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**TELOMERASE REVERSE TRANSCRIPTASE IN
ATHEROSCLEROSIS**

DISSERTATION

**A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the College of Medicine at the University of Kentucky**

By

Hua Qing

Lexington, Kentucky

**Director: Dr. Dennis C. Bruemmer, Professor of Pharmacology and Nutritional
Sciences**

Lexington, Kentucky

2017

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ABSTRACT OF DISSERTATION

TELOMERASE REVERSE TRANSCRIPTASE IN ATHEROSCLEROSIS

Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase and the limiting factor for the enzyme activity. The expression of TERT and telomerase activity is increased in atherosclerotic plaques. However, the role of TERT dysregulation during atherosclerosis formation remains unknown.

The work herein first identified a multi-tiered regulation of TERT expression in smooth muscle cells (SMC) through histone deacetylase (HDAC) inhibition. HDAC inhibition induces TERT transcription and promoter activation. At the protein level in contrast, HDAC inhibition decreases TERT protein abundance through enhanced degradation, which decreases telomerase activity and induces senescence. Furthermore, during vascular remodeling in vivo, TERT protein expression in the neointima is prevented by HDAC inhibition. These data illustrate a differential regulation of TERT transcription and protein stability by HDAC inhibition. TERT is highly expressed in replicating SMC of atherosclerotic and neointimal lesions. Using a model of guidewire-induced arterial injury, neointima formation was reduced in TERT-deficient mice. Studies in SMC isolated from TERT-deficient and TERT overexpressing mice with normal telomere length established that TERT is necessary and sufficient for cell proliferation. TERT deficiency did not induce a senescent phenotype but resulted in G1 arrest albeit hyperphosphorylation of the retinoblastoma protein. This proliferative arrest was associated with stable silencing of the E2F1-dependent S-phase gene expression program which could not be reversed by ectopic overexpression of E2F1. Chromatin immunoprecipitation and accessibility assays revealed that TERT was recruited to E2F1 target sites to increase chromatin accessibility for E2F1 by facilitating the acquisition of permissive histone modifications. These data indicate

a mitogenic effect of TERT on SMC growth and neointima formation through epigenetic regulation of proliferative gene expression. Furthermore, TERT expression is induced in activated macrophages during experimental and human atherosclerosis formation. To investigate the role for TERT in lesional macrophages and the subsequent effect on atherosclerosis formation, TERT-deficient mice were crossbred with LDL-receptor-deficient (LDLr^{-/-}) mice to generate first generation G1TERT^{-/-}LDLr^{-/-} offsprings, which were then further intercrossed to obtain third generation G3TERT^{-/-}LDLr^{-/-} mice. G1TERT^{-/-}LDLr^{-/-} mice revealed no telomere shortening while severe telomere attrition was evident in G3TERT^{-/-}LDLr^{-/-} mice. When fed an atherogenic diet, G1TERT^{-/-}LDLr^{-/-} and G3TERT^{-/-}LDLr^{-/-} mice were both protected from atherosclerosis formation compared to their wild-type controls, indicating that genetic TERT-deletion prevents atherosclerosis, and formation of the disease is not affected by telomere attrition. Similarly, atherosclerosis development was decreased in chimeric LDLr^{-/-} mice with TERT deletion in hematopoietic stem cells after bone marrow transplantation. TERT deficiency reduced macrophage accumulation in atherosclerotic lesions and altered chemokine expression, including CXCL1/2/3, CCL3, CCL5, CCL21, CCR7, IL-6, and IL-1 α . In isolated macrophages, gene ontology (GO) enrichment analysis of silenced inflammatory genes indicated that TERT positively regulates signal transducer and activator of transcription (STAT) cascade, which was confirmed by the decreased tyrosine phosphorylation of STAT3 protein resulting from TERT deletion. These findings indicate genetic TERT deficiency decreases atherosclerosis formation by silencing inflammatory chemokine transcription through inactivation of the STAT3 signaling pathway in activated macrophages.

In conclusion, the dysregulation of TERT expression within atherosclerotic plaques plays a causative role for vascular remodeling, including injury-induced neointima formation and hypercholesterolemia-induced atherosclerosis, through inducing SMC proliferation and a pro-inflammatory phenotype in infiltrating macrophages. These findings unveil a mechanism of TERT exacerbating the pathological vascular remodeling, which may provide a novel therapeutic target to combating vascular diseases.

KEYWORDS: Telomerase, Vascular remodeling, Cell proliferation, Smooth muscle, Macrophage, Inflammation

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2017-4-21

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**TELOMERASE REVERSE TRANSCRIPTASE IN
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1 CHAPTER ONE

Introduction

1.1 Atherosclerosis

1.1.1 Introduction

Atherosclerosis is the most common pathological process leading to cardiovascular diseases. Fatty streaks, which can start in childhood, are the initial atherosclerotic lesions as a result of macrophage infiltration and foam cell accumulation. Fatty streaks seldom cause clinical events but gradually evolve into complicated atheromatous plaques with a collection of immune cells, vascular smooth muscle cells (VSMC), necrotic cores, calcified regions, modified lipids, and foam cells. The chronic expansion of plaques narrows the lumen of the blood vessel and eventually causes obstruction, resulting in ischemia of downstream tissues. The clinical manifestations as a consequence of insufficient blood supply include myocardial infarction, stroke, abdominal aortic aneurysms or lower limb ischemia depending on the location of blocked vessel, which determines for a large part the extent of morbidity and mortality¹.

1.1.2 Smooth Muscle Cell Proliferation in Atherosclerosis

VSMC are primarily distributed in the medial layer of the artery. Disturbed laminar flow activates endothelium resulting in increased permeability of lipoproteins and inflammatory dysfunction. Under inflammatory stimulation, medial VSMC migrate into the intima and undergo dedifferentiation from a quiescent, contractile phenotype towards a synthetic, proliferative state that contributes to “intimal thickenings”. Therefore, abundant VSMC are found in the intima of specific sites with low shear stress that are more susceptible to atherosclerosis formation. Compared to the medial VSMC, the intimal VSMC are more mobile and proliferate, and they produce pro-inflammatory mediators that attract other immune cells to the site, thus creating the initial nidus for the development of atherosclerotic lesions².

Following activation and the subsequent inflammatory response of the endothelial cell (EC), VSMC can proliferate under the stimulation of multiple growth factors, including platelet-derived growth factor, transforming growth factor- β , angiotensin II, epidermal growth factor and insulin-like growth factor 1³. These various growth factors initiate signals converging to a cascade of biochemical events in the G1 phase of the cell cycle that permit cells to cross the G1/S checkpoint into the S (synthesis) phase. For example, the transition of cells from G1 to S phase in the cell cycle requires increased phosphorylation of retinoblastoma tumor suppressor protein (Rb). In a hypophosphorylated state, Rb sequesters the E2 factor (E2F) family of transcription factors, which are critical regulators of G1/S transition, thus activates the transcription of genes encoding

the enzymatic and structural machinery for DNA synthesis and chromosomal replication. Rb phosphorylation and subsequent E2F release are tightly controlled by multiple positive regulators, such as cyclin and cyclin-dependent kinase (CDK) complexes, and many negative effectors⁴. There are two families that prevent the progression of the cell cycle, the CIP/KIP (CDK-interacting protein/Kinase inhibitory protein) family and the INK4 (Inhibitor of Kinase 4) family. Members of both families prevent progression of the cell cycle by inactivating cyclin-CDK complexes. However, CIP/KIP inhibitors suppress the entire spectrum of CDKs, while INK4 family mainly inhibits CDK4/6 and arrests the cell cycle in the G1 phase. The CIP/KIP family includes p21, p27, and p57. p21 is activated by p53, which is triggered by DNA damage. The INK4 family includes p15, p16, p18, and p19⁵. These positive and negative regulators of cell cycle, as well as their targets, are outlined in Figure 1.1. The balance between the positive and negative branches of cell cycle regulators is of paramount importance to VSMC proliferation in the injured arterial wall and the initiation of atherosclerosis formation. Macrophages in Atherosclerosis

Atherosclerosis is a nonresolving inflammatory condition with continual recruitment of circulating monocytes into the intimal atherosclerotic lesions, where they differentiate into macrophages and scavenge cholesterol to become foam cells. During this process, both the lineage commitment and the functions of macrophages in atherosclerotic lesions are also altered. These phenotypic alterations of macrophages play important roles at all stages of lesion progression⁶.

It has been recognized that macrophages in atherosclerotic plaques originate primarily from circulating monocytes instead of local proliferation of tissue-resident macrophages⁷. Monocytes are derived from hematopoietic stem cells (HSC), self-renewing stem cells in bone marrow that continuously replenish all blood cell lineages. Differentiation of HSC produces common myeloid progenitor cells (CMP) and common lymphocyte progenitor cells (CLP), which undergo further differentiations while gradually losing their self-renewing potential and give rise to all types of blood cells along multiple lineages. CMP are the precursor of granulocytes, macrophage progenitors (GMP), megakaryocyte and erythroid progenitors (MEP), and macrophage and dendritic cell progenitors (MDP). MDP are the immediate precursor of monocytes, macrophages and dendritic cells but not granulocytes. Granulocytes, composed of basophils, eosinophils and neutrophils, are derived from GMP⁸. The entire blood cell collection with various flavors all as descendants of HSC (the process is known as Hematopoiesis) is illustrated in Figure 1.2. Monocytosis as a result of a series of successive proliferation/differentiation events from HSC, and MDP and an increase in the number of circulating monocytes, could thus heighten the chance of monocytes entering the arterial intima and expedite the development of atherosclerosis. There are two subtypes of monocytes, Ly6C⁺ and Ly6C⁻⁹. During inflammation, Ly6C⁺ monocytes infiltrate into the tissue and differentiate into M1 macrophages, which contribute to tissue degradation and T cell activation and are distinguishable from other groups of macrophages by the secretion of pro-inflammatory cytokines such as TNF α and IL-6¹⁰. In a steady state, Ly6C⁺

monocytes differentiate into Ly6C⁺ monocytes, which patrol the lumen through crawling on the resting endothelium of blood vessels in healthy tissues. Ly6C⁺ monocytes are more likely to mature to M2 macrophages, which secrete anti-inflammatory cytokines and contribute to tissue repair¹¹.

Macrophages are the dominant immune cells in atherosclerotic lesions at all stages of the disease. The inflammatory macrophage phenotype induced by interferon- γ (IFN- γ) and the toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) has been classically termed the M1 type. These stimuli initiate a strong inflammatory program in the macrophage, which includes production of proinflammatory cytokines and chemokines, inducible nitric oxide synthase (NOS) producing large quantities of nitric oxide (NO), and production of reactive oxygen species to aid in pathogen destruction. An alternative form of macrophages named M2 subtype are induced by interleukin-4 (IL-4) and IL-13 and produce proresolving molecules, such as IL-10 and transforming growth factor- β . The M2 macrophages are involved in tissue remodeling and repair¹². However, since the distinctive characteristics for classifying the M1 and M2 subtypes are derived from experiments in vitro, it is still an open question whether this classification bears any significance when applied to in vivo conditions. The phenotype of the lesional macrophages, which are exposed to a plethora of stimuli, is more complex as it constantly responds to dynamic cues from the microenvironment by intracellular signal-relaying cascades and therefore could change with the progression of the disease. Nevertheless, the pro-inflammatory macrophages have been shown to be the dominant phenotype advanced human endarterectomy lesions, where IFN-

γ is more abundant than IL-4¹³. The inflammatory responses of macrophages are the result of an intricate network of signal transductions and gene expressions. For example, activation of the TLR pathway triggers nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and Mitogen-activated protein kinases (MAPK) to initiate their respective signaling network, which amplify the original signal into the expression of thousands of inflammation-related genes¹⁴. Therefore, the net proatherogenic effect of lesional macrophages is likely due to the highly complex crosstalk between various cytokines and their signaling effectors, which can be differentially triggered by a broad spectrum of stimuli. The process accompanying proinflammatory macrophage activation mainly includes three inflammatory pathways: signal transducer and activator of transcription (STAT) molecules activated by IFN γ binding to its receptor, NF κ B and MAPK triggered by the activation of TLR or tumor necrosis factor (TNF) receptor^{15, 16}. Activation of STAT protein is an essential corresponding downstream effect of TLR4 and IFN γ signaling pathways¹⁷. Therefore, the STAT proteins are described as central signaling molecules that control cellular adaption and functional reprogramming in response to environmental stimuli or stress¹⁸. A large number of cytokines, growth factors, and hormones function as the ligand to activate STAT pathways by binding to their membrane receptors, which subsequently triggers the phosphorylation of Janus kinase (JAK). JAK proteins are receptor-associated protein tyrosine kinases that activate STAT proteins by tyrosine phosphorylation. The STAT proteins are latent transcription factors residing in the cytoplasm which translocate to the nucleus when phosphorylated and activate transcription of target

genes¹⁹. The JAK/STAT signaling pathway is actively involved in regulating atherosclerosis formation. Secretion of pro-atherosclerotic cytokines and chemokines by macrophages in atherosclerotic lesions, which is a critical pathogenic step has been specifically attributed to activation of the IL-6/STAT3 signaling pathway²⁰⁻²⁴.

1.2 Telomerase Reverse Transcriptase

1.2.1 Regulation of Telomerase Reverse Transcriptase

1.2.1.1 Introduction

Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase, which is a ribonucleoprotein that synthesizes telomere repeat sequences to protect telomeres from replicative erosion²⁵. TERT is the limiting factor for telomerase activity in both cancer and normal somatic cells. It is critical for the maintenance and stabilization of linear chromosomes and the regulation of cellular senescence and immortalization²⁶. In the biological system, TERT expression is closely regulated in a context-dependent manner via a multi-tiered regulatory network involving genetic and epigenetic controls, post-transcriptional regulation and post-translational modification²⁷. The transcriptional control is the primary and rate-limiting regulation of TERT expression in many cells, for example, TERT is transcriptionally repressed in most human somatic cells and is upregulated through TERT promoter activation in most cancer cells²⁸. The TERT promoter contains binding sites for a number of transcription factors involved in cellular proliferation, apoptosis, differentiation, inflammation, and

tumorigenesis^{27, 29}. For example, our previous studies have uncovered disease-relevant/pathogenic VSMC proliferation as a result of TERT up-regulation by binding of Ets-1 or E2F1 to the TERT promoter^{30, 31}. Alternative transcript splicing of TERT as a result of the post-transcriptional regulation has been documented in many human tissues and cell lines, and likely plays a role in the regulation of telomerase activity and other cellular functions by TERT. The human TERT gene contains 16 exons and can be spliced into multiple isoforms. None of the identified alternatively spliced forms of TERT gene has reverse transcriptase activity, thus they cannot reactivate telomerase and elongate telomeres. Although it is assumed that most variants are degraded and unlikely translated into proteins, some TERT isoforms have been shown to be potentially oncogenic³². The observation that the abundance of TERT protein does not translate into telomerase activity leads to the discovery of post-translational modifications, e.g., the phosphorylation of specific serine, threonine, or tyrosine residues, as an important regulatory mechanism for TERT activity. These phosphorylation events facilitate TERT nuclear translocation thereby allowing for assembly of active telomerase to elongate telomeres³³.

1.2.1.2. Epigenetic Regulation of TERT Expression

Among the multilayered control of TERT expression and function described above, the chromatin environment and epigenetic status of the endogenous TERT locus are also pivotal for the regulation of TERT mRNA level. Epigenetic regulation of TERT expression is mainly achieved through tagging of the histone core

complexes and methylation of the CpG loci near the promoter region³⁴. In eukaryotes, genomic DNA is wrapped around histone octamers each composed of two tetramers of H2A, H2B, H3 and H4 subunits to form structural units known as nucleosomes, which can be further condensed into higher-order chromosomal architectures. Such tightly bundled configuration blocks the access of promoters and regulatory elements on the DNA by transcription-associated factors and the transcriptional machinery. To allow dynamic access to condensed DNA, chromatin and nucleosomes can be dynamically restructured in a process called chromatin remodeling, in which various covalent labels are added to or removed from the histone proteins that signal to the ATP-powered remodeling complexes which actually perform the restructuring³⁵. The N-terminal tails of the histones are subject to a variety of covalent modifications including acetylation, methylation, phosphorylation, and ubiquitination³⁶. Histone hyperacetylation, which can be enhanced by unchecked activity of histone acetyltransferase (HAT), marks active transcription due to the uncoiling of DNA and histones. On the other hand, removal of these acetyl groups by histone deacetylases (HDAC) reduces accessibility of the modified regions by allowing the chromatin to acquire a more compact conformation³⁶. HDAC can be inhibited by a class of competitive inhibitors, which facilitates stable histone acetylation resulting in enhanced nucleosomal unwrapping and activates gene transcription within chromatin regions that are otherwise heavily condensed³⁷. In human somatic cells that are terminally differentiated, the TERT gene locus lies within a compact, nuclease-resistant chromatin domain and remains inactive. The fact that HDAC inhibitors can

increase TERT transcription, as shown in multiple studies, suggests that native chromatin environment, i.e., formation of heterochromatins, is critical for the tight regulation of TERT during cell proliferation and differentiation³⁸⁻⁴¹.

1.2.2 Function of TERT

1.2.2.1 Telomeric Functions of TERT

Because of the “end replication problem”, the failure of the DNA replication machinery to replicate the end of linear chromosomes, chromatin erosion at the termini occurs in each round of DNA replication cycles in eukaryotic organisms. Telomeres, the repetitive DNA-protein complexes at the end of chromosomes, not only act as a buffer to counteract replication-associated shortening, but also conceal linear chromosome ends from being falsely identified as strand breaks and subject to inappropriate repair which usually results in detrimental chromosomal fusions⁴². Over time telomeres become critically shortened with successive cell divisions, which triggers replicative senescence, a status in which cells permanently exit cell cycle. As a result, normal somatic cells can only proliferate for a limited number of times/generations predetermined by their telomere length, the molecular basis for cellular senescence and normal human aging⁴³. Telomerase is an enzyme that maintains the integrity of telomeres by adding telomeric repeats to chromosome ends. Telomerase is composed of a catalytic subunit TERT, an RNA-containing subunit and associated proteins. The telomerase RNA serves as the template for reverse transcription by TERT²⁵. In humans, telomerase is ubiquitously expressed during the first weeks of

embryogenesis, after which it is generally repressed throughout embryonic development and remains inactive in normal somatic human cells, while in activated lymphocytes, germ line cells, stem cells, and cancer cells, the enzyme activity is maintained⁴⁴. Although TERT transcription is undetectable in matured somatic cells, its level is elevated during telomerase reactivation. Furthermore, telomerase activity can be fully restored by ectopic expression of TERT. Therefore, TERT is recognized as the most important limiting subunit for telomerase activity²⁶. Transcriptional regulation of TERT expression is suggested to be the dominant regulatory mechanism for the silencing of telomerase during cellular differentiation as well as its reactivation during carcinogenesis²⁷. Moreover, TERT-deficient mice exhibit significantly telomere erosion and phenotypes associated with telomere shortening, such as the loss of tissue renewal, in late generations of the TERT-deficient lineage obtained by successive cross-breeding of heterozygous TERT-deficient mice⁴⁵. Thus, TERT is necessary and crucial for telomerase activity and the maintenance of telomere integrity.

1.2.2.2 Non-Telomeric Functions of TERT

In addition to telomere elongation, accumulating evidence indicates that TERT also has fundamental functions that are independent of its enzymatic activity. These non-telomeric functions of TERT are broadly associated with the regulation of gene expression, modulation of cellular signaling, cell cycle regulation, reducing reactive oxygen species release, protection of mitochondria, inhibition of apoptosis, promotion of cellular and organismal survival, and

modulation of DNA damage response⁴⁶⁻⁴⁸. In addition, TERT was found to shuttle between different cellular compartments where specific localization can change under a variety of conditions, such as during the progression through different cell-cycle stages, cellular transformation and when the cells suffer from extensive DNA damage⁴⁹. This observation further reinforces the notion of telomere-independent functions of TERT involved in different cell signaling pathways when targeted to different locations or responding to different stimulations.

Among these telomere-independent activities of TERT, the role of TERT in gene transcription has been investigated in detail. Experiments of global expression profiling or single specific gene analysis have shown that TERT affects the expression of hundreds of genes, which involve in diverse cellular processes including growth control, energy metabolism, apoptosis, DNA damage response, wound healing, tumor metastasis and angiogenesis⁵⁰⁻⁵⁶. Similarly, our previous study also identified an extra-telomeric role of TERT in macrophage expression of matrix metalloproteinase-2 (MMP-2) during abdominal aortic aneurysm formation⁵⁷. To date, how TERT is involved in gene transcription is largely unknown. It has been suggested that TERT may function as transcription effector to recruit other transcription factors. For example, TERT interacts directly with the nuclear factor NF- κ B p65 subunit to stimulate inflammatory gene expressions⁵⁸. More emerging evidence indicates that TERT participates in the modulation of chromatin architecture. For example, TERT alters histone tail modification and chromatin in cells lacking TERT is more susceptible to micrococcal nuclease digestion⁵⁹. TERT also interacts with transcription activator BRG1, which is a

component of ATP-dependent chromatin remodeling complexes and has helicase and ATPase activities. The interaction between TERT and BRG1 regulates the expression of genes involved in the Wnt signaling pathway and contributes to heterochromatin assembly at locations distinct from telomeres⁶⁰. These findings imply novel molecular mechanisms of TERT in many essential cellular processes by targeting genes and pathways beyond its well defined function in telomere biology.

In summary, the canonical function of TERT is telomere elongation as a component of telomerase. However, accumulating evidence indicates various non-telomeric functions of TERT, including regulating the expression of a broad spectrum of genes. As a transcription regulator, TERT may be present in different protein complexes and play essential parts in modulating chromatin structures (Figure 1.3). However, the mechanisms by which TERT controls gene transcription remain controversial and require further investigation.

1.2.3 Telomere and Telomerase Homeostasis in Atherosclerosis

1.2.3.1 Introduction

Telomere attrition serves as a key checkpoint in the control of cell proliferation by triggering replicative senescence. Therefore, telomere-based senescence has been closely implicated in cancer development and aging⁴⁴. Telomerase activity and TERT expression are upregulated in most cancer cells in support of unrestrained cellular proliferation. On the other hand, lack

of telomerase activity in human somatic tissues and concomitant telomere erosion correlates with age-related pathologies^{61, 62}. Telomere-associated gene mutations, e.g. TERT or TERC, result in accelerated telomere shortening, which is the cause of three human disorders, dyskeratosis congenita⁶³, multiple different diseases collectively characterized by bone marrow failure⁶⁴, and idiopathic pulmonary fibrosis⁶⁵. Their clinical presentation is associated with an impaired proliferative capacity of cells in tissues requiring constant renewal, i.e. skin, oral mucosa and bone marrow, indicating the capacity to maintain adequate telomere length is vital for tissues with high proliferative potential. Telomerase reactivation by overexpressing TERT in cancer-resistant mice extends lifespan, supporting the hypothesis that repressed telomerase supports cancer resistance at the cost of longevity⁶⁶. Conversely, this trade-off for cancer resistance increases the risk for age-related degenerative diseases⁶⁷. Generally, telomeres erode with age, and reflect a cell's past proliferative history and future propensity for apoptosis, senescence, and transformation. However, it remains controversial whether telomere shortening contributes to the aging process and aging-related diseases, including atherosclerosis⁶⁸. Moreover, contrary to the general concept of the aging-associated telomere attrition, telomeres are actually elongated in the atherosclerotic plaques, a result of increased TERT expression and activated telomerase, the extent of which correlates with the severity grade of atherosclerosis⁶⁹⁻⁷¹. Therefore, during the pathogenesis of specifically atherosclerosis formation, telomeres may change differently in peripheral blood leukocytes and cells within local tissues. Also, together with elongated telomeres,

the upregulated TERT expression and reactivated telomerase within the arterial intima suggest a functional involvement of the telomeric system in vascular remodeling.

1.2.3.2 Telomere and Telomerase Homeostasis in Endothelial Cells

Aging is a major risk factor for the initiation and the development of atherosclerosis. There is growing evidence about telomere biology and cell senescence involved in the pathogenesis of cardiovascular diseases. Telomere shortening in EC was observed during atherosclerosis formation, which is considered to be caused by oxidative stress as a consequence of the disturbed blood flow⁷². Meanwhile, EC in human atherosclerotic lesions present a senescence-associated phenotype which can be rescued by ectopic expression of TERT using viral transduction⁷³. Chronic oxidative stress and diminished activation of PI3K/AKT pathway may be the underlying mechanism bridging telomeric aberrations and loss of EC viability⁷⁴. However, until now there is little proof to show a direct effect of TERT on stress-induced premature senescence of EC during atherosclerosis formation.

1.2.3.3 Telomere and Telomerase Homeostasis in Smooth Muscle Cells

The differentiation and maturation of human somatic cells occur with the suppression of TERT expression and telomerase activity. As telomeres shorten with each replication cycle, these cells gradually lose replicative competency and

permanently exit cell cycle⁴³. However, accumulating evidence suggests that during tissue renewal and healing processes, expression of TERT can be induced in response to various environmental cues, which enhances cell multiplication in the time of need²⁷. Similarly, inducible expression of TERT has been shown in proliferating SMC during neointima and atherosclerosis formation^{30, 31}. Multiple mechanistic models that link the activation of telomerase and proliferation of VSMC have been proposed. Minamino et al. demonstrated that TERT activation through phosphorylation and the subsequent nuclear translocation accounts for telomerase-driven cell life span⁷⁵. In addition, our previous study has identified that TERT is the key factor that mediates the restenosis-preventing effect of everolimus, which coats the stent and limits tissue overgrowth. SMC proliferation is dependent on the expression of TERT which facilitates E2F1 binding to S-phase gene promoters⁷⁶.

VSMC within human atherosclerotic plaques displays a senescence phenotype with a limited proliferative capacity⁷⁷. These cells present low level of TERT expression and short telomeres⁷⁸, indicating that a TERT-associated premature cellular senescence phenotype may participate in the pathogenesis of human atherosclerosis.

1.2.3.4 Telomere and Telomerase Homeostasis in Inflammation

Age-associated immune deficiency is considered a major contributory factor to the increased frequency of morbidity and mortality among the elderly. Adaptive immunity of ageing individuals deteriorates because of a progressive decline of

global T cell and B cell populations, possibly as a consequence of the exhaustion of the available pools of naïve lymphocytes and along with their predecessors. The innate immunity of aging individuals, however, is relatively well preserved although age-dependent alterations are frequently observed⁷⁹. Inflammation plays a crucial role for the development of atherosclerosis. The presence of short LTL in human atherosclerosis can be mainly attributed to increased leukocyte turnover during inflammation and the following accelerated telomere loss per replication⁸⁰. Similarly, telomere shortening in T lymphocytes and accelerated leukocyte turnover are evident in patients with chronic infection and inflammatory disease⁸¹,⁸². Therefore, telomere shortening is considered to be a feature of immunosenescence characterized by a decreased ability to produce antibodies, the decline in phagocytosis and cellular migration, as well as a reduction of cell numbers⁸³. Although atherosclerosis is an age-related disease, telomere erosion in mice shows an atheroprotective effect. The late generation TERC-knockout mice on an apolipoprotein E-deficient background developed fewer atherosclerotic lesions, which has been associated with an impaired proliferative capacity of macrophages and lymphocytes⁸⁴. Therefore, telomere attrition might cause cellular senescence, and apoptosis in different cell types, but have variable effects on complex disease processes in a tissue-specific pattern.

