomere homeostasis is maintained by TERT, the reverse transcriptase responsible for telomere elongation\textsuperscript{72}, together with an associated RNA component (Terc)\textsuperscript{73}. TERT adds telomeric repeats after DNA replication has taken place\textsuperscript{48, 74}, but TERT activation can also be initiated at transcriptional and post-translational levels\textsuperscript{57}. Serine/threonine kinases Akt and PKC positively regulate TERT, activating enzyme activity and increasing telomere length\textsuperscript{47}. c-Myc, a transcription factor that regulates as many as 15\% of all known human genes\textsuperscript{72} also promotes TERT transcription leading to increased TERT protein levels and activity\textsuperscript{72, 75}.

Pim-1, a serine/threonine kinase, promotes cell proliferation and survival in conjunction with c-Myc\textsuperscript{34, 76, 77}. Previous studies from our group demonstrated enhanced myocardial regeneration by genetically engineering CPCs to overexpress Pim-1\textsuperscript{40} but the underlying cellular and molecular basis of Pim-1-mediated effects upon CPCs remain obscure. Understanding the molecular basis for Pim-1 enhancement of CPC activity is essential to delineate the mechanistic basis of Pim-1 activity and determine the potential of Pim-1-modified CPCs for incorporation into protocols for augmented clinical treatment of heart failure. Findings presented in this chapter demonstrate telomere length is transiently increased in CPCs overexpressing Pim-1 (CPCeP) correlated to acceleration of mitotic rate and decreased cell cycle time. Telomeric lengthening and telomerase activity stimulated by Pim-1 is dependent upon c-Myc activation and protects CPCs from doxorubicin induced telomere attrition. Revealing these
underlying mechanisms of telomere preservation and acceleration of mitosis by Pim-1 offers exciting new potential therapeutic interventions for CPC-mediated cardiac regeneration in heart failure.
METHODS

CARDIAC PROGENITOR CELL ISOLATION, CULTURE, AND TRANSDUCTION

CPCs were isolated as previously described\(^8,9\). CPCs were plated (0.5\(\times\)10\(^4\) cells per well) in 6 well plates, transduced with lentivirus (multiplicity of infection=10), expanded, and isolated by fluorescently activated cell sorting (FACS) to enrich for GFP\(^+\) cells. Cells were then maintained and passaged in CPC media\(^8\).

IMMUNOBLOT ANALYSIS

Whole cell lysates isolated from CPCs were prepared in 1X SDS sample buffer\(^76\). Lysates were sonicated briefly then boiled for 5 minutes and used or stored at -80C. Samples were loaded into an Invitrogen 4-12\% Tris-Glycine mini-gel and run at 150V for 1.5 hours on an Invitrogen electrophoresis apparatus. Separated proteins were transferred to a PVDF membrane pre-incubated in methanol then blocked for 1 hour with 5\% dry non-fat milk in TBS-T (50 \(\text{mM} \) Tris-HCl (pH 7.6)/150 \(\text{mM} \) NaCl/ 0.1\% Tween 20). After transfer, the membrane was probed with primary antibodies overnight at 4\(^\circ\)C with gentle agitation in blocking buffer. Primary antibodies include: TERT (Abcam, ab5181) and GAPDH (Chemicon). The next day blots were washed with TBS-T three times and probed with fluorescent, HRP, or alkaline phosphatase-conjugated secondary antibodies 1:1000 in blocking solution (Jackson Immuno Research Laboratories) for 2 hours at room temperature. Following three washes with TBS-T blots were
scanned using a Typhoon 9410 (GE Healthcare) and signal quantitated using NIH Image J software.

**IMMUNOCYTOCHEMISTRY**

CPCs on two chamber glass slides were fixed in 4% paraformaldehyde then washed 3 times in PBS. Cells were permeabilized with 0.1M Glycine 0.2% Triton X for 2 minutes followed by three washes in PBS. Cells were then blocked in 10% horse serum/PBS for 1 hour, and then primary antibodies were applied in blocking buffer. A section accompanied all stainings without primary antibody as a negative control. Primary antibody information is provided in Table 3.1.

**REAL TIME PCR**

Total RNA was isolated from CPCs using Quick RNA MiniPrep (Zymo Research) and reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (Biorad). RT-PCR was performed in duplicate using iQ SyBR Green (Biorad) according to the manufacturer’s protocol. Data are standardized to 18S ribosomal RNA and are the averages of three experiments +/- S.E.M., primer sequences are provided in Table 3.2.

**TELOMERE LENGTH MEASUREMENTS**

Telomere length was analyzed by fluorescent in situ hybridization (Q-FISH) and confocal microscopy. PNA probe was purchased from DAKO (K5325) and used according to manufacturer’s protocol on cells fixed with 3:1 methanol/ acetic acid fixative and dried on glass chamber slides. Telomere signal was
acquired in each nucleus using Leica LCS confocal software. To control for variation slides with defined amounts of fluorescence were utilized to acquire appropriate signal intensity.

**Telomeric Repeat Amplification Protocol**

For whole cell TRAP assays, CPC plates were scraped in 3-[3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS) buffer then centrifuged at 4°C. Protein concentration was determined using Bradford assay then manufacturer’s protocol was followed for RT-PCR (Millipore S7701). Each group was subjected to heat inactivation as negative control. TSR8 positive controls with known TERT activity were used as a positive control and to create standard curve and assign TRAP activity units. Results are averages of three independent experiments in triplicate +/- SEM.

**Doxorubicin Treatment, Pharmacologic Inhibitors, Caspase 8, and Cell Cycling**

CPCs were treated for 4 hours with 1 μM of Dox in CPC growth media. Small molecule c-Myc inhibitor 10058-F4 (Sigma, 50μM) was administered to CPCs and continually passaged in the presence of inhibitor for indicated passages. MST 312, telomerase inhibitor (Sigma, 1 μM) was administered to CPCs and continually passaged in the presence of inhibitor for indicated passages. CPC apoptosis was determined by Caspase 8 activity (Clontech Laboratories, APT129). 250 ng/ml of staurosporine in 2.5% FBS media was used.
as a positive control for cell death. CPC proliferation was determined by BrdU incorporation, one hour before the end of the experiment CPCs were incubated with BrdU (10 µM) then fixed in 4% paraformaldehyde. For CyQuant, 1000 CPCs were plated and allowed to adhere to a 96 well dish. CyQuant solution was added at indicated time points and measured according to manufacturer’s protocol (Invitrogen, Catalog number C35006). Doubling time was calculated using the following formula $DT = \log N/\log 2$, where N is the cell number of the harvested CPCs at the end of the experiment divided by the initial number of cells seeded.

**PROXIMITY LIGATION ASSAY**

The PLA kit was purchased from Olink Biosciences and the manufacturer’s protocol was followed. Briefly, primary antibodies were applied after an hour block in 10% horse serum and incubated overnight at 4°C. The following day the slides were washed, then plus and minus PLA probes were applied in blocking buffer and incubated at 37°C. Next the slides were treated with ligation solution for 30 minutes followed by 90 minutes of amplification solution all at 37°C. After 3 washes slides were treated with Sytox Blue and cover slipped in Vecta-shield mounting medium.

**STATISTICAL ANALYSIS**

All data are expressed as mean ± SEM. Statistical analysis was performed using Student’s T-test and ANOVA with Tukey’s posthoc analysis as appropriate.
Telomere length distribution was determined using Kolmogorov-Smirnov test. P values less than 0.05 are considered significant.
RESULTS

INCREASED MITOTIC RATE IN CPCEP

Cell proliferation increases in CPCEP early after transduction and subsequent passaging (1.37 fold) measured by Cyquant in vitro DNA content assay. Interestingly, by 18 passages post infection the cell proliferation rates of CPCEPs are equal to that of GFP overexpressing control CPCs (CPCE, Figure 1.3A). Likewise, doubling time in early passage CPCEP is significantly shorter than CPCE (29.4 hours vs. 36.3 hours), however doubling time increases upon passaging and normalizes with CPCE at later passages (Figure 1.3B). Pim-1 levels remain high throughout passaging (Figure 1.4A) therefore decreases in doubling time in CPCEP are not an effect of diminished Pim-1 levels. To determine the effects of Pim-1 on CPCs derived from hearts of older mice, CPCs were isolated from 13-month-old FVB mice. Similar to CPCEP, older CPCs overexpressing Pim-1 (OCPCeP) display an increase in cellular proliferation over control (OCPCe) when measured by CyQuant (Figure 1.5, 1.11 and 1.29 fold at days 3 and 5, respectively). Enhanced proliferation in OCPCeP translates to a significant decrease in doubling time compared to OCPCe (Figure 1.3 C, 44.3 hours vs. 51.9 hours). CPCs overexpressing a kinase dead form of Pim-1 (CPCEPDN) cycle at a rate similar to CPCE suggesting that kinase activity is necessary for decreased doubling time. Interestingly, doubling time in CPCs lentivirally overexpressing the enzyme required for lengthening telomeres, telomerase (CPCTERT) is similar to that of CPCEP (Figure 1.3C, 31.6 hours).
**TELOMERE ELONGATION IN CPCs WITH SHORT TELOMERES**

CPC growth is regulated by telomere length and TERT activity\(^78\) therefore telomere length in OCPCe was measured by quantitative fluorescent in situ hybridization (QFISH). OCPCe have an average telomere length of 25.8 relative fluorescent units (rfus, Figure 1.6A), however OCPCeP possess significantly longer telomeres on average of 74.02 rfus (Figure 1.6B). Average telomere length of CPCs isolated from hearts of 2-month-old FVB mice is 46.06 rfus, as measured by QFISH, confirming that CPCs from older mice have relatively shorter telomeres (Figure 1.6C). Short telomeres are known to signal TERT\(^79\) therefore we investigated the ability of Pim-1 to stimulate TERT on short telomeres. OCPCeP possess increased levels of *TERT* gene, TERT protein, and TERT activity (Figure 1.6D-F, Figure 1.4A).

**ACUTE TELOMERE LENGTHENING IN CPCeP NORMALIZES THROUGH CULTURE PASSAGING**

Increases in mitotic rate (Figure 1.3) and telomere length (Figure 1.4) are evident soon after Pim-1 overexpression in cultured CPCs, so persistence of these enhancements was subsequently determined by serial passaging of CPCeP versus CPCe in culture. Comparative telomere measurements based upon rfus show a significant increase of 2.1 fold for CPCeP versus CPCe from passages 4-6 (Figure 1.7A and 1.7F; \(p<0.01\)) demonstrating that Pim-1 can increase telomere length even in CPCs derived from hearts of young mice. However, the increased rfus detected for CPCeP telomere length is lost with...
continued serial passage, becoming comparable between CPCeP and CPCe by passages 11-12 in culture (Figure 1.7C and 1.7D). Participation of Pim-1 in preservation of telomere length is evident in CPCePDN that show significant 23% loss of rfus at early passages (p<0.01, Figure 1.7E). Telomere extension depends upon TERT activity since CPCeP treated with TERT inhibitor MST 312 show no increase in rfus compared to CPCe (Figure 1.8). Collectively these results establish Pim-1 as a mediator of TERT activity that can promote transient telomere elongation early after cell modification that correlates temporally with increased mitotic activity.

*TELOMERE EXTENSION IN CPCeP DEPENDS UPON INCREASED TERT ACTIVITY*

Telomere average length determined by rfus from serial passage of CPCeP shows early augmentation at passages 4-6 that subsequently normalizes to that of CPCe by passages 11-12 and is maintained comparably between CPCeP and CPCe until passages 20-22, which is considered late passage (Figure 1.9A). Whereas the acute telomere lengthening in early passage CPCs correlates with significant increases in TERT activity (2.99 fold), this elevation of TERT is not present in CPCe, CPCePDN, or mid to late passage CPCeP (Figure 1.9 and Figure 1.4A). Specifically, TERT protein levels decrease 5.54 fold in CPCeP at passages 11-12 relative to early passage coincident with telomere length normalization. Again, TERT activity is augmented by Pim-1 kinase as demonstrated by significant reductions resulting from knockdown using short hairpin lentivirus for Pim-1 (shPim) in both CPCe and CPCeP (Figure 1.9D).
Tumor suppressor protein p53 levels are minimal and comparable to CPCe in early passages. However, p53 precipitously increases in CPCeP through serial passaging (42.6% more in p11-12 and 21.1% more in p20-22, Figure 1.9E and 1.4C) consistent with decreases in telomere length and TERT activity in later passages. Taken together the data suggest TERT is upregulated early in CPCeP leading to increased telomere length and accelerated mitosis. This effect reverses upon serial passaging resulting in increased p53, reduced TERT, a slowing mitotic rate, and diminished telomeres (Figure 1.9F).

