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Thoracic Aortic Aging and Smooth Muscle Cell Phenotype

Jason Benjamin Wheeler
Medical University of South Carolina

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By

Jason Benjamin Wheeler

A thesis submitted to the faculty of the Medial University of South Carolina in partial fulfillment of the requirements for the degree of Master of Science in the College of Graduate Studies.

Department of Molecular Cellular Biology and Pathobiology

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Approved by:

Chairman, Advisory Committee

Jeffrey Jones

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To my fiancé, Elizabeth, without whom I could not have endured so many years in school.

To my parents, who encouraged me to pursue what makes me happy.

To my friends, who made me feel capable and supported.

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Table of Contents

Acknowledgements	Page iii
List of Tables	Page v
List of Figures	Page vi
Abstract	Page vii
Introduction	Page 1
Hypothesis and Specific Aims	Page 2
REVIEW OF THE LITERATURE	
Chapter 1: The Aorta and Extracellular Matrix Homeostasis	Page 3
Chapter 2: TGF-β Signaling is Increased in Aging and Aortopathies	Page 9
EXPERIMENTAL METHODS, RESULTS AND DISCUSSION	
Chapter 3: Methods Establishing a Model of Aortic Aging and Measuring Contraction	Page 15
Chapter 4: A Mouse Model of Aortic Aging and a Novel Contractile Deficit with Age	Page 20
Chapter 5: Me**th**ods for Measuring SMC Phenotype Parameters	Page 33
Chapter 6: Aortic SMC Phenotype is Not Stable with Age	Page 37
Chapter 7: Summary and Future Directions	Page 51
List of References	Page 55

List of Tables

Table 6.1 ECM Remodeling Genes Examined by PCR Array Page 42

List of Figures

Figure 4.1 Thoracic aortic diameter, wall thickness, and lumen perimeter with age. Page 22

Figure 4.2 Thoracic aortic structure and composition changes with age. Page 23

Figure 4.3 Mechanical properties of young and old aortas. Page 25

Figure 4.4 Aortic contractility decreases with age. Page 26

Figure 4.5 Collagen gel contraction by aortic smooth muscle cells decreases with age. Page 27

Figure 6.1 Thoracic aortic SMC proliferation is decreased with aging. Page 39

Figure 6.2 Thoracic aortic SMC migration is decreased with aging. Page 40

Figure 6.3 Thoracic aortic SMC adhesion is decreased with aging. Page 41

Figure 6.4 Gene expression profile of ECM remodeling genes with aging. Page 43

Figure 6.5 Aortic SMC collagen isoform expression with aging. Page 44

Figure 6.6 Expression of TGF-β signaling pathway components with aging. Page 45

Figure 7.1 Summary: A New Understanding of Aortic Aging Page 54

JASON BENJAMIN WHEELER. Thoracic Aortic Aging and Smooth Muscle Cell Phenotype. (Under the direction of JEFFREY JONES)

Background: The thoracic aorta undergoes structural and mechanical changes with age, including dilation and increased collagen leading to decreased compliance. However, how age-dependent changes in resident cells mediate this aging process is not well understood. Previous studies have demonstrated a reduction in both *ex vivo* aortic contraction and *in vitro* collagen gel contraction by aortic smooth muscle cells (SMCs) from old mice relative to young, suggesting a deficit in aortic SMC contractility with age. Vascular SMCs are known to shift out of a contractile phenotype, with disease or mechanical injury, to remodel the extracellular matrix (ECM). Therefore, we hypothesized that aortic SMCs undergo a phenotype change with age that contributes to aortic structural and mechanical changes with aging.

Methods/Results: Aortic SMCs were cultured from thoracic explants harvested from 6 month ("young", n=6) and 21 month ("old", n=6) C57 mice. Phenotype was defined by assessing proliferation, migration, adhesion, and gene expression. When measured over 7 days, old aortic SMCs displayed reduced proliferation relative to young aortic SMCs. In a modified Boyden chamber, migration by old aortic SMCs was reduced compared to young (13.4±1.0 vs. 28.8±4.8 migrated cells). Old aortic SMCs were less adherent to a poly-D-lysine surface after mechanical washing relative to young (12.6±1.3% vs. 23.3±1.9% adherence). A PCR array was used to measure the expression of ECM remodeling genes, including ECM proteins (collagens), matrix metalloproteinases (MMPs), and tissue inhibitors of MMPs. Increased expression of these genes produced a distinct genotypic profile in old aortic SMCs relative to young.