Immune cells are unique among normal somatic cells as the activated phenotype can up-regulate telomerase and limit telomere attrition in the process of continued cell proliferation. Telomerase activity and TERT expression in immune cells have been demonstrated to be elevated during viral or bacterial

infection, as well as in a variety of chronic diseases related with low-grade inflammation, including atherosclerosis, idiopathic pulmonary fibrosis, rheumatoid arthritis, and systemic lupus erythematosus⁸⁵. Consistent with the dysregulation of TERT and telomerase in both acute and chronic scenarios of inflammation, telomerase activity and TERT expression have been shown to regulate the secretion of inflammatory cytokines^{58, 86-89}.

Considering these collectively findings described above, TERT protein expression and telomerase activity are inducible in various cell types circulating in the vessel and telomere shortening in genetic models may impact vascular dysfunction. However, whether TERT deficiency and loss of telomerase activity play a causal role in vascular remodeling and atherosclerosis formation is incompletely understood.

1.3 Hypothesis and Specific Aims

The cellular mechanisms underlying vascular remodeling during atherosclerosis formation include endothelial cell activation and subsequent macrophage infiltration. Among the many proteins and enzymes implicated in tissue repair, telomerase is recognized as one of the most important proteins involved in remodeling processes. However, its role in inflammation and vascular disease remains unknown. Our recent studies have confirmed an increased expression of TERT in atherosclerosis and after vascular injury^{30, 31, 71, 32, 72}. TERT is highly induced by mitogenetic and atherogenic stimulation in SMC and macrophages. However, the role of TERT dysregulation within the arterial intima

and the consequence of inducible vascular TERT overexpression in the process of vascular remodeling remains unknown.

Aim One: TERT transcription is dependent on chromatin remodeling, and HDAC inhibition prevents atherosclerosis formation and neointima formation. Therefore, the first aim is to define whether and how pharmacological HDAC inhibition targets TERT expression during proliferative vascular remodeling.

Aim Two: Aberrant proliferation of VSMC in response to injury induces pathological vascular remodeling during atherosclerosis and neointima formation. Telomerase is the rate-limiting factor for cell replication and tissue renewal whose physiological roles in vascular diseases remains to be determined. Therefore, the second aim is to determine whether TERT affects proliferative vascular remodeling and to identify the molecular mechanism of TERT-driven VSMC proliferation.

Aim Three: TERT is highly induced by atherogenic lipoproteins and inflammatory cytokines in macrophages. Thus, we will continue to investigate the function of TERT overexpression in macrophages during atherosclerosis formation. The third aim is to define the role and mechanisms of TERT in macrophage activation and the consequent development of atherosclerosis.

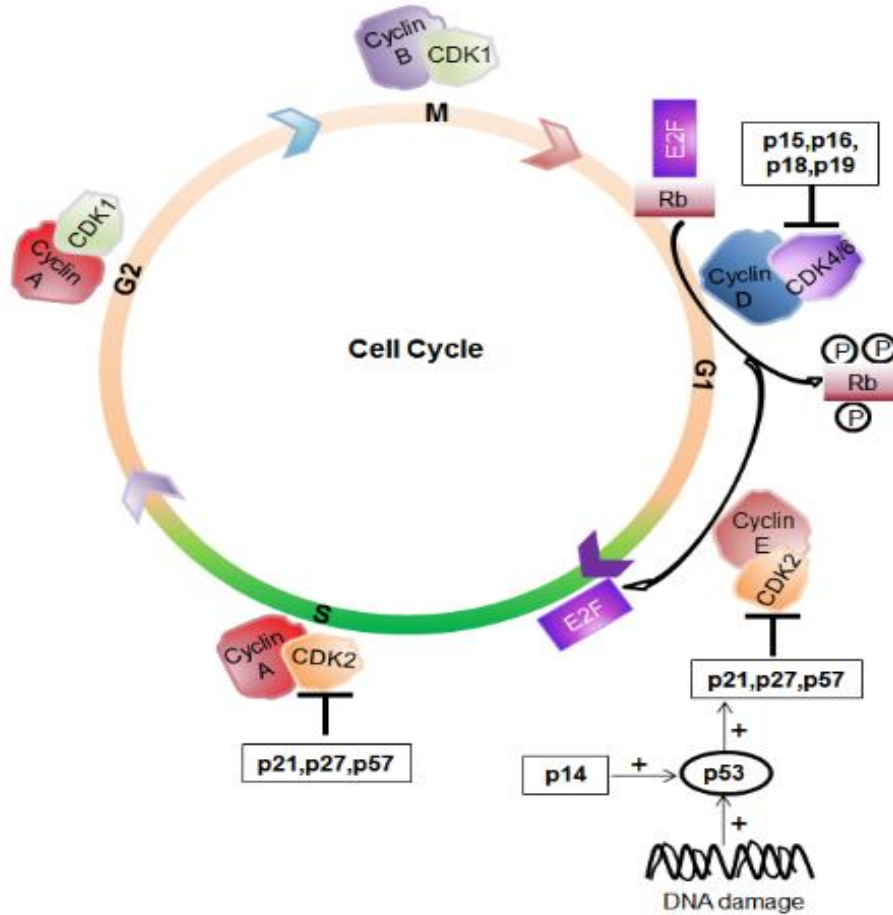


Figure 1.1 The Regulation of Cell Cycle.

Cyclins and cyclin-dependent kinase (CDK) complexes are the positive regulators to drive cell cycle forward. Conversely, the INK4 family (p15, p16, p18, and p19) and CIP/KIP family (p21, p27, and p57) are the main inhibitors to inactivate cyclin-CDK complexes and halt the progression of the cell cycle. Besides, P53, triggered by DNA damage, is another negative master to stop the cell cycle by promoting P21 production. p14 prevents the degradation of p53, thus links to the p53/p21 pathway to inhibit cell-cycle progression.

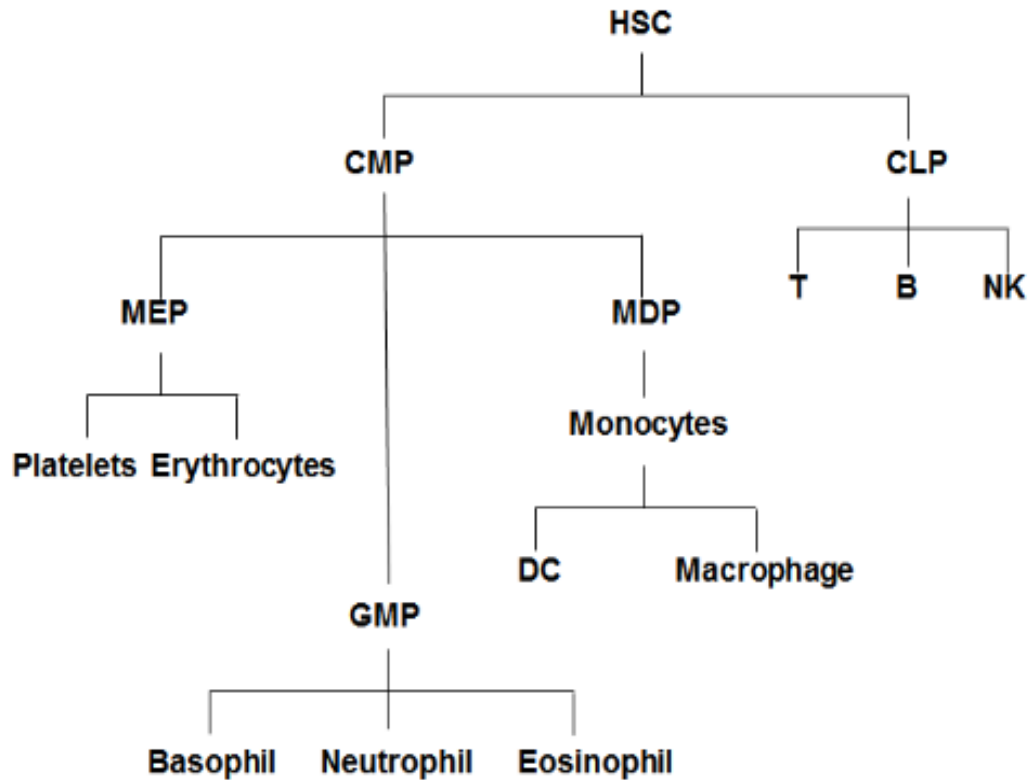


Figure 1.2 Development to Macrophages from Hematopoietic Stem Cells.

The figure illustrates the differentiation pathways of hematopoietic stem cells (HSC) that give rise to the various hematopoietic cell lineages. CLP, common lymphoid progenitors; CMP, common myeloid progenitors; GMP, granulocyte and macrophage progenitors; MDP, macrophage and dendritic cell progenitors; MEP, megakaryocyte and erythroid progenitors; DC, dendritic cell; NK, natural killer.

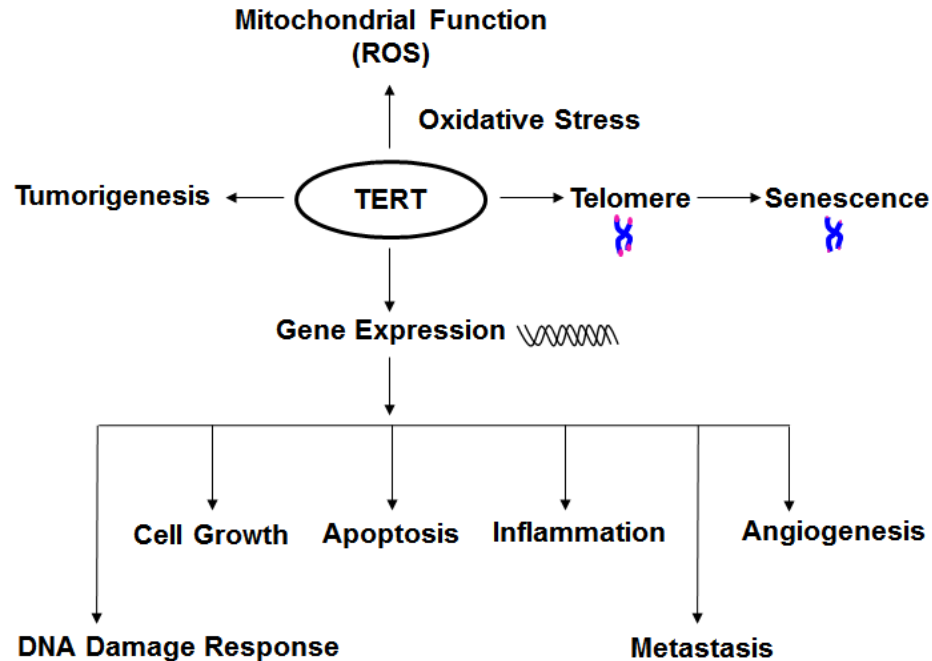


Figure 1.3 Functions of Telomerase Reverse Transcriptase

The canonical function of telomerase reverse transcriptase (TERT) is the maintenance of telomere integrity. Alternatively, TERT may function as a transcription cofactor or participate in chromatin remodeling, thus playing a role in the regulation of gene expression. The genes that might be regulated by TERT are involved in many essential cellular functions and biological processes, such as cell growth, DNA damage response, apoptosis, inflammation, angiogenesis, and metastasis. These extra-telomeric functions of TERT may play an important part in tumorigenesis. In addition, under oxidative stress, TERT translocates from nucleus to mitochondria, which could modify mitochondrial functions such as its metabolic capacity, energy efficiency and reactive oxygen species (ROS) production by the electron transferring complexes.

2 CHAPTER TWO

Differential Regulation of Telomerase Reverse Transcriptase Promoter Activation and Protein Degradation by Histone Deacetylase Inhibition

2.1 Abstract

Telomerase reverse transcriptase (TERT) maintains telomeres and is rate limiting for replicative life span. While most somatic tissues silence TERT transcription resulting in telomere shortening, cells derived from cancer or cardiovascular diseases express TERT and activate telomerase. In the present study, we demonstrate that histone deacetylase (HDAC) inhibition induces TERT transcription and promoter activation. At the protein level in contrast, HDAC inhibition decreases TERT protein abundance through enhanced degradation, which decreases telomerase activity and induces senescence. Finally, we demonstrate that HDAC inhibition decreases TERT expression during vascular remodeling *in vivo*. These data illustrate a differential regulation of TERT transcription and protein stability by HDAC inhibition and suggest that TERT may constitute an important target for the anti-proliferative efficacy of HDAC inhibitors.

2.2 Introduction

Telomeres are repetitive DNA sequences which protect the ends of chromosomes and are maintained by the catalytic activity of telomerase reverse

transcriptase (TERT)⁹⁰. Since most adult somatic cells transcriptionally repress TERT, telomeres shorten progressively during tissue renewal⁹¹. This telomere shortening has been suggested to limit the replicative potential and ultimately induce cellular senescence⁹². Conversely, TERT expression is induced and telomerase is activated during cancer and atherosclerosis development, both being the most prevalent human diseases^{70, 71, 93}. The observation that TERT overexpression induces cancer formation^{94, 95} and provides an unlimited mitotic potential⁹⁶ has suggested a permissive role for TERT to sustain cancer cell proliferation. Consequently, there has been significant interest to develop targeted TERT therapeutic approaches, which are currently being tested in clinical trials⁹⁷.

While a critical contribution of TERT to oncogenesis has been well-described, its role in atherosclerosis and neointima formation remains unknown. TERT expression and telomerase activity are induced during atherosclerosis formation and in response to vascular injury^{30, 70, 71}. At the cellular level, mitogenic stimulation induces TERT transcription and telomerase activity in vascular smooth muscle cells (VSMC)⁹⁸. In vitro, this transient expression of TERT is both necessary and sufficient for the proliferation of VSMC^{30, 31}, pointing to an important function of TERT for the proliferative response underlying vascular disease. Because TERT transcription is dependent on chromatin remodeling⁹⁹, we investigated in the present study whether inhibition of histone deacetylation induces TERT transcription. Furthermore, since histone deacetylase (HDAC) inhibition prevents atherosclerosis formation and neointima formation^{100, 101}, we evaluated whether pharmacological HDAC inhibition targets TERT expression

during proliferative vascular remodeling. We provide direct evidence that inhibition of histone deacetylation activates TERT transcription but decreases its protein stability, ultimately resulting in repression of telomerase activity and senescence, mechanisms that likely contribute to the inhibition of neointima formation by HDAC inhibitors.

2.3 Materials and Methods

Cell Culture

Rat aortic vascular smooth muscle cells (VSMC) were purchased from Lonza and maintained in DMEM supplemented with 10% FBS. Cells were starved in serum-free DMEM for 24 hours, pretreated with Scriptaid (Enzo Life Sciences) at the indicated doses for 30 min and stimulated with 10% FBS for the indicated time points. For protein stability assays, 10 µg/ml cycloheximide (Sigma) was added to cell cultures to inhibit protein synthesis, and cytosolic and nuclear proteins were harvested subsequently at the indicated time points.

Quantitative Real-time RT-PCR

Total RNA was isolated with TRIzol® (Invitrogen) and reverse transcribed into cDNA using SuperScript II (Invitrogen). RNA expression of target genes was quantified using an iQ™ SYBR Green Supermix (BioRad). Primers used in this study are as follows: TERT, forward: 5'-GTACAGCTGCTGAGGTCATTCTT-3'; reverse: 5'-AAACACTGGGTCTGCTTCTTTTT-3'; P16, forward: 5'-

GGTCACCGACAGGCATAACTTC-3'; reverse: 5'-
AAAGGAGGGCTGAGGCCTAA-3'; P21, forward: 5'-
CCGGGAGAACATGTATTTTGGT-3'; reverse: 5'-
GGGTGTAAAGATTCGGGACAGT-3'; GADD45, forward: 5'-
TGGTGACGAACCCACATTCA-3'; reverse: 5'-ACTGGCACCCACTGATCCAT-3';
RPL3A, forward: 5'-GTACGCTGTGAGGGCATCAA-3'; reverse: 5'-
CTCGAGACGGGTTGGTGTTC-3'. Samples were analyzed in triplicate and
normalized to expression values of the housekeeping gene RPL3A. Data were
calculated using the $2^{-\Delta\Delta CT}$ method¹⁰².

HDAC siRNA Transfection

siRNA experiments were performed using the SMARTpool technology (Dharmacon RNA Technologies), which provides a mix of four different proprietary siRNAs specific for the target gene. VSMC were transfected with HDAC1, HDAC2, HDAC3 or scrambled siRNA (50 nM) using Lipofectamine™ RNAiMAX (Invitrogen) for 6 hours. Following transfection, cells were recovered in growth media overnight, serum-deprived for 24 hours and stimulated with growth media for 24 hours before collecting RNA.

Plasmids, Transient Transfections and Luciferase Assays

The TERT promoter constructs have been described previously³¹. VSMC were transiently transfected with 1.0 µg TERT promoter construct using

Lipofectamine 2000 (Invitrogen) in OPTI-MEM. Following transfection, cells were serum deprived, treated with 6 μ M Scriptaid and stimulated with complete growth media for 24 hours. Luciferase activities were analyzed using a Dual Luciferase Reporter Assay (Promega). Transfection efficiency was normalized to *Renilla* luciferase activities generated by cotransfection of pRL-null-renilla.

Western Blotting

Cytosolic and nuclear fractions were isolated using a Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) and protein was subjected to routine immunoblotting using the following antibodies: TERT (Rockland 600-401-252S), TBP (Abcam ab818) and GAPDH (Sigma G9545). Expression of GAPDH and TBP was used to assess for equal protein loading for cytosolic and nuclear compartments, respectively.

Telomerase Activity Analysis

Telomerase activity was measured using a quantitative Telomerase Detection Kit (Allied Biotech Inc.) per manufacturer's instructions. Telomerase activity was quantified by real-time PCR using whole-cell extract containing 0.5 μ g of protein.

Senescence Analysis

VSMC were treated with Scriptaid at the indicated doses for 48 hours and stained overnight for β -galactosidase activity using a Senescent Cell Histochemical Staining Kit (Sigma CS0030) according to the manufacturer's instructions.

Endovascular Femoral Artery Guide Wire Injury

Guide wire endothelial denudation injuries were performed in C57BL/6J mice (The Jackson Laboratory) at 8 weeks of age as described³⁰. Twelve mice were injected with Scriptaid or DMSO the day before injury, on the day of the injury, and every other day thereafter for 28 days. The dose of Scriptaid (3.5 μ g/g body weight intraperitoneally) used in this study was based on the doses used in our previous Scriptaid study¹⁰¹. All procedures on mice were approved by the institutional animal care and use committee at the University of Kentucky.

2.4 Results

2.4.1 TERT Transcription Is Induced by HDAC Inhibition

To first evaluate whether histone acetylation affects TERT transcription, we employed the HDAC inhibitor Scriptaid, which potently suppresses histone deacetylation in vascular cells and inhibits VSMC proliferation^{101, 104}. As depicted in Figure 2.1 A-B, pre-treatment of VSMC with previously published doses of the HDAC inhibitor Scriptaid induced TERT mRNA expression in a time- and dose-dependent manner¹⁰⁵. At the employed doses and kinetics Scriptaid did not induce

apoptosis, as assessed by Annexin V and propidium iodide staining (data not shown). In order to establish specificity of this pharmacological approach and to identify specific HDAC that regulate TERT mRNA expression, we next employed siRNA-knockdown of HDAC1, 2 and 3 in VSMC. Depletion of HDAC1, but not HDAC2 or HDAC3, significantly increased TERT transcript levels (Figure 2.1 C). To further evaluate the mechanism by which HDAC inhibition regulates TERT transcription, we next analyzed TERT promoter activity in Scriptaid-treated VSMC. Transient transfection of a luciferase reporter driven by the full-length TERT promoter revealed a solid induction of TERT promoter activity in response to HDAC inhibition by Scriptaid (Figure 2.1 D). Collectively, these findings confirm that pharmacological HDAC inhibition and HDAC1 depletion induce TERT transcription in VSMC.

2.4.2 HDAC Inhibition Decreases TERT Protein Levels and Suppresses

Telomerase Activity

We next analyzed whether the induction of TERT transcription by HDAC inhibition translated into increased TERT protein expression levels. However, surprisingly Scriptaid treatment potently decreased both nuclear (Figure 2.2 A) and cytoplasmic (Figure 2.2 B) TERT protein levels in a dose-dependent fashion. Since TERT is the limiting factor for telomerase activity, we further analyzed whether HDAC inhibition reduced the enzymatic activity of TERT. Consistent with the noted decline in TERT protein mass, inhibition of HDAC activity by Scriptaid significantly attenuated telomerase activity in VSMC (Figure 2.2 C). Albeit inducing TERT

transcription, these observations indicate that HDAC inhibition ultimately decreases TERT protein mass resulting in a decline of telomerase activity.

2.4.3 HDAC Inhibition Accelerates TERT Protein Degradation

Since Scriptaid decreased both nuclear and cytoplasmic TERT expression, we reasoned that HDAC inhibition might decrease protein stability rather than enhance nuclear export. Consistent with this notion, HDAC inhibitors have been previously reported to trigger protein degradation¹⁰⁶. To address this question, we pretreated VSMC with vehicle or Scriptaid and performed a chase experiment after inhibiting protein *de novo* synthesis with cycloheximide. As depicted in Figure 2.3, Scriptaid treatment significantly decreased TERT protein stability compared to vehicle treatment. Therefore, HDAC inhibition may destabilize TERT protein through post-translational modifications, an effect that ultimately prevails over increased transcription induced by histone acetylation.

2.4.4 HDAC Inhibition Induces VSMC Senescence

Depletion of telomerase activity induces telomere shortening and replicative senescence⁹². Therefore, we next investigated whether the depletion of TERT induced by HDAC inhibition resulted in cellular senescence. As expected and depicted in Figure 2.4 A, Scriptaid treatment of VSMC induced β -galactosidase expression, a well-established biomarker of senescence¹⁰⁷. Furthermore, Scriptaid treatment induced a gene expression program implicated in cellular senescence, including transcript levels of p16, p21 and GADD45 (Figure 2.4 B). In concert,

these experiments demonstrate that the decline in telomerase activity induced by HDAC inhibition is associated with VSMC senescence.

2.4.5 HDAC Inhibition Reduces TERT Expression in Neointima Following Vascular Injury

Pharmacological HDAC inhibitors suppress cell proliferation¹⁰⁸, and in our previous studies we demonstrated that HDAC inhibition by Scriptaid attenuates the proliferative response underlying pathological neointima formation¹⁰¹. Based on our in vitro data, we hypothesized that HDAC inhibition might suppress TERT expression in vivo during pathological neointima formation in response to vascular injury. To address this question, we analyzed TERT expression in femoral arteries of mice treated either with vehicle (DMSO) or Scriptaid and subjected to vascular injury using a model of endothelial denudation injury¹⁰¹. As depicted in Figure 2.5 A, nuclear TERT expression was highly induced during neointima formation. In contrast, the inhibition of neointima formation by the HDAC inhibitor was associated with significantly decreased vascular TERT expression, even after normalization for the reduced neointimal area (Figure 2.5 B). This finding establishes that TERT expression in the neointima is suppressed by HDAC inhibition, which may contribute to the prevention of neointimal VSMC proliferation observed with HDAC inhibition.

2.5 Discussion

Inhibition of histone deacetylation through pharmacological approaches is currently being evaluated in clinical trials as a novel promising anti-proliferative therapy¹⁰⁹. During vascular remodeling, HDAC inhibitors potently prevent neointima formation and inhibit aberrant VSMC proliferation¹⁰¹. Although telomerase activation constitutes a key mechanism implicated in the replicative potential of a cell⁹¹⁻⁹³, the regulation of TERT by HDAC inhibition and its importance as pharmacological target for the anti-mitotic efficacy of HDAC inhibitors in vascular cell types have previously not been evaluated. In the present study, we demonstrate that both pharmacological inhibition and genetic depletion of HDAC activate TERT transcription. However, TERT protein levels and telomerase activity are potently inhibited by HDAC inhibition due to accelerated protein degradation. This inhibition of TERT expression results in cellular senescence, an effect that likely contributes to the anti-proliferative efficacy of HDAC inhibitors during neointima formation in response to vascular injury.

TERT constitutes the limiting factor for the catalytic activity of telomerase¹¹⁰. While TERT expression is inherently associated with aberrant cell proliferation⁹³, most normal somatic cells epigenetically silence TERT promoter activity by DNA methylation and histone deacetylation^{111, 112}. However, during the activation of malignant cell proliferation, transcription factors sequester HDAC from the TERT promoter allowing subsequent histone acetylation and transcription from the promoter¹¹³. Similarly as during cancer formation, aberrant VSMC proliferation constitutes a hallmark of atherosclerosis development and neointimal remodeling².

During this proliferative process, VSMC transcriptionally induce TERT expression and telomerase activity, which are required for their mitotic response^{30, 31}. To our knowledge, data presented here are the first to establish that inhibition of HDAC activity or HDAC1 depletion activates TERT transcription in VSMC. Our observation that TERT promoter activation is induced by HDAC inhibition is consistent with several prior studies^{38, 39, 111}. Furthermore, siRNA-mediated knock-down confirmed specificity for HDAC1 in the repression of TERT transcription in VSMC. Conversely, overexpression of HDAC1 has been reported to lead to the repression of the TERT promoter in cancer cells³⁹. However, the regulation of TERT by histone deacetylation remains controversial, since TERT transcription has also been reported to be repressed by pharmacological HDAC inhibitors^{40, 114}. Therefore, the mechanisms underlying TERT transcription are likely dependent on the environmental cues and specific for various tissues.

Although Scriptaid treatment of VSMC activated the promoter, TERT activity was reduced and TERT protein degradation enhanced by HDAC inhibition. In addition to activating transcription by histone tail modification, HDAC inhibitors are well-established to induce post-translational modification, including protein degradation through ubiquitination^{115, 116}. Moreover, while TERT expression is primarily regulated at the transcriptional level, posttranslational modification and proteasome-mediated degradation of TERT have been reported to play an important role for telomerase inactivation^{117, 118}. In support of a differential regulation of TERT transcript levels and protein abundance by HDAC inhibition is a recent study in glioma cells that reported decreased TERT activity albeit

increased TERT transcription in response to HDAC inhibition¹¹⁹. The molecular mechanism underlying enhanced TERT protein degradation by HDAC inhibition is likely indirect through increased lysine acetylation of non-histone proteins, thereby regulating protein stability. One reasonable hypothesis supported by the literature for future investigation may involve increased acetylation of the TERT chaperone HSP90 by HDAC inhibition¹²⁰, an effect that would result in enhanced protein degradation of its well-established client TERT¹¹⁷.