**TERT interacts with Pim-1**

Pim-1 and TERT interact directly as assessed by co-immunoprecipitation of whole cell lysates at passage 7 (Figure 1.10B). These interactions appear to be independent of kinase activity as Pim-1 also pulled down TERT in CPCePDN (Figure 1.10B). To identify the localization of these interactions proximity ligation assays (PLAs) were performed on CPCe and CPCeP. TERT and Pim-1 interact in all three cell types however there is dramatically more interactions in CPCeP (Figure 1.10A, red dots). Pim-1/TERT PLA is negative in the absence of primary antibodies, and PLA signal is dramatically reduced in CPCePs treated with Pim-1 kinase inhibitor Quercetagetin (Q) or when Pim-1 is deleted with silencing RNA specific to Pim-1 (siPim-1, Figure 1.10C-H). TERT is potentially phosphorylated on several serine and threonine residues$^{80,81}$; however the exact TERT serine or threonine (pSer-TERT or pThr-TERT) is unknown. Therefore PLA was performed using antibodies to TERT and phospho-threonine or phospho-serine. pThr-TERT
interactions are abundant in CPCe, CPCeP, and CPCePDN (Figure 1.11A-D) and appear throughout the nucleus and cytoplasm. Interestingly, pThr-TERT appears more often in low passage CPCeP, pThr/TERT interactions diminish at passage 11 (Figure 1.11B and 1.11C). pSer-TERT similarly to pThr-TERT is common throughout the cell, however pSer-TERT interactions are diminished in the CPCePDN, suggesting that Pim-1 phosphorylates a serine residue on TERT. To corroborate the interactions demonstrated by PLA, phospho-TERT was assayed in CPCeP +Q and CPCeP +siPim-1, pSer/pThr-TERT interactions are substantially diminished in the presence of both pharmacologic inhibitor and genetic deletion (Figure 1.10D and G). Collectively, decreased telomere length, diminished TERT activity, reduced Pim-1-TERT and pSer-TERT interactions in CPCePDN suggest Pim-1 interacts with TERT, phosphorylating a serine residue leading to TERT activation and telomere elongation.

**c-MYC INHIBITION BLOCKS PIM-1 MEDIATED TELOMERE LENGTHENING**

Previous studies from our lab and others have described a synergistic interaction between c-Myc and Pim-1 to promote CPC proliferation and survival. c-Myc is up 4.4 fold in early passage CPCeP (P4-7, Figure 1.12A). CPCs were treated with a c-Myc small molecule inhibitor 10058-F4 (MycI, 50 µM) following infection and FACS sort. TERT and c-Myc target eLF4 mRNA are significantly downregulated, verifying c-Myc inhibition (Figure 1.12B) in low passage CPCe and CPCeP. TERT activity is known to be activated by c-Myc, thus TRAP activity was measured in CPCe and CPCeP. TERT activity is
significantly decreased in both CPCe and CPCeP treated with MycI, however the severity of reduction is far greater in CPCeP (Figure 1.12C, 4.52 fold decrease). Telomere length remains unchanged in CPCe and CPCeP treated with vehicle, (dimethyl sulfoxide, DMSO) (Figure 1.12D and E), however when passaged in the presence of MycI telomere length in CPCeP, rather than increasing is significantly diminished compared to vehicle (Figure 1.12G). Telomere length is diminished in MycI treated CPCe (Figure 1.12F) as well, indicating a pivotal role for c-Myc in Pim-1 induced telomere maintenance.

**TELOMERE LENGTH PRESERVATION AFTER DOXORUBICIN**

Doxorubicin (Dox) is a known cardiotoxic anthracycline that acts to deplete the CPC pool through the induction of oxidative stresses leading to cardiomyopathy. To test the functional relevance of Pim-1 dependent telomere lengthening we treated CPCe and CPCeP with Dox (1µM, 4 hours) at passages where telomere lengths are comparable between groups. TERT expression increases with Dox administration, but Dox treated CPCeP contain significantly more TERT than CPCe treated (1.7 fold over CPCe Dox, Figure 1.13A), concurrent with TERT activity (1.28 fold over CPCe+ Dox, Figure 1.13B). Dox induced 2.98 fold more apoptosis as measured by Caspase 8 activity in CPCe, while Dox has no effect on CPCeP Caspase 8 activity (Figure 1.13C). CPC proliferation measured by BrdU incorporation reveals similar levels without treatment in CPCe and CPCeP; interestingly both CPCe and CPCeP incorporate more BrdU when treated with Dox, indicating both lines synthesize DNA at the
same rate (Figure 1.14B). Despite elevated TERT activity, CPCe telomere length is significantly diminished upon Dox treatment (Figure 1.13D and 1.13F), however Dox induced telomere attrition is prevented in CPCeP (Figure 1.13E and 1.13G). Similar to Figure 6, telomere lengthening is dependent upon c-Myc activity as MycI prevents telomere length preservation (Figure 1.13H and 1.13I).
DISCUSSION

Current excitement related to clinical implementation of regenerative medicine is tempered by limitations in efficiency of stem cell-mediated repair, including low efficiency engraftment, persistence, and expansion of adoptively transferred cell populations. A clinical trial infusing autologous cardiac progenitor cells (CPCs) in patients suffering from myocardial infarction (MI) damage with ejection fraction <40% shows partial recovery of myocardial structure and function, but the fate of donated cells cannot be readily assessed\(^ \text{16} \). Furthermore, patient age and disease progression are likely to compromise proliferative potential and survival of CPCs in the setting of regenerative therapy. Thus strategies aimed at extending cellular replicative lifespan may be essential to reinvigorate CPCs derived from aged or chronically stressed cardiac tissue samples to reconstitute the damaged myocardium. Extrapolating interpretations from experimental animal MI models ranging from mice to pigs treated with autologous stem cells reveal consistent benefits in myocardial structure and function despite significant losses of donated cells shortly after adoptive transfer\(^ \text{83-85} \). However, molecular interventions to enhance survival and persistence of adoptively transferred stem cells improve restoration of myocardial performance and reverse pathologic damage relative to control cells alone\(^ \text{86, 87} \). Specifically, genetic modification of CPCs with Pim-1 expression leads to superior cellular regeneration while concurrently increasing cell numbers necessary to improve myocardial structure and function as shown using either
murine or human derived CPCs\textsuperscript{40, 41}. Telomere length of CPCeP engrafted into damaged myocardium was found to be significantly longer by 2.02 and 2.34 fold relative to CPCe using either murine\textsuperscript{40, 41} or human-derived\textsuperscript{40, 41} CPCs respectively. In addition, CPCeP exhibit a two fold increase in asymmetric chromatid segregation together with enhanced proliferation, thereby increasing the number of committed cells available for myocardial repair\textsuperscript{88}. Results presented here are consistent with the premise that myocardial Pim-1 expression prolongs the proliferative phase of CPC growth resulting in a hyperplastic phenotype\textsuperscript{76}. The mechanistic basis for beneficial effects of Pim-1 engineering likely rests, in part, with the capacity for proliferation coupled with maintenance of telomere length, both characteristics of youthful myocardial cells. Collectively, antagonism of CPC aging phenotype together with enhanced survival-signaling make Pim-1 an attractive molecular interventional approach to enhance CPC-mediated myocardial regeneration.

Precedents in highly mitotic cells support critical roles for c-Myc in acceleration of proliferation. Immortalization of human and mouse fibroblasts occurs with ectopic expression of c-Myc in the presence of activated Ras, however overexpression of c-Myc alone increases cellular proliferation without transformation\textsuperscript{89, 90}. CDC25a, a CDK activating phosphatase and target of c-Myc promotes G1/S transition by targeting and activating cyclin/CDK2 complexes\textsuperscript{91, 92}. Interestingly, fibroblasts enter S-phase 2-3 hours faster when cells overexpress CDC25a, demonstrating that CDC25a is sufficient to stimulate DNA synthesis\textsuperscript{92}. G1/S transitions are promoted through Pim-1 dependent increases in CDC25a.
activity and the inhibitory actions of p21 phosphorylation\textsuperscript{93}. Pim-1 synergistically cooperates with c-Myc to promote proliferation in neoplastic cell lines and CPCs\textsuperscript{76, 94}. Future studies will aim to elucidate the relationship between Pim-1 and CDC25a in CPC mitotic clock regulation.

Dysfunctional telomeres signal cell senescence and cell cycle arrest, however mitosis can be rescued through re-activation of TERT\textsuperscript{95}. Shortened telomeres limit proliferation of cells, slowing the mitotic clock, that in time cripples the regenerative capacity of tissues\textsuperscript{53}. Senescent CPCs are characterized by short telomeres, increased p53, cell cycle arrest, and heart failure due to the inability of CPCs to replenish apoptotic cardiomyocytes\textsuperscript{78, 96}. TERT knockout mice suffer from decreased cardiomyocyte mitosis, increased p53 induced apoptosis and dilated cardiomyopathy\textsuperscript{49} consistent with hearts that have exhausted their CPC reserve. Telomere lengthening due to enhanced TERT activity delays the aging process by allowing more rounds of progenitor cell mitosis and increasing the longevity of the animal\textsuperscript{97}. The molecular basis for our results is consistent with the postulate that transient telomere elongation and accelerated cell cycle hinge on the expression of TERT and p53 throughout passaging, as c-Myc and Pim-1 overexpression remain constant in CPCeP (Figure 1.7F). Shortly after CPCs are transduced with Pim-1, TERT is upregulated and activated thereby lengthening telomeres. Concurrently Pim-1 drives mitosis and diminishes population-doubling time. Acute cell cycle rate increases and telomere lengthening normalizes as TERT activity and expression levels decrease and p53 levels increase. Elevated p53 expression prompted in
response to diminished telomere length slows mitosis, thereby inhibiting CPC immortalization. Engineering CPCs ex vivo with Pim-1 to activate TERT and stimulate mitosis is an attractive means of opposing the deleterious effects of aging that is not perpetual and does not lead to cellular immortalization.

Cardiomyopathy in patients treated with doxorubicin is postulated to result from depletion of the CPC population that, in turn, diminishes replacement of damaged myocytes leading to ventricular dilation, wall thinning, and premature mortality. Mechanistically, doxorubicin acts as an oxidative stressor creating DNA damage and promoting apoptosis. Variability in cardiotoxic response to doxorubicin treatment among patients may be a consequence of individual differences in telomere length and TERT activity in the CPCs. Telomere attrition produced by doxorubicin administration is blunted in CPCeP, offering a novel molecular therapeutic intervention for doxorubicin-induced cardiotoxic damage as well as enhancement of regenerative potential for CPC derived from aged or chronically stressed myocardium.

In conclusion, as clinical trials utilizing autologous progenitor cell transplantation advance, the quality of progenitor cells administered is of critical importance. The cellular and molecular phenotypes of the CPCs will determine their efficacy in cardiac repair. Results from this study suggest that Pim-1 promotes a short-lived increase in mitotic rate in conjunction with an acute lengthening of CPC telomeres, characteristics of youthful CPCs. Augmenting the regenerative potential of CPCs through molecular engineering with Pim-1 enhances proliferation and antagonizes the phenotypic characteristics of aging,
providing a mechanistic basis for observations of enhanced regeneration using CPCeP in the damaged myocardium.
Figure 1.3 Temporary increases in proliferation and reduced CPC doubling time with Pim-1 overexpression.