Conclusions: With aging, aortic SMCs exhibit reduced proliferation, migration, and adhesion. Furthermore, old aortic SMCs have upregulated expression of ECM remodeling genes, including collagens. Together, these results suggest that altered aortic SMC phenotype plays an active role in aortic structural and mechanical changes with age.

Introduction

The thoracic aorta undergoes many structural and mechanical changes with aging, including dilation, increased collagen content, and increased stiffness. These age-related changes have been increasingly recognized to adversely impact normal aortic function and may even affect the risk of aortopathies, including aneurysm. In particular, increased aortic stiffness has been identified as an independent risk factor for developing cardiovascular disease and clinical guidelines on screening for aortic stiffness have been established. Interestingly, recent research suggests that there may be overlapping signaling pathways involved in aortic aging and aortic aneurysm. Considering the rapid growth of the elderly population, which will double in the next 20 years, there is a need for greater understanding of normal aortic aging processes. Most reports of human aortic aging are limited to characterizations of histologic and stiffness changes with age, with little description of changes at the cellular level or potential biochemical mechanisms. Moreover, no non-transgenic animal model of aortic aging that recapitulates the hallmarks of human aortic aging has been described in the literature, significantly hindering the ability to study these aspects of aortic aging. Therefore, our objectives were to develop a non-transgenic mouse model of aortic aging and identify cellular and biochemical level changes associated with aging. Accordingly, this thesis will review the research that established a non-transgenic mouse model of aortic aging, identified a novel age-related contractile deficit, and implicated a phenotype shift in the aortic smooth muscle cell as a mediator of aortic aging. Lastly, it will conclude with a discussion of future experimental steps to further elucidate the mechanisms of aortic aging.

Hypothesis and Specific Aims

HYPOTHESIS: Aortic SMCs undergo a phenotype shift with age that contributes to aortic structural and mechanical changes with aging.

SPECIFIC AIM 1: Establish that aging is accompanied by ECM and cellular changes that directly affect the compliance and contractility of the thoracic aorta. a) Examine aortic geometry and wall structure by *in vivo* microscopy and histologic sections to demonstrate changes in aortic diameter, wall thickness, collagen and elastin content, and cellular content with age. **b**) Demonstrate impaired mechanical function of the aorta with age by measuring passive compliance and active contractility *ex vivo*. In addition, determine the contribution of age-related changes in aortic medial collagen and elastin content to passive compliance. **c**) Demonstrate that aortic smooth muscle contractility is reduced at the cellular level using a collagen gel contraction assay in the presence of a broad matrix metalloproteinase (MMP) inhibitor.

SPECIFIC AIM 2: Demonstrate that aortic SMCs undergo a phenotype shift with age. a) Establish smooth muscle cell phenotype is altered with age through *in vitro* assays for the following parameters: proliferation, migration, adhesion, and gene expression. **b**) Demonstrate increased aortic SMC expression of collagen and transforming growth factor beta (TGF-β) signaling pathway components.

REVIEW OF THE LITERATURE

Chapter 1: The Aorta and Extracellular Homeostasis

Structural Anatomy of the Aorta

The thoracic aorta is a large elastic artery with a tri-layer wall composed of the *tunica intima*, *tunica media* and *tunica adventitia*.[1] These three layers are divided by a sheet of elastin fiber, the internal and external elastic laminae.[2] The functions of each layer are determined by its endogenous components.[3] Proper function of each layer within the thoracic aorta depends upon dynamic and tightly regulated homeostatic systems that preserve its microstructural organization.[4] The *tunica intima* is the innermost layer and consists of a layer of simple squamous epithelium, known as endothelium, affixed to a basement membrane resting on a thin connective tissue matrix. Endothelial cells regulate the diffusion of solutes and water, coagulation along the luminal surface, and transduce adrenergic stimulation. The intimal layer extends from the luminal surface to an outer border lined by an internal elastic membrane that marks the transition to the middle layer. The middle layer, or *tunica media*, is the thickest layer and comprised of vascular smooth muscle cells (VSMCs), fibroblasts, collagen, several helical elastic laminae and ground substance (a gel of glycosaminoglycans, glycoproteins and proteoglycans). It is this middle layer that is predominantly responsible for the biomechanical characteristics of the aorta. Alternating, concentric sheets of smooth muscle and elastin, known as lamellar units, are intercalated radially by collagen and provide both distensibility and tensile strength.[2] Medial lamellar content is greatest in the ascending thoracic aorta and decreases by approximately one-half at the level of the abdominal aorta, progressively reducing the elastin to collagen ratio.[2, 5] The *tunica adventitia* is the outermost layer that coats the media with a