HDAC inhibitors target TERT expression in cancer cells^{40, 121} and have recently provided promising results in phase II clinical trials^{122, 123}; however, their efficacy in human vascular disease remains unknown. In preclinical models, HDAC targeting stabilizes atherosclerosis and prevents proliferative remodeling during neointima formation following endovascular injury^{100, 101}. In this study, we extend these observations and demonstrate that HDAC inhibition downregulates TERT expression during neointima formation *in vivo*. Specific targeting of telomerase activity in a rat model of vascular injury has recently been demonstrated to prevent neointima formation¹²⁴. Furthermore, TERT depletion is well-established to inhibit VSMC proliferation and induce cellular senescence^{30, 31}, consistent with our observation that Scriptaid-dependent downregulation of TERT protein abundance increased beta-galactosidase staining and senescence-associated gene expression. Therefore, although the inhibition of VSMC proliferation and neointima formation by Scriptaid in our previous studies likely involves targeting of multiple mitogenic proteins¹⁰¹, TERT repression may play an important role for the anti-proliferative efficacy of HDAC inhibitors.

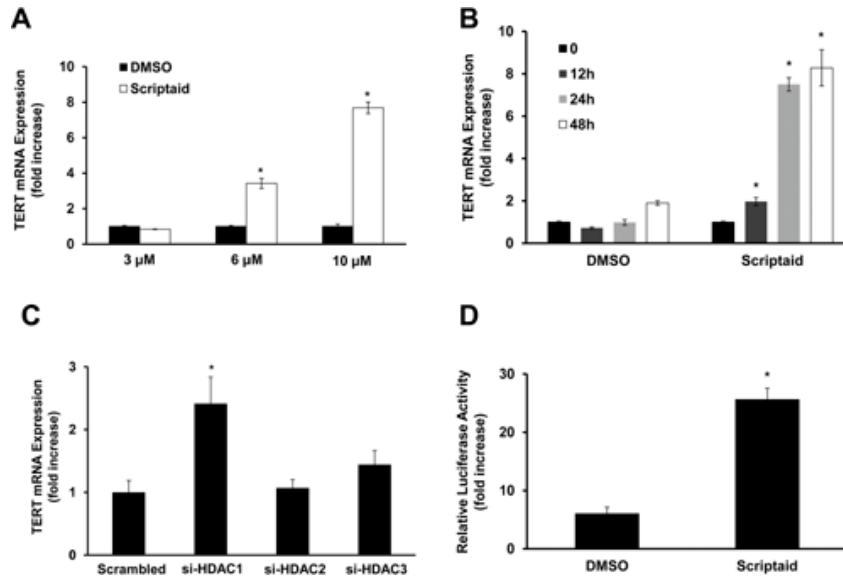


Figure 2.1 HDAC Inhibition Induces TERT Transcription.

A-B, Scriptaid increases TERT mRNA expression in a dose- (A) and time-dependent (B) manner. TERT mRNA expression levels were analyzed by quantitative real-time RT-PCR and normalized to transcript levels of the housekeeping gene RPL3A. Values are mean \pm SEM (* $P < 0.05$ vs. DMSO treatment). C, HDAC1 siRNA increases TERT mRNA expression. VSMC were transfected with siRNA targeting HDAC1, 2 and 3, or Scrambled siRNA control. Values are mean \pm SEM (* $P < 0.05$ vs. scrambled siRNA). D, Scriptaid activates the TERT promoter. VSMC were treated with 6 μ M Scriptaid or DMSO after transfection with a TERT promoter construct driving a luciferase reporter. Transfection efficiency was adjusted by normalizing firefly luciferase activities to *Renilla* luciferase activities generated by cotransfection with pRL-CMV. Values are mean \pm SEM (* $P < 0.05$ vs. DMSO).

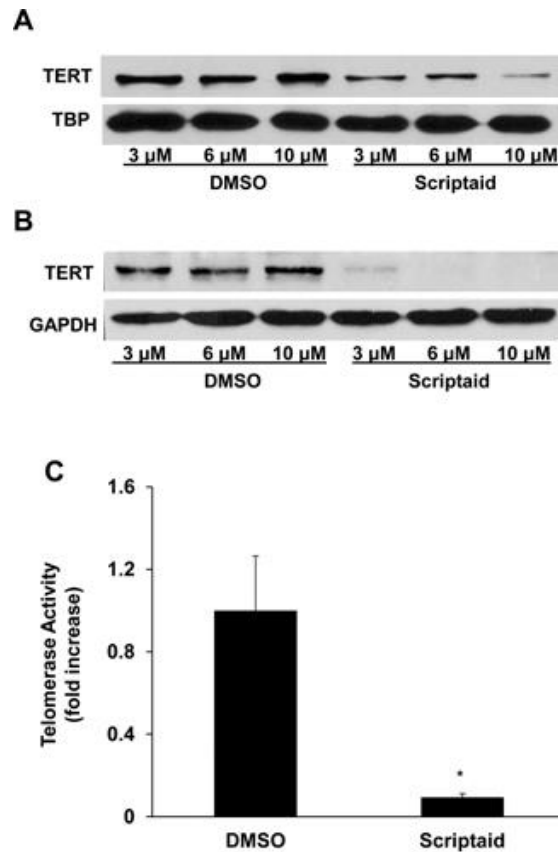


Figure 2.2 HDAC Inhibition Decreases TERT Protein Levels and Telomerase Activity.

A-B, Scriptaid down-regulates TERT protein expression. TERT protein expression levels were analyzed by Western blotting in nuclear (A) and cytoplasmic (B) fractions harvested after 24 hours of Scriptaid or DMSO treatment. Cohybridization for TBP or GAPDH was performed to control for equal protein loading. The autoradiographs shown are representative of 3 independently performed experiments. C. Scriptaid decreases telomerase activity in VSMC. Telomerase activity was analyzed 48 hours after 3 μ M Scriptaid or DMSO treatment. Values are mean \pm SEM (* P < 0.05 vs. DMSO).

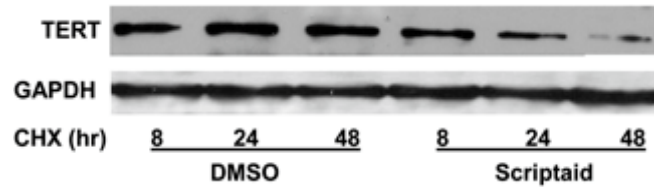


Figure 2.3 HDAC Inhibition Accelerates TERT Protein Degradation.

After Scriptaid or DMSO pre-treatment, cycloheximide (CHX 10 μ g/ml) was added to inhibit protein synthesis and whole cell lysates were harvested at the indicated time points. The autoradiographs shown are representative of 3 independently performed experiments.

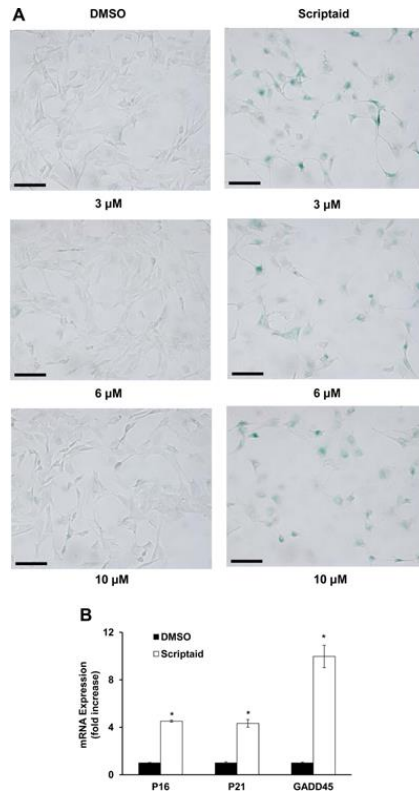


Figure 2.4 HDAC Inhibition Induces VSMC Senescence.

A, Scriptaid enhances senescence-associated β -galactosidase activity. Serum-deprived VSMC were pretreated with Scriptaid or DMSO at the indicated doses for 30 minutes, and then stimulated with 10% FBS for 48 hours. Scale bars, 100 μ M. B, Scriptaid induces senescence-associated gene expression. mRNA was harvested 24 hours after 6 μ M Scriptaid or DMSO treatment. P16, P21, and GADD45 mRNA expression levels were analyzed by quantitative real-time PCR and normalized to transcript levels of the housekeeping gene RPL3A. Values are mean \pm SEM (* $P < 0.05$ vs. DMSO treatment).

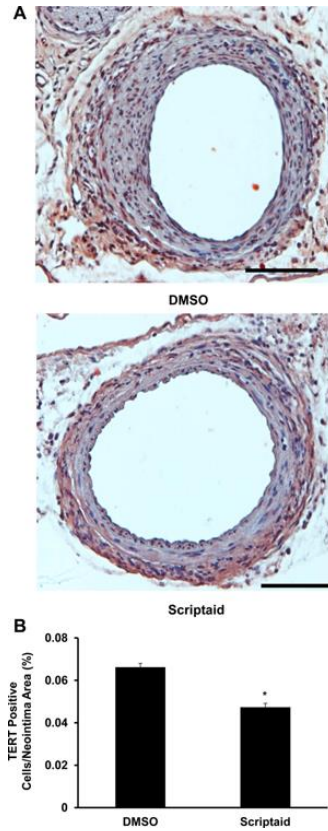


Figure 2.5 HDAC Inhibition Reduces TERT Expression in the Neointima Following Vascular Injury.

A, Representative TERT staining in the neointima after vascular injury. Tissues were stained for TERT (red) and nuclei counterstained with hematoxylin (blue). Scale bars, 100 μ M. B, Quantification of TERT-positive cells in the neointima. The percentage of TERT-positive cells to the neointima area was calculated as the amount of TERT-positive cells divided by the neointima area. Cross sections with the same distance from sutures were examined (n= 3 per group). Values are mean \pm SEM (* P < 0.05 vs. DMSO treatment).

3 CHAPTER THREE

Telomerase Reverse Transcriptase Deficiency Prevents Neointima Formation Through Chromatin Silencing of E2F1 Target Genes

3.1 Abstract

Objective: Aberrant proliferation of smooth muscle cells (SMC) in response to injury induces pathological vascular remodeling during atherosclerosis and neointima formation. Telomerase is rate limiting for tissue renewal and cell replication; however, the physiological role of telomerase in vascular diseases remains to be determined. The goal of the present study was to determine whether telomerase reverse transcriptase (TERT) affects proliferative vascular remodeling and to define the molecular mechanism by which TERT supports SMC proliferation.

Approach and Results: We first demonstrate high levels of TERT expression in replicating SMC of atherosclerotic and neointimal lesions. Using a model of guide-wire induced arterial injury, we demonstrate decreased neointima formation in TERT-deficient mice. Studies in SMC isolated from TERT-deficient and TERT overexpressing mice with normal telomere length established that TERT is necessary and sufficient for cell proliferation. TERT-deficiency did not induce a senescent phenotype but resulted in G1 arrest albeit hyperphosphorylation of the retinoblastoma protein. This proliferative arrest was associated with stable

silencing of the E2F1-dependent S phase gene expression program and not reversed by ectopic overexpression of E2F1. Finally, chromatin immunoprecipitation and accessibility assays revealed that TERT is recruited to E2F1 target sites and promotes chromatin accessibility for E2F1 by facilitating the acquisition of permissive histone modifications.

Conclusions: These data indicate a previously unrecognized role for TERT in neointima formation through epigenetic regulation of proliferative gene expression in SMC.

3.2 Introduction

Telomerase has been implicated in multiple developmental processes including cell activation, differentiation, proliferation, and apoptosis⁴³. The catalytic activity of telomerase maintains telomeres, hexanucleotide DNA-protein complexes that protect the ends of chromosomes, and is rate-limiting for tissue renewal¹²⁵. Since most somatic cells repress the telomerase reverse transcriptase (TERT) catalytic core, telomeres shorten progressively during tissue renewal ultimately limiting lifespan⁹¹. In addition to the extension of telomeres, mounting evidence suggests that TERT exhibits a direct regulatory role in the activation of gene expression without apparent involvement of its well-established function in telomere homeostasis¹²⁶. Early profiling experiments first documented that TERT induces differential gene expression⁵⁵. More recent support for a non-canonical activity of TERT derives from the observation that TERT induces cell proliferation independently of its catalytic activity and function to elongate telomeres^{55, 94, 127-129}.

Although the mechanisms underlying this non-canonical, mitogenic function of TERT remain elusive, TERT has been demonstrated to activate specific transcriptional programs and associates with chromatin at locations distant to telomeres, indicating a possible function of TERT in the epigenetic control of gene expression^{60, 130}.

Aberrant cell proliferation constitutes an important mechanism underlying proliferative vascular remodeling during atherosclerosis and neointima formation². Although telomerase is recognized as one of the most ancestral proteins in the control of mammalian cell proliferation, it remains to be investigated whether TERT causally contributes to these cardiovascular diseases. TERT expression is low in quiescent SMC of the normal vasculature^{71, 78}, but increased during neointima formation and atherosclerosis development^{30, 70, 71}. In cultured cells, TERT is induced in both activated macrophages and in proliferating SMC^{30, 70, 71, 98}. In SMC, the transient increase in TERT abundance in response to growth factor stimulation serves a mitogenic role since inhibition of TERT by oligonucleotide or pharmacological targeting inhibits SMC proliferation^{30, 31, 70, 75, 98, 131}. Furthermore, acute telomerase downregulation using TERT antisense RNA decreases proliferation of SMC isolated from genetically hypertensive rats¹³². Although recent genetic experiments established that the mitogenic activity of TERT requires neither telomere extension nor the catalytic activity^{55, 127-129}, the molecular mechanisms by which TERT supports cellular proliferation remain elusive.

In this study, we employed a genetic model of TERT-deficiency to investigate the transcriptional mechanisms underlying the mitogenic activity of

TERT during proliferative vascular remodeling. Our studies reveal that TERT deletion prevents neointima formation and SMC proliferation by inducing a silenced chromatin environment at S phase gene promoters.

3.3 Materials and Methods

Immunohistochemical Staining

For immunohistochemical staining of TERT expression in human coronary atherosclerotic lesions, segments of coronary arteries were harvested from explanted hearts during cardiac transplantation as described⁷¹. Tissues were fixed in 4% phosphatebuffered formaldehyde and embedded in paraffin, and transverse sections (8 μ m) were collected. Serial paraffin-embedded sections were incubated with a mouse monoclonal TERT antibody (ab5181, Abcam) or mouse monoclonal anti-smooth muscle α -actin antibody (ab18147, Abcam) followed by biotinylated goat anti-mouse IgG (BA-9200, Vector Laboratories) and counterstaining with hematoxylin. For immunofluorescent colocalization studies, tissues were incubated with antibodies raised against TERT (GTX82476, GeneTex), PCNA (ab2426, abcam) and α -SMA (ab7817, abcam), followed by incubation with Alexa Fluor 647-conjugated goat anti-chicken, Alexa Fluor 488-conjugated goat anti-mouse, and Alexa Fluor 594-conjugated goat anti-rabbit antibodies (A-21449, A11001 & A11012, Invitrogen) and counterstaining with DAPI. Using a similar protocol, paraffin-embedded sections of mouse femoral arteries subjected to guide wire-induced injury or sham surgery were stained with PCNA (ab18197, abcam) or TERT (MA5-16034, Thermo) followed by incubation with Alexa Fluor 488-

conjugated goat anti-mouse and Alexa Fluor 594-conjugated goat anti-rabbit antibodies (A11001 & A11012, Invitrogen) and counterstaining with DAPI.

Cell Culture

Primary human coronary artery SMC and growth media were commercially obtained (Lonza) and cultured per the manufacturer's instructions. Primary murine aortic vascular SMC were isolated from mice as described. TERT^{-/-} mice were obtained from The Jackson Laboratory (TERT^{-/-} mice¹³³, stock number 005423, The Jackson Laboratory) and maintained using heterozygote parental breeders⁵⁷. SMC were isolated from the first generation offspring littermate G1TERT^{+/+} and G1TERT^{-/-} mice. To obtain third generation G3TERT^{-/-} offsprings, G1TERT^{-/-} mice were interbred using cousin mating strategies. Mice overexpressing a murine TERT transgene (referred to as TERTtransgenic) under control of the ubiquitin C promoter were kindly provided by Drs. Richard Hodes and Jeffrey Chiang¹³³. Mice hemizygous for this transgene were interbred with C57BL/6J mice obtained from the Jackson Laboratory (C57BL/6J, Stock number 000664, The Jackson Laboratory) to generate littermate TERT wildtype mice (referred to as TERTwildtype). For all cell culture experiments, cells passage three to nine were used. Cells were grown to 60-70% confluence and serum deprived in 0.4% FBS (Invitrogen) for at least 24 hours, followed by stimulation with 10% FBS for the indicated time. All human and animal studies were performed with the approval of the University of Kentucky, Institutional Review Board or Institutional Animal Care and Use Committee, respectively.

TERT siRNA Transfection

siRNA experiments were performed using the SMARTpool technology (Dharmacon RNA Technologies), which provides a mix of four different proprietary siRNAs specific for human TERT. Human coronary artery SMC were transfected with TERT or scrambled siRNA (50 nM) using LipofectamineTM RNAiMAX (Invitrogen) for 6 hours.

Adenoviral Infection

Human coronary artery SMC, or G1TERT^{+/+} and G1TERT^{-/-} SMC were transduced with the indicated plaque-forming units (PFU)/cell of adenovirus overexpressing GFP (Adx-GFP), TERT (Adx-TERT/GFP), or E2F1 (Adx-E2F1). Transduction efficiency was verified at > 90% using fluorescence microscopy and Western Blotting. For 5-bromodeoxyuridine incorporation (BrdU) assays, SMC were incubated with BrdU reagent for 12 hours after 24-hours of adenoviral infection.

Proliferation Assays

For all proliferation assays, cells were serum-deprived for 24 hours and stimulated with 10% FBS for 48 hours. SMC proliferation was analyzed by cell counting using a hemocytometer. BrdU assays (Millipore) were performed per manufacturer's instructions. For analysis of cell cycle distribution, cells were trypsinized, fixed in ice cold 70% ethanol, treated with 40 µg/mL RNase (in PBS)

for 30 minutes at 37°C, and labeled with 50 µg/mL propidium iodide (in PBS). Cell cycle distribution was determined using a FACSCalibur sorting system (Becton Dickinson). For all data shown, individual experiments were performed at a minimum in triplicate using different preparations of cells.

Endovascular Femoral Artery Guidewire Injury

Guidewire-induced endothelial denudation injuries were performed on the left femoral artery of littermate G1TERT+/+ and G1TERT-/- mice (n=8/group) at 8-12 weeks of age with a 0.25-mm SilverSpeed-10 hydrophilic guidewire (Micro Therapeutics Inc, Irvine, Calif) as described¹³⁴. The denudation injury was accomplished with 4 passages of the wire. Mice were euthanized 28 days after injury, and femoral arteries were isolated and embedded in paraffin for tissue analysis. Sections were stained with an elastic Verhoeffvan Gieson staining kit (Sigma Aldrich) to visualize the internal and external elastic lamina. The intimal and medial areas were measured by computerized morphometry using Image-Pro Plus 7.0 software (MediaCybernetics) as described¹³⁴.

Western Blotting

Western blotting was performed as previously described¹³⁴ using the following antibodies: Phospho-Rb (Ser807/811) (9308, Cell Signaling), MCM7 (sc-9966, Santa Cruz Biotechnology), Acetyl-Histone H3 (Lys9) (9469, Cell Signaling),

TERT (600-401-252, Rockland), E2F1 (05-379, Upstate), CBP (7389, Cell Signaling) and GAPDH (sc-25778, Santa Cruz Biotechnology).

Quantitative RT-PCR

RNA isolation and quantitative real-time RT-PCR analyses were performed as recently described. Primer sequences are listed in Table I. mRNA expression levels were analyzed in triplicate and normalized to housekeeping genes TFIIB (murine) or TBP (human). For quantitative real-time RT-PCR analyses, data were calculated using the $2^{-\Delta\Delta C_t}$ method¹⁰².

Immunoprecipitation

Human coronary artery SMC extracts were immunoprecipitated overnight with 2 μ g rabbit IgG (2729, Cell Signaling), anti-TERT (600-401-252, Rockland), or anti-E2F1 (sc-193, Santa Cruz), followed by 4 hours incubation with protein A agarose beads (9863, Cell Signaling). The protein A-antigen-antibody complexes were washed five times with 1 x cell lysis buffer, resuspended with 3x SDS sample buffer, and heated at 100°C for 5min. Samples were then analyzed by Western Immunoblotting with indicated antibodies.

TERT shRNA Lentiviral Transduction

SMARTvector human lentiviral TERT shRNA (5548171, GE Dharmacon) was employed to deplete TERT expression (referred to as shTERT). SMARTvector

empty vector control (S-005000-01, GE Dharmacon) was used as negative control (referred to as shCTL). Human coronary artery SMC were infected with lentivirus at an MOI of 5 for 24 hours. Infection efficiency was verified at >90% after 48 hours infection by mRNA quantification using real-time PCR.

Chromatin Immunoprecipitation Assays

SMC were stimulated with FBS for 10 hours following 24 hours of serum-deprived starvation. Cells were then collected for Chromatin Immunoprecipitation (ChIP) assays using the MAGnify Chromatin Immunoprecipitation System (Invitrogen) according to manufacturer's instructions. 50µg of chromatin was immunoprecipitated with 5µg of antibody raised against E2F1 (sc-193x, Santa Cruz Biotechnology), TERT (582000, Calbiochem), Acetyl-Histone H3 (Lys9) (9649, Cell Signaling) or CBP (7389, Cell Signaling). Rabbit polyclonal IgG (Invitrogen) was used as a negative control. Immunoprecipitated DNA was amplified by quantitative RT-PCR using SYBR green. Primers used for quantitative PCR amplify nucleotides -346 to -176bp of the murine MCM7 promoter, -356 to -102bp of the murine cyclin A promoter, -305 to -97bp of the murine PCNA promoter, -355 to -190bp of the human MCM7 promoter, -250 to -100bp of the human cyclin A promoter, and -547 to -700bp of the human PCNA. In addition, primers targeting loci upstream of E2F1 binding sites were used as negative controls. Primer sequences are listed in Table I.

Chromatin Accessibility by Real-Time PCR (ChART-PCR)

G1TERT^{+/+} and G1TERT^{-/-} SMC were serum-deprived and stimulated with FBS for 12 hours. Following stimulation, nuclei were harvested using the protocol described by Rao et al. ¹³⁵and digested using *Cac8I* restriction enzyme (New England BioLabs). Digested DNA was purified using the QIAquick Gel Extraction Kit (QIAGEN) per manufacturer's instructions. 20ng of recovered DNA was analyzed by quantitative PCR using SYBR green. Primers used amplify nucleotides -346 to -176bp of the murine MCM7 promoter and encompass two E2F1 binding sites and three *Cac8I* restriction sites. Decreased PCR amplification of this sequence indicates *Cac8I* digestion and increased chromatin accessibility. Results were normalized using primers which amplify upstream sequences - 620 to -513bp of the murine MCM7 promoter lacking *Cac8I* restriction sites (listed in Table I) and expressed as percent accessibility.

Telomere Length Analysis

For quantification of telomere length using real-time PCR, samples were run in triplicate with 35 ng of DNA per reaction using an iCycler and SYBR Green I system (Bio-Rad) as described. Telomere repeat copy number data were normalized to 36B4 as single-copy gene. The primer sequences are listed in Table I.

Senescent Staining

Murine SMC were seeded at a density of 8×10^5 cells per well on a 6-well plate and serum-deprived in DMEM containing 0.4% FBS. After 24 hours, senescent staining was performed as previously described using the Senescent Cells Histochemical Staining Kit (Sigma Aldrich).

Statistical Analysis

To compare two groups on a continuous response variable, we used a 2-sample Student *t* test or Mann–Whitney *U* test as appropriate. One-way ANOVA was used to compare multiple groups, followed by Tukey post hoc analysis. All data are presented as mean \pm SEM.

3.4 Results

3.4.1 TERT Is Expressed in Replicating Neointimal Smooth Muscle Cells

TERT protein expression was first analyzed in neointimal SMC of human coronary artery atherosclerotic lesions from hearts explanted during cardiac transplantation. While TERT expression is considered negligible in normal arteries^{71, 78}, we confirmed abundant TERT expression in the SMC-rich neointimal region of the fibroatheroma (Figure 3.1 A). Furthermore, confocal microscopy confirmed that TERT expression colocalizes with proliferating cell nuclear antigen (PCNA), a cofactor of DNA polymerase required for replication (Figure 3.1 B). Similarly, TERT was highly expressed in replicating SMC during the proliferative

response following vascular injury of the mouse femoral artery (Figure 3.1 C). Therefore, this upregulation of TERT expression may indicate an important role in vascular remodeling underlying neointima formation.

3.4.2 TERT Is Required for Smooth Muscle Cell Proliferation and Neointima Formation

Inhibition of TERT has previously been shown to inhibit SMC proliferation^{75, 98, 131}. Using siRNA-mediated TERT knock-down and adenoviral-mediated overexpression of TERT (Appendices Figure A.1A-C), we corroborated that TERT is both necessary and sufficient for SMC proliferation (Figure 3.2 A-B). To advance these studies to *in vivo* models, we next isolated primary aortic SMC from TERT-deficient and TERT overexpressing mice¹³³. Consistent with the well-described phenotype^{26, 136-138}, the first generation G1TERT^{-/-} progeny from heterozygote founder mice exhibits normal telomere length (Appendices Figure A.2) and none of the phenotypes associated with telomere attrition. However, homozygous intercrossing of G1TERT^{-/-} mice results in progressive telomere loss in third generation G3TERT^{-/-} mice (Appendices Figure A.2). To further complement this genetic TERT depletion model, we studied proliferation of SMC isolated from C57BL/6 mice overexpressing a murine TERT transgene under control of the ubiquitin C promoter (referred to as TERTtransgenic)¹³³. When exponentially growing aortic SMC from these mice were analyzed (Figure 3.2 C), cells isolated from hemizygous TERTtransgenic mice displayed a marked growth advantage compared to their littermate controls (referred to as TERTwildtype). Conversely,

SMC isolated from G1TERT^{-/-} mice exhibited a complete growth arrest compared to G1TERT^{+/+} cells. Interestingly, the growth arrest occurred to a similar extent in G1 and G3TERT^{-/-} SMC, indicating that this mitogenic defect is primarily due to TERT deletion and does not require telomere attrition. To further corroborate that the mitogenic arrest is due to TERT deficiency, we overexpressed TERT in G1TERT^{-/-} SMC, which rescued proliferation (Figure 3.2 D). Based on these data and to investigate the regulation of SMC proliferation by TERT *in vivo*, we next analyzed the proliferative response underlying neointima formation in G1TERT^{-/-} mice. As depicted in Figure 3.3 A and B, neointima formation was significantly decreased in G1TERT^{-/-} mice compared to their wildtype G1TERT^{+/+} littermates. Collectively, these results indicate that TERT deletion decreases SMC proliferation *in vitro* and during neointima formation *in vivo*.