A. CPC proliferation determined by Cyquant assay. *p<0.05 vs. CPCe at same passage.  
B and C. Calculated CPC doubling time determined by Cyquant and Trypan blue cell counts. **p<0.01 vs. CPCe, # p<0.05 vs. OCPCE. CPCs overexpressing eGFP (CPCe), Pim-1 (CPCeP), CPCs isolated from 13 month old FVB mice overexpressing eGFP (OCPCE), Pim-1 (OCPCEP), CPCs overexpressing a kinase dead mutant of Pim-1 (CPCePDN), and CPCs overexpressing TERT (CPCTERT).
**Figure 1.4: Pim-1 overexpression remains unchanged throughout passaging.**

**A-C.** Immunoblots of CPCe and CPCeP at varying passages and probed using antibodies against the indicated proteins. + indicates whole lysates from Hela cells, e is CPCe and eP is CPCeP. **B.** + Indicates whole cell lysates from CPCs infected with lentivirus overexpressing TERT.
Figure 1.5: Increased cycling in CPCs isolated from 13 month old hearts and infected with Pim-1.

Cyquant assay performed on isolated older CPCs infected with lentivirus overexpressing GFP (OCPCe) or Pim-1 (OCPCeP). #p=0.09 vs. OCPCe day 3, *p<0.05 vs. OCPCe day 5. Results are from three independent experiments.
Figure 1.6: Telomerase activation and telomere lengthening in CPCs from aged hearts.

A-B. CPCs isolated from 13 month old mouse hearts and infected with eGFP or Pim-1 (OCPCe or OCPCeP, respectively) were plated and subjected to QFISH to determine telomere length (dashed red lines indicate averages). C. Telomere lengths of CPCs isolated from 2-month-old FVB hearts demonstrate telomere lengths in younger CPCs. D. TERT gene determined by RT-PCR. E. TERT expression levels in OCPCe and OCPCeP three passages after infection. F. TERT activity measured by TRAP assay. Dashed red lines represent average telomere lengths. Results are mean +/- SEM. *p<0.05 vs. OCPCe, ** p<0.01 vs. OCPCe, telomere lengths between OCPCe and OCPCeP are significantly different p<0.001 measured by KS test. Results are from three independent experiments.
**Figure 1.7**: Telomere length is increased in CPCeP.

**A and B.** Telomere lengths in early passages (p4-6) CPCe and CPCeP measured by QFISH (**p<0.01**). **C and D.** Telomere lengths in later passages (p11-13) of CPCe and CPCeP, telomere lengths are no longer statistically different. **E.** Telomere length is significantly decreased in early passage CPCePDN (**p<0.01** vs. CPCe by KS test). Dashed red lines represent average telomere lengths. **F.** CPCe and CPCeP telomeres visualized by QFISH (white foci), nuclei are stained using To-Pro-3 (blue). Results are from three independent experiments.
Figure 1.8: CPCeP telomere lengthening is dependent on TERT activity.

A-D. Early passage CPCs treated with either DMSO or TERT inhibitor (MST-312, 1µM). *p<0.05 vs. CPCe DMSO, #p<0.05 vs. CPCe or CPCeP DMSO. Results are from three independent experiments.
Figure 1.9: Telomere lengthening in CPCeP is due to acute increases in TERT activity.

A. Average telomere lengths in CPCe and CPCeP throughout 22 passages, **p<0.001 vs. CPCe passages 4-6. B. TERT protein expression at varying passages, values are fold change divided by low passage CPCe (4-6). C. Telomerase activity of CPCe and CPCeP from various passages post transduction. D. Telomerase activity in low passage CPCePDN (p 4-6) and after short hairpin deletion of Pim-1 in CPCe and CPCeP (shPim). E. p53 protein expression at multiple passages determined by immunoblot and standardized to GAPDH expression. F. Summary of mechanism for transient telomere lengthening and increased mitotic rate in CPCeP. N=3. **p<0.01, *p<0.05 determined by KS test, student’s t-test, or two way ANOVA where applicable.
Figure 1.10: Pim-1 interacts with TERT.

A. Pim-1 and TERT interactions demonstrated by PLA for Pim-1 and TERT (red).
B. Co-immunoprecipitation of Pim-1 and TERT. C PLA performed on CPCeP without primary antibodies; this control was performed with every assay to confirm specific staining. D and E. PLAs of Pim-1 and TERT (red), Pim-1 and pS/pT and Tert (red) in CPCeP treated with Pim-1 inhibitor (Q, 10μM for 24 hours). F. Pim-1 expression knocked down with a Pim-1 specific siRNA. Lane 1 is CPCeP, lane 2 is CPCe+ siPim-1, and lane 3 is CPCeP +siPim-1. G and H. Pim-1 and TERT (red) and pS/pT and Tert CPCeP treated with siPim-1.
Figure 1.11: TERT is a target of Pim-1.

A-D. Phosphorylated TERT on threonine residues and serine residues (E-H, red), cell body is visualized using GFP (green), and nuclei are stained with Sytox (white). Early passage CPCeP (P4) demonstrates increased pThr/TERT and pSer/TERT that diminishes over passaging (CPCeP p11).
Figure 1.12: Myc Inhibition blocks Pim-1 mediated telomere elongation.

A. Expression of c-Myc by immunoblot, GAPDH is utilized as a loading control.
B. RT-PCR of c-Myc target genes TERT and eLF4 (control) in vehicle (DMSO) CPCeP or CPCe and CPCeP +Mycl (50 µM for 3 passages) values are fold change over vehicle treated CPCe. C. TERT activity in vehicle and Mycl CPCe and CPCeP. (***p<0.01 vs. CPCeP vehicle treated, $p<0.05$ CPCe vehicle vs. CPCe Mycl treated). D and E. Telomere lengths of vehicle treated CPCe and CPCeP and F and G. Myc I treated, telomere length determined by QFISH (**p<0.0001 vs. vehicle CPCe, #p=0.002 vs. Mycl CPCe, and $p<0.0001$ vs. CPCeP vehicle determined by KS test).
Figure 1.13: Telomere length is preserved in CPCEPs challenged with Doxorubicin.

A. Fold change increase in TERT protein in CPCEP after Dox treatment (p13-15, 4 hours 1 µM Dox). B. TERT activity in CPCs 4 hours after Dox measured by TRAP, values are fold change over Dox treated CPCE (*p<0.05). C. Caspase 8 activity was determined after Dox treatment; values are fold change over untreated CPCe. D-G. QFISH analysis in untreated and Dox treated CPCE and CPCEP at the same passages. H and I. Telomere length measurements in CPCE and CPCEP treated with Dox and Mycl. (*p<0.01 vs. untreated CPCE, **p<0.001 vs. untreated CPCE). Results are averages of three independent experiments.
Figure 1.14. TERT expression and DNA incorporation after Dox treatment.

A. Immunoblots of CPCe and CPCeP treated with Dox and probed for indicated antibodies + indicates whole cell Hela lysates. B. DNA synthesis measured by BrdU incorporation in DOX treated and untreated CPCs. $ p<0.05 $ vs UT CPCe.
CHAPTER 2.

CARDIOMYOCYTE TELOMERE LENGTHENING MEDIATED BY PIM-1
INTRODUCTION

Aging, metabolic disorders, environmental stresses, nutrition, and genetics all contribute to the increasing prevalence of heart disease in the United States\(^4\). The deterioration of myocardial function over time is due to a combination of diminished regenerative capabilities, together with increasing accumulation of senescent cardiomyocytes possessing marginal functional performance\(^{100}\). The following maladaptive compensatory responses result in decreased hemodynamic function, marginal cardiac output, and heart failure\(^{101}\). Repopulating the myocardium with new functional cardiomyocytes through adoptive transfer approaches may serve to delay pathology and possibly reverse dysfunction\(^{40, 41}\). Alternatively, intervening on the existing cardiomyocytes through molecular interventions may antagonize the aging phenotype and prolong the functional lifespan of cardiomyocytes.

Chromosomal instability, cellular senescence, and apoptosis increase with age and are a result of telomeric shortening\(^96\). Cardiomyocyte telomere attrition stems from limited mitosis and endogenous oxidative stresses\(^{55, 102}\) and has a direct correlation with incidences of heart disease\(^{11, 96}\). TERT directly antagonizes cellular senescence by lengthening telomeres\(^{55}\). Both expression and activity of TERT diminish as mice\(^{50}\) and humans age\(^{102}\); in cardiomyocytes TERT expression is high in neonates and drops precipitously with age, though persists throughout life\(^{51}\). Thus stimulating the telomere/telomerase axis has become an attractive avenue to combat the effects of aging in order to reverse
heart failure.

Mechanistically, TERT is molecularly regulatable by both induction and repression signals. Akt\textsuperscript{103}, PKC\textsuperscript{81}, and IGF1\textsuperscript{104} are known to stimulate TERT activity, and transcription factors c-Myc\textsuperscript{72, 75} and ETS-2\textsuperscript{105} are capable of promoting TERT transcription. Inversely, activation of the transforming growth factor-1β (TGF1β) pathway represses TERT transcription and inhibits TERT activity resulting in shortened telomeres\textsuperscript{61, 106}. TGF1β is a soluble secreted ligand that acts upon binding with a heteromeric complex of receptors (TGFR1 and TGFR2) which contain cytoplasmic serine-threonine kinase domains\textsuperscript{107}. Binding results in phosphorylation of cytoplasmic SMAD proteins. Once phosphorylated SMADs translocate to the nucleus where they act as transcription factors\textsuperscript{108}. Specifically, receptor activated SMAD-3 is an inhibitory transcription factor that inhibits TERT transcription\textsuperscript{106}. Cardiac TGF1β signaling is associated with decreased cellular proliferation\textsuperscript{109}, cardiomyocyte hypertrophy\textsuperscript{110, 111}, and fibrosis\textsuperscript{112}.

The cardioprotective kinase Pim-1 has been shown to increase myocyte cellularity \textit{in vivo}\textsuperscript{1}, promote CPC cycling\textsuperscript{76} and act on mitochondria to promote survival via intrinsic pathways\textsuperscript{39}. Initial studies from our lab describe how myocardial specific overexpression of Pim-1 (Pim-wt) prevents maladaptive cardiac remodelling by promoting myocyte survival and enhancing endogenous repair through CPC regeneration\textsuperscript{1, 20, 40}. This chapter describes the effect of Pim-1, in concert with TERT to transiently lengthen telomeres in neonatal cardiomyocytes. Increased telomere length correlates with increased
proliferation and hyperplasticity, in contrast mice with global Pim-1 elimination (Pim-KO) possess short telomeres early in development together with significant increases in TGF1β. *In vitro* analysis of neonatal rat ventricular cardiomyocytes (NRCMs) reveals that TGF1β overexpression results in diminished TERT expression, activity, and telomere length. Telomeres are lengthened upon administration of TGF1β receptor inhibitor, in addition to increased telomere lengths observed with Pim-1 expression. Collectively, these results provide novel mechanistic insight for how Pim-1/TGF1β maintain cardiomyocyte telomere/TERT homeostasis.
METHODS

ANIMAL STUDIES AND CELL CULTURE

The Institutional Animal Care Committee of San Diego State University approved all animal protocols. Myocardial infarction, heart isolation, and perfusion were performed as previously described\textsuperscript{76}. Primary neonatal rat cardiomyocytes were isolated as described previously\textsuperscript{1, 113}. NRCMs were infected in a milliliter of M199 media supplemented with 2% FBS for 2 hours. Next, myocytes were washed in PBS and given 10% FBS media for 24 hours before they were harvested for experimentation. NRCMs were treated with quercetin (10\(\mu\)M) at the time of infection. Small interfering RNA (siRNA) targeted against Telomerase was purchased from Applied Biosystems and transfected with HiPerfect transfection reagent (Qiagen) following manufacturers protocol. Two days after siTERT or siScramble transfection NRCMs were infected with adenovirus’ overexpressing GFP or Pim-1/GFP (Pim-wt).