thin layer of loose connective tissue made by fibroblasts, the primary cell type in the adventitia. The adventitia functions as a fibroblast and stem cell progenitor reservoir, allowing rapid influx of these cells into the aortic wall when needed (e.g. injury).[6] Traversing along the human thoracic aorta within the adventitia are the vasa vasorum, which are smaller blood vessels that penetrate into the thick media to supply nutrition beyond that provided by diffusion from the luminal blood. Within this tri-laminar structure common to all arteries, the extracellular matrix (ECM) and cellular components of the aorta interact and mediate normal aortic function.

Extracellular Matrix

The ECM is a vital network of supportive fibrous proteins that confers biomechanical strength and organization to virtually every tissue within the body. Recent studies have revealed that the ECM is not simply a passive scaffold onto which cells attach and move, but a dynamic regulator of tissue growth and development. Within the aorta, the ECM is critical to withstanding the range of blood pressures and shear forces acting on vessel walls. To maintain the structural integrity of the vascular wall and properly regulate the behavior of its constituent cells, ECM components are continually renewed by resident cells in a highly regulated process of remodeling that delicately balances matrix protein deposition and degradation by proteases. Dysregulation of these homeostatic systems can result in deleterious ECM remodeling, which can impair normal aortic function. The two most abundant and studied aortic ECM components in the settings of aneurysmal disease and aging are elastin and collagen.

Elastin is the most abundant ECM component in the aortic wall, forming as much as 50% of the dry weight of the thoracic aorta.[7] During aortic development, VSMCs synthesize and arrange concentric rings of elastic fiber around the arterial lumen.[8] Mechanical stretch or pressure also stimulates elastin synthesis by medial VSMCs.[9] This macroaggregate fiber is secreted as

soluble tropoelastin, stabilized by intermolecular cross-linking, and polymerized onto a pre-formed microfibril network. Elastic fibers allow the aorta to comply with increased hemodynamic stress during systole, and through its recoil capacity, maintain sufficient diastolic blood pressure.[10]

After elastin, collagen is the second most abundant ECM component within the aortic media and adventitia. Collagen acts as the counterpart to elastin in that its tensile strength and stiffness oppose the compliance of the elastic lamellae. Many types of collagen exist within the aortic wall and may be categorized by their morphology into either fibrillar or non-fibrillar collagens. Types I, II and III are fibrillar collagens and the predominant types. Types IV, V and VI are nonfibrillar collagens and are major components of the endothelial basement membrane and interstitial tissues, and are a primary component of the aortic adventitia.[11] The integrity of the aortic wall is primarily attributed to collagen types I and III.[12] Thus, increased collagen proteolysis due to an imbalance of synthesis and degradation would adversely affect aortic integrity and indeed has been reported in human aortic aneurysms.[13]

Microfibrillar proteins also play significant structural and signaling roles that are essential to normal aortic development and homeostasis. Microfibrils contribute significantly to aortic wall integrity by acting to reinforce compliant tissues much like collagen. Fibrillins and fibulins are the two main microfibrils in the aortic ECM. The role of fibrillin 1 (FBN1) in vascular physiology is well described.[14] Fibrillins direct aortic morphogenesis during fetal development. Within the adult aorta, fibrillin 1 interacts with collagen and elastin, forming a strong supportive scaffold around an elastin protein core, and is essential for proper elastin organization. Recent research on the role of FBN1 in the aorta has identified its crucial role in regulating the sequestration and activity of various ECM and growth factors.[15] In particular, FBN1 levels

modulate the bioavailability of transforming growth factor-beta (TGF-β), a major signaling protein influencing vascular ECM remodeling.[16]