3.4.3 TERT Deletion Induces Cell Cycle Arrest and Silences E2F1 Target Gene Expression

We next sought to identify the mechanisms responsible for the growth arrest of G1TERT^{-/-} SMC. Using real-time PCR for sensitive quantification of telomere lengths, we first confirmed that G1TERT^{-/-} SMC did not display measurable evidence of telomere attrition (Appendices Figure A.2). Furthermore, TERT deficiency did not induce senescence-associated β -galactosidase staining or gene expression (Appendices Figure A.3 A-B). From these data we inferred that the growth arrest induced by TERT-deficiency was likely independent of the well-established function of TERT to extend telomeres and prevent cellular

senescence. Therefore, we next analyzed DNA synthesis and cell cycle distribution. These studies revealed that TERT deficiency inhibited mitogen-induced DNA synthesis (Figure 3.4 A) and arrested SMC in the G₀/G₁ phase of the cell cycle to reduce S phase entry (Figure 3.4 B-C). This transition from G₁ → S phase is mediated through phosphorylation of the retinoblastoma protein (Rb)¹³⁹. However, TERT depletion did not result in decreased Rb phosphorylation but rather hyperphosphorylation (Figure 3.4 D-E), indicating that TERT deletion alters DNA synthesis likely through a mechanism downstream of Rb phosphorylation.

Rb phosphorylation releases the transcription factor E2F1, which transactivates promoters required for S phase entry and ensuing DNA replication, including the *bona fide* E2F1 target genes cyclin A, PCNA, and minichromosome maintenance proteins (MCM)^{140, 141}. Although Rb was hyperphosphorylated in G₁TERT-deficient cells, downstream expression of these three E2F1 target genes was silenced (Figure 3.4 F). This differential S phase gene expression profile occurred in the absence of changes in E2F1 expression levels (Appendices Figure A.4). Similarly, other activating E2F family members were not affected by deletion of the TERT locus (Appendices Figure A.3.B). To further complement this finding, we employed siRNA-mediated knockdown of TERT in primary human coronary artery SMC. As depicted in Figure 3.4 G-I, the induction of MCM7 mRNA and protein expression in response to mitogenic stimulation was attenuated in TERT-depleted SMC. Complementary data was obtained for the E2F1 target genes cyclin A and PCNA (data not shown).

3.4.4 TERT Is Recruited to Chromatin and Required for E2F1 Binding to Canonical Sites

The silencing of E2F1 target gene expression in TERT-deficient SMC indicated that TERT might facilitate E2F1-dependent transcription. To assess this possibility, we first confirmed that E2F1 and TERT physically associate in human coronary artery SMC (Figure 3.5 A). However, to more directly test whether TERT is recruited to canonical E2F1 motifs, we performed chromatin immunoprecipitation (ChIP) assays using primer pairs that cover the E2F1 sites in the cyclin A, PCNA, and MCM7 promoters. As shown in Figure 3.5 B, mitogenic stimulation of G1TERT^{+/+} SMC induced not only E2F1 binding to its target site in the MCM7 promoter but also a potent and inducible co-recruitment of TERT. Further evaluation of the chromatin complex demonstrated that this recruitment of E2F1 and TERT was associated with increased acetylation of histone H3 (K9Ac-H3), a typical chromatin modification of gene activation¹⁴². To investigate whether TERT is required for E2F1 binding to chromatin and to validate the role of TERT in the regulation of E2F1-dependent transcription, we next analyzed whether TERT deficiency affects E2F1 recruitment to its response elements within the MCM7, Cyclin A, and PCNA promoters. As depicted in Figure 3.5 C, inducible TERT binding to E2F1 canonical sites in the MCM7 promoter was negligible in human coronary artery SMC depleted of TERT using a lentiviral approach (Appendices Figure A.1 D). Similarly, no TERT binding was observed at upstream non-canonical promoter regions (Appendices Figure A.6), confirming specificity of the ChIP assay. Comparable results were observed for the cyclin A and PCNA

promoters (data not shown). As further shown in Figure 3.5 D-F, this mitogen-induced E2F1 recruitment to the MCM7, cyclin A, and PCNA promoters was significantly reduced upon TERT depletion. A complementary impairment of E2F1 binding to its target sites was observed in G1TERT^{-/-} murine cells (data not shown). These studies confirm that TERT supports E2F1 binding to its target sites within promoters of genes required for entry into S-phase of the cell cycle.

3.4.5 TERT-Deficiency Decreases Chromatin Accessibility and Histone Acetylation

We next examined chromatin accessibility specifically at E2F1 binding sites using a Chromatin accessibility by Real-Time PCR (ChART-PCR) assay¹³⁵. Briefly, this highly sensitive assay to quantify chromatin accessibility combines DNase hypersensitivity with specific restriction enzyme digestion followed by PCR amplification, thereby providing a quantitative chromatin accessibility assessment at a specific transcription factor binding site. Genomic DNA from G1TERT^{-/-} and G1TERT^{+/+} SMC was subjected to DNase I, meningococcal nuclease, and *Cac8I* restriction enzyme digestion followed by real-time PCR using primers encompassing the E2F1 motif in the MCM7 promoter (Figure 3.6 A). The restriction enzyme *Cac8I* specifically cleaves the E2F1 motif TTTCGCGC, and accessible euchromatin will not be amplified by PCR since the primers cannot hybridize to the E2F1 site due to cleavage by the restriction enzyme. As shown in Figure 3.6 B, TERT-deficiency was associated with significantly reduced chromatin accessibility at the E2F1 site relative to G1TERT^{+/+} SMC. Since our data established that

TERT occupancy of E2F1 sites is associated with the enrichment of histone H3 acetylation, we further analyzed whether TERT-deficiency modulates the recruitment of permissive histone acetylation at E2F1 sites. As shown in Figure 3.6 C, FBS stimulation of human coronary artery SMC resulted in a profound increase in lysine 9 histone H3 acetylation of the E2F1 response elements within the MCM7 promoters. In contrast, this inducible histone acetylation was completely abolished in human coronary artery SMC transduced with TERT shRNA lentivirus. Similarly, mitogen-induced lysine 9 histone H3 acetylation was reduced at the protein level in G1TERT^{-/-} SMC (Figure 3.6 D-E), a finding that may at least in part contribute to the silenced chromatin state at E2F1 sites in TERT-deficient cells. Considering that the p300/CREB-binding protein (CBP) coactivator family confers histone acetyltransferase activity⁴¹ that is required for E2F1 activation of its S phase target genes⁴², we investigated whether CBP contributes to histone H3 acetylation during TERT-dependent E2F1 recruitment. A direct TERT-CBP interaction was first confirmed by immunoprecipitation (Figure 3.6 F). Furthermore, mitogen-induced CBP recruitment to E2F1 target chromatin within the MCM7 promoter was confirmed by ChIP. In contrast, inducible CBP recruitment was markedly impaired upon TERT deletion (Figure 3.6 G). Decreased H3 acetylation and altered CBP recruitment upon TERT depletion were also observed at the cyclin A and PCNA (data not shown). These findings suggest that TERT facilitates CBP recruitment and subsequent Histone H3 acetylation to disrupt repressive chromatin at E2F1 target chromatin.

To finally corroborate decreased chromatin accessibility as a possible mechanism underlying silencing of E2F1 target genes, we overexpressed E2F1 in G1TERT^{-/-} SMC. A silenced chromatin state accompanying TERT deficiency should produce a permanent insensitivity to E2F1-dependent proliferation, since E2F1 would be unable to access its consensus sites. As depicted in Figure 3.6 H-J, adenoviral-mediated transduction of E2F1 (Appendices Figure A.5) in SMC wildtype for the TERT locus increased expression of its target gene MCM7 expression and the ensuing cell proliferation and DNA synthesis. In contrast, this activity of ectopic E2F1 to induce target gene transcription and cell proliferation was altered in TERT-deficient SMC. Collectively, these experiments identify TERT as a component of the chromatin complex at E2F1 sites and establish a previously unrecognized role for TERT to activate E2F1-dependent gene promoters through chromatin modulation.

3.5 Discussion

Altered SMC function constitutes a pivotal component of pathological vascular remodeling and neointima formation^{2, 143}. In the present study, we illustrate a previously unrecognized mechanism that supports the mitogenic phenotype of SMC. The inducible expression of TERT in proliferating SMC during vascular remodeling and the requirement for SMC proliferation *in vitro* and neointima formation *in vivo* establish an important role for TERT in vascular biology. TERT is required for the activation of an S phase gene expression program, associates with chromatin at E2F1 consensus sites, and facilitates the

acquisition of a permissive chromatin environment through CBP-mediated histone acetylation. Collectively, these experiments support a novel role for TERT in the epigenetic control of SMC proliferation.

While expression of the catalytic subunit of telomerase TERT is considered to be negligible in the normal vasculature^{71, 78}, we observed that TERT is induced in proliferating SMC during the neointimal remodeling process resulting from atherosclerosis formation or vascular injury. Using loss and gain-of-function approaches combined with genetic TERT modulation in mice, our data demonstrate a causal relationship between TERT expression and the mitogenic response. Similarly, prior studies using oligonucleotide or pharmacological targeting of TERT documented decreased SMC proliferation^{75, 98, 131}. To extend these observations, we studied SMC proliferation in primary cells isolated from G1TERT-deficient mice *in vitro* and neointima formation *in vivo* in mice deficient for the TERT locus. This murine model has been widely employed to exploit telomere-independent functions of TERT in G1TERT^{-/-} mice with normal telomere lengths as well as phenotypes that occur as a result of progressive telomere attrition in third generation G3TERT^{-/-} mice^{131, 144-147}. Conversely, constitutive expression of TERT in transgenic mice does not elongate telomeres¹³⁶ since TERT only elongates critically short telomeres^{57, 148}. Therefore, this transgenic model provides another means to study TERT activities that occur independently of its function to extend telomeres^{94, 127-129}. In our experiments, TERT-deficiency in the presence of normal telomere length in G1TERT^{-/-} mice induced growth arrest of SMC and reduced neointima formation. In contrast, TERT overexpression in SMC

isolated from transgenic mice elicited a clear growth advantage. From these observations we infer that the SMC growth arrest induced by TERT deficiency does not depend on telomere attrition but rather on TERT function. Consistent with this notion is the accumulating evidence that TERT induces, for example, stem cell and cancer proliferation through telomere-independent mechanisms that do not require the catalytic activity^{94, 127-130}. Furthermore, proliferating cancer cells express splice variants deficient in catalytic activity, and expression of these variants in telomerase-negative cells induces cell proliferation¹⁴⁹, pointing to a novel non-canonical function of TERT in the regulation of cell proliferation.

G1TERT-deficient SMC did not display senescence-associated changes in their phenotype or gene expression, further supporting a telomere-independent mechanism of growth arrest. Instead, cell cycle analysis revealed that TERT-deficiency induced a G1→S phase arrest associated with decreased DNA synthesis. Similarly, acute knock-down of TERT in cancer cells induces cell cycle arrest without telomere shortening while TERT overexpression drives epithelial cells into S phase^{150, 151}. In proliferating cells, phosphorylation of the Rb protein constitutes a critical first step for S phase entry¹³⁹, which in turn releases its active transcriptional repression of E2F1^{139, 141}. In TERT-deficient SMC we observed hyperphosphorylation of Rb, which would be expected to release E2F1-dependent repression, induce target gene transcription, and facilitate S phase entry. Contrary to this assumption, mitotic E2F1 target genes required for DNA synthesis were silenced in TERT-deficient SMC, albeit increased Rb phosphorylation. This unanticipated requirement of TERT for E2F1-dependent transcription was neither

the result of decreased E2F1 protein expression nor reversible by ectopic E2F1 overexpression, indicating a stable transcriptional silencing in response to TERT deletion that cannot be attributed to a lack of E2F1 availability. Alternatively, a model in which E2F1 is present but unable to gain access to cognate binding sites due to condensed chromatin in TERT-deficient SMC appears conceivable. The ensuing altered transcription of E2F1-dependent genes in turn may provide a positive feedback to mechanisms that are upstream of Rb and induce its phosphorylation to overcome the proliferative arrest. Consistent with this concept is our observation that altered expression of E2F1 target genes in TERT-deficient SMC is associated with decreased E2F1 binding to their respective consensus motifs. To our knowledge these are the first data to demonstrate that E2F1-dependent activation of S phase genes requires TERT expression. These findings complement the more widely appreciated function of telomerase in maintaining telomere repeats and provide a molecular mechanism underlying the well-described, mitotic function of TERT.

Using CHIP assays, we demonstrate that TERT is recruited along with E2F1 to consensus sites within differentially regulated target genes. This inducible TERT occupancy of E2F1 sites is associated with the enrichment of CBP and permissive histone H3 acetylation on lysine 9 (K9Ac-H3). To confirm that TERT recruitment to E2F1 cognate elements is functionally important for the activation of chromatin, we probed for acetylated histone marks and chromatin accessibility. These studies revealed that TERT deletion decreases histone acetylation at E2F1 sites and reduces chromatin accessibility at the E2F1 site. Therefore, it is unlikely that the

mechanism by which TERT activates E2F1-target promoters is through direct DNA binding but rather involves chromatin remodeling and/or facilitated recruitment of other chromatin-modifying enzymes. CBP supports histone acetyltransferase activity required for E2F1 binding¹⁵². Our observations support that the association of TERT with CBP facilitates its recruitment to chromatin and local histone acetylation, which releases repressive chromatin. This hypothesis is further supported by the recent finding that TERT regulates Wnt-dependent transcription in stem cells through chromatin association and interaction with the chromatin modifier BRG1⁶⁰. BRG1 constitutes the energy-providing subunit of multiple chromatin-modifying enzymatic complexes to disrupt the chromatin architecture of target promoters and recruit histone acetyltransferases, which represent critical steps for E2F1-induced promoter activation¹⁵². Considering our observation that TERT depletion decreased chromatin accessibility and histone acetylation at E2F1 sites, it is possible that TERT serves as a chaperone to facilitate the recruitment of BRG1 and/or histone acetyltransferase activity to E2F1 target sites. In addition to this mechanism, TERT has been reported to stabilize transcription factors through inhibition of ubiquitination¹⁵³. However, since E2F1 protein levels were unaffected by TERT deletion we would consider this mechanism unlikely to explain the chromatin remodeling of TERT-deficient SMC.

Based on our data, we conclude that transient re-expression of TERT during cell cycle progression provides a mechanism for the transcription of S phase cell cycle-regulatory genes. TERT is recruited to canonical E2F1 target genes and induces a permissive chromatin environment. *In vivo*, this mechanism is required

for the proliferative response of SMC to induce neointima formation. Further investigation of this novel epigenetic function of TERT and characterization of TERT-regulated gene expression programs will likely yield new insights into how TERT functions as a regulator of cell proliferation.

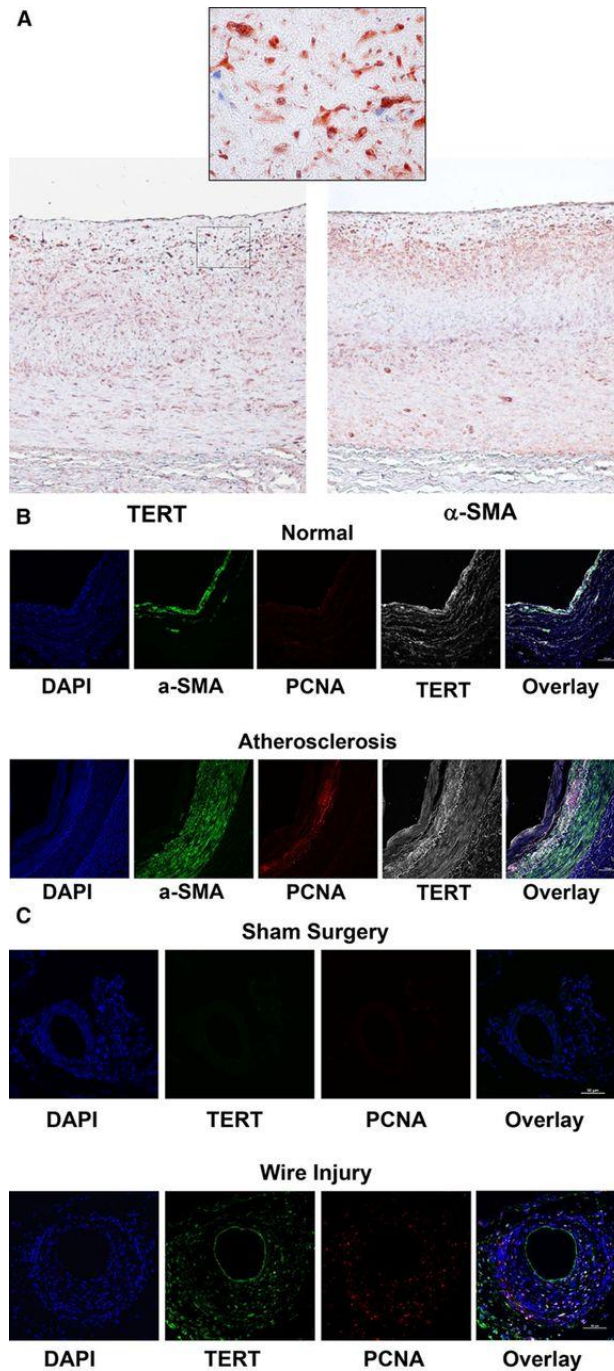


Figure 3.1 TERT is Expressed in Replicating Smooth Muscle Cells.

A, Expression of TERT in SMC of human coronary artery atherosclerotic lesions. Sections of an early stage, SMC-rich fibroatheroma of a human coronary artery were immunostained for TERT and α -smooth muscle actin. Low resolution images (100X) and high resolution image (1000X) of the boxed area showing

TERT staining. B, Co-localization of TERT and PCNA in SMC of atherosclerotic lesions. TERT expression in proliferating SMC was detected by immunofluorescence staining of TERT (white), PCNA (red), and α -SMA (green) with nuclei counterstained with DAPI (blue) in sections of atherosclerotic coronary arteries compared with normal controls. Scale bar 100 μ M. C, Co-expression of TERT and PCNA in the murine neointima following guide wire-induced femoral artery injury. Tissues were stained for TERT (green), PCNA (red), and nuclei counterstained with DAPI (blue). Representative immunofluorescence staining of TERT and PCNA expression within injury-induced neointima or sham arteries. Scale bar 50 μ M.

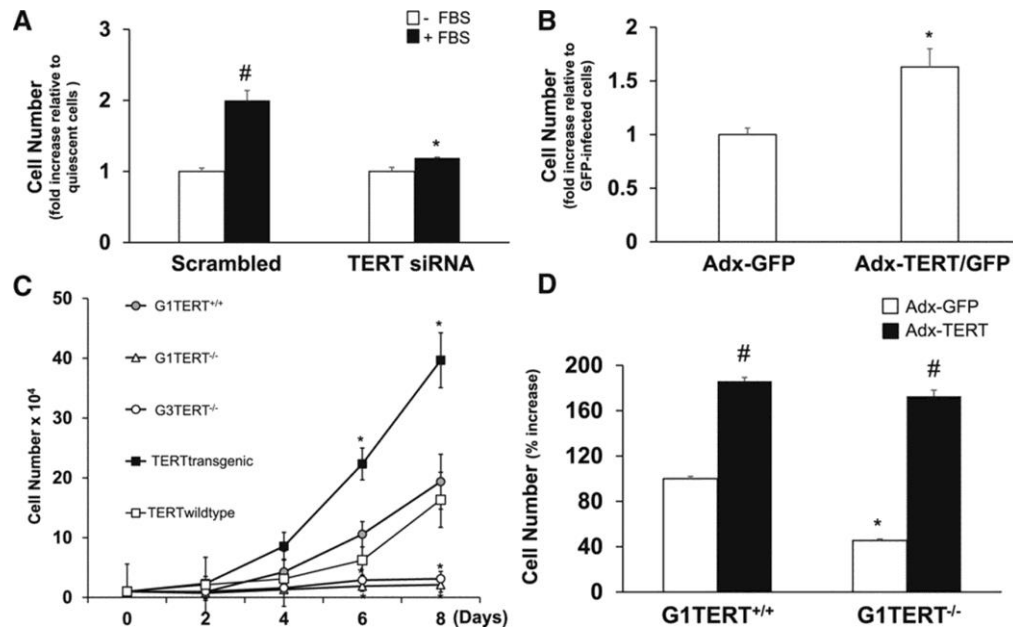


Figure 3.2 TERT is Required for Smooth Muscle Cell Proliferation.

A, siRNA-induced TERT knockdown decreases SMC proliferation. Human coronary artery SMC were transfected with TERT siRNA and serum-deprived for 24 hours. Synchronized cells were then stimulated with FBS for 48 hours and counted using a hemocytometer. Cell numbers are presented as mean \pm SEM relative to quiescent cells (# $P < 0.05$ versus quiescent, * $P < 0.05$ versus scrambled). B, Adenoviral-mediated overexpression of TERT promotes SMC proliferation. Serum-deprived human coronary artery SMC were transduced for 24 hours with 100 plaque-forming units (PFU) of adenoviral constructs overexpressing GFP/TERT (Adx-TERT/GFP) or GFP (Adx-GFP) as control. Following transduction, proliferation was analyzed after 48 hours by cell counting using a hemocytometer. Data are presented as mean \pm SEM relative to quiescent GFP-infected cells (* $P < 0.05$ versus Adx-GFP). C, Proliferation of vascular SMC isolated from TERT overexpressing (TERTtransgenic), littermate wildtype (TERTwildtype), first generation G1TERT^{-/-}, littermate wildtype G1TERT^{+/+}, and third generation G3TERT^{-/-} mice (n=8 mice per group). Serum-deprived SMC were stimulated with FBS and counted at the indicated time points using a hemocytometer. Data are

presented as mean \pm SEM (*P<0.05 versus the respective wildtype control mice). D, TERT overexpression rescues the mitogenic defect of TERT-deficient SMC. Murine SMC from G1TERT^{-/-} or G1TERT^{+/+} mice were infected with 100 PFU of Adx-TERT/GFP or Adx-GFP. Forty-eight hours after transduction, proliferation was analyzed by cell counting using a hemacytometer. Values are fold increase relative to G1TERT^{+/+} transduced by Adx-GFP and presented as mean \pm SEM (#P<0.05 versus Adx-GFP, *P<0.05 versus G1TERT^{+/+}).

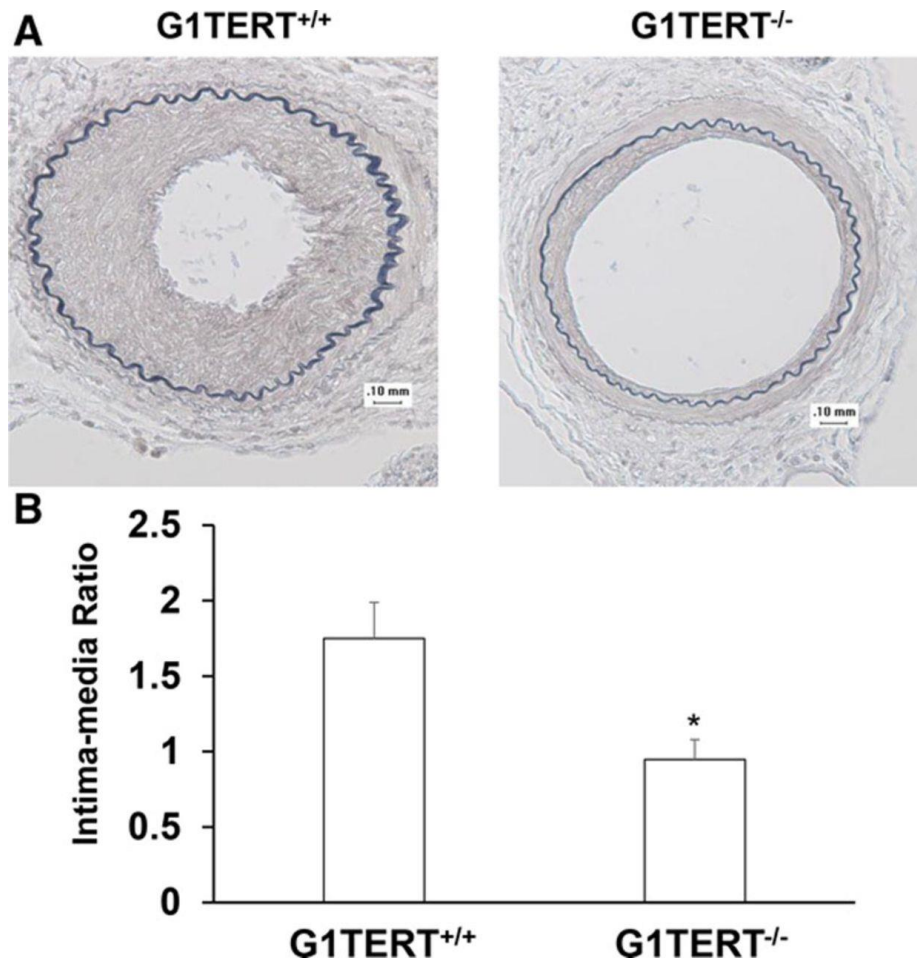


Figure 3.3 TERT Deficiency Attenuates Neointima Formation.

A, Photomicrographs of neointima formation in the femoral arteries of G1TERT^{+/+} and G1TERT^{-/-} mice. Guide wire-induced endothelial denudation injuries were performed and tissues harvested after 28 days (n=8 per group). Tissues were stained using Verhoeff-van Gieson staining to visualize the internal and external elastic lamina. Scale bar represents 0.1 mm. B, The ratio of intima to media was calculated as the intimal area divided by the medial area. Data are presented as mean ± SEM (**P*<0.05 versus G1TERT^{+/+}).

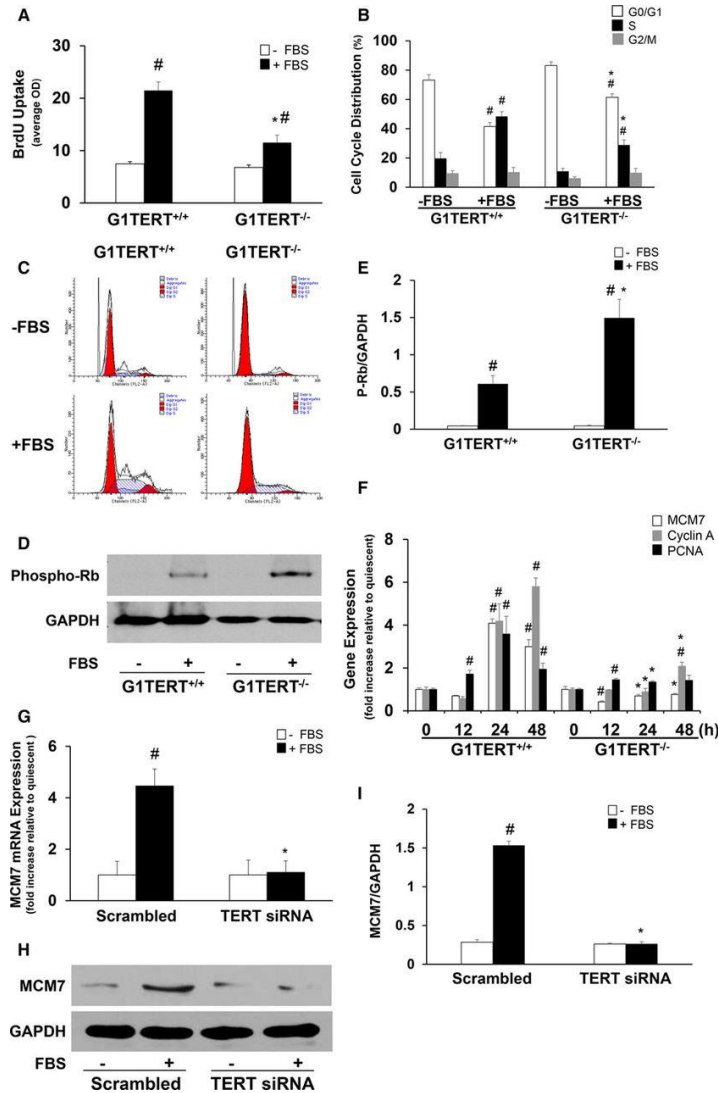


Figure 3.4 TERT Deletion Induces Cell Cycle Arrest and is Required for E2F1 Target Gene Expression.