IMMUNOBLOT ANALYSIS

Whole cell lysates isolated from whole hearts and NRCMs were prepared in 1X SDS sample buffer and prepared as described in Chapter 1 Methods. Primary antibodies: TRF1 (Abcam, 1423), c-Myc (Santa Cruz, sc-42), TERT (Abcam, ab5181), TGFR1 (Abcam, 31013), Smad-3 (Abcam, 75512), and TGF1\(\beta\) (Abcam, 66043).
**IMMUNOCYTOCHEMISTRY**

NRCMs on 2 chamber glass slides were paraformaldehyde fixed then washed in PBS. Cells were permeablized with 0.1M Glycine, 0.2% Triton X100 for 2 minutes followed by a PBS wash. Cells were then blocked in 10% horse serum/PBS for 1 hour, and then primary TGF1β (Abcam, 66043) and sarcomeric actin (Sigma) antibodies were applied in blocking buffer. A section not given primary antibody as a negative control accompanied all stainings. When inspected these slides did not have staining indicating specific staining for that primary antibody.

**TELOMERE LENGTH MEASUREMENTS**

Telomere length was analyzed by quantitative fluorescent in situ hybridization (Q-FISH) and confocal microscopy. Telomere PNA probe was purchased from DAKO (K5325) and manufacturer’s protocol was followed, permeabilization of paraffin sections was accomplished using 15 minutes of boiling 10mM citrate followed by 9 minutes of proteinase K digestion.

**TELOMERIC REPEAT AMPLIFICATION PROTOCOL**

Telomerase activity was measured by quantitative polymerase chain reaction (Millipore S7701) according to manufacturers protocol for in vivo heart samples. For whole cell NRCM TRAP assays, NRCM plates were scraped in 3- [3- cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS) buffer then centrifuged at 4°C. Protein concentration was determined using Bradford
assay, and then manufacturer’s protocol was followed for PCR reactions (Millipore S7700). RNase was treated to each group as negative control. PCR products were run on Invitrogen 20% TBE gels, and OD determined with NIH Image J. Results are averages of three independent experiments in triplicate +/- SEM.

**TGF1β Administration and Inhibition**

NRCMs were treated with TGF1β (4ng/ml) and LY-364947 (TGFRI 10μM) at time of adenoviral infection.

**Statistical Analysis**

All data are expressed as mean ± SEM. Statistical analysis was performed using Student’s T-test and ANOVA with Tukey’s posthoc analysis as appropriate. Telomere length distribution was determined using Kolmogorov-Smirnov test. P values less than 0.05 were considered significant.
RESULTS

TELOMERE LENGTHENING IN PIM-WT MYOCYTES

Cardiomyocyte telomere length assessed by QFISH (Figure 2.1A) in Pim-wt, Pim-1 global knockout (Pim-KO), and non-transgenic (NTG) myocardial sections demonstrate a profound increase in telomere length in Pim-wt cardiomyocytes at 2 days and 2 weeks of age (Figure 2.1B-E). Myocyte cross sectional area analysis reveals an inverse relationship between telomere length and myocyte size at 2 days, 2 weeks, and 3 months of age in all mouse lines measured. Telomere length is longest in 2 day Pim-wt myocytes in all myocyte areas investigated, yet larger myocytes possess telomeres significantly shorter than telomeres in smaller myocytes (Figure 2.1B). Suggesting smaller cells with longer telomeres are phenotypically younger and possibly newly differentiated. Telomere lengths in 2 day Pim-KO mice are significantly shorter compared to NTG throughout all myocyte sizes (Figure 2.1B). Increased telomere length is preserved in Pim-wt myocytes at 2 weeks, while telomere lengths in Pim-KO myocytes normalize to NTG telomere lengths (Figure 2.1C). Interestingly, at 3 months of age telomere lengths in Pim-wt myocytes diminish to those of NTG (Figure 2.1D). On average, Pim-wt myocyte telomeres are dramatically longer than NTG and Pim-KO at 2 day and 2 weeks of age (Figure 2.1E). At 3 months the myocytes possess telomeres of similar length to NTG control. Consistent with telomere length, TERT activity in Pim-wt myocardial samples measured by TRAP is significantly greater than NTG at 2 days and 2 weeks. TERT activity in
all hearts tested diminishes as the animal ages and becomes comparable at 3 months of age.

**TELOMERE LENGTH DETERMINANTS AND MARKERS OF CELLULAR AGING**

Telomere lengthening is dynamically regulated by several factors known to promote cell proliferation and survival in addition to telomere maintenance. c-Myc, a transcription factor known to upregulate TERT transcription is significantly downregulated in Pim-KO left ventricular samples at 2 days of age (Figure 2.2A, 38.6% of NTG). Levels of c-Myc were comparable between NTG and Pim-wt at 2 days of age (Figure 2.2A). TERT expression levels are downregulated in Pim-wt (65.0% of NTG, p=0.09) and Pim-KO hearts (48.7% of NTG, p<0.05, Figure 2.2A) despite elevated activity levels in the Pim-wt. Cellular aging and apoptosis markers p53 and Check point kinase-2 (Chk2) are upregulated in Pim-KO hearts (1.23 fold and 1.48 fold respectively) while p53 is significantly downregulated (63.2%) and Chk2 is unchanged in Pim-wt hearts compared to 2 day NTG controls (Figure 2.2A). c-Myc expression levels dramatically increase with Pim-1 overexpression at 2 weeks of age (2.58 fold) consistent with previous studies linking a synergistic relationship between Pim-1 and c-Myc. Inversely c-Myc remains decreased in Pim-KO heart samples compared to NTG (Figure 2.2B, 70.4%). TERT and p53 expression normalized after 2 weeks in Pim-wt hearts yet TERT remained reduced (55.9%) in Pim-KO hearts at 2 weeks. Chk2 protein expression is significantly below that of NTG in both Pim-wt and Pim-KO hearts indicating a reduction in apoptotic signaling at 2 weeks (Figure
2B, 15.6% and 53.5% respectively). c-Myc and TERT protein levels are elevated above NTG in Pim-wt hearts despite telomere length normalization at this time point (Figure 2.2C, 4.26 and 3.39 fold). p53 protein levels in Pim-KO hearts are reduced at 3 month indicating a stabilization in apoptotic signaling at this age (Figure 2C, 45.6%).

**Pim-1 interacts with TERT**

To investigate if Pim-1 directly interacts with TERT proximity ligation assays (PLAs) were performed. Pim-1 interacts with TERT throughout development in NTG and Pim-wt myocardial sections (Figures 2.3A). Pim-1/TERT interactions are considerably increased in younger hearts and decline with age consistent with the expression profiles of these proteins previously established1, 11. These interactions occur in the nucleus and cytoplasm of cardiomyocytes and interstitial cells of NTG and Pim-wt hearts but are absent in Pim-KO hearts (Figure 2.3A and data not shown).

To determine whether a short transient overexpression of Pim-1 is sufficient to mimic results found in transgenic mouse hearts, adenovirus overexpressing Pim-1 (Pim-wt) and enhanced green flourescent protein (GFP) control were utilized to infect NRCMs. Interactions between Pim-1 and TERT persisted in NRCMs and these interactions can be amplified with Pim-1 overexpression (Figure 2.3B). Of note, GFP and Pim-wt NRCMs contain similar levels of phospho-TERT (Serine and Threonine), however Pim-wt NRCMs
possess higher incidences of nuclear phosphorylated TERT (Figure 2.3C, yellow arrows) suggesting activated TERT.

**ACUTE TELOMERE ELONGATION WITH PIM-1 OVEREXPRESSION**

NRCMs were infected with GFP and Pim-wt adenovirus and QFish (Figure 2.4A and B) and TRAP assays were performed (Figure 2.4C). Pim-wt infection resulted in a significant induction of TERT activity (1.75 fold, Figure 2.4C). Increased TERT activity coincides with longer telomeres in Pim-wt NRCMs after 24 hours of adenoviral infection (Figures 2.4D and E, 27.8% increase over GFP). Decreased telomere length is observed when NRCMs are infected with a kinase dead adenovirus of Pim-1 (Pim-DN, Figure 2.4F, 83.1% of GFP fluorescence). To demonstrate that Pim-1 is activating TERT to lengthen telomeres a siRNA specific to TERT was transfected into NRCMs. HiPerfect Transfection reagent (Qiagen) requires minimal media, thus NRCMS transfected with a scramble siRNA have a small reduction in telomere length when compared to previous full media adenoviral infections (Figures 2.4 and 2.5). Knockdown of TERT results in a minimal reduction in GFP infected NRCM telomere length (Figure 2.5A and B) and completely abolishes the telomere lengthening effects in the Pim-wt NRCMs (Figure 2.5C and D). Interestingly, a severe telomere erosion occurs with TERT knockdown in addition to Pim-DN infection (Figure 2.5 E and F 44.4% of siScr Pim DN). In addition to diminished telomere length, siTERT reduced TERT gene expression and attenuated Pim-wt induced TERT activity (Figure 2.5G and H).
TGF1β INHIBITION PROMOTES TERT ACTIVITY AND TELOMERE ELONGATION

To investigate the in vivo signaling involved in neonatal Pim-KO telomere erosion, an apoptotic gene array was performed on 2 day NTG, Pim-wt, and Pim-KO hearts. Although many genes are altered, TGF1β was prominently upregulated in Pim-KO hearts (data not shown). To validate apoptotic array results, a qPCR was performed with primers specific to TGF1β. qPCR tracings demonstrate a significant upregulation of TGF1β transcript in Pim-KO neonate hearts (Figure 2.6A). Consistent with in vivo results, TGF1β is upregulated 3.33 fold in control NRCMs treated with Pim-1 inhibitor Quercitagitin, (Q, Figure 2.6B) and remains unchanged when treated with LY-364947, a TGF1β receptor inhibitor (TGFRI). Pim-wt NRCMs possess significantly less TGF1β at basal levels and when treated with Q (49.1% and 27.9% respectively, Figure 5b), however treatment with TGFRI has little effect on Pim-wt NRCMs. TGF1β increases 2.58 fold in Pim-DN NRCMs with a slight, but not significant, increase upon administration of Q (Figure 2.6B). The most significant increase in TGF1β was as a result of TGFRI treatment together with Pim-DN (37.8 fold). TGF1β is known to disrupt TERT activity by signaling through Smad3 transcription factors 61, 106, thus we examined TERT activity in the presence of TGFRI. TGFRI treatment induces TERT activity 1.89 fold in GFP, 1.19 fold in Pim-wt, and 2.83 fold in Pim-DN infected NRCMs (Figure 2.6C). TGFRI stimulated TERT activity leads to increased telomere lengths in GFP and Pim-wt NRCMs (2.45 and 1.89 fold over untreated, Figure 2.6E and 2.6F). TGFRI induced telomere lengthening
is independent of Pim-1 activity as Pim-DN NRCMs possess 2.46 fold longer telomeres over untreated (Figure 2.6G).

TGF1β is known to promote brain natriuretic peptide (BNP) transcription in cardiac myocytes\textsuperscript{114}, therefore qPCR with primers specific for BNP were utilized to ascertain if recombinant TGF1β was functional in our system. GFP infected NRCMs upregulated BNP 6.52 fold over untreated NRCMs (Figure 2.7A). To assess the effect of TGF1β on TERT activity, a TRAP assay was performed. GFP and Pim-wt NRCMs have a reduction in TERT activity (Figure 2.7B) with a significant decrease in Pim-wt infected NRCMs (2.89 fold), while not affecting TERT activity in Pim-DN NRCMs (data not shown). Telomere length is significantly diminished in Pim-wt and Pim-DN NRCMs treated with TGF1β (2.70 and 1.47 fold, Figure 2.7E). Collectively, TGF1β expression reduces TERT activity and diminishes telomeres, while inhibition of TGF1β stimulates TERT activity and lengthens cardiomyocyte telomeres.