Vascular Smooth Muscle Cells

There are three primary cell types within the thoracic aortic wall: vascular smooth muscle cells, fibroblasts and endothelial cells. Each of these types is capable of altering the aortic ECM through production of proteolytic enzymes, ECM component proteins, and proteolytic inhibitors. Thus, all of the cell types present in the aorta likely contribute to vascular ECM homeostasis. However, VSMCs are the largest cell population by percent in the aortic media, which is the layer which primarily determines the biomechanical properties of the aorta. Thus, this cell type may exert the most influence over normal aortic function. VSMCs are capable of both contraction and ECM protein synthesis. Smooth muscle cell contraction is involved in several cell functions important in aortic wall homeostasis: cell shape and length, alignment and migration. Additionally, the ability of these cells to synthesize, secrete, repair, and organize ECM proteins (collagen, elastin and fibrillin) is vital to wall integrity. Thus, VSMCs are uniquely equipped to regulate medial structure through both contraction and ECM synthesis. Through direct interaction of VSMCs with various ECM proteins, these cells can integrate mechanical stimuli into cellular (contraction) and extracellular (secretion) responses, remodeling the aorta to meet current physiological demands and maintain normal function. For example, decreased mechanical tension on VSMCs has been reported to alter multiple signaling pathways.[17] Cumulatively, these studies exemplify the interdependent and interactive cell environment within the aortic wall that may be disrupted in the context of aging.

Extracellular Matrix Homeostasis

Vascular homeostasis maintains both the vascular ECM and its constituent cells in a physiological state that maximizes the functional integrity of the aorta while adapting to individual hemodynamic demands. Aortic ECM components confer mechanical stability and hemo-propulsive elasticity to the aortic wall. Additionally, ECM components modulate the bioavailability and activity of several growth factors, for example the pro-fibrotic transforming growth factor-beta (TGF-β) signaling family. To achieve homeostasis, resident cells in the thoracic aorta integrate and respond to a variety of mechanical inputs and biochemical signals with alterations in cellular function including proliferation, migration, and ECM modification. Thus, ECM structure and resident cells participate in a continuous feedback cycle. With respect to the synthesis of ECM components, the transcriptional profiles of aortic wall cells must preserve a delicate balance between deposition and degradation. Tightly regulated, alternating cycles of synthesis and degradation, known as remodeling, are mediated by several families of proteolytic enzymes and their endogenous inhibitors. An increase in expression of proteolysis inhibitors could result in accumulation of ECM proteins, while an increase in expression of proteinases would favor ECM degradation. Adaptive shifts in the relative abundance of these mediators alter the structural composition and organization of the vascular ECM, in turn influencing the phenotype of resident cells and vice versa. Changes in cellular phenotype are therefore relevant to ECM homeostasis. Thus, understanding the regulation of aortic cell phenotype may have significant implications toward therapeutically addressing age-related alterations in aortic ECM homeostasis.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are the most studied family of proteolytic enzymes involved in ECM homeostasis and implicated in aortopathies, such as aneurysm. This family of proteinases contains over 25 members with a wide range of target substrates, including virtually all ECM proteins. All of the major cell types within the aorta are capable of producing MMPs. The aortic ECM is directly degraded by these MMPs in response to growth factors (e.g. transforming growth factor-beta, TGF-β), inflammation, oxidative stress, and matrix degradation products. Elevated TGF-β signaling is associated with alterations in MMP expression and activity.[18-20] All MMPs, except membrane bound MMPs, are secreted as proenzymes and require proteolytic cleavage, in some cases by other MMPs, for their release and activation.[21] To prevent cyclic MMP activation and unregulated proteolysis, a delicate balance of MMPs and endogenous tissue inhibitors of MMPs (TIMPs) is maintained, as well as other pre- and post-transcriptional regulatory mechanisms. Indeed, the local balance of MMPs and TIMPs within the aortic wall determines the overall effect on the vascular ECM, either deposition or degradation. Somewhat paradoxically, increased MMP activity can release sequestered pro-fibrotic factors, such as TGF-B, and result in ECM accumulation.