A, TERT-deficiency decreases DNA synthesis. SMC from G1TERT^{+/+} or G1TERT^{-/-} mice were serum-deprived and stimulated with FBS. After 24 hours BrdU incorporation was quantified. Values are mean \pm SEM (# P <0.05 versus quiescent, * P <0.05 versus G1TERT^{+/+}). B-C, TERT-deficiency induces cell cycle arrest. SMC were prepared as in A, and cell cycle distribution was analyzed by flow cytometry following propidium iodide staining. The percentages of cells in G0/G1, S, and G2/M phases (B) were calculated from flow cytometric plots of cell cycle distribution (C). Values are

mean \pm SEM ($\#P < 0.05$ versus quiescent, $*P < 0.05$ versus G1TERT+/+). Data were collected from three independently performed experiments (n=3 per group). D-E, TERT deletion induces mitogen-induced hyperphosphorylation of Rb. Cells were prepared as described in A. Whole cell lysate was blotted for phospho-Rb (Ser 807/811) and GAPDH as loading control. The autoradiograms shown (D) are representative of three independently performed experiments using different cell preparations. Densitometric quantification (E) normalized to GAPDH. Values are mean \pm SEM ($\#P < 0.05$ versus quiescent, $*P < 0.05$ versus G1TERT+/+) F, Silencing of S phase gene expression in G1TERT-/- SMC. SMC were stimulated with FBS as described above. mRNA expression of MCM7, cyclin A, and PCNA was analyzed by quantitative real-time PCR and normalized to transcript levels of the housekeeping gene TFIIB. Values are mean \pm SEM ($\#P < 0.05$ versus 0h, $*P < 0.05$ versus G1TERT+/+ at the same time point). G-I, siRNA-induced TERT deletion suppresses MCM7 expression in human coronary artery SMC. SMC were stimulated with FBS following transfection with TERT siRNA or scrambled control. MCM7 transcript and protein expression levels were analyzed by real-time PCR (G) and Western Blotting (H-I) after 12 and 24 hours, respectively. Densitometric quantification (I) normalized to GAPDH. Values are mean \pm SEM ($\#P < 0.05$ versus quiescent, $*P < 0.05$ versus Scrambled).

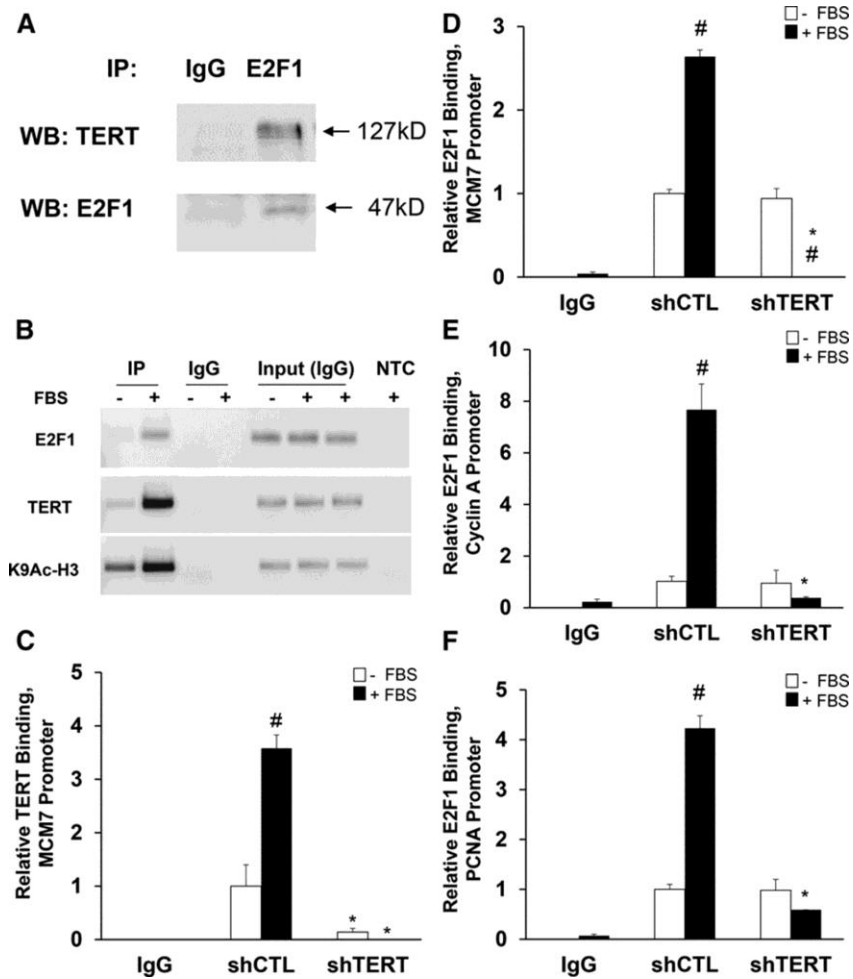


Figure 3.5 TERT is Recruited to Chromatin and Required for E2F1 Binding to Target Promoters.

A, Interaction of TERT and E2F1 in human coronary artery SMC. Cell extracts were immunoprecipitated (IP) with control IgG or anti-E2F1 antibodies. Co-immunoprecipitated protein were visualized by Western Blotting (WB) using anti-TERT and anti-E2F1 antibodies. B, Mitogen-induced recruitment of E2F1, TERT, and lysine 9-acetylated histone H3 (K9Ac-H3) to target chromatin of the MCM7 promoter. Representative gel images of ChIP experiments performed in quiescent G1TERT^{+/+} SMC stimulated with FBS for 10 hours. Cells were harvested for ChIP using antibodies against E2F1, TERT, or K9Ac-H3 (indicated as IP). Immunoprecipitation with respective non-immune IgG served as control (indicated as IgG). Chromatin Input and non-template (NTC) are depicted

as additional controls. The precipitated chromatin was then amplified using primer pairs covering the E2F1 binding sites within the MCM7 promoter. C, Mitogen-induced TERT recruitment to E2F1 target chromatin. Forty-eight hours after lentiviral transduction with either non-targeting shRNA control (shCTL) particles or TERT shRNA (shTERT) particles, human coronary artery SMC were stimulated with FBS following 24 hour serum-free starvation and then harvested for ChIP assays. Chromatin immunoprecipitation was performed using antibody against TERT. Promoter binding was quantified by real-time PCR using primers covering E2F1 binding sites within the MCM7 promoter. Cycle threshold (C_t) values were normalized to C_t values of input samples and presented as mean \pm SEM (# P <0.05 versus quiescent, * P <0.05 versus shCTL). D-F, TERT depletion abolishes mitogen-induced E2F1 binding to the promoters of MCM7 (D), cyclin A (E), and PCNA (F). Human coronary artery SMC were prepared as in described in (C). ChIP was performed using anti-E2F1 antibody. Quantification of immunoprecipitated chromatin by real-time PCR using primers covering E2F1 binding sites within the promoter regions of MCM7, cyclin A, and PCNA. Data are presented as mean \pm SEM (# P <0.05 versus quiescent, * P <0.05 versus shCTL).

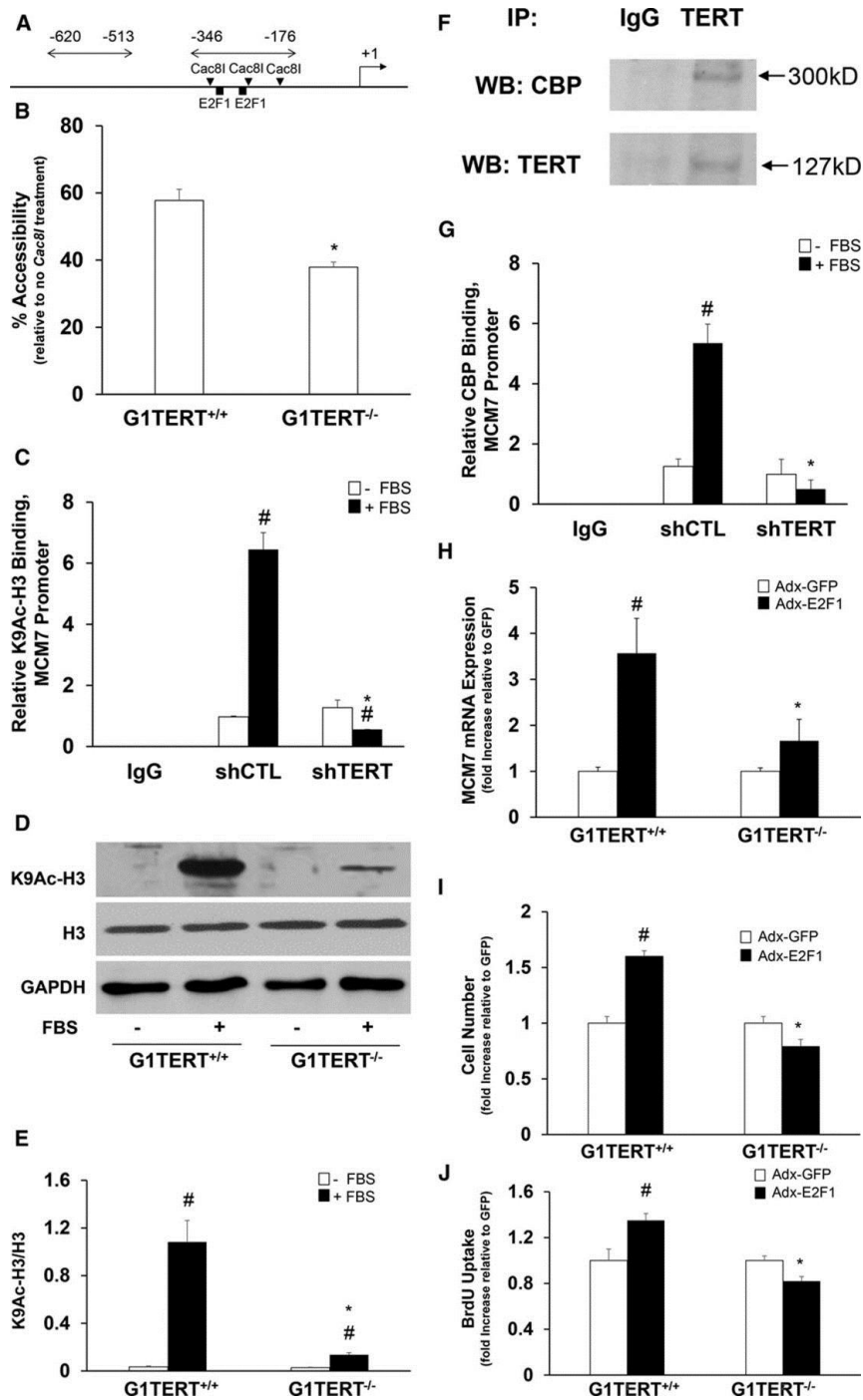


Figure 3.6. TERT-Deficiency Decreases Chromatin Accessibility and Histone Acetylation.

A, Schematic illustration of locations of primers, *Cac8I* restriction sites, and E2F1 binding sites on MCM7 promoter. Sequences amplified by primers (arrows), *Cac8I* restriction sites (-312, -244, and -182 from the

transcription initiation site, triangles), and E2F1 binding sites (-297 and -250 from the transcription initiation site, squares) are noted. The transcription initiation site is marked as +1. B, TERT deletion decreases DNA accessibility of E2F1 binding sites within the MCM7 promoter. Nuclei isolated from FBS-stimulated SMC of G1TERT^{+/+} or G1TERT^{-/-} mice were digested with *Cac8I* restriction enzyme and purified for ChART-PCR. Primers used for real-time PCR amplify sequences between nucleotides -346 and -176 in the MCM7 promoter, which encompass two E2F1 binding sites and three *Cac8I* restriction sites. Results were normalized against amplification of a control sequence upstream of the MCM7 promoter (nucleotides -620 to -513) with no *Cac8I* restriction sites. Data are presented as mean \pm SEM relative to uncut samples ($*P < 0.05$ versus G1TERT^{+/+}). C, TERT depletion abolishes mitogen-induced histone H3 acetylation at the E2F1 response elements within the MCM7 promoter. Human coronary artery SMC were treated as Figure 5C. ChIP was performed using an antibody against acetylated Histone H3 (Lys9). Quantification of immunoprecipitated chromatin was performed by real-time PCR using primers covering E2F1 binding sites within the promoters of MCM7. Data are presented as mean \pm SEM ($\#P < 0.05$ versus quiescent, $*P < 0.05$ versus shCTL). D-E, TERT-deficiency decreases acetylation of histone H3. Vascular SMC from G1TERT^{+/+} and G1TERT^{-/-} mice were stimulated with FBS following 24 hour serum-free starvation and protein was harvested. Western Blotting was carried out using antibody against acetylated Histone H3 (Lys9). Histone H3 and GAPDH were used as loading controls. Densitometric quantification (E) normalized to H3. Data are presented as mean \pm SEM ($\#P < 0.05$ versus quiescent, $*P < 0.05$ versus G1TERT^{+/+}). F, Interaction of TERT and CBP in human coronary artery SMC. Immunoprecipitation (IP) of TERT from cell lysates followed by Western Blotting (WB) with anti-CBP. The same blot was subsequently stripped and re-probed with anti-TERT antibody. G, TERT

depletion reduces CBP recruitment to the E2F1 response element in the MCM7 promoter. Human coronary artery SMC were treated as in Figure 5C. ChIP was performed using anti-CBP antibody. Quantification of immunoprecipitated chromatin was performed by real-time PCR using primers covering E2F1 binding sites within the MCM7 promoter. Data are presented as mean \pm SEM (# P <0.05 versus quiescent, * P <0.05 versus shCTL). H-J, The proliferative defect induced by TERT-deletion is not reversed by overexpression of E2F1. Vascular SMC from G1TERT+/+ and G1TERT-/- mice were transduced with 25 PFU/cell adenovirus overexpressing E2F1 or control GFP. MCM7 mRNA expression was analyzed by real-time RT-PCR after 24 hours (H). Cell proliferation was analyzed after 24 hours by cell counting using a hemocytometer (I) or by BrdU incorporation (J). Data are presented as mean \pm SEM (# P <0.05 versus Adx-GFP, * P <0.05 versus G1TERT+/+).

4 CHAPTER FOUR

Telomerase Reverse Transcriptase Deficiency Prevents Atherosclerosis Formation through an Inhibition of Macrophage-Related Inflammation

4.1 Abstract

Objective: Telomerase Reverse Transcriptase (TERT), the catalytic subunit of telomerase, supports critical cellular processes required for tissue remodeling. Previous studies established that TERT expression is induced in activated macrophages and during experimental and human atherosclerosis formation. In the present study, we investigated the role of TERT in atherosclerosis development and macrophage inflammation.

Approach and Results: TERT-deficient mice were crossbred with LDL-receptor-deficient (LDLr^{-/-}) mice to generate first generation G1TERT^{-/-}LDLr^{-/-} offsprings, which were then successively intercrossed to obtain the third generation G3TERT^{-/-}LDLr^{-/-} mice. G1TERT^{-/-}LDLr^{-/-} mice displayed no telomere shortening while severe telomere attrition was evident in G3TERT^{-/-}LDLr^{-/-} mice. When fed on an atherogenic diet, G1TERT^{-/-}LDLr^{-/-} and G3TERT^{-/-}LDLr^{-/-} mice were both protected from atherosclerosis formation compared to their wildtype controls, indicating that genetic TERT-deletion prevents atherosclerosis, and formation of the disease is not affected by telomere attrition. Similarly, atherosclerosis development was decreased in chimeric LDLr^{-/-} mice with TERT deletion in

hematopoietic stem cells after bone marrow transplantation. TERT deficiency reduced macrophage accumulation in atherosclerotic lesions and altered chemokine expression, including CXCL1/2/3, CCL3, CCL5, CCL21, CCR7, IL-6, and IL-1 α . In isolated macrophages, sequence analysis of silenced inflammatory gene promoters indicated that TERT deletion altered signal transducer and activator of transcription 3 (STAT3)-dependent chemokine expression and recruitment of phosphorylated STAT3 to its target promoters. Mechanistically, TERT expression was necessary and sufficient to maintain STAT3 phosphorylation.

Conclusions: Our data support that genetic TERT deficiency decreases atherosclerosis formation by silencing inflammatory chemokine transcription through inactivation of the STAT3 signaling pathway.

4.2 Introduction

Telomeres are repetitive DNA-protein complexes that protect the ends of chromosomes and are maintained by the enzyme telomerase⁴⁴. TERT confers the catalytic activity and is the limiting factor for telomerase activation^{26, 110}. Overexpression of TERT in cancer and stem cells confers a virtually unlimited replicative capacity^{61, 128, 154}. In contrast, most somatic cells are thought to suppress telomerase activity by silencing the TERT gene, resulting in telomere shortening and limited replicative potential⁴⁴. While this traditional role of TERT in maintaining telomeres is firmly established, mounting evidence points to a second role of TERT in the control of cell signaling and gene expression that is

independent of its reverse transcriptase activity and ability to elongate telomeres⁴⁷. For example, overexpression of TERT has been demonstrated to activate gene transcription by chromatin modulation⁶⁰. Also, multiple studies have revealed that TERT activates various signaling pathways, including MAP-kinase⁸⁹ and NF- κ B signaling⁵⁸, through a telomere maintenance-independent mechanism.

Leukocyte telomere length shortens with age in humans, and telomere dysfunction has been linked to a host of pathologies including dementia, cardiovascular events, asthma, diabetes, and stroke^{43, 67}. Therefore there has been an increasing interest in the reactivation of telomerase, the enzyme to elongate telomeres, to protect against cellular senescence and age-related diseases⁶². However, in contrast to the circulating leukocyte telomere shortening that is generally associated with cardiovascular risk¹⁵⁵, long telomere length⁶⁹ and telomerase activation^{70, 71} are observed in leukocytes isolated directly from human unstable coronary artery plaques. Furthermore, atherosclerosis is decreased in apoE-deficient mice with short telomeres due to lack of the telomerase component that provides the RNA template to the enzyme⁸⁴. Collectively, these results challenge the general dogma that telomerase activation may prevent vascular cell dysfunction and atherosclerosis development.

Considering this important function of telomerase in cell biology, surprisingly little is known about the function of telomerase and telomere-related molecular processes in atherosclerosis. TERT expression is thought to be essential in preventing EC senescence^{72, 73}. Our laboratory has further demonstrated that TERT is inducible in proliferating vascular smooth muscle cells

(VSMC) and represents a mitogenic effect^{30, 31}. Furthermore, our recent studies confirmed an increased TERT expression in macrophages of human atherosclerotic lesions⁷¹, indicating a potentially important function of TERT in vascular remodeling. However, the role of TERT in atherosclerosis has not been investigated.

4.3 Materials and Methods

Immunohistochemical Staining

For immunohistochemical staining of TERT expression in human coronary atherosclerotic lesions, segments of coronary arteries were harvested from explanted hearts during cardiac transplantation as described. Tissues were fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin, and transverse sections (8 μ m) were collected. Serial paraffin-embedded sections were incubated with a rabbit monoclonal TERT antibody (600-401-252S, Rockland) or mouse monoclonal CD68 antibody (ab955, Abcam), followed by biotinylated goat anti-rabbit IgG (BA-1000, Vector Laboratories) or biotinylated goat anti-mouse IgG (BA-9200, Vector Laboratories) and counterstaining with hematoxylin. For immunofluorescent colocalization studies, tissues were incubated with antibodies raised against TERT (600-401-252S, Rockland) or p-STAT3 (9145S, Cell Signaling), and CD68 (ab955, Abcam), followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse, and Alexa Fluor 594-conjugated goat anti-rabbit antibodies (A11001 & A11012, Invitrogen) and counterstaining with DAPI.

Mice

Littermate TERT^{+/+} and TERT^{-/-} mice on a mixed C57BL/6J background were used (The Jackson Laboratory, Stock Number 005423). First generation G1TERT^{-/-} mice derived from heterozygote founder mice were further mated to generate late generation, i.e. third generation G3TERT^{-/-} mice. G1TERT^{-/-} and G3TERT^{-/-} with their respective littermate were crossbred with male low-density lipoprotein receptor-deficient mice (LDLr^{-/-}, The Jackson Laboratory) to generate G1 and G3TERT^{-/-}LDLr^{-/-} mice. Study mice and their littermate controls were fed a saturated fat-enriched diet for 12 weeks to induce atherosclerosis formation (Harlan Teklad TD.88137).

Bone marrow transplantation (BMT) was performed by repopulating lethally irradiated male LDLr^{-/-} mice (The Jackson Laboratory) with bone marrow-derived cells of male first-generation TERT-deficient mice (TERT^{-/-}, The Jackson Laboratory, B6.129S-Terttm1Yjc/J, stock number 005423) or their wild-type littermates (TERT^{+/+}). Briefly, mice were maintained on water containing antibiotics (sulfamethoxazole/trimethoprim) for one week before BMT until four weeks after BMT. Recipient mice were irradiated with a total of 900 Rads from a cesium source that was delivered in 2 doses within 3 to 4 hours. Bone marrow-derived cells of male TERT^{+/+} or TERT^{-/-} mice were obtained from the tibias and femurs of donor mice and were injected into the tail vein of 8-week-old irradiated male LDLr^{-/-} recipient mice (1.2×10⁷ cells per mouse, n=10 with TERT^{+/+} and n=7 with TERT^{-/-}). After four weeks of recovery, a saturated fat-enriched diet was fed for 12 weeks until the end of the study (Harlan Teklad TD.88137). All studies were

performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Atherosclerosis Quantification

En face atherosclerosis was quantified as lesion area on the intimal surface of aortic arches. The entire aorta from the aortic root to the common iliac arteries was removed from the sacrificed mice. The intimal surface is exposed by a longitudinal cut through the inner curvature of the aortic arch and down the anterior aspect of the remaining aorta. The tissue is then pinned to a dark surface, and the lesions are observed through a dissecting microscope. The area of atherosclerotic lesions was quantified and represented as percent of the total intimal surface.

Quantification of Macrophage and Foam Cell Accumulation

Aortic roots were frozen in OCT media (Tissue-Tek, Sakura Finetek, Torrance, CA) and serial 10 μm sections were cut as previously described¹⁰³. Accumulation of lipids in lesions was visualized by staining with Oil Red O. Macrophages were detected by immunohistochemistry staining using anti-CD68 antibody (ab955, Abcam). Percentage of macrophage and lipid (Oil Red O positive) area in aortic root plaques was calculated using computer-assisted image analysis (Image-Pro, Media Cybernetics, Rockville, MD).

Telomere Length Quantification

For quantification of telomere length using real-time PCR, DNA was isolated from bone marrow differentiated macrophages (BMDM) of G1TERT+/+, G1TERT-/-, G3TERT-/-, was isolated harvested from TERT-/- samples were run in triplicate with 35 ng of DNA per reaction using an iCycler and SYBR Green I system (Bio-Rad) as described. Telomere repeat copy number data were normalized to 36B4 as the single-copy gene. Below lists the primer sequences.

tel 1: 5'-GTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3';

tel 2: 5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3';

36B4 forward: 5'-CAACCCAGCTCTGGAGAAAC-3';

36B4 reverse: 5'-AAGCCTGGAAGAAGGAGGTC-3'.

Quantitative fluorescence in situ hybridization on metaphase chromosomes was performed using Cy-3 labeled (CCCTAA) PNA probe (Telomere PNA Fish Kit/Cy3, Dako, K5326) to analyze the telomeric sequences in BMDM. Cells were counterstained with DAPI to visualize the chromosomes.

Quantification of Total Cholesterol Concentration and Lipoprotein-Cholesterol Distribution

Total plasma cholesterol concentration and lipoprotein-cholesterol distribution were analyzed as previously described. Briefly, total plasma cholesterol was measured by an enzymatic colorimetric method using the Wako Cholesterol E kit (Wako Chemicals, Richmond, VA). Lipoprotein-cholesterol

distribution was detected by size exclusion chromatography using a fast performance liquid chromatographic machine (Pharmacia GE Healthcare Life Sciences, Pittsburgh, PA).

Cell Culture

Mouse BMDM were isolated from the femurs of mice and differentiated using Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 20% fetal bovine serum and 30% L929 cell-conditioned medium for ten days. BMDM isolated from G1TERT^{-/-} mice or wild-type littermates (n = 6/group) were treated with 100 ng/ml lipopolysaccharide (LPS) for the indicated time before RNA or whole cell lysate collection.

NanoString Gene Expression Analysis

Inflammatory gene expression was profiled using nCounter GX mouse inflammation kit consisting of 184 inflammation-related genes and six internal reference genes. Briefly, RNA was collected from cells with Trizol (Invitrogen, Grand Island, NY). 10ng of purified RNA was hybridized against the nCounter GX mouse inflammation kit (NanoString Technologies, Seattle, WA) and data were analyzed using nSolver Analysis Software (NanoString Technologies, Seattle, WA). The geometric mean of the code counts for the positive control genes was used for normalization.

Gene Ontology Enrichment Analysis

LPS-induced inflammatory genes changed more than 1.5 fold by TERT deletion are included in Gene Ontology (GO) biological process enrichment analysis. Enrichment analysis was performed using the Gene Ontology Consortium ([Http: //geneontology.org/page/go-enrichment-analysis](http://geneontology.org/page/go-enrichment-analysis)) based on biological process terms.

Western Blotting

Western blotting was performed as described using antibodies against p-STAT3 (Tyr705) (9131, cell signaling), total STAT3 (12640, cell signaling), and GAPDH (FL 335, Santa Cruz).