**TELOMERE LENGTH PRESERVATION AFTER MYOCARDIAL INFARCTION BY PIM-1**

Previous studies have reported increased ki-67 positive myocytes, decreased TUNEL positive myocytes, and reduced infarct size in Pim-wt hearts 7 days post MI\textsuperscript{1}. Small myocytes with long telomeres are capable of cycling in addition to withstanding the oxidative stresses that are common in the infarct. Therefore, QFish was performed on heart sections 7 days post MI. Small myocytes in Pim-wt border zones possess longer telomeres than NTG control myocytes of similar size (Figure 2.6A 1.89 Fold <400\textmu m and 1.75 Fold 401-
600µm). Pim-wt hearts contain increased TERT protein specifically in the infarct portion of the heart, have elevated pTERT evidenced by PLA in the border zone, and possess more TERT activity in the infarct. Taken together, 7 days post MI, cardiac specific Pim-1 overexpression results in an infarct zone containing increased TERT expression and activity with myocytes possessing longer telomeres.
DISCUSSION

Age associated cardiac decompensation is a consequence of dying cardiomyocytes that are no longer replenished by the senescent CPC population\textsuperscript{11}. The strain produced by the increase in dysfunctional myocytes results in myocardial dilation, a condition where large hypertrophied myocytes compose a majority of the myocardium\textsuperscript{115}. In the elderly, this commonly precedes diastolic heart failure and is a leading cause of mortality\textsuperscript{115}. Senescent cardiomyocytes possess short telomeres and inactive TERT, suggesting an intriguing target to develop a therapeutic in order to re-activate TERT. Active TERT will target short telomeres, lengthening them, decreasing chromosomal instability, apoptosis, and rejuvenating cardiomyocyte function. In addition, lengthening telomeres has the ability to stimulate small transient amplifying myocytes to re-enter the cell cycle\textsuperscript{55}.

Recent studies from our lab have established that engineering progenitor cells with Pim-1 limits the damage associated with MI through cardiomyocyte addition and paracrine stimulation\textsuperscript{40, 41, 116}. Results described within provide further mechanistic evidence for the cardioprotective and anti-aging effect observed upon Pim-1 expression. Re-activation of TERT, downregulation of p53, and increased telomere length provides an explanation for the hyperplastic phenotype seen early in development as well as the cardioprotective effects after MI observed upon Pim-1 overexpression\textsuperscript{1, 2}. Genetic deletion of Pim-1 results in no overt phenotype\textsuperscript{117}, however knockout mice are unable to withstand cardiac
insult\textsuperscript{1, 2}. When Pim-KO mice are subjected to MI the myocardium quickly dilates due to increased cardiomyocyte apoptosis\textsuperscript{1}. Pim-KO results in larger infarct sizes compared to control in addition to significant decreases in CPC mobilization\textsuperscript{1, 76}. It appears that susceptibility to insult may be due to increased p53 and Chk2 expression together with decreased telomere length observed early in development (Figures 2.1 and 2.2). In addition, shortened telomeres lead to metabolic failure through decreased mitochondrial respiration and biogenesis\textsuperscript{44}, interestingly, Pim-1 plays a protective role at the mitochondria\textsuperscript{39}.

Induction of TGF1\(\beta\) upon Pim-1 ablation or dysfunction is a novel insight and provides further mechanistic detail into the cardioprotective role of Pim-1. TGF1\(\beta\) is a well established negative regulator of cell cycling\textsuperscript{118} through repression of c-Myc\textsuperscript{119} and induction of Kip/Cip cell cycle inhibitors\textsuperscript{120}. TGF1\(\beta\) can be expressed in cardiomyocytes and when TGF1\(\beta\) is present hypertrophy is induced both \textit{in vivo} and \textit{in vitro}\textsuperscript{114, 121}. Cardiomyocyte hypertrophy is accompanied by increased collagen deposition and fibrosis when stimulated with TGF1\(\beta\)\textsuperscript{111, 122}. Conversely, mice lacking TGF1\(\beta\) are resistant to hypertrophy when stimulated by Angiotensin II or trans-aortic constriciton (TAC)\textsuperscript{111}. In humans, TGF1\(\beta\) is increased in patients with multiple forms of heart disease, including idiopathic cardiomyopathy\textsuperscript{123}, MI\textsuperscript{124}, and hypertension\textsuperscript{126}. Interestingly, TGF1\(\beta\) is commonly utilized to differentiate stem cells into cardiomyocytes, unfortunately little is known mechanistically of how TGF1\(\beta\) induces differentiation\textsuperscript{126, 127}. Multiple phenotypes associated with Pim-1 dysfunction or ablation correlate with
those seen with TGF1β expression in the heart, and are listed above. Inversely, Pim-wt hearts are resistant to fibrosis caused by TAC in vivo, and when NRCMs were subjected to endothelin-1 stimulation, Pim-wt adenovirus prevented hypertropy. This chapter demonstrates that Pim-KO results in increased TGF1β at two days of age and correlates with a decrease in telomere length. TGF1β induced telomere attrition was recapitulated in vitro on NRCMs and was coincident with reductions in TERT activity (Figure 2.6). In addition, blocking TGF1β signaling using a TGF1β receptor 1 inhibitor activated TERT and lengthened telomeres, in addition to Pim-wt dependent telomere elongation. The toxic effects of TGF1β on cardiomyocyte telomeres is an unforeseen consequence of cardiomyopathy, thus manipulating TGF1β signaling through receptor inhibition or Pim-1 expression represent novel targets to antagonize the aging phenotype.
Figure 2.1: Telomere Length is increased in developing Pim-wt myocytes.

A. QFISH of telomeres (white) in a 3-month-old Pim-wt heart, myocytes are identified using an antibody to tropomyosin (red) and Topro (blue) to identify nuclei. B. Quantitation of cardiomyocyte telomere length in neonatal hearts. C. 2 week old hearts and D. 3 month old hearts, organized by myocyte size. All myocyte sizes are in µm². E. Graph demonstrating an average increase in telomere length in 2 day and 2-week-old Pim-wt hearts. F. Telomerase activity quantified using the TRAP assay. N=3 hearts, *p<0.05 vs. same cell size in NTG **p<0.01 vs. same cell size in NTG. $p<0.05 vs 2 day Pim-wt.
Figure 2.2: Cellular aging markers over time.

A. Quantitation of 2 day, B. 2 week, C. and 3 month old whole heart lysates. Immunoblots are probed with antibodies against c-Myc, TERT, p53, and Chk2. *p<0.05 vs. NTG, #p<0.05 vs. Pim-wt. $p=0.06 vs. NTG. N=4 hearts.
Figure 2.3: Pim-1 interacts with TERT in vivo and in vitro.

A. Proximity ligation assays (PLAs) performed on NTG, Pim-wt, and Pim-KO hearts at 2 days, 2 weeks, and 3 months of age. Green dots represent in vivo interactions, desmin staining is in red, and nuclei are stained with Topro (blue).

B. PLA for Pim-1 and TERT interactions performed on isolated NRCMS infected with adenovirus overexpressing GFP (GFP) or Pim-1 (Pim-wt). GFP (green) expression delineates the cell body and Pim-1/TERT interactions are red.

C. Increased nuclear phosphorylated TERT (yellow arrows) in Pim-wt NRCMs demonstrated by PLA (red dots).
Figure 2.4: TERT/Pim-1 interactions lead to increased TERT activity and telomere lengthening.

A-B. QFISH performed on NRCMs infected with GFP or Pim-wt adenovirus. C. Telomerase activity assessed by TRAP assay in adenovirally infected NRCMs. D-F. Telomere lengths determined by QFISH in NRCMs adenovirally infected with viruses, dashed vertical red line indicates average telomere fluorescence. Results are averages of three independent experiments. **p<0.01 vs. GFP.
Figure 2.5: Telomere lengthening mediated by Pim-1 is dependent upon TERT.

A-F. Telomere fluorescence determined by QFISH in NRCMS transfected with scramble siRNA (siScr) or siRNA-targeting TERT then infected with indicated viruses. Dashed vertical red line indicates average telomere fluorescence. *p<0.05 vs. GFP siScr, **p<0.01 vs. GFP siScr, #p<0.01 vs. Pim-wt siScr. G. TERT activity determined by Trap assay *p<0.05 vs. GFP siScr, #p<0.05 vs. Pim-wt siScr. H. qPCR verifying TERT knockdown in siTERT transfected NRCMs. Results are averages of three independent experiments.
Figure 2.6: TGF1β inhibition lengthens telomeres in addition to Pim-1.

A. qPCR tracings demonstrating increased TGF1β expression in Pim-KO neonate hearts. n=4 hearts  

B. TGF1β gene expression in NRCMs infected with adenovirus in combination with LY-364947 (TGFRI 10µM) TGFRI.  

C. TERT activity determined by TRAP assay on NRCMs adenovirally infected and either treated or untreated with TGFRI.  

D-F. Telomere lengths of NRCMs infected with indicated adenovirus and treated with TGFRI. **p<0.01 vs. GFP untreated, $p<0.05$ vs. untreated. Results are averages of three independent experiments.
Figure 2.7. Decreased TERT activity and diminished telomere length upon TGF1β administration.

A. BNP, a known TGF1β target is upregulated when TGF1β is administered to NRCMs for 24 hours (4 ng/ml). B. TERT activity in GFP and Pim-wt NRCMs treated with DMSO or TGF1β for 24 hours. C-E. Diminished telomere lengths in Pim-wt and Pim-DN NRCMs. Results are averages of three independent experiments.
Figure 2.8: Telomere length preservation after myocardial infarction by Pim-1.

A. Telomere fluorescence of border zone cardiomyocytes 7 days after MI in NTG, Pim-wt, and Pim-KO hearts. B. Immunoblot analysis of remote and infarct whole cell lysates taken 7 days after MI. C. PLA analysis of Pim-1 and TERT, pSer/TERT, and pThr/ TERT (green) in border zone cardiomyocytes (red). D. TERT activity assessed by TRAP assay, **p<0.01 vs. NTG infarct samples. N=4 hearts.
CHAPTER 3.

SHELTERIN COMPLEX INFLUENCED BY PIM-1 IN CARDIAC PROGENITOR CELLS AND CARDIOMYOCYTES
Chapter 3 represents the future directions portion of this dissertation. In an attempt to further understand the mechanism of Pim-1 signaling on telomeres, several telomere related factors were analyzed. The following is a brief description of the shelterin complex and telomere-associated proteins, a graphical representation and table are provided to facilitate reading (Figure 3.1B and Table 3.1).

The nucleoprotein complex known as shelterin consists of telomere-associated proteins (Figure 3.1) and is essential to protect the chromosome from critical shortening caused in part by oxidative stresses and DNA replication\textsuperscript{65, 68}. Mutations in the shelterin complex frequently result in unregulated cell growth in both germ and somatic cells\textsuperscript{66}. Regulation by posttranscriptional modification, localization, and phosphorylation of these telomere related proteins protect the telomere from unregulated telomere shortening\textsuperscript{65, 66, 68, 69}.

Shelterin consists of Telomere Repeat binding Factors 1 and 2 (TRF1 and TRF2), Protector Of Telomeres 1 (POT1), TRF1 and TRF2- Interacting Nuclear protein 2 (TIN2), and POT1 and Tin2 Organizing Protein (PTOP1, Figure 3.1B, Table 3.1), the presence and localization of these proteins throughout the cell determines TERT access to the telomere\textsuperscript{65, 69, 129}. TRF1 and TRF2 homodimerize to telomere specific 5'-TTAGGG-3' repeats, through Myb DNA binding domains present on both factors\textsuperscript{71, 130}. Their binding inhibits TERT access and protects against non-replicative telomere attrition. TRF1 is
necessary for efficient telomere replication and successful mitosis\textsuperscript{131}, as TRF1 null mutants are embryonic lethal. In addition, upon phosphorylation of TRF1, a substrate of Akt, telomeres paradoxically become shorter\textsuperscript{132}. TRF2 maintains telomeres by forming T-Loops, the telomeric secondary structure important in preventing chromosomal fusions\textsuperscript{133}. TRF2 inhibits Ataxia Telangiectasia Mutated (ATM) dependent non-homologous end joining (NHEJ), preventing genomic instability\textsuperscript{134}. Loss of TRF2 is exceptionally toxic to cells; null mutants upregulate Ligase 4 dependent NHEJ and premature cellular senescence occurs\textsuperscript{135}. Unlike TRF1 and TRF2, POT1 binds to single stranded 5’-TTAGGG-3’ repeats on the 3’ overhang preventing chromosomal fusions and regulates access of TERT through interactions with PTOP\textsuperscript{136, 137}. The shelterin linchpin, PTOP (also known as TPP1, TINT1, or PIP1) binds Tin2 and POT1 limiting TERT access to the telomere and protects the complex from degradation signaling\textsuperscript{138}. Conversely, the POT1-PTOP heterodimer recruits and increases TERT processivity on critically short telomeres\textsuperscript{139}. Acting as a link between single and double stranded telomeres, Tin2 binds and stabilizes double stranded DNA binding proteins with PTOP-POT1, finalizing the complex (Figure 3.1B)\textsuperscript{140-142}. Tin2 regulates telomeres in several ways, in conjunction with PTOP, Tin2 recruits TERT\textsuperscript{139, 140}; inversely Tin2 can inhibit (ADP ribosyl)ate polymerases (PARPs) preventing TRF1 degradation\textsuperscript{143}. Together, the shelterin complex maintains the telomere by both recruiting and inhibiting TERT access to the telomere.