Chapter 2: TGF-β Signaling is Increased in Aging and Aortopathies

"Canonical" TGF-β Signaling Pathway

Transforming growth factor-β is a soluble cytokine secreted by cells in the form of a large latent complex (LLC) composed of a homodimer of mature TGF-β peptide, a homodimer of TGF-β's inactive cleaved peptide fragment (latent associated protein, LAP), and latent transforming growth factor binding protein (LTBP).[22] Motifs within fibrillin-1 interact with LTBP and target the LLC to the ECM.[23] Thus, the ECM serves to sequester and concentrate TGF-β in locations where it may be rapidly activated when needed.[24] Indeed, the ECM is no longer thought to be a passive structural support but rather a dynamic regulator of growth factor bioavailability and signaling.[25, 26]

Mature TGF-β (types 1–3) is activated by release from the LAP through multiple mechanisms including: direct proteolysis, by non-proteolytic dissociation mediated by thrombospondin-1 or integrin αvβ6, as well as exposure to reactive oxygen species, or low pH.[27] Once activated, TGF-β predominantly forms homodimers that can now interact with and bind a TGF-β receptor in the first step of the signaling cascade.[22] Transforming growth factor-β receptors have been subdivided into three types. Type-I (also known as Activin receptor-like kinase 5/ALK-5 or TGFBRI) and type-II are the primary receptors of the classical - or "canonical"-pathway and both possess serine/threonine kinase activity. Type-III (also known as betaglycan) is an accessory receptor that binds TGF-β and presents it to the type - I and II receptor complex.[22] Other receptors that bind and signal in response to TGF-β include endoglin (type-III receptor) and the Activin receptor-like kinase 1(ALK-1), a type - I receptor family member.

After release from the ECM, mature TGF-β first binds a homodimer of the type-II receptor inducing an autophosphorylation event. This, in turn, recruits a homodimer of the type - I receptor forming the complete ligand-receptor complex. The type-II receptor then activates the type - I receptor via transphosphorylation.[28] The kinase domain of the activated type - I receptor propagates the intracellular signal through the phosphorylation of specific receptor-regulated Smad proteins (R-Smads; Smad 1, 2, 3, 5, and 8), which are the second messengers of the canonical TGF-β signaling pathway. For example, activation of the type - I receptor TGFBRI, results in the phosphorylation of Smad 2 or 3; while activation of the type - I receptor ALK-1 results in the phosphorylation of Smad 1, 5 or 8. The choice of Smad is likely tissue-specific and context-dependent. The phosphorylated R-Smad then interacts with a common Smad or "co-Smad" (Smad4), which induces translocation of the complex to the nucleus. The nuclear Smad complex along with multiple co-regulatory factors form a transcription regulating complex capable of activating or repressing TGF-β associated genes [29, 30] (Figure 2.1). Activation of the TGF-β system stimulates a number of diverse cellular processes, such as cell growth, proliferation and apoptosis and therefore requires strict regulation at multiple levels. An example of this regulation, is the negative feedback of inhibitory Smads (I-Smads; Smad6 and 7) induced by TGF-β stimulation.[31] Smad6 exerts its effects by binding directly to type - I receptors and blunting R-Smad phosphorylation.[32] Smad6 also inhibits signaling by competing with Smad4 for receptor Smad binding sites, reducing nuclear translocation.[33] Smad7 inhibits TGF-β signaling by targeting TGFBRI and II for ubiquitination and subsequent degradation, through the recruitment of Smurfs 1 and 2 (Smad ubiquitination regulatory factor 1 and 2).[34-36] Additionally, many regulatory proteins influence the bioavailability of TGF- β, such as the structurally related scavenging proteoglycans decorin and biglycan, which bind and reduce its availability for signaling.[37-39]

TGF-β Signaling in Aneurysm and Aging

Recent research suggests that TGF-β signaling is involved in aortic aneurysm and aortic aging. Marfan syndrome (MFS) and Loeys-Dietz syndrome (LDS) are well described heritable disorders characterized by connective tissue defects and aortic aneurysms.[40] A mutation in fibrillin-1 (FBN1), a protein component of microfibrils, accounts for more than 90 % of MFS.[41] Fibrillin-1 was demonstrated through multiple studies to interact with and sequester latent transforming growth factor-beta (TGF-β) within the extracellular matrix (ECM) [3 –6]. In 2003, Neptune et al. hypothesized that the loss of microfibrils may have an effect on the sequestration of TGF-β within the ECM and demonstrated that TGF-β signaling was markedly activated within lung tissue of a mouse MFS model.[42] Furthermore, the emphysematous lung phenotype of the MFS mice was restored to wild type with anti-TGF-β antibody, strongly suggesting that TGF-β signaling dysregulation contributed to the pathogenesis of MFS.[42] Subsequently in 2005, Loeys and Dietz identified mutations within type-I (TGFBRI) or II (TGFBRII) TGF-β receptors in a cohort of patients with a phenotype similar to MFS but without FBN1 mutations.[43] Interestingly, despite mutated receptors incapable of propagating signal, patients with Loeys-Dietz syndrome (LDS) paradoxically exhibited indications of increased TGF-β signaling: increased expression of collagen and connective tissue growth factor (CTGF), similar to MFS patients.[43] Interestingly, dysregulation of TGF-β signaling and MMPs has been reported in the context of aortic aging. Wang et al. described increased MMP-2 mediated TGF-β signaling using aged rat aortic homogenates and immunostaining, in association with increased aortic collagen content.[44] Furthermore, in vitro studies implicated the aortic SMC as the cell type responsible for TGF-β activation. Increased MMP-2 activity with age has also been reported in primate and human aortas.[44-46] Therefore, normal aortic aging processes could result in an aortic substrate more susceptible to aortopathies, particularly aneurysm.