Flow Cytometry

For phenotypic identification of hematopoietic stem cells (HSC), progenitor cells, and mature hematopoietic cells, cells were stained with cell surface markers and analyzed by flow cytometry. Bone marrow cells were stained with the differentiated lineage cell markers (CD5, CD3, B220, Mac-1, Gr-1, and Ter119 from BD Pharmingen, San Jose, CA) and stem cell markers (Sca-1 from Invitrogen, Grand Island, NY, c-kit from BD Pharmingen, San Jose, CA). The HSC-enriched population is negative for the lineage markers but positive for Sca-1 and c-kit (Lin-Sca-1+c-kit+). Committed progenitor cells, macrophage and dendritic progenitors (MDP) were identified by the markers of CD16/CD32 (eBioscience,

San Diego, CA), and CD115 (BD Pharmingen, San Jose, CA) and were identified as Lin-Sca-1- c-kit+ CD16/CD32+CD115+. Dead cells were excluded by propidium iodide selection. Bone marrow cells were analyzed and sorted on a BD FACS Aria II flow cytometer (Becton Dickinson, San Jose, CA). Each experimental group was sorted independently. Flow cytometry and fluorescence-activated cell sorting (FACS) data were analyzed using FlowJo software (Tree Star, Ashland, OR)

For identification of mature hematopoietic cells in blood, granulocytes were identified as CD3e-CD19-NK1.1-CD11b+Ly6G^{hi}; total monocytes were identified as CD3e-CD19-NK1.1-Ly6G-CD11b+CD115+, and monocyte subsets were identified as Ly6C⁺ monocytes or Ly6C⁻ monocytes by Ly6C fluorescence intensity gated on total monocytes. Antibodies used for staining are as follows: APC-Cy7 mouse CD11c, FITC mouse Ly6C, PE-cy7 mouse Ly6G, Percp-cy5.5 mouse CD11b (BD Biosciences, San Jose, CA); PE anti-mouse CD115 (Biolegend, San Diego, CA). Antibodies were used according to the manufacturer's protocol. Cellular fluorescence was assessed with FACSCalibur (BD Biosciences, San Jose, CA) and data were analyzed with Cell Quest software (version 3.3, Becton Dickinson, San Jose, CA).

Transfection of HEK 293 Cells

HEK 293 cells were transiently transfected with a TERT or green fluorescent protein expression vector using Lipofectamine 2000 (Invitrogen, Grand Island, NY) as described. 24 hours after transfection, cells were treated with LPS (1ug/ml) or IL-6 (10ng/ml) for 30 minutes before whole cell lysates were collected.

Statistical Analysis

Results were represented as means or medians depending on the distribution of data. Unpaired Student's t-test was used to compare the means between two independent groups on a single variable. The effect of TERT on atherosclerosis was compared using the Kruskal-Wallis test followed by Dunn Test post hoc analysis. P values <0.05 were considered to be statistically significant.

4.4 Results

4.4.1 TERT Is Expressed in Macrophages from Atherosclerotic Lesions in Human Coronary Artery

To confirm that TERT is expressed in complex atherosclerotic lesions in vivo, we performed immunohistochemical analysis on human coronary arteries. Consistent with our earlier report, we found significant TERT expression within the atherosclerotic lesions of human coronary arteries whereas in healthy arteries its presence is negligible. Unusually high levels of TERT expression were observed in the macrophage-rich shoulder region of advanced atherosclerotic lesions. As shown by immunofluorescent staining, TERT colocalized with macrophage CD68 in the atherosclerotic lesions (Figure 4.1. A-B). These observations confirm a pathological overexpression of TERT by macrophages in human atherosclerotic lesions.

4.4.2 Telomere Length Is Preserved in Early Generation of TERT-Deficient Mice

TERT^{-/-} mice (The Jackson Laboratory, Stock Number 005423) were used to test whether TERT deletion affects atherosclerosis formation. First generation G1TERT^{-/-} mice derived from heterozygote founder mice exhibited normal telomere lengths and no phenotypes associated with telomere attrition. However, intercrossing of G1TERT^{-/-} mice results in progressive telomere loss in subsequent generations. Therefore, this unique system is useful in two aspects: it allowed the investigation of telomere-independent functions of TERT in G1TERT^{-/-} mice, as well as the phenotypes that occur as a result of telomere attrition in the third generation G3TERT^{-/-} mice. The described alterations of telomere attrition are documented in Figure 4.2. A-C.

4.4.3 Early and Late Generations of TERT-Deficient Mice Show Comparable Reduction in Atherosclerosis Formation

While TERT expression is upregulated in the atherosclerotic lesions, it is not known whether TERT expression contributes to atherosclerosis development. To address this question, we generated an atherosclerosis mouse model by crossbreeding G1TERT^{-/-} or G3TERT^{-/-} mice with LDLr^{-/-} and fed a high-cholesterol diet. G1TERT^{-/-}LDLr^{-/-} mice developed fewer atherosclerotic lesions compared to littermate G1TERT^{+/+}LDLr^{-/-} mice, but no difference was found between G1 and G3TERT^{-/-} LDLr^{-/-} mice (Figure 4.3. A-E), indicating that TERT deletion in the presence of normal telomere length decreases atherosclerosis

through a mechanism that is not associated with telomere shortening. TERT deletion did not affect plasma cholesterol concentration and lipoprotein distribution (Figure 4.4. A-B), indicating an intrinsic role of TERT to promote atherosclerosis formation.

4.4.4 TERT Deficiency in Bone Marrow Prevents Atherosclerosis Formation and Macrophage Recruitment in LDLr^{-/-} Mice

Given that the expression of TERT by infiltrating macrophages in atherosclerotic plaques was significantly higher compared to that of normal control arteries, and whole body TERT deletion ameliorated atherosclerotic formation, we hypothesize that the aberrant TERT expression in macrophages is key to the development of atherosclerosis through enhanced vascular inflammation. Bone marrow of TERT-null donors was transplanted to LDLr^{-/-} mice which resulted in the depletion of TERT in the hematopoietic lineage. As depicted in Figure 4.5.A-D, macrophage infiltration and aortic lipid content were decreased in these chimeric mice after 12 weeks of the atherogenic diet, indicating that the selective deletion of TERT in bone marrow prevented atherosclerosis formation by decreasing macrophage recruitment into the arterial wall.

As monocytosis resulted from hypercholesterolemia-induced hematopoiesis contributes to atherosclerosis, we next quantified bone marrow resident HSC and MDP populations as well as the leukocyte and monocyte subsets sampled from systemic circulation. Bone marrow deletion of TERT did not change the counts of all the cell sets examined (Figure 4.6. A-D). Therefore, the proliferation of HSC and

MDP, as well as the corresponding monocyte differentiation, are not the potential mechanisms for TERT-induced atherosclerosis formation.

4.4.5 Inflammatory Genes Are Silenced in G1TERT^{-/-} Macrophages

The observations that TERT deletion reduces atherosclerosis formation in mice with normal telomeres and monocyte counts, led us to hypothesize that TERT induces atherosclerosis through mechanisms other than the well-established functions of TERT in telomere maintenance. Since TERT has recently emerged as a transcriptional regulator to modulate the expression of genes independently of its telomerase activity, we employed the NanoString technique to analyze transcript levels of 184 inflammatory genes in BMDM induced by LPS treatment. As shown in Figure 4.7. A-C, multiple genes associated with atherosclerosis formation were found to be expressed at significantly lower levels in G1TERT^{-/-} BMDM. These include a host of inflammatory cytokines known to be involved in atherosclerosis formation, including IL-6, TNF α , IL-1 α and IL-1 β , and various members of the CC/CXC chemokine family. These findings indicate that TERT may be required for the expression of inflammatory genes in macrophages without affecting telomere homeostasis.

4.4.6 TERT Activates STAT3 Signaling

As shown in Figure 4.8. GO enrichment analysis of TERT-regulated target genes using the “biological processes” terms revealed that significantly enriched

processes ($P \leq 0.01$) include chemotaxis, cellular response to IL-1 or IFN γ , and STAT cascade activation. These hypothesis-generating data indicate that tyrosine phosphorylation of STAT protein may constitute an underlying mechanism of TERT-regulated chemokine and cytokine secretion in macrophages. To address this question, we next investigated whether TERT-deletion in G1TERT $^{-/-}$ BMDM affected STAT3 phosphorylation as the underlying transcriptional mechanism of TERT-regulated chemokine and cytokine expression. As depicted in Figure 4.9. A-D, LPS-induced STAT3 phosphorylation was reduced in G1TERT $^{-/-}$ BMDM. Using a transgenic mouse model of TERT overexpression (TERT-Tg, kindly provided by Dr. Chiang) we confirmed that TERT overexpression in BMDM activated STAT3 phosphorylation. In addition, STAT3 activation by TERT was also observed in human 293 cells stimulated with either LPS or IL-6. Furthermore, we identified decreased p-STAT3 levels in atherosclerotic plaque-associated macrophages in LDLr $^{-/-}$ mice with a bone marrow deletion of TERT (Figure 4.10. A-B).

4.5 Discussion

Macrophages are a critical source of the chronic inflammation and vascular remodeling processes that are key to the initiation and development of atherosclerosis formation¹⁵⁶. Among the mechanisms that orchestrate tissue remodeling and repair, telomerase activation is thought to control essential cellular functions including cell activation, replicative lifespan, differentiation, and proliferation¹⁵⁷. An upregulated expression of TERT, the catalytic subunit of telomerase, was observed in activated macrophages during atherosclerosis

formation⁷¹. However, the mechanism that underlies this regulation and functional consequences remains unknown. In the current study, we demonstrate that genetic TERT depletion prevents atherosclerosis formation in both early and late generation TERT-deficient mice. Based on these observation, we infer that TERT-deficiency protects from atherosclerosis through mechanisms other than telomere attrition. Similarly, atherosclerosis development was decreased in chimeric LDLr^{-/-} mice with TERT deletion in hematopoietic stem cells following bone marrow transplantation. In isolated macrophages, TERT deficiency reduced the expression of multiple chemokines, which are well known to be causally involved in the formation of atherosclerosis. All regulated target genes are STAT3-induced genes, consistent with results obtained from our GO enrichment analysis. Furthermore, we documented a decrease of STAT3 phosphorylation in TERT-deficient BMDM while TERT overexpression enhanced STAT3 phosphorylation in mouse BMDM and in human 293 cells. Finally, decreased STAT3 activation was confirmed *in vivo* by a decrease of STAT3 phosphorylation in macrophages of atherosclerotic plaques isolated from LDLr^{-/-} mice with TERT-depleted bone marrow. These findings suggest that genetic TERT deficiency reduces atherosclerosis formation by silencing inflammatory chemokine transcription through altered STAT3-dependent signaling.

Telomere length shortening with successive cell divisions has been proposed to serve as biomarker of biological aging⁶⁷. Therefore, there has been a growing interest in utilizing telomere extension as a preventive strategy against age-related diseases, including atherosclerosis⁶². However, previous studies have

identified that telomere length and telomerase activity are increased in human atherosclerosis, particularly in leukocytes isolated directly from unstable coronary artery plaques^{69, 70}. Consistent with this finding, we observed increased TERT expression in macrophages isolated from human atherosclerotic lesions⁷¹. Importantly, TERT deficiency in this macrophage population was shown to protect mice against atherosclerosis. Furthermore, atherosclerosis was decreased to a similar extent in LDLr^{-/-} mice crossbred with either early generation or late generation of TERT^{-/-} mice. These data indicate that the mechanism of atherosclerosis prevention associated with genetic TERT deletion is dependent on TERT deletion but independent of telomere length maintenance because telomere length in the early generation of TERT^{-/-} mice was indistinguishable from the wild-type littermates. In addition to our findings, numerous studies have identified telomere-independent functions of TERT involved in essential biological processes, including cellular proliferation, differentiation, apoptosis, and DNA repair response⁴⁸. Therefore, it is possible that TERT functions through an alternative mechanism to regulate the viability and function of cells within the arterial wall, ultimately affecting the outcome of atherosclerotic development. However, the similar degree of atherosclerosis prevention in G1 and G3 TERT-deficient mice cannot be interpreted by telomere length variation during the process of atherosclerosis formation. Increased telomere uncapping following telomere erosion could provoke important consequences, including chromosomal end-to-end fusions, aneuploidy, and impaired stress responses¹⁵⁸. Moreover, to date, there is no evidence from human studies showing that telomere attrition in

leukocytes causes atherosclerosis. In contrast to the general notion that short telomere length constitutes a hallmark of cardiovascular events⁶⁸, there was a decrease in atherosclerosis in mice with short telomeres due to the proliferative limitation of immune cells⁸⁴. Therefore, the role of telomere shortening in atherosclerosis is inconclusive. Our current results indicate that TERT deletion attenuates atherosclerosis formation through a mechanism that is likely unrelated to telomere length regulation.

TERT expression in vascular cells, including VSMC and endothelial cells (EC), is critical for cellular functions and adaptations during vascular remodeling and may exert anti-atherosclerotic effects through the protection against cellular senescence^{30, 31, 72, 73}. Therefore, the absence of TERT in these resident cells in our whole-body TERT deletion experiments may confound our mechanistic interpretation on the pro-inflammatory role of TERT specifically in macrophages. Consequently, chimeric LDLr^{-/-} mice with hematopoietic deletion of TERT using bone marrow transplantation enabled us to extend these observations and determine whether TERT-dependent activation of macrophages contributes to atherosclerosis formation¹⁵⁹. Similarly, bone marrow deletion of TERT suppresses atherosclerosis development and macrophage accumulation within the atherosclerotic lesion, implying a macrophage-specific effect of TERT to promote atherosclerosis formation. TERT expression in macrophages is induced during the activation of immune cells. Multiple studies have revealed that TERT activates various inflammatory signaling pathways, including MAP-kinase⁸⁹ and NF-κB signaling⁵⁸, and an effect that is mediated through telomere maintenance-

independent mechanisms. In line with these findings, our current study shows that TERT deletion reduces the expression of multiple inflammatory cytokines and prevents STAT3 activation. STAT3 signaling pathway is activated by a broad range of growth factors and cytokines¹⁶⁰. Following phosphorylation, activated STAT3 transiently translocates from the cytoplasm to the nucleus, where it functions as a core transcription factor regulating the expression of a large number of genes associated with proliferation, survival, and inflammation¹⁶¹. During innate immunity, STAT3 serves as an essential downstream target of Toll-like receptor 4 (TLR4)¹⁶² and IFN γ pathways¹⁶³ and is involved in the extensive network of the inflammatory signaling pathways, including MAP-kinase¹⁶, NF- κ B¹⁶⁴, and PI3K signaling¹⁶⁵. Therefore, STAT3 is increasingly recognized as a central signaling molecule to control cellular adaption in response to environmental stimuli or stress responses in EC, VSMC, and inflammatory cells¹⁶⁶. In macrophages, STAT3 activation polarizes macrophages from the pro-inflammatory M1 to the anti-inflammatory M2 type and exacerbates or reduces inflammation in a context-dependent manner^{24, 167, 168}. Given the multiple functions of STAT3 in various biological processes and the complex interactive network between STAT3 and other inflammatory signaling pathways, our study demonstrates a novel permissive effect of TERT on pro-inflammatory macrophages through the activation of STAT3.

Telomerase activity is upregulated in response to cytokine-induced proliferation and cell cycle activation in human hematopoietic cells¹⁶⁹. Moreover, ectopic overexpression of TERT is directly associated with the proliferative capacity of stem cells and may exert an additional role in lineage differentiation¹⁷⁰.

¹⁷¹. Therefore, we examined the proliferation and differentiation of HSC, progenitor cells, peripheral leukocytes and monocytes in LDLr^{-/-} mice reconstituted with TERT^{-/-} bone marrow. As expected, bone marrow deletion of TERT did not affect the numbers or composition of these cell lineages. Previously it has been demonstrated that critical telomere shortening reduces the response to hematopoietic ablation¹⁵⁸ and HSC engraftment¹⁷². However, in our studies telomere shortening was not evident in leukocytes isolated from first generation TERT-deficient mice¹⁷³. The dissociation of telomerase activity and telomere maintenance in human HSC was also noted by Wang et al.¹⁷⁴. Furthermore, multiple studies have identified that the prolonged self-renewal of HSC is not limited by TERT deletion^{173, 175}. All these findings indicate that stem cells from early-generation TERT^{-/-} mice with normal telomere lengths can provide efficient hematopoietic recovery with an adequate amount of functional daughter cells. Therefore, TERT contribution to atherosclerosis formation is unlikely a result of alterations in leukocytosis or monocytosis, a well-established mechanism for atherosclerosis formation^{7, 176-178}.

In summary, the current study demonstrates a TERT-dependent activation of the STAT3 signaling pathway and pro-inflammatory macrophage phenotype, which consequently contributes to atherosclerosis formation. Our findings provide novel insights into the mechanisms controlling inflammatory gene expression and characterize TERT as a previously unrecognized regulator of atherosclerosis.

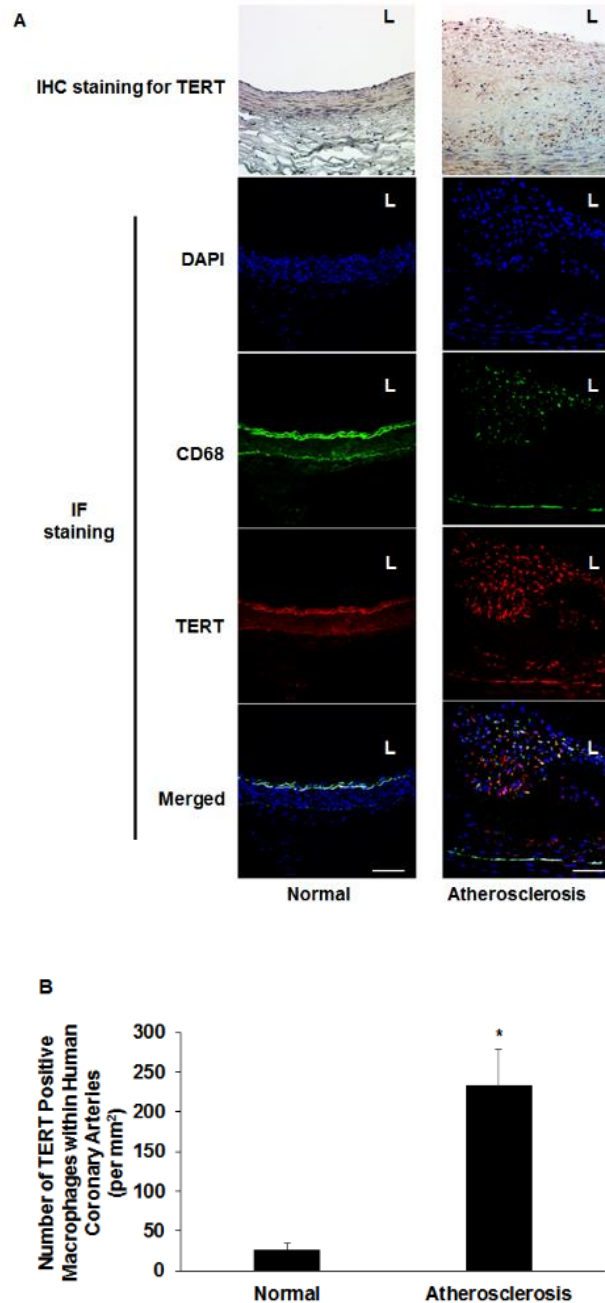


Figure 4.1 TERT is Expressed in Macrophages of Human Coronary Artery Atherosclerotic Lesions.

A. Representative IHC and IF images of serial sections of human coronary arteries from healthy or atherosclerosis patient. Segments of healthy (Left) and atherosclerotic (Right) human coronary arteries were harvested during an

autopsy. For immunohistochemistry staining (IHC), paraffin-embedded sections were stained for TERT, followed by hematoxylin counterstaining. TERT positive staining is red. Note the lack of TERT staining in normal coronary arteries compared with the abundant immunoreactivity against TERT in the shoulder region of atherosclerotic lesions. The serial sections of those used for IHC were applied for immunofluorescence staining (IF) to detect TERT and macrophage co-localization in the atherosclerotic lesion. Sections were stained for macrophage marker CD68 (green), TERT (red), and counterstained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei (blue, lower left panel). The merged images show the composite staining. The co-localization of CD68 and TERT immunoreactivity is yellow. Scale bar, 100 μ m. B. Quantification of TERT-positive macrophages within human coronary artery atherosclerotic lesion. Paraffin-embedded sections of healthy (n=5) and atherosclerotic (n=3) human coronary arteries were stained for TERT and macrophage marker CD68 by IF as described in A. The number of TERT-positive macrophages within the atherosclerotic lesions was counted from four equally distributed sites on each coronary artery segment using software ImageJ. Results are presented as mean \pm SEM (*P<0.05 vs. normal control).

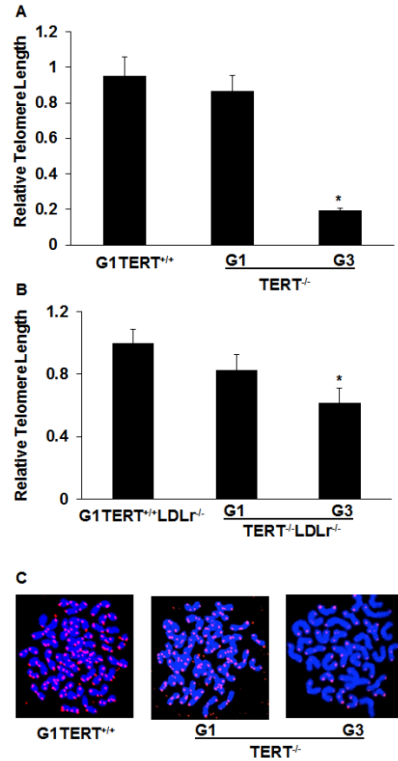


Figure 4.2 Telomere Lengths are Preserved in G1TERT^{-/-} mice but Significantly Decreased in G3TERT^{-/-} Mice.

A-B. Quantification of telomere lengths by real-time PCR in bone marrow differentiated macrophages from (A) TERT^{+/+}, G1TERT^{-/-} and G3TERT^{-/-} mice (n=3 per group) and (B) TERT^{+/+}LDLr^{-/-}, G1TERT^{-/-}LDLr^{-/-} and G3TERT^{-/-}LDLr^{-/-} mice (n=5 per group). The copy number of telomere repeats was normalized to that of a single-copy gene (36B4). Values are mean ± SEM (* p<0.05 vs. TERT^{+/+}). C. Representative telomeric fluorescence in situ hybridization assay on metaphase chromosomes of TERT^{+/+}, G1TERT^{-/-} and G3TERT^{-/-} bone marrow differentiated macrophages. DAPI-stained chromosomes and Cy-3-labeled telomeres are shown in blue and red, respectively.

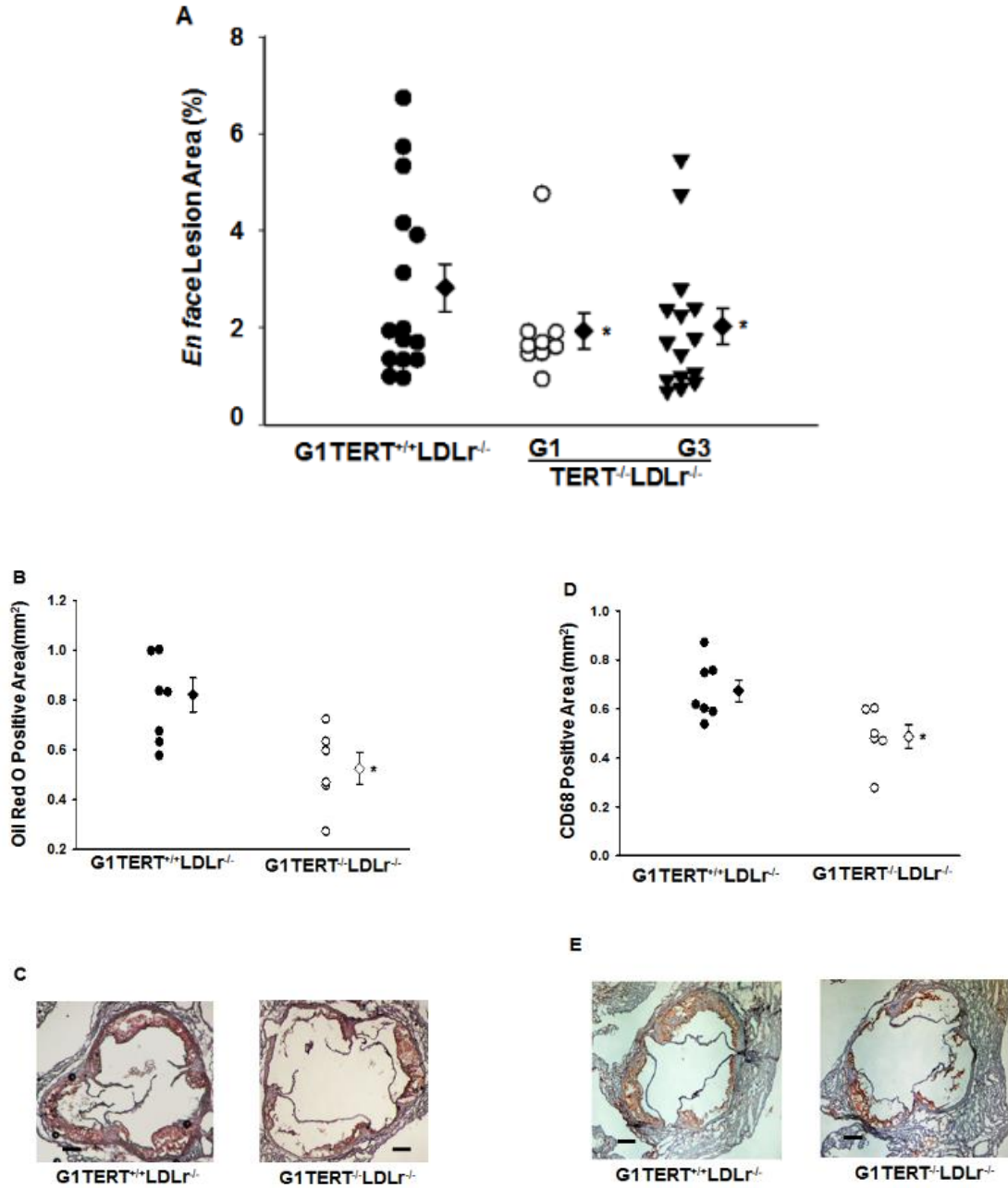


Figure 4.3 Early and Late Generation of TERT-Deficient Mice Present Comparable Reduction in Atherosclerosis Formation.

A. TERT^{-/-}LDLr^{-/-} mice developed fewer atherosclerotic lesions compared with TERT^{+/+}LDLr^{-/-} mice but no difference was found between G1TERT^{-/-}LDLr^{-/-} and G3TERT^{-/-}LDLr^{-/-}. Thoracic atherosclerotic lesion size was measured by en face analysis. The effect of TERT on atherosclerosis was compared using

the Kruskal-Wallis test followed by Dunn Test post-hoc analysis. Circles and triangles represent individual mice; diamonds represent medians (* $p < 0.05$ vs. G1TERT^{+/+}LDLr^{-/-}). B. Quantification of lipid content from oil red O staining. Circles represent individual mice; diamonds represent medians (* $p < 0.05$ vs. G1TERT^{+/+}LDLr^{-/-}). C. Representative Oil Red O staining of aortic root sections. Neutral lipids are stained red. Scale bar, 200 μ M. D. Quantification of macrophage content from macrophage immunostaining. Circles represent individual mice; diamonds represent medians (* $p < 0.05$ vs. G1TERT^{+/+}LDLr^{-/-}). E. Representative immunostaining of aortic root sections using rabbit anti-mouse CD68 antibody to label macrophages. Positive cells are stained red. Scale bar, 200 μ M.