DNA is constantly bombarded with genotoxic consisting of reactive oxygen species (ROS), produced during metabolic respiration. These oxidative
stresses frequently produce DNA damage in the form of DNA double strand breaks (DSB)\textsuperscript{144}. Eukaryotic cells prefer the process of NHEJ as opposed to homologous recombination (HR) preferred by single celled organisms to facilitate DSB repair\textsuperscript{129}. NHEJ requires several factors including Ku86 (Ku80 in yeast) and DNA dependent protein kinase (DNApK)\textsuperscript{145, 146}. DNApK is the catalytic subunit necessary for DSB repair, however new insights pinpoint DNApK as a mediator of telomere stability and chromosomal integrity\textsuperscript{147}. Telomeric shortening and chromosomal fusions have been reported in cells deficient in Ku86 or DNApK\textsuperscript{146, 148}. However, when expressed Ku86 and DNApK may prevent telomeric attrition, cellular senescence, and promote proliferation possibly by recruiting and enhancing TERT processivity through a direct Ku-TERT interaction\textsuperscript{149, 150}.

The proto-oncogenic transcription factor c-Myc orchestrates multiple facets of cellular proliferation, survival, and differentiation\textsuperscript{151}. c-Myc dimerizes with Max proteins then binds to E box DNA sequences promoting transcription of numerous target genes, many known to promote G1-S transition\textsuperscript{72}. Endogenous c-Myc is necessary for normal heart development and is upregulated in response to cardiomyopathic stimuli\textsuperscript{152, 153}. However, deregulated c-Myc is known to promote neoplastic transformation in conjunction with other unregulated oncogenes\textsuperscript{154}. In the context of telomere biology, c-Myc is known to promote \textit{TERT} transcription through the 29 specific Myc/Max E box sequences, as a consequence of mRNA production TERT protein and activity is induced\textsuperscript{75}. In addition to c-Myc as a transcriptional activator of TERT, c-Myc also interacts with TRF1, freeing TRF1 from the telomere resulting in telomere lengthening\textsuperscript{155}.\textsuperscript{75}
Chapter 1 demonstrates the necessity of c-Myc activity for Pim-1 dependent telomere lengthening and increased TERT activity in CPCS.

PINX1, initially identified with PINX3 (FBX4), through yeast two-hybrid screens as proteins that interact with TRF1, and sufficiently inhibit TERT activity, arresting cellular proliferation and accelerating apoptosis \(^{156}\). Mutational analysis revealed that TERT is inhibited by interactions with the C-terminus of PINX1, as truncated C-terminal PinX1 was sufficient to inhibit TERT activity and increase crisis \(^{157}\). *In vivo* analysis revealed that PINX1 null cells injected into nude mice formed tumors rapidly, after only two weeks establishing PINX1 as a potent inhibitor of TERT with a role in cellular immortalization \(^{156}\). Collectively, these studies suggest that TRF1 recruits PINX1 to the telomere and once there binds to TERT to inhibit telomere elongation \(^{156}\). Conversely, TRF1-PINX1 absent short telomeres allow TERT access and subsequent telomere elongation \(^{158}\).

FBX4 also known as PINX3 is an E3 ubiquitin ligase that specifically ubiquitinates TRF1, marking the telomere factor for proteasomal degradation \(^{159}\). FBX4 was found to interact with αβ crystallin to form a complex termed SCIP \(^{160}\). The complex ubiquitinates Cyclin D allowing the cell cycle to progress \(^{161}\), interestingly, αβ crystallin is not necessary for FBX4 to ubiquitinate TRF1 \(^{162}\). By removing TRF1 from the telomere, TERT accesses and elongates telomeres, thus FBX4 is critical in regulating telomere maintenance \(^{159}\). The mechanism signaling FBX4 localization to the nucleus is unknown, it is hypothesized that FBX4 phosphorylation \(^{161}\) or another form of posttranslational modification signals FBX4 to short telomeres.
Tankyrase 1 and 2 are PARPs that accumulate in the cytoplasm and translocate to the nucleus where they specifically poly (ADP ribosyl)ate TRF1, releasing it from the telomere\textsuperscript{163, 164}. Ribosylated TRF1 exits the nucleus where it is marked for proteasomal degradation\textsuperscript{165}. Tankyrase has two isoforms 1 and 2 both of which appear to have similar functions\textsuperscript{166}, however mice with genetic deletion of both isoforms are embryonic lethal\textsuperscript{167}. Tankyrase inhibition on cells is not lethal either by interfering RNA or pharmacologics, but telomere attrition and premature senescence have been observed\textsuperscript{168}. Elongated telomeres without increases in TERT activity are a consequence of Tankyrase overexpression \textit{in vitro} and \textit{in vivo}\textsuperscript{169}. Some other substrates of Tankyrases include DNApK which maintains protein stability when ribosylated\textsuperscript{168} and NuMA which utilizes ribosylation to promote mitosis by stabilizing spindle poles\textsuperscript{170, 171}.

Herein, this chapter focuses on delineating a possible mechanism for the results demonstrated in Chapters 1. This mechanism concentrates on the shelterin complex and some telomere related proteins that are modulated upon Pim-1 overexpression. Understanding the mechanism of how Pim-1 drives TERT activity in order to lengthen telomeres will further the safe development of therapies to antagonize aging and treat heart disease through CPC mediated cardiac regeneration.
METHODS

CARDIAC PROGENITOR CELL ISOLATION, CULTURE, AND TRANSDUCTIONS

CPCs were isolated as previously described\(^8,9\), and then CPCs were cultured and transduced as described in Chapter 1.

IMMUNOBLOT ANALYSIS

Whole cell lysates isolated from CPCe, CPCeP, or CPCePDN were prepared in 1X SDS sample buffer and prepared as described in Chapter 1 Methods. Total Akt antibody was purchased from Santa Cruz and phospho 473 Akt was purchased from CST. All other primary antibodies are detailed in Table 3.1.

RT-qPCR

RNA was extracted and Q-PCR was performed as described in Chapter 1. Primer sequences are provided in Table 3.2. Results are the averages of three independent experiments +/- S.E.M.

IMMUNOCYTOCHEMISTRY

Immunocytochemistry on CPCs was performed as described in Chapter 1. Primary antibody information is provided in Table 3.1.

NUCLEAR/ CYTOPLASMIC FRACTIONATION

Nuclear/cytoplasmic fractionation was performed using an Ambion Inc. PARIS kit (AM1921) according to manufacture’s protocol with the addition of
three additional nuclear pellet washes with cell fractionation buffer to ensure minimal fraction contamination.

*Telomere Length Measurements*

Telomere length was performed as described in Chapter 1. Results are the averages of three independent experiments +/- S.E.M.

*Caspase 8 Activity Assay*

CPC apoptosis was determined by Caspase 8 activity (Clontech Laboratories, APT129). 250 ng/ml of staurosporine in 2.5% FBS media was used as a positive control for cell death. Results are averages of three independent experiments +/- S.E.M.

*Statistical Analysis*

All data are expressed as mean ± SEM. Statistical analysis was performed using Student’s T-test and ANOVA with Tukey’s posthoc analysis as appropriate. P values less than 0.05 were considered significant.
RESULTS

SHELTERIN COMPLEX EXPRESSION WITH PIM-1 OVEREXPRESSION.

CPCeP and CPCe controls were analyzed by immunoblot for components of the shelterin complex. TRF1 and TRF2 protein were upregulated (1.4 and 2.56 fold), while TIN2 remained unchanged in CPCeP compared to CPCe (Fig. 3.1). POT1 is upregulated 3.11 fold while PTOP is significantly decreased (3.32 fold). DNA damage signaling factor and TERT recruiter, Ku86 is upregulated 2.5 fold over CPCe and expression of DNApK remains unchanged. CPCeP possess 6.6 fold more c-Myc, consistent with investigations in murine hearts overexpressing Pim-176. TERT inhibitor, PINX1 is significantly downregulated (2.79 fold) whereas FBX4 increased 4.4 fold in CPCeP. Both isoforms of Tankyrase increase by 1.7 and 1.5 fold respectively, unexpectedly CPCePs contained 2.75 fold less TERT protein (Figure 3.1 A and B).

RT-qPCR analysis of telomere related factors revealed posttranscriptional regulation in CPCeP. TRF1, TRF2, Ku86, and c-Myc transcript levels increased significantly (2.12, 1.93, 1.41, and 2.01 fold respectively Figure 3.2A) in conjunction with protein, however Tin2, POT1, PTOP, DNApK, PARP1, FBX4, and Tankyrase 2 all had similar transcript levels compared to CPCe control. Analogous to their protein levels transcript levels of Tankyrase 1 and TERT are significantly reduced (77.3% and 26% of CPCe, respectively). Importantly, PinX1 mRNA is upregulated 7.58 fold despite having 2.79 fold less PinX1 protein versus CPCe, suggesting Pim-1 dependent PINX1 translational inhibition or
enhanced degradation (Figure 3.2A). Shelterin components TRF1 and POT1 have significantly less transcript in CPCePDN (1.84 and 1.74 fold), however CPCePDN had 2.13 fold more TRF2, substantially more than CPCe. DNApK and Ku86 are significantly downregulated (2.89 and 2.59 fold) whereas PARP1 is upregulated (1.46 fold) in the CPCePDN recapitulating shortened telomere phenotypes\textsuperscript{172} (Figure 3.2B). c-Myc mRNA is considerably upregulated in the CPCePDN (3.02 fold) similar to CPCeP, however unlike CPCeP, PINX1, FBX4, and Tankyrase 1 are significantly downregulated (2.03, 6.67, and 4.12 fold, respectively). TERT transcripts are significantly downregulated in CPCePDN similar to the levels in CPCePs (2.64 fold, Figure 3.2B). Together these results indicate a role for Pim-1 in telomere related factor protein and mRNA expression.

**Localization of Telomere Proteins**

Telomere maintenance is dynamically regulated by mechanisms dependent on shelterin factor’s expression and localization\textsuperscript{69}. Nuclear/cytoplasmic fractions were prepared in conjunction with immunoblot and immunocytochemistry (ICC) analysis to determine localization of telomere related proteins (Figure 3.3 A-I). TRF1 and TRF2 are expressed predominantly outside the nucleus in CPCePs suggesting that they are targeted for degradation (2.02 and 2.4 fold decrease Figure 3.3A-C). Tin2 exists throughout the cell but is 1.61 fold more nuclear localized in CPCeP; Figure 3.3D insets demonstrate co-localization of Tin2 with DNApK consistent with localization on the telomere. POT1 exists primarily in the nucleus (Figure 3.3A and 3.3E), however CPCeP
possess 2.52 fold more nuclear POT1 than control. PTOP, the link between the double and single stranded telomere is significantly decreased in the nuclear fraction of CPCeP cells (2.42 fold), an unexpected finding considering increased nuclear localization of Tin2 and POT1. Nuclear DNAPK and Ku86 are significantly increased (2.7 and 2.1 fold, Figure 3.3A), Figure 3.3D suggests that DNAPK is localized on the telomere together with Tin2, while Ku86 is substantially upregulated throughout the nucleus (Figure 3.3F). Nuclear c-Myc is more abundant (1.79 fold) suggesting increased c-Myc mediated transcription of target genes (Figures 3.3A and 3.3G). PINX1 and FBX4 are significantly enriched in the cytoplasm away from their targets (4.86 fold and 2.64 fold respectively, Figure 3.3H and 3.3I). PINX1 is not apparent in the nucleus of CPCeP cells suggesting no PINX1 mediated TERT inhibition (Figure 3.3H). Nuclear Tankyrase 1 and 2 are significantly reduced, in addition nuclear Tankyrase 1 and 2 tended to be co-expressed with TRF1 (Figure 3.3B, inset), suggesting polyADP ribosylation\textsuperscript{164, 165}. TERT expression is dramatically reduced in whole CPC lysates, however upon fractionation analysis TERT was found to be predominantly in the nucleus (3.1 fold over CPCe, Figure 3.3A and 3.3H).