Functional and Cardiovascular Risks Associated with Aortic Aging and Aortopathies

Both aortopathies and normal aortic aging are associated with impaired aortic function, which may also impact cardiovascular disease risk. One functional change associated with both aortic aging and aortopathies is decreased compliance.[4] The compliance of the aorta allows it to distend during systole and briefly store a majority of the stroke volume. Elastin lamellae store some of the ventricular contraction energy, which through elastic recoil, modifies the pressure pulse generated by the left ventricle into continuous flow to the peripheral circulation, simultaneously maintaining adequate diastolic blood pressure.[47] Among the aortic branches dependent upon this recoil force are the coronary arteries, which conduct the majority of their blood flow during diastole when the aortic pressure exceeds the ventricular pressure, particularly to the left ventricle. Unfortunately, decreases in aortic compliance negatively affect both diastolic and systolic blood pressure.[47] The normal aorta counters falling diastolic blood pressure due to peripheral run-off with elastic recoil that adds stored stroke volume thereby sustaining consistent diastolic pressure. Thus, diastolic pressure falls rapidly in the setting of a non-compliant aorta. Moreover, without the storage of blood volume within a compliant aorta, this volume is immediately conveyed systemically, adding to systolic pressure. The abnormally increased difference in systolic and diastolic pressure, known as pulse pressure, is a risk factor for coronary artery disease and contributes to increased cardiac workload.[48] Combined with the potential for reduced coronary perfusion pressure during diastole, reduced aortic compliance with age may increase cardiac oxygen demand, reduce output and predispose to cardiovascular disease.[49]

Significance of Age-Related Research and Aortic Changes with Age

Current population projections suggest that life expectancy is increasing as well as the median age of our population. This is driven in part by the "Baby Boomer" generation, who started reaching 65 years of age in 2011. In the next 20 years, the number of people in the United States over 65 years old, the average age of diagnosis with a thoracic aortic aneurysm, is expected to double.[50, 51] Therefore, research on normal aortic aging processes is increasingly relevant and may significantly inform future therapeutic approaches for aneurysm.

It has been well described that aging is associated with changes in human aortic geometry, even in the absence of aortopathy and cardiovascular risk factors such as hypertension.[52-54] Redheuil and colleagues reported an age-dependent increase in the diameter and thickness of the human thoracic aorta in healthy patients.[55-57] This dilation, according to the Law of LaPlace, would increase wall stress even without increases in blood pressure, suggesting perhaps that increased thickness with age is a compensatory response to increased wall stress and when insufficient may contribute to aneurysm risk.[58] Furthermore, aging is known to be associated with fibrosis in a number of organs including the aortic wall.[59, 60] In particular, an increase in collagen content has been reported to occur in aortas from older subjects even in the absence of hypertension, which due to the mechanical properties of collagen is believed to contribute to increased aortic stiffness or reduced compliance with age.[61-63] Although total elastin content has been reported to be similar in aortas from young and older human subjects, the abundance of elastin relative to other ECM components is reduced in the aortas from old subjects.[61, 64] In addition, the organization of ECM components within the aorta has been reported to decrease with age and perhaps contribute to stiffness.[65, 66] Interestingly, alterations within the aortic ECM may initiate inflammatory and calcification process that can

also influence vascular stiffness.[67] Changes in SMC phenotype have also been reported in association with age-related alterations in the aortic ECM, suggesting these cells may mediate aortic aging.[68] Together, these age-related changes in the structure and composition of the aorta, in association with changes in SMC phenotype, impair normal function and may also contribute to an aortic substrate that is more susceptible to aortopathies, including aneurysm and rupture.[69, 70]