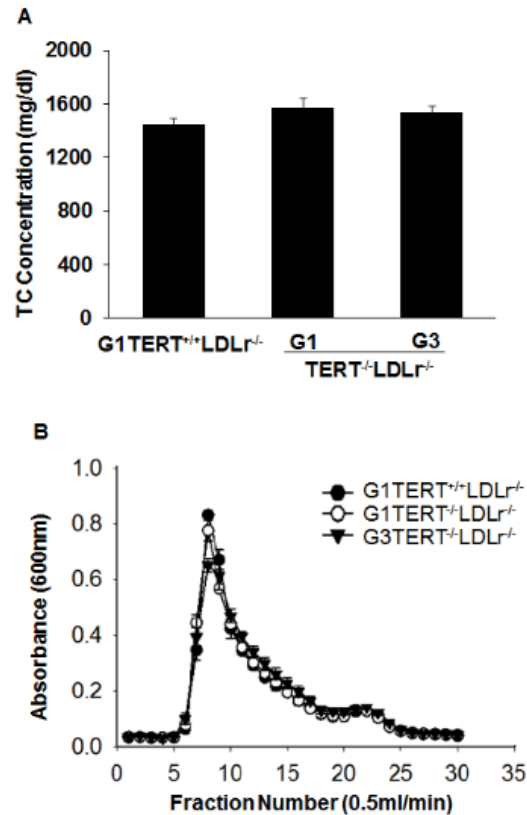


Figure 4.4 TERT Deletion does not Affect Plasma Cholesterol Concentration and Lipoprotein Distribution.

A. Total plasma cholesterol (TC) concentration measured by an enzymatic colorimetric method. Values are mean \pm SEM. B. Lipoprotein cholesterol distribution analyzed by size exclusion chromatography. Values represent each fraction by mean \pm SEM.

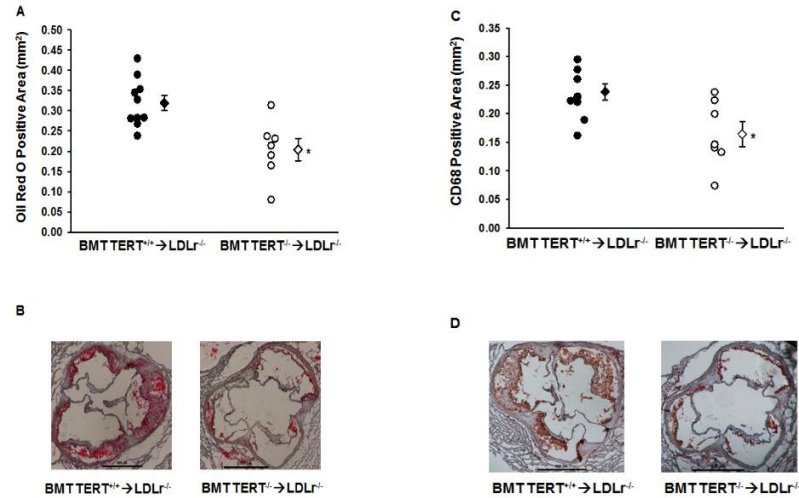


Figure 4.5 Early and late generation of TERT-deficient mice present comparable reduction in atherosclerosis formation.

Bone marrow transplantation (BMT) was applied to conditionally delete TERT expression in the hematopoietic system. LDLr^{-/-} mice reconstituted with wildtype or TERT-deficient bone marrow were fed on an atherogenic diet for 12 weeks, then atherosclerosis was analyzed by quantification of lipid and macrophage content in the aortic roots. A. Quantification of lipid content from Oil Red O staining. Circles represent individual mice; diamonds represent medians (* p<0.05 vs. BMT G1TERT^{+/+}LDLr^{-/-}). B. Representative Oil Red O staining of aortic root sections. Neutral lipids are stained red. Scale bar, 200µM. C. Quantification of macrophage content from macrophage immunostaining. Circles represent individual mice; diamonds represent medians (* p<0.05 vs. BMT G1TERT^{+/+}LDLr^{-/-}). D. Representative immunostaining of aortic root sections using rabbit anti-mouse CD68 antibody to label macrophages. Positive cells are stained red. Scale bar, 200µM.

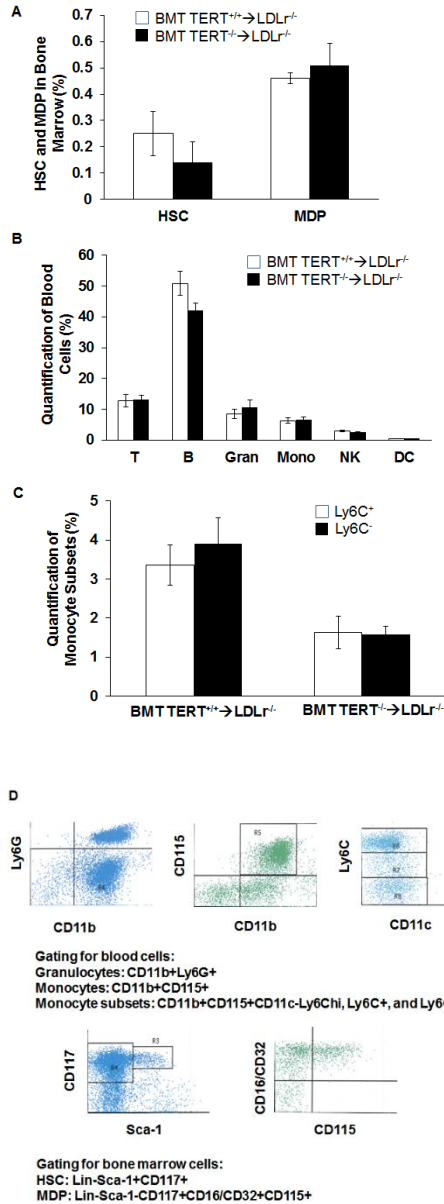


Figure 4.6 Bone Marrow Deletion of TERT Does not Alter the Counts of HSC, MDP, and Blood Cells.

A. Quantification of hematopoietic stem cells (HSC) and macrophage and dendritic progenitors (MDP) in bone marrow of chimeric LDLr^{-/-} mice received TERT^{+/+} or TERT^{-/-} bone marrow (n=10 per group) using flow cytometry. Values are mean ± SEM B. Quantification of differential white blood cell populations in LDLr^{-/-} mice reconstituted with TERT^{+/+} or TERT^{-/-} bone marrow (n=10 per group) analyzed by flow cytometry. T, T cells; B, B cells; Gran, granulocytes; Mono, monocytes;

NK, natural killer T cells; DC, dendritic cells. Values are mean \pm SEM. C. Quantification of monocyte subsets in the blood of LDLr^{-/-} mice reconstituted with TERT^{+/+} or TERT^{-/-} bone marrow (n=10 per group) by flow cytometry. Values are mean \pm SEM. D. Gating strategy for identification of HSC, MDP, and leukocytes by flow cytometry.

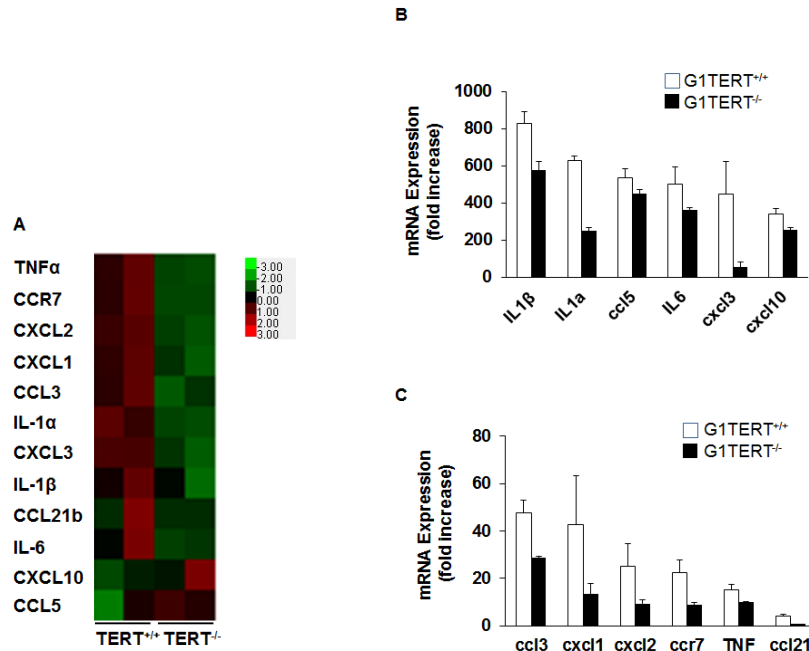


Figure 4.7 TERT-Deficient Macrophages Display Silenced Inflammatory Gene Expression.

A. G1TERT^{-/-} or G1TERT^{+/+} BMDM were treated with 100ng/ml LPS for 8 hours. Inflammatory gene expression was analyzed by the NanoString nCounter Analysis System. A. Heat map of LPS-induced genes changed more than two-fold with TERT deletion. Each colored square on the heat map represents the relative transcript abundance (in log₂ space) of each gene with the high expression being red and the low expression being green. Each column represents one group (n=3 per group) and each row shows the transcript level of one gene. B-C. NanoString expression graphs of atherosclerosis-related inflammatory genes that changed more than two-fold with TERT deletion. Data are presented as fold increase relative to untreated cells. Values are mean \pm SEM.

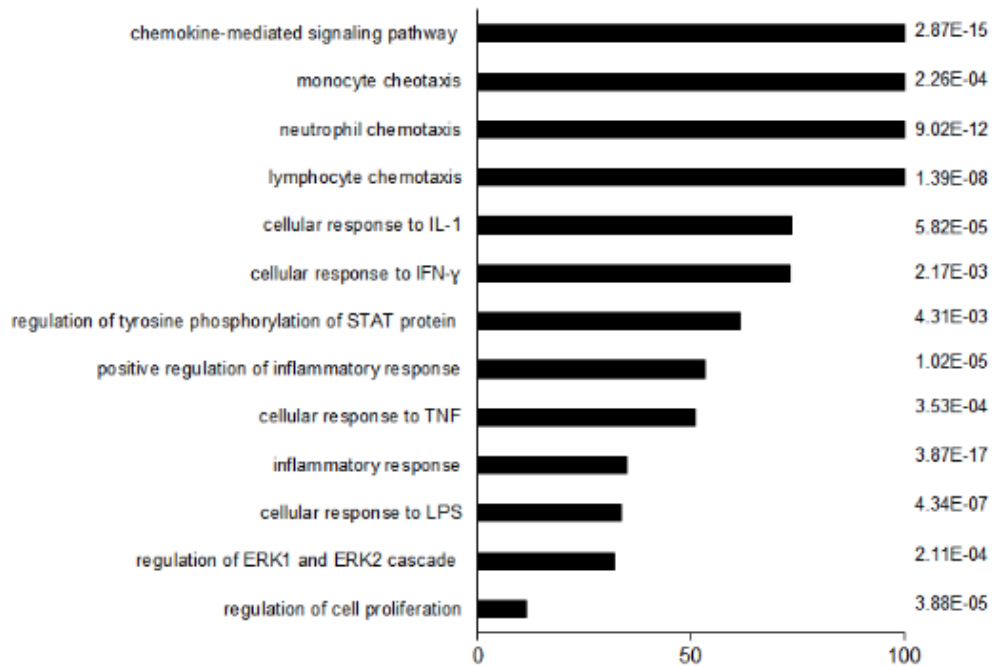


Figure 4.8 Gene Ontology Enrichment Analysis of TERT-Target Genes.

LPS-induced inflammatory genes changed more than 1.5 fold by TERT deletion are included in gene ontology (GO) biological process enrichment analysis. Enrichment analysis was performed using the Gene Ontology Consortium (<http://geneontology.org/page/go-enrichment-analysis>) based on biological process terms. P-value of every enriched biological process is indicated at the end of each column. IL-1: interleukin-1; interferon-gamma: IFN γ ; TNF: tumor necrosis factor; lipopolysaccharide: LPS: lipopolysaccharides; ERK: extracellular signal-regulated kinases.

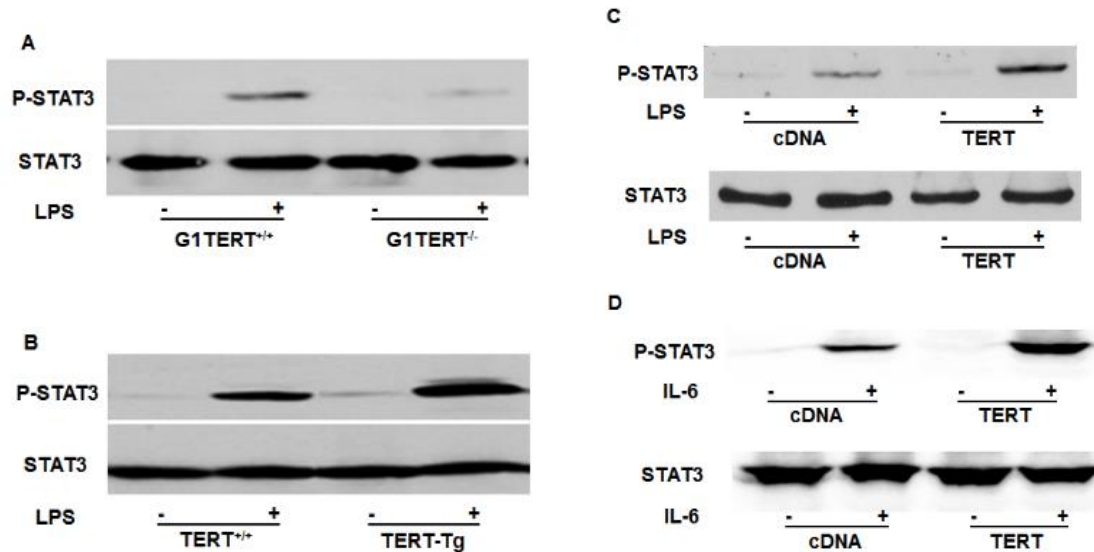


Figure 4.9 TERT Activates STAT3 Signaling Pathway.

A. Inhibition of STAT3 phosphorylation in TERT-deficient BMDM. G1TERT^{-/-} and G1TERT^{+/+} BMDM were stimulated with 100ng/ml LPS for 1 hour; P-STAT3 and total STAT3 protein expression were detected by Western blot analysis. B. Enhanced STAT3 phosphorylation in the BMDM overexpressing TERT (TERT-Tg). TERT-Tg and TERT^{+/+} BMDM were treated with 100ng/ml LPS for 1 hour; P-STAT3 and total STAT3 protein expression were detected by Western blot analysis. C-D. Increased STAT3 phosphorylation in HEK 293 cells with TERT overexpression. HEK 293 cells were transfected with plasmids that overexpress TERT. 24 hours after transfection, cells were treated with 1 μ g/ml LPS (C) or 10ng/ml IL-6 (D) for 30 minutes before whole cell lysates were collected. P-STAT3 and total STAT3 protein expression were detected by Western blot analysis.

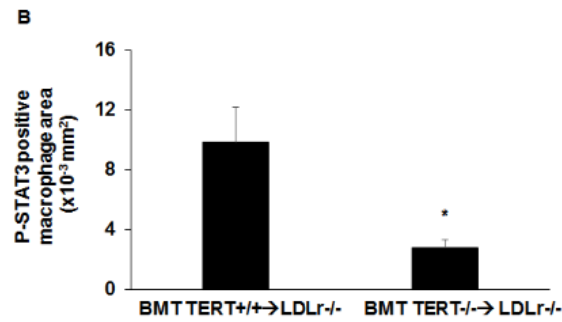
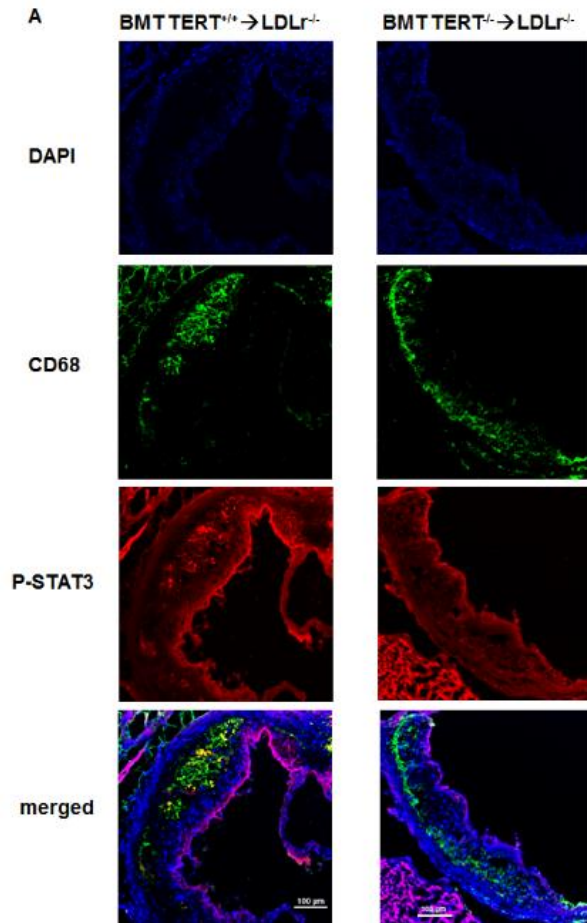


Figure 4.10 Reduced STAT3 Activation within the Macrophages in the Atherosclerotic Lesions of LDLr^{-/-} Mice with Bone Marrow Deletion of TERT

A. Representative images of the p-STAT3 and CD68 co-staining in the sections of aortic roots from LDLr^{-/-} mice transplanted with TERT^{+/+} or TERT^{-/-} bone marrow. Sections were stained for macrophage marker CD68 (green), p-STAT3 (red), and counterstained with 4', 6-diamidino-2-phenylindole (DAPI) to visualize nuclei (blue). The merged images show the composite staining. The colocalization of CD68 and p-STAT3 immunoreactivity is yellow. Scale bar, 100µm.

B. Quantification of p-STAT3-positive macrophages within the aortic atherosclerotic lesion. The area of p-STAT3 positive macrophages within the atherosclerotic lesions was counted from three equally distributed sites on each segment using software ImageJ. Results are presented as mean ± SEM (*P<0.05 vs. BMT TERT^{+/+}→LDLr^{-/-}).

5 CHAPTER FIVE

General Discussion

5.1 Summary

Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase and the limiting factor for enzymatic activity. Expression of TERT and telomerase activity is increased in atherosclerotic plaques. However, the role of TERT dysregulation during atherosclerosis formation remains unknown.

The work herein first identified a multi-tiered regulation of TERT expression in VSMC through HDAC inhibition. In VSMC treated with a small molecular HDAC inhibitor, the abundance of TERT transcript increased through transactivation of the TERT promoter. At the protein level, in contrast, HDAC inhibition accelerated the turnover of TERT towards degradation, which reduced overall telomerase activity and induced senescence. Furthermore, during vascular remodeling, TERT protein expression in the neointima was suppressed by HDAC inhibition. These data illustrate a differential regulation of TERT transcription and protein stability by HDAC inhibition. Moreover, the current study found that TERT deficiency reduces neointima formation following guidewire-induced arterial injury. Studies in SMC isolated from TERT-deficient and TERT overexpressing mice established that TERT is necessary and sufficient for cell proliferation without affecting telomere length. TERT deficiency induced cell cycle arrest at G1 phase without causing senescence albeit hyperphosphorylation of the retinoblastoma protein. This

proliferative arrest was associated with stable silencing of the E2F1-dependent S-phase gene expression program which could not be reversed by ectopic overexpression of E2F1. Chromatin immunoprecipitation and accessibility assays revealed that TERT was recruited to chromatin regions containing E2F1 binding sites and facilitated the permissive histone modifications to allow the access of these locations to the transcription factor. These data indicate a promotional effect of TERT on SMC growth and neointima formation through epigenetic regulation of proliferative gene expression. Also, we investigated the function of TERT in macrophages during atherosclerosis formation. TERT expression was upregulated in activated macrophages during atherosclerosis formation. To study the role of TERT in this process, we crossbred TERT-deficient mice with LDL-receptor-deficient (LDLR^{-/-}) mice to generate first generation G1TERT^{-/-}LDLR^{-/-} offsprings, which were then intercrossed to obtain the third generation G3TERT^{-/-}LDLR^{-/-} mice. G1TERT^{-/-}LDLR^{-/-} mice exhibited no telomere shortening while severe telomere attrition was evident in G3TERT^{-/-}LDLR^{-/-} mice. When fed an atherogenic diet, G1TERT^{-/-}LDLR^{-/-} and G3TERT^{-/-}LDLR^{-/-} mice were both protected from atherosclerosis formation compared to their wild-type controls, indicating that genetic TERT-deletion prevents atherosclerosis, and formation of the disease is not affected by telomere attrition. Similarly, atherosclerotic development was decreased in chimeric LDLR^{-/-} mice with stem cell deletion of TERT generated by bone marrow transplantation. TERT deficiency reduced the accumulation of macrophages in atherosclerotic lesions and inhibited the expression of proinflammatory cytokines, including CXCL1/2/3, CCL3, CCL5, CCL21, CCR7, IL-

6, and IL-1 α . Gene ontology (GO) enrichment analysis of inflammatory genes altered by TERT deletion identified an overrepresentation of STAT3-dependent, which was confirmed by western blotting showing a decreased tyrosine phosphorylation of the STAT3 protein in TERT-deficient BMDM. These findings suggest that the protective effect of TERT deletion on atherosclerosis formation is likely a result of suppressed STAT3 signaling in macrophages, which silences the expression of a variety of proinflammatory cytokines.

In conclusion, the current study identifies that TERT dysregulation within atherosclerotic plaques plays a causative role in atherosclerosis and injury-induced neointima formation by promoting VSMC proliferation and macrophage inflammatory response. These findings identify a novel mechanism by which TERT might exacerbate pathological vascular remodeling under diseased conditions, which may provide a new therapeutic target.

5.2 TERT Dysregulation During Vascular Remodeling

5.2.1 TERT Expression Is Up-Regulated in the VSMC and Macrophages During Vascular Remodeling

Telomeres get shortened with each cell division due to the “end replication problem”⁴². Therefore, the inverse relationship between telomere attrition and age makes telomere length to be proposed as a candidate biomarker of aging⁶⁸, which is a major risk factor for atherosclerosis¹⁷⁹. Consequently, leukocyte telomere attrition is suggested to correlate with vascular aging and cardiovascular events,

although the clinical association has not been consistent⁶⁸. Furthermore, telomere length measured in peripheral leukocytes is not a surrogate marker for other tissues¹⁸⁰. In line with this concept is Huzen's finding that atherosclerosis patients had shorter leukocyte telomeres, but longer plaque telomeres that are related to plaque characteristics and development of restenosis following endarterectomy⁶⁹. Therefore, the relationship between short leukocyte telomere length and atherosclerosis severity is inconclusive, and telomere integrity in local tissue may change differently from that of peripheral blood cells.

Telomerase, an enzyme with its RNA template and reverse transcriptase, is the primary mechanism to prevent telomere erosion by synthesizing telomeric DNA²⁵. Telomerase activity and the expression of its catalytic subunit TERT are suppressed in differentiated cells^{29, 181}. In line with this concept, expression of TERT and telomerase activity was found to be negligible in the normal vasculature. In the pathogenic vascular remodeling process preceding atherosclerosis and injury-induced neointima formation, however, TERT expression and telomerase was reactivated^{30, 31, 71}. Our findings are consistent with an earlier study in which an increased TERT expression and telomerase activity were identified in the aorta of hypertension¹³². Data published by Matthews et al. also noted that TERT expression was readily detectable in lesional macrophages⁷⁸. Similarly, neutrophils isolated from coronary artery plaques of patients with unstable angina was found to have abnormal telomerase activity⁷⁰. In contrast, EC in the aortic wall during aneurysm formation¹⁸² and VSMC in the atherosclerotic fibrous cap⁷⁸ were thought to express low levels of TERT rendering the telomerase inert.

However, a technical drawback was acknowledged in these two studies, that both measured TERT and telomerase expression using immunohistochemistry staining, which only provides the semi-quantitative assessment thus presents a risk of experimental bias. With repeated analysis in three different mouse models, our group provides compelling evidence for the reactivation of telomerase and TERT expression in the artery during vascular remodeling.

5.2.2 Multi-Layer Control of TERT Expression During Vascular Remodeling

Transcriptional control constitutes the primary mechanism of regulation of TERT expression¹⁸³. Our previous studies observed a profound induction of TERT expression and telomerase activity in the neointima following vascular injury mediated by Ets-1 or E2F1 dependent transactivation of the TERT promoter^{30, 31}; besides, a similar change in TERT expression and telomerase activity was detected in atherosclerotic lesional macrophages as a result of NF-kB signaling⁷¹. This early research from our group indicates that pro-atherogenic mediators upregulate TERT transcription through promoter activation.

In addition to transcriptional regulation, TERT expression is also subject to modulation via post-transcriptional, post-translational modifications, as well as epigenetic mechanisms²⁷. In the current study, we illustrate a differential control at the transcript versus protein levels of TERT expression by HDAC inhibition. Quiescent somatic cells epigenetically silence the TERT promoter activity by histone deacetylation³⁸, an effect that is reversed during malignant cell growth due to HDAC sequestration⁴¹. Our observation of enhanced TERT promoter activity

upon HDAC inhibition is consistent with several prior studies^{38, 39, 111}. However, we also noticed a decrease in the abundance of TERT protein and telomerase activity due to enhanced degradation of the TERT protein. The two-stage control of TERT expression by HDAC inhibition might be a result of enhanced global acetylation of nonhistone proteins following HDAC inhibitor treatment. HDAC inhibitors have been shown to promote protein degradation through post-translational modification in addition to the classical epigenetic mechanism^{115, 116}. Moreover, although TERT expression is primarily regulated at the transcriptional level, posttranslational modification and proteasome-mediated degradation of TERT have been reported to play a significant role in telomerase inactivation^{117, 118}. Therefore, our findings support the notion that TERT expression is subject to a coordinated multi-layer control of expression, in which HDAC inhibitor-induced protein acetylation may modulate levels of epigenetic and post-translational regulation of TERT protein expression.