Chapter 1 demonstrates Pim-1 interacts with TERT, in addition, TRF1 is pulled down with Pim-1 and appears to have less affinity in CPCePDN. These interactions were not detected in IGG controls or IPs performed with an irrelevant antibody (Figure 3.4). The inability of TRF1 to interact with Pim-1 in CPCePDN suggests that phosphorylation is needed for interactions.
Prolonged Nuclear TRF1 Results in Senescence and Apoptosis

Nuclear localization of FBX4 results in ubiquitination of TRF1 and telomere elongation\(^{159}\). To determine if Pim-1 induced FBX4 to ubiquitinate TRF1 a silencing RNA was utilized to efficiently knockdown FBX4 (Figures 3.5A and 3.5B). FBX4 knockdown results in nuclear accumulation of TRF1 (Figure 3.6A) and an unusual flattening phenotype (Figure 3.6B). Telomere length is severely diminished in HT1080 cell lines when FBX4 is knocked down\(^{159}\), yet remained unchanged in CPCeP despite the abnormal phenotype (Figure 3.6C), possibly due to the relatively small amount of nuclear TRF1 at basal levels (Figure 3.3B). Several cardiac genes were analyzed to determine if CPCs were differentiating in response to FBX4 silencing. All cardiac lineage markers were negative (data not shown). Interestingly, \(p16\) transcripts are significantly upregulated in siFBX4 CPCePs suggesting that the CPCs are exiting the cell cycle and becoming senescent. To confirm this, ICC of p16 demonstrated the presence of p16 protein only in siFBX4 treated (Figure 3.6E) and not siScramble treated nor CPCePs treated with a siRNA specific for p16 (Figure 3.5C). In addition to expressing p16, siFBX4 treated CPCs fail to proliferate and are substantially more apoptotic demonstrated by CyQuant DNA incorporation assay and Caspase 8 activity assay (Figures 3.6F and 3.6G).

Pim-1 overexpression transiently lengthens telomeres upon initial infection, which normalizes upon subsequent passages (Chapter 1). Increased telomere length correlates with an acute reduction in doubling time therefore we hypothesized that other known proliferative molecules might be involved in
normalization. One such CPC cell cycle regulator, Akt\textsuperscript{173} appeared to be involved. Akt signaling mediated by phosphorylation on Serine 473 diminished at passages similar to those when cell cycle time and telomere length normalize to control. Interestingly, pAkt levels do not statistically change in CPCe demonstrating that reductions in pAkt are CPCeP specific (Figure 3.7).
DISCUSSION

**Pim-1 and Shelterin**

Collectively, this chapter illustrates the dynamic control of the shelterin complex and its associated factors by Pim-1. These factors can be influenced in many ways in addition to expression. Subcellular localization, phosphorylation and ubiquitination of telomeric proteins determine function. For example an abundance of TRF2 in the cytoplasm has little effect on TERT inhibition. Many factors are known to act on TRF1, promoting degradation signaling either through heterodimerization\textsuperscript{155}, ubiquitination\textsuperscript{159}, poly(ADP) ribosylation\textsuperscript{164} or phosphorylation\textsuperscript{132}. c-Myc promotes telomere elongation by physically interacting with TRF1, possibly by dimerization\textsuperscript{174}. This interaction removes TRF1 from the telomere and allows TERT to access and elongate. FBX4 is an E3 ubiquitin ligase that specifically ubiquitinates TRF1 marking it for degradation\textsuperscript{159}. Figure 3.3I demonstrates the punctate cytoplasmic localization of FBX4 in CPCePs, an interesting localization consistent with a recent study implicating 14-3-3 in facilitating phosphorylation dependent FBX4 dimerization, which is necessary for FBX4 activity\textsuperscript{175}. Barbash et al. demonstrate phosphorylation of FBX4 by GSK3β facilitating dimerization leading to increased FBX4 activity and accelerated Cyclin D degradation\textsuperscript{160}. Pim-1 targets a similar consensus sequence to GSK3β, in fact they are both known to phosphorylate Cdc25a\textsuperscript{176} \textsuperscript{20}. In addition, Pim-1 is known to mediate Cyclin E expression, another Cyclin known to facilitate G1/S transition\textsuperscript{76}. FBX4 plays a critical role in cell cycle regulation, DNA damage
responses, and telomere maintenance, thus a possible FBX4/Pim-1 interaction is the subject of on-going studies. PARP signaling by Tankyrases 1 and 2 acts specifically on TRF1, TRF1 and Tankyrase co-localize, which implies ribosylation and TRF1 degradation. In order for Tankyrase to enter the nucleus it must be recruited by TRF1, long telomeres recruit TRF1 to the nucleus, which would provide an explanation for the increases in TRF1 mRNA, and protein (Figures 3.1-3.3). Tankyrase 1 is regulated by phosphorylated during mitosis\textsuperscript{163}, which would suggest a possible role for Pim-1 in regulating another telomere related factor’s activity, specifically on TRF1. Microtubule (-) ends are anchored by NuMA when phosphorylated by Pim-1, during mitosis phosphorylated NuMA recruits and is ribosylated by Tankyrase 1 which is necessary to stabilize microtubules and successfully segregate sister chromatids\textsuperscript{171}.

TRF1 is a substrate for many kinases, in various cell types, and can be phosphorylated in response to different stimuli. Phosphorylation results in telomere elongation in some circumstances and telomere shortening in others\textsuperscript{132, 177, 178}. The dominant view in phosphorylation dependent regulation of TRF1 is that during S phase TRF1 is dephosphorylated and TERT can access the telomere\textsuperscript{179}. After mitosis kinases like Akt, Casein Kinase 2, or Plk1 phosphorylate TRF1, phosphorylation is necessary for telomere binding and telomere preservation.

TRF2 is necessary in maintaining chromosomal integrity during all stages of the cell cycle\textsuperscript{71}. TRF2 binds to telomeres and possesses the potential to inhibit TERT at the distal ends of telomeres, and TRF2 is abundantly expressed
on long telomeres\textsuperscript{180}. In the absence of DNA damage, TRF2 is primarily regulated by telomeric repeats. Upon genotoxic stress either through senescence signaling or pharmacologic treatment, TRF2 is localized on telomere ends where it prevents chromosomal aberrations\textsuperscript{181-183}. CPCePs express 2.56 fold more TRF2 in conjunction with significantly longer telomeres, suggesting that TRF2 is bound to telomeres but does not prevent TERT access (Figures 3.1 and 3.3). Upon Dox induced DNA damage TRF2 becomes nuclear localized suggesting a protective role of TRF2 in response to Dox (data not shown). DNA damage induced TRF2 modifications include phosphorylation by ATM and ribosylation by PARP1; both result in prevention of chromosomal aberrations\textsuperscript{184, 185}. TRF2 is regulated by p53 in response to critically short telomeres and progression into cellular senescence\textsuperscript{186}. When cells have reached the end of their replicative lifespan p53 is upregulated, which activates an E3 ubiquitin ligase that specifically targets TRF2 degradation in the proteasome\textsuperscript{186}. In many cell types p53 is down regulated by Pim-1\textsuperscript{1, 187} and up regulated upon doxorubicin administration\textsuperscript{188}. These alterations in p53 expression would account for the elevated levels TRF2 at basal and post Dox conditions (Figures 3.1, and data not shown).

Nuclear Tin2 is essential for cell survival and telomere maintenance, by binding TRF1 and TRF2 Tin2 creates a strong connection that works to inhibit telomere erosion and prevents modifications of TRF1 and TRF2\textsuperscript{65}. CPCePs possess more nuclear Tin2 as a consequence of longer telomeres. Figure 3.3D
illustrates Tin2 expression on telomeres in CPCeP, and this localization most likely serves to protect telomere erosion.

Under basal conditions PTOP anchors POT1 to Tin2 as shown in Figure 3.1B, these interactions bridge single stranded telomeres to the double stranded complex\(^{189}\). PTOP/ POT1 disassembly leads to telomere elongation in various cancer cell lines\(^{190, 191}\). Evidence from this current study supports this phenomenon, in CPCePs, POT1 is significantly increased whereas PTOP expression is the reverse suggesting an open telomere phenotype typical for TERT elongation\(^{138}\). Interestingly, PTOP levels are substantially greater in the cytoplasm of Pim-1 overexpressing cells, increased cytoplasmic accumulation begs the question of a possible non-telomeric function for PTOP outside the nucleus.

**Pim-1 and DNA Damage Associated Factors**

The exact roles of DNApK at the telomere is not clear, however it is known that DNApK on its own does not activate telomerase, signal telomerase to the telomere, or lengthen telomeres\(^{192, 193}\). DNApK is involved in DNA capping and without DNApK chromosomal fusions are abundant, most situations involve recruitment of DNApK by Ku86 that in turn is recruited by TRF1\(^{148, 194}\). Once recruited to telomeric ends it is hypothesized that DNApK blocks further processing. Due to its size (470 kDs) one would speculate DNApK accumulation at telomeric termini will serve as a powerful means of protection. Figure 3.3A and 3.3D demonstrate the increased nuclear localization of DNApK in CPCePs
with additional co-localization of DNAPK at the telomere (Figure 3.3D, inset). Emerging research into DNAPK activity and function reveals a broader function for DNAPK outside of DNA damage response\textsuperscript{195}. DNAPK may function to regulate cell cycle\textsuperscript{196} and apoptosis\textsuperscript{197}, if so phosphorylation dependent DNAPK activation by Pim-1 would be an interesting mechanism explaining pro-proliferative and anti-apoptotic signaling in cells overexpressing Pim-1.

**Pim-1 and Telomere Associated Factors**

PinX1 is well characterized as a potent inhibitor of TERT\textsuperscript{158}, because of this PinX1 expression is thought to be a prognostic marker for tumorigenicity\textsuperscript{198}. Endogenous expression in non-tumorigenic cells is thought to be low and functions to inhibit transformation when recruited to the telomere by TRF1\textsuperscript{158}. The importance of c-Myc in Pim-1 overexpressing cells is well documented (Chapter 1), with co-expression resulting in a pro-proliferative and anti-apoptotic phenotype with CPCs having a particular emphasis in self-renewal\textsuperscript{76}. c-Myc is inversely expressed in regard to PinX1 in CPCePs, specifically c-Myc is 6-fold upregulated during basal conditions and down over 4 fold upon treatment with Dox (data not shown). Collectively, these results indicate that Pim-1 overexpressing cells are up regulating c-Myc, which increases TERT activity in the absence of PinX1. Results in Chapter 1 demonstrate further the necessity of c-Myc activity on telomere preservation. Antagonizing cellular senescence by modifying the Shelterin complex expression is an exciting intervention to extend the lifespan of CPCs and ultimately the myocardium.
# FIGURES

## Table 3.1: Telomere related proteins and functions.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Full Name</th>
<th>Size (kDa)</th>
<th>Normal Function</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pim-1</td>
<td>Proviral integration Site for Moloney Virus</td>
<td>34, 44</td>
<td>Proto oncogene, cell survival and proliferation</td>
<td>Cell Signaling</td>
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<tr>
<td>TRF1</td>
<td>Telomere repeat binding factor 1</td>
<td>66</td>
<td>Telomerase repressor, binds to double stranded telomeric DNA</td>
<td>Abcam</td>
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<tr>
<td>TRF2</td>
<td>Telomere repeat binding factor 2</td>
<td>65</td>
<td>Telomerase repressor, binds to double stranded telomeric DNA, important in T loops formation</td>
<td>Cell Signaling</td>
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<tr>
<td>TIN2</td>
<td>TRF1 interacting nuclear protein</td>
<td>40</td>
<td>Controls Tankyrase activity</td>
<td>Santa Cruz</td>
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<tr>
<td>POT1</td>
<td>Protection of telomeres 1</td>
<td>65</td>
<td>Bind single stranded telomere, protect from endogenous nucleases</td>
<td>Abcam</td>
</tr>
<tr>
<td>PTOP</td>
<td>POT1 and TIN2 organizing protein</td>
<td>57</td>
<td>Strengthens interaction between POT1 and TRF1</td>
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<tr>
<td>DNA pK</td>
<td>DNA protein kinase</td>
<td>460</td>
<td>End capping and creating T-Loops</td>
<td>Santa Cruz</td>
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<td>Ku86</td>
<td>DNA protein kinase</td>
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<td>Parp</td>
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<td>Poly(ADP-Ribosylation of TRF2</td>
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<td>46</td>
<td>TRF1 ubiquitin ligase specifically targets TRF1</td>
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<td>Tankyrase1/2</td>
<td>TRF1-interacting ankyrin related ADP-ribose polymerase</td>
<td>1.170, 2.130</td>
<td>ADP-ribose polymerase, targets TRF1 sends it out of the nucleus</td>
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<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
<td>98</td>
<td>Ribonucleoprotein that lengthen telomeres</td>
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Table 3.2. Primers used in this Dissertation.