5.3 TERT in Atherosclerosis Formation

5.3.1 Telomere-Independent Function of TERT in Vascular Remodeling

The mitogenic phenotype of VSMC triggers the intimal hyperplasia at the early stage of atherosclerosis³. In the present study, we illustrate a previously unrecognized epigenetic regulation of TERT over E2F1 target genes in proliferating VSMC. TERT associates with histone acetyltransferase P300 to open chromatin regions at E2F1 consensus sites and facilitates E2F1 target gene transcription to induce cell cycle progression. As macrophage-mediated

inflammation plays a fundamental role in all stages of disease from initiation through progression and, ultimately, the thrombotic complications of atherosclerosis, we continued to investigate the macrophage-specific function of TERT. Our data show that TERT deficiency suppresses atherosclerosis formation through silencing STAT3-regulated inflammatory genes in macrophages. Moreover, preliminary results on the function of TERT in VSMC and macrophages were corroborated by similar findings from in vivo experiments in the early generation of TERT-deficient mice with normal telomere length, suggesting a telomere-independent function of TERT during vascular remodeling. In concert with these observations was our previous study that also documented that TERT-deficient macrophages per se could prevent MMP-2 transcription and aortic aneurysm formation with no significant telomere erosion⁵⁷. In support of our findings of this novel function of TERT, results from other groups show that TERT modulates the chromatin state¹⁸⁴, interacts with DNA-modifying enzymes⁶⁰, and activates a variety of genes regulating proliferation^{55, 152, 185}, apoptosis^{46, 148, 186, 187}, inflammation^{58, 86, 188}, angiogenesis^{189, 190}, and stem cell mobilization^{191, 192}. Therefore, TERT may regulate cell function not only through canonic telomere elongation but also through non-canonical mechanisms that require further investigation.

5.3.2 TERT Facilitates Chromatin Remodeling

Using chromatin immunoprecipitation assays, we demonstrate that TERT promotes E2F1 target gene transcription by increasing chromatin accessibility

through p300-mediated histone acetylation. Other groups also identified that TERT modulates chromatin architecture through interaction with BRG1 (Brahma-related gene 1)^{60, 184}, a subunit of chromatin remodeling complex SWI/SNF with ATPase activity. The model explains the synergistic effects of histone acetylation and ATP-dependent chromatin remodeling complexes on transcriptional activation, during which additional chromatin remodeling complexes, including the SWI/SNF complex, are recruited to restructure the nucleosomes and expose the binding sites of transcriptional factors³⁵. In line with this concept, accumulating evidence supports that the p300 coactivator interacts with the chromatin-modifying BRG1 complex during transcriptional activation to stimulate histone acetylation^{193, 194}. Specifically, Zhang et al. characterized a BAF (BRG1-associated factors)-mediated interaction of p300 and BRG1 during transcriptional regulation¹⁹⁵. Consistent with these findings, our observation of TERT binding to p300 may provide a mechanism by which TERT permits transcription of proliferative genes. Therefore, our current findings identify a novel role of TERT in the epigenetic regulation of VSMC proliferation.

5.4 Future Study

5.4.1 Define the Core Transcriptional Network of TERT During Atherosclerosis Formation

Our study characterizes TERT as a transcriptional coactivator to generate permissive chromatin through association with p300 to promote histone H3 acetylation. Histone acetylation marks genes that are actively transcribed¹⁹⁶.

Therefore, the binding of TERT to the enhancer-associated protein p300 supports that TERT serves as a bridge between the core transcriptional machinery and the functional regulatory elements during transcriptional activation. A similar mechanism of chromatin regulation has been described by the interaction between BRG1 and TERT to facilitate the transcription of Wnt target genes⁶⁰. Furthermore, TERT has been shown to regulate transcription via an association with the transcriptional machinery. For example, TERT increases RNA pol III binding to target genomic regions¹⁹⁷; TERT physically binds to p65 and affects genome-wide p65 occupancy of target chromatin⁵⁸. Furthermore, many studies have implied that TERT binds to various promoter regions and activates target gene transcription. For example, TERT stimulates RNA polymerase I transcription and enhances ribosomal biogenesis¹⁹⁸. Based on these findings, we hypothesize that TERT is involved in a transcriptional network that ultimately controls essential cellular functions, including proliferation, inflammation, apoptosis, and DNA damage response pathways. Given that TERT is highly expressed and exhibits potent telomerase activity within the atherosclerotic lesions, it will be important to understand related mechanisms by which TERT regulates cellular functions and accelerates disease progression.

5.4.2 Define the Pharmacological Application of TERT for Atherosclerosis Treatment

A hallmark of atherosclerosis development and neointimal remodeling is aberrant VSMC proliferation², which can be effectively suppressed by HDAC

inhibitors¹⁰¹. HDAC inhibitors have been characterized as a unique category of anti-cancer drugs with four HDAC inhibitors approved by the United States Food and Drug Administration. HDAC inhibitors target TERT expression in cancer cells⁴⁰¹²¹. Our current study further demonstrates a novel mechanism underlying the anti-mitotic efficacy of HDAC inhibitors during neointima formation involving the suppression of TERT expression. Moreover, TERT dysregulation within the arterial wall contributes to atherosclerosis and neointima formation by promoting VSMC proliferation and macrophage inflammatory responses. These findings prompt an intriguing question: Is TERT a reliable pharmacological target for atherosclerosis therapy? There are a variety of telomerase-inhibiting compounds under investigation for cancer therapy since most cancer cells exhibit high telomerase activity compared to normal somatic cells. However, there is currently no TERT targeting compound suitable for selectively targeting tumor cells without affecting the normal stem cell population, highlighting the importance of cell-specific targeting for future pharmacological development.

In summary, the current study highlights an aberrant over-expression of TERT within atherosclerotic lesions and a novel role of TERT in VSMC mitosis and macrophage inflammation. However, in order to design potent TERT-targeting therapeutics for vascular diseases and avoid potential side effects, a gap for future development remains. In particular, understanding mechanisms of gene regulation by TERT and pathways of cell-specific targeting of TERT will constitute important avenues for future research.

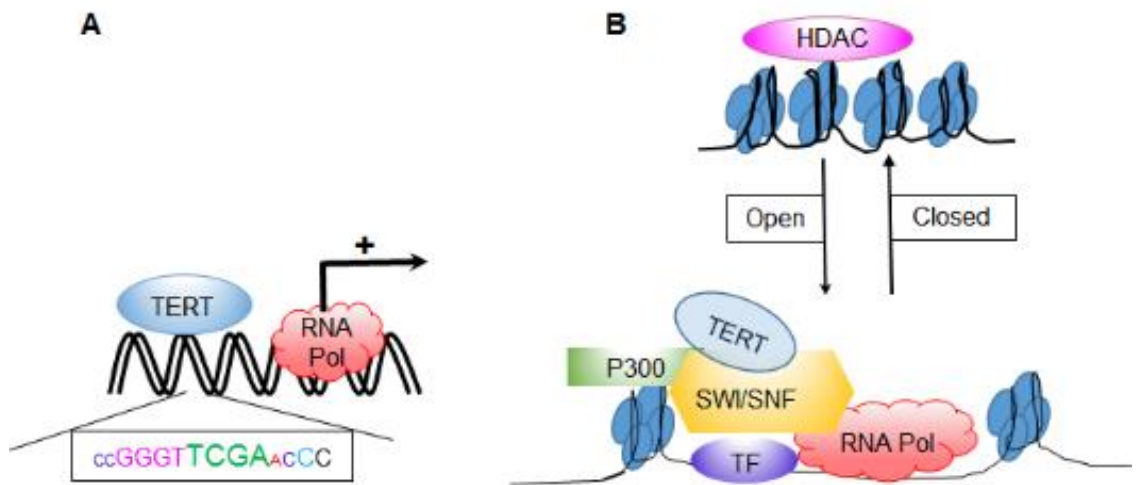


Figure 5.1 Proposed TERT Function Over Transcription Regulation.

TERT plays a role in bridging the core transcriptional machinery to the functional regulatory elements during transcriptional activation. A, TERT acts as a transcriptional factor binding to the DNA motif to activate target promoters. The nucleotides in color are the presumed sequence motif of TERT in hESC-1 cells based on genome-wide ChIP-seq results¹⁹⁷. B, TERT recruits transcriptional coactivators to the core machinery thus enhancing transcriptional efficiency.

APPENDICES

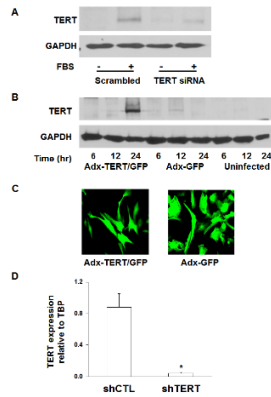


Figure A.1 A, TERT depletion using siRNA-mediated knock-down. Human coronary artery SMC were transfected with TERT siRNA or scrambled control, serum deprived for 24 hours, and stimulated with FBS for 48 hours. TERT expression was analyzed by Western Blotting. Co-hybridization for GAPDH was used to control for equal protein loading. B-C, Adenovirus-mediated overexpression of TERT in human coronary artery SMC. Cells were transduced for the indicated time points with 30 PFU of adenoviral constructs and analyzed for TERT expression by Western Blotting (B). Transduction efficiency of co-expressed GFP was confirmed by fluorescent microscopy at 50 PFU (C). The autoradiograms and images shown are representative of at least three independently performed experiments using different cell preparations. (D) TERT depletion using a lentiviral vector expressing TERT shRNA. Human coronary artery SMC were transduced with lentiviral vector expressing TERT shRNA (shTERT) or nontargeting control (shCTL) for 24 hours. mRNA expression of TERT was analyzed 48 hours after transduction by quantitative real-time PCR and normalized to transcript levels of the housekeeping gene TBP. Values are mean \pm SEM (* P <0.05 versus shCTL).



Figure A.2 Telomere lengths of SMC isolated from G1TERT^{+/+}, G1TERT^{-/-} and G3TERT^{-/-} mice. Quantification of telomere lengths in G1TERT^{+/+}, G1TERT^{-/-} and G3TERT^{-/-} SMC using real-time PCR (n=3 mice per group). Data are presented as fold change relative to 36B4 (mean ± SEM, *P < 0.05 versus G1TERT^{+/+}).

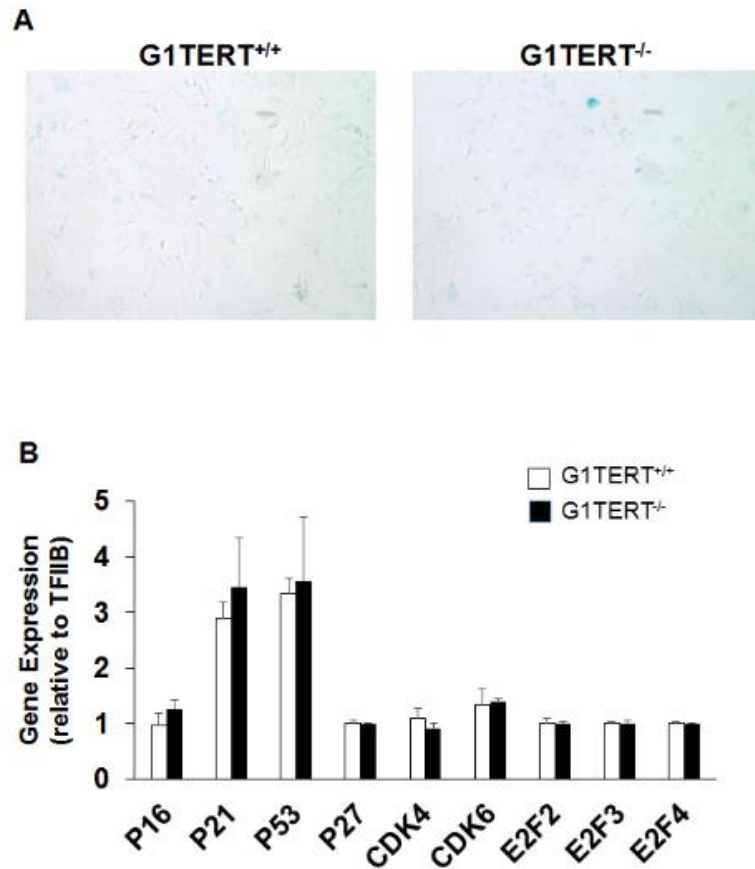


Figure A.3. A, G1TERT^{+/+} and G1TERT^{-/-} SMC were stained for senescence-associated b-galactosidase activity (objective magnification, x20). The images shown are representative of three independently performed experiments using different cell preparations. B, Transcript levels of p16, p21, p53, p27, CDK4, CDK6, E2F2, E2F3, and E2F4 in G1TERT^{+/+} and G1TERT^{-/-} SMC. mRNA expression levels of the indicated genes were analyzed by real-time RT-PCR and normalized to transcript levels of the housekeeping gene transcription factor TFIIB. Data are presented as mean \pm SEM, (n=4/group).

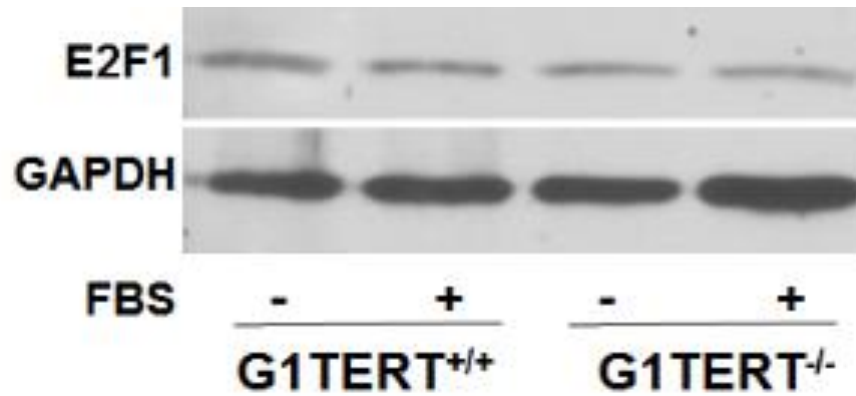


Figure A.4 TERT deletion does not affect E2F1 expression in SMC. Western blotting for E2F1 expression in whole cell lysates isolated from quiescent SMC (- FBS) stimulated with FBS (+ FBS). Co-hybridization for GAPDH was used to control for equal protein loading. The autoradiogram shown is representative of three independently performed experiments using different cell preparations.

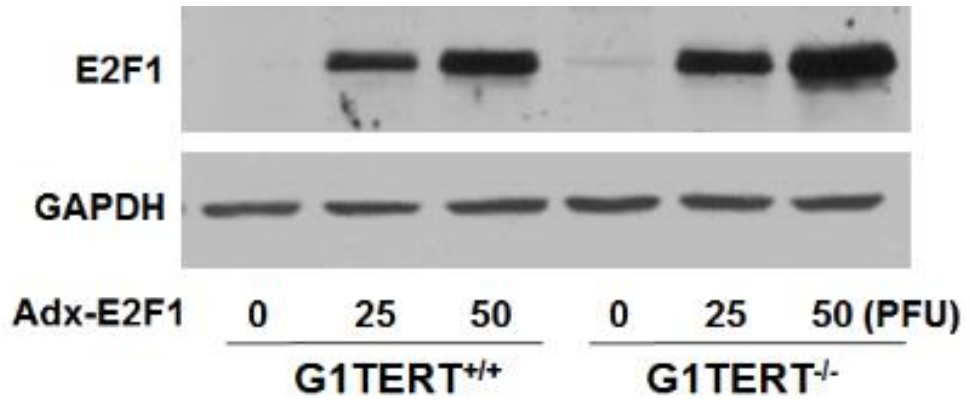


Figure A.5 TERT deletion does not affect E2F1 expression in SMC. Western blotting for E2F1 expression in whole cell lysates isolated from quiescent SMC (- FBS) stimulated with FBS (+ FBS). Co-hybridization for GAPDH was used to control for equal protein loading. The autoradiogram shown is representative of three independently performed experiments using different cell preparations.

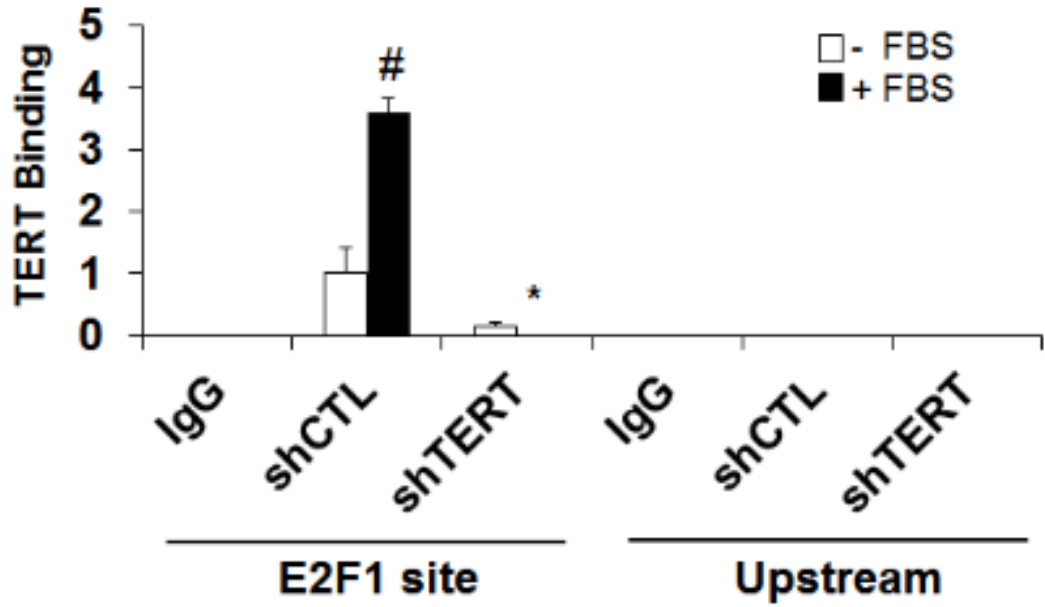


Figure A.6 TERT binding is dependent on the presence of the E2F1 binding site in the MCM7 promoter. Chromatin upstream of the E2F1 promoter sequence was not immunoprecipitated by the TERT antibody. Data are presented as mean \pm SEM (# P <0.05 versus quiescent shCTL, * P <0.05 versus FBS treated shCTL).

Table I: Primers used in this study

Name	Use	Sequence
Murine MCM7	RT-PCR	F: 5'-GGCACAGGGCTTACTCTCAG-3' R: 5'-AGGAGACTGGTCCACACCAC-3'
Murine Cyclin A	RT-PCR	F: 5'-CTTGCTGCGGGTAAAGAGAC-3' R: 5'-CGCAGCAGAAGCTCAAGGC-3'
Murine PCNA	RT-PCR	F: 5'-TTGGAATCCCAGAACAGGAG-3' R: 5'-ATTGCCAAGCTCTCCACTTG-3'
Human MCM7	RT-PCR	F: 5'-TGTGGGGCAGAGACCTAC-3' R: 5'-CTGGGCAATCCTTGTGTT-3'
Murine P53	RT-PCR	F: 5'-CCTCTGAGCCAGGAGACATTTTC-3' R: 5'-AAGCCCAGGTGGAAGCCATAGTTG-3'
Murine Rb	RT-PCR	F: 5'-CCTTGAACCTGCTTGTCCCTC-3' R: 5'-GGGCAAGGGAGGTAGATTTC-3'
Murine P16	RT-PCR	F: 5'-CTTTGTGTACCGCTGGGAAC-3' R: 5'-CGCTAGCATCGCTAGAAGTG-3'
Murine P21	RT-PCR	F: 5'-GTACTTCTCTGCCCTGCTGC-3' R: 5'-GCTTGGAGTGATAGAAATGGTCAGG-3'
Murine P27	RT-PCR	F: 5'-GCCGGGATATGGAAGAAG-3' R: 5'-ATGCCGGTCCTCAGAGTT-3'
Murine CDK4	RT-PCR	F: 5'-GCTGCCACTCGATATGAACC-3' R: 5'-CCTTAACAAGGCCACCTCAC-3'
Murine CDK6	RT-PCR	F: 5'-GAGAAGGACAGCCTGAGTCG-3' R: 5'-CCTCACTGGTCTGCACTCG-3'
Murine E2F2	RT-PCR	F: 5'-ACGGCGCAACCTACAAAGAG-3' R: 5'-GTCTGCGTGTAAGCGAAGT-3'
Murine E2F3	RT-PCR	F: 5'-AAACGCGGTATGATACGTCCC-3'

		R: 5'-CCATCAGGAGACTGGCTCAG-3'
Murine E2F4	RT-PCR	F: 5'-CTCACCACCAAGTTCGTGTC-3' R: 5'-TCTCGATCAGACCGATGCCTT-3'
Murine TFIIB	RT-PCR	F: 5'-CTCTCCCAAGAGTCACATGTCC-3' R: 5'-CAATAACTCGGTCCCCTACAAC-3'
Human TBP	RT-PCR	F: 5'-GGAGAGTTCTGGGATTGTACCGC-3' R: 5'-ATATTCGGCGTTTTCGGGCAC-3'
E2F1 target sites within the murine MCM7 promoter	ChIP	F: 5'-GCTTTAAGAAACACTCCTCCCACAC-3' R: 5'-GCCAGCCCCTAACTTTAACCAATCAA-3'
E2F1 target sites within the murine PCNA promoter	ChIP	F: 5'-CCTCCCAGGCTCCTACCC-3' R: 5'-GGCCTACAGCGACAACCTACC-3'
E2F1 target sites within the murine Cyclin A promoter	ChIP	F: 5'-CCACTGAGCAGCAGAGATCGGC-3' R: 5'-GGCGGGAGGAGCGTAGAGC-3'
Upstream sequence of E2F1 binding sites in the murine MCM7 promoter	ChIP	F: 5'-CACATTGCGTGAGATGGTTC-3' R: 5'-GGTGGCCACAACCTTAGAAA-3'
Upstream sequence of E2F1 binding sites in the murine PCNA promoter	ChIP	F: 5'-CGGGTTCATCTTCAGCCTAA-3' R: 5'-GCGTCGCTCTGAACTTTTCT-3'
Upstream sequence of E2F1 binding	ChIP	F: 5'-AGGACAATTGGGACAGCATT-3' R: 5'-CTAGGCAGGAGCGTATGGAT-3'

sites in the murine		
Cyclin A promoter		
E2F1 target sites		
within the human	ChIP	F: 5'- ATGTTGGCCAGGCTGATTTC -3' R: 5'- CTCCCAGGACTGGTTCATTG-3'
MCM7 promoter		
E2F1 target sites in		
the human PCNA	ChIP	F: 5'- CTGGCTGCTGCGCGA-3' R: 5'- CACCACCCGCTTTGTGACT-3'
promoter		
E2F1 target sites in		
the human Cyclin A	ChIP	F:5'-TCACCTGGCTTGTCAGTCAC-3' R:5'-GACAGAGCTGGGGCTTGG-3'
promoter		
Upstream sequence		
of E2F1 binding	ChIP	F: 5'-GTAGAGGCGGAGTTTCACCA-3' R: 5'-CTCCCAGGACTGGTTCATTG-3'
sites in the human		
MCM7 promoter		
Upstream sequence		
of E2F1 binding	ChIP	F: 5'-TGTGATGCTATGTTTTAAAGAGGTA-3' R: 5'- AATTTTATGCCACGTACATCTTTTATC-3'
sites in the human		
PCNA promoter		
Upstream sequence		
of E2F1 binding	ChIP	F: 5'-CGCTTTCATTGGTCCATTTC-3' R: 5'-CCGGCCAAAGAATAGTCGTA-3'
sites in the human		
Cyclin A promoter		
E2F1 binding sites		
in the murine	ChART	F: 5'-GCTTTAAGAAACACTCCTCCCACAC-3'
MCM7 promoter	PCR	R: 5'-GCCAGCCCCTAACTTTAACCAATCAA-3'
(-346 to -176)		

upstream sequence		
of the murine	ChART	F:5'-ACATCACGACCACCACTGTC-3'
MCM7 promoter (-620 to -513)	PCR	R: 5'-GGGGTAGGGTAGCTGGGTAG-3'
Telomere	Telomere	tel 1: 5'-GTTTTTGGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3'
	Length	tel 2: 5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3'
	Analysis	
36B4	Telomere	F: 5'-CAACCCAGCTCTGGAGAAAC-3'
	Length	R: 5'-AAGCCTGGAAGAAGGAGGTC-3'
	Analysis	

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EDUCATION

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PUBLICATIONS

Peer-Reviewed Publications

1. Endorf EB, **Qing H**, Aono J, Terami N, Doyon G, Hyzny E, Jones KL, Findeisen HM, Bruemmer D. (2017) Telomerase Reverse Transcriptase Deficiency Prevents Neointima Formation Through Chromatin Silencing of E2F1 Target Genes. *Arterioscler Thromb Vasc Biol.* 37(2):301-311. PMID 27932351
2. Aono J, Ruiz-Rodriguez E, **Qing H**, Findeisen HM, Jones KL, Heywood EB, Bruemmer D. (2016) Telomerase Inhibition by Everolimus Suppresses Smooth Muscle Cell Proliferation and Neointima Formation Through Epigenetic Gene Silencing. *JACC Basic Transl Sci.* 1 (1-2):49-60. PMID 27127803
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Non-Peer Reviewed Publications

1. **Qing H.**, Zhang S. (2008) The effect of high-dose intravenous methylprednisolone pulse therapy for infiltrative ophthalmopathy. *Journal of endocrine surgery* **2**(2): 109-111

2. Wei Y., **Qing H.**, Duan L, Mei M, Cheng Q, Li Q. (2008) The tumor necrosis factor- α inhibits retinal binding protein 4 secretion in mature 3T3-L1 adipocytes. *Journal of Chongqing Medical University* **33**(5): 575-577
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6. **Qing H.**, Li Q., Lou S., Zhang S. (2005) Impact of early malnutrition on pancreatic β -cell function of rats at adult stage. *Chongqing Medical Journal* **34**(1): 21-23
7. **Qing H.**, Luo R., Li Q. (2005) Comparison between efficacy of nateglinide and repaglinide in improving type 2 diabetes pancreatic β -cell function. *Journal of Chongqing Medical University* **30**(3): 930-933
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9. Luo S., Li Q., **Qing H.** (2004) Malnutrition during the early phase of life causes insulin resistance and impaired glucose tolerance in adult rat. *Chinese Journal of Diabetes* **12**(4): 300-302

WORK EXPERIENCE

2004~2008

Resident

Department of Internal Medicine, the First Affiliated Hospital of Chongqing

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2008~2010

Attending Physician.

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2010~2013

Post Doctor

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PRESENTATIONS AND POSTERS

Presentation: Telomerase in Atherosclerosis

Pharmacology and Nutritional Sciences seminar 2016

Presentation: Telomerase in Atherosclerosis

Pharmacology and Nutritional Sciences seminar 2015

Poster: Telomerase in Atherosclerosis

Cardiovascular Research day, University of Kentucky Gill Heart Institute 2015

Poster: Telomerase in Atherosclerosis

DOM Research Day, University of Pittsburgh Department of Medicine 2016

Poster: Poster: Telomerase in Atherosclerosis

25th Annual Fellows Research Day, University of Pittsburgh 2017

PROFESSIONAL AFFILIATIONS

2015~ Present Member, American Association for the Advancement of Science
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2013~ Present Member, American Heart Association (AHA)

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