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<th>Reverse Primer 3’-5’</th>
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<td>Tank2</td>
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<tr>
<td>TERT</td>
<td>TGAACACAGGCCACCGAC</td>
<td>GTTCCTCCGCCGGGCTG</td>
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Figure 3.1: Telomere related proteins in CPCeP.

A. Immunoblot analysis performed on passage 10 CPCe and CPCeP and probed for the indicated antibodies. Values are fold change CPCeP expression / CPCe expression.

B. Schematic of telomere related proteins with arrows indicating the effect of Pim-1 overexpression. Results are mean +/- SEM. *p<0.05 vs. CPCe. **p<0.01 vs. CPCe N=4 different passage whole CPC lysates.
**Figure 3.2: Shelterin Gene Expression with and without Dox Treatment.**

**A.** RT-qPCR analysis for indicated genes in CPCe, CPCeP, and **B.** CPCePDN with and without Dox treatment (1 µM for 4 hours). Results are mean +/- SEM. *p<0.05 vs. CPCe, **p<0.01 vs. CPCe, $p<0.05$ vs. untreated CPC. N=4.
**Figure 3.3: Localization of telomere related proteins.**

**A.** Distribution of telomere related proteins determined by nuclear/ cytoplasmic fractionation and immunoblot analysis. Values are fold change over the respective CPCe fraction. **B-I.** Representative immunofluorescent images of indicated telomeric factors in CPCe and CPCeP. Insets in B demonstrate co-localization of Tankyrase (red) and TRF1 (green) in CPCeP that is absent in CPCe. Insets in D demonstrate co-localization Tin2 (red) and DNApK (green) in CPCeP that is absent in CPCe. Results are mean +/- SEM. *p<0.05 vs. CPCe. ** p<0.01 vs. CPCe # p=0.06 vs. CPCe. N=4 fractionation preps.
Figure 3.4: Pim-1 interacts with TRF1.

Immunoprecipitations pulling down Pim-1 followed by IB for Pim-1 in CPCeP and GFP in CPCePDN to identify exogenous Pim-1/GFP fusion. Antibodies against TERT, TRF1, and Pim-1 are used to identify interactions. Lane 1 is IGG control, lanes 2-4 are IPs for Pim-1 in CPCeP (GFP is not fused in CPCeP), lanes 5-7 are IPs for myc tag in CPCeP, lanes 8-10 are IPs for Pim-1 in CPCePDN, lane 12 is whole cell CPCePDN, and lane 13 is whole cell CPCeP. Pim-1/TERT interactions are discussed in Chapter 1.
Figure 3.5: FBX4 is efficiently knocked down using silencing RNA specific for FBX4.

A. RT-PCR of CPCe and CPCeP transfected with a scramble siRNA (siScr) and siRNA directed toward FBX4 (siFBX4). Values are normalized to 18S RNA, and are expressed as fold change over CPCe +siScr values. B. Immunoblot of CPCeP +Scr and CPCeP +siFBX4 probed with antibodies against FBX4 and GAPDH as loading control. C. CPCeP +siScr and CPCeP +siP16 transfected with siRNA directed toward p16 to verify p16 (red) antibody specificity, nuclei are identified with Sytox blue (green). Si transfections were performed on three independent experiments. **p<0.01 vs. CPCe and CPCeP siScr.
Figure 3.6: Senescence and apoptosis are induced by FBX4 knockdown.

A. TRF1 becomes localized to the nucleus in CPCeP transfected with siScr or siFBX4. Yellow boxes indicate the cells magnified to the right. B. CPCs have lost their spindle appearance and now possess cellular outgrowths. C. Telomere length measured by QFISH, CPCeP transfected with siScr or siFBX4 are not significantly different. D. RT-PCR analysis with p16 primers. E. ICC of siFBX4 treated cells for p16 (red), and GFP (white). F. CyQuant analysis of CPCeP to assess proliferation rate. G. Caspase 8 activity analysis of CPCeP treated with siScr or siFBX4 **p<0.01 vs. CPCeP +siScr. Results are averages of three independent experiments.
Figure 3.7: Phospho Akt levels diminish in CPCeP in late passages.

Phospho Akt on Serine 473 (pAkt473), and total Akt (tAkt), and GAPDH as loading control detected by immunoblot with quantitation below. CPCe quantitation is in blue and CPCeP is in red. + indicates HeLa cell positive control.
CONCLUSION OF THE DISSERTATION

Heart disease continues to be the leading cause of death in the western world, in fact the mean life expectancy for men and women would increase by as much as 7 years if all forms of CVD were abolished\textsuperscript{4}. The prevalence of CVD increases exponentially in the elderly population, making age a major risk factor for heart disease\textsuperscript{199}. According to the American Heart Association, the elderly account for 80\% of patients with coronary heart disease, 75\% of patients with congestive heart failure, and 70\% of patients with atrial fibrillation\textsuperscript{200}. Thus, reversing the aging cardiac phenotype would dramatically the burden of CVD in the health care system.

Initial reports of adoptively transferred stem cells into humans are positive, however the improvements are minimal and many question the ability of these protocols to produce long term recovery\textsuperscript{15, 16}. Limited regeneration stems from the inability of aged or diseased CPCs to engraft into the damaged myocardium, proliferate, survive, and differentiate into functional cardiac cells. Engineering CPCs with Pim-1 has been shown to enhance cardiac regeneration, but little was known mechanistically about how CPCeP maintained a superior regenerative capacity\textsuperscript{40, 201}. The data provided within this dissertation demonstrate that Pim-1 is able to reverse an aging phenotype by activating TERT in CPCs, thereby lengthening their telomeres (Figures 1.6 and 1.7).

The use of a lentivirus to express Pim-1 has been controversial due to the possibility of integration into a proto-oncogenic area of the genome and
subsequent chromosomal instability. These safety concerns can be tapered, because the effects of Pim-1 expression are acute, increased CPC proliferation and telomeric lengthening occurs early after infection and returns to control in later passages (Figure 1.3). These results further demonstrate the effectiveness of Pim-1 as a cardioprotective therapeutic with little risk of oncogenic transformation.

Dox is a commonly administered chemotherapeutic, that can cause cardiomyopathy\textsuperscript{202}. Dox induced cardiomyopathy is thought to stem from an induction of free radicals that attack all the cells in the heart\textsuperscript{203}. Oxidative stresses are known to decrease telomere length, and diminished telomeres lead to apoptosis\textsuperscript{204}. However, it is thought that Dox acts on the telomeres of CPCs preferentially, leading to severe CPC death and a diminished CPC pool that is unable to replace damaged myocytes\textsuperscript{13, 98}. Due to the relative success of Dox in treating various cancers, several methods aimed at diminishing collateral damage have been proposed. Administration of Dox together with antioxidants and metal ion chelators have been successful in treating some patients\textsuperscript{202}. Alternatively, CPC injections to replenish the damaged CPCs have been successful in mouse models\textsuperscript{13}. CPCePs are able to inhibit doxorubicin induced telomere attrition by activating TERT (Figure 1.13). In addition, CPCePs are resistant to doxorubicin induced apoptosis but this is most likely due to telomere protection and peripheral Pim-1 survival signaling at the mitochondria.

Pim-1 is a constitutively active kinase with many substrates, and data within this dissertation reveal two novel interacting proteins, TERT and TRF1
(Figure 1.11 and Figure 3.4). These interactions provide mechanistic insight into how Pim-1 protects the telomere and stimulates telomere lengthening. CPCePs maintain a high level of Pim-1 expression throughout passaging (Figure 1.4), thus the spatial-temporal expression of these other proteins most likely regulates their access to the telomere. Additionally, Pim-1 synergistically interacts with c-Myc, and this interaction is necessary for Pim-1 dependent telomere lengthening and TERT activity (Figure 1.12). Pim-1 overexpression dynamically regulates multiple genes related to telomere maintenance, elucidating the consequences of this will lead to combinatorial therapies to enhance cardiac regeneration.

As we age, DNA replication associated with karyokinesis and endogenous oxidative stresses erode the telomeres in cardiomyocytes\(^{205}\). Diminished telomeres lead to myocyte senescence and apoptosis, resulting in fewer cells and a poorly functioning heart\(^{206}\). The ability of Pim-1 to preserve and lengthen telomeres represents a potential prophylactic that may be administered to individuals with a history of heart disease. Implementing Pim-1 as a preventative therapy directly to the heart relies partially on the efficacy and safety of adeno-associated viruses (AAV). Currently, several phase I/II clinical trials are taking place, including one utilizing an AAV1-SERCA2a virus to treat heart failure\(^{207,208}\). A Pim-1 expressing AAV-9 has been utilized to treat diabetic cardiomyopathy in mice\(^{42}\), together with resistance to oncogenic transformation results presented within this dissertation, it may be possible to move AAV-Pim-1 studies into larger animals and humans.
MI is a catastrophic event that results in cellular necrosis, apoptosis, and fibrosis due to an influx of pro-death and pro-fibrosis molecules. One pro-fibrotic signaling molecule known to be up-regulated following MI is TGF1β. TGF1β activates TGF1β receptors 1 and 2 that phosphorylate several downstream SMAD transcription factors. Phosphorylated SMAD3 can act as a repressive transcription factor against TERT, diminishing TERT levels, and subsequently shortening telomeres. Chapter 2 implicates increased TGF1β as a mediator for decreased telomere length in Pim-KO neonate mice (Figure 2.1). When TGF1β receptor is blocked using pharmacological inhibitors, TGF1β is unable to inhibit TERT transcription. By blocking this pathway TERT lengthens telomeres to greater lengths than those observed with Pim-1 alone (Figure 2.6). These results suggest a combinational therapy and/or prophylactic using Pim-1 in addition to TGF1β receptor blocker to preserve and lengthen telomeres and prevent CVD. However, this depends on the caveat that both Pim-1 and TGF1β receptor inhibitor can be safely and effectively administered specifically to the myocardium.

The consequences of aging are inescapable, but slowing down the aging process is an exciting avenue for potential therapeutics. Future research harnessing the potential of Pim-1 mediated anti-aging effects rests with the safe administration of respective factors by either AAV vectors, additional virus types under the control of a myocardial specific promoter, plasmids capable of being transfected inside a beating heart, or a small molecule activator. The impaired stem cell pool observed in the elderly suggests that delivering Pim-1 will likely
need to be coupled with an adoptive transfer of CPCs, in order to facilitate cardiac regeneration. This strategy would most likely involve the isolation of CPCs from an elderly patient, transformation with pro-survival and pro-proliferative factors such as Pim-1 in a laboratory, followed by injection into the damaged myocardium. Successful interventions like these will not only restore the heart from injury, but will also return the heart to a more youthful phenotype free from heart disease.
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