However, relatively little is known about the mechanisms behind aortic aging. It is not well understood which cell types play critical roles in aortic aging process and how these cells are altered with aging. Within the aortic literature, the majority of reports of human aortic aging are limited to characterizations of histologic and stiffness changes with age, with little description of changes at the cellular level or potential biochemical mechanisms that produce or contribute to these age-related changes. Moreover, no non-transgenic animal model of aortic aging that recapitulates the hallmarks of human aortic aging has been validated in the literature, significantly hindering the ability to study these aspects of aortic aging. Therefore, our objectives were to develop a non-transgenic mouse model of aortic aging and identify cellular and biochemical level changes associated with aging.

EXPERIMENTAL METHODS, RESULTS AND DISCUSSION

Chapter 3: Methods Establishing a Model of Aortic Aging and Measuring Contraction

Overview

The goals of this study were to determine the effects of age on the geometry, the cellular and ECM composition, and the relationship between ECM composition and mechanical characteristics of the thoracic aorta.

Animals

All procedures were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (2012) and approved by the institutional animal care and use committee of the Medical University of South Carolina. C57BL6 wild-type male and female mice (N=103, 50 male, 53 female) aged 6 (n=55) and 21 (n=48) months were obtained from the National Institute on Aging mouse colony at Charles River Laboratories International (Wilmington, MA, USA). Equal numbers of male and female mice were used in each study. Old mice were heavier than young mice (33.5±0.9 versus 28.5±0.8 g; old, n=38; young, n=42). Tibia length was measured with a digital caliper after incubating 48 hours in 2 mol/L potassium hydroxide and was similar between the old and young groups (18.2±0.1 mm versus 18.0±0.2 mm; old (n=9) versus young (n=19), respectively).

In-Vivo Aortic Diameter Measurement

Anesthetized mice (2% isoflurane) were intubated and mechanically ventilated.[71] A left posterolateral thoracotomy was performed at the sixth intercostal space. Images of the exposed

descending thoracic aorta were captured on a calibrated video microscopy system (PAXcam using PAX-it software version 7.6; MIS Inc) and then were used to measure external aortic diameter, as described previously.[71]

Histology and Quantitative Analysis

Descending thoracic aortas from young (n=10) and old (n=10) mice were harvested from the level of the eighth rib to the diaphragm and fixed in 10% formalin for 48 hours and then transferred to 70% ethanol for storage at 4°C. Fixed aortic segments were embedded in paraffin and sectioned at 2-μm thickness. Sequential sections were stained with picrosirius red for collagen, Verhoeff-Van Gieson for elastin, and hematoxylin and eosin to visualize cell nuclei for quantification. Aortic tissue sections were visualized on a Zeiss Axioskop 2 microscope (Carl Zeiss MicroImaging), and digital images were acquired (Axiocam MRc camera and AxioVision software, version 4.8). All quantitative image analysis was performed using SigmaScan Pro version 5.0 (Systat Software Inc) and Adobe Photoshop CS5 (Adobe). For each study, analysis of histology sections was performed using at least 3 randomly chosen high-power fields from 3 different sections from each young (n=10) and old (n=10) mouse. Aortic medial thickness was measured from the internal elastic lamina to the adventitial border. Aortic lumen perimeter was calculated by summation of the edge lengths of a digital overlay filling the vessel lumen. Collagen and elastin volume fraction in the medial layer was determined as the ratio of the area stained by picrosirius red and Verhoeff-Van Gieson stain, respectively, divided by the total area of tissue within each high-power field. The volume fraction of thin (green–yellow birefringence) and thick (red–orange birefringence) collagen fibers was measured in picrosirius red–stained sections illuminated by polarized light. Cell density (in cells/mm^2) in the medial aortic wall was calculated by counting the number of nuclei in 0.0023-mm^2 areas of medial tissue from 3

sections from young (n=10) and old (n=10) mice and dividing by the total area of tissue examined.

Aortic Ring Preparation and Measurement of Passive Tension

Descending thoracic aortas from young (n=25) and old (n=24) mice were harvested and immediately placed in cold Krebs-Hanseleit buffer (118 mmol/L NaCl, 4.6 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, 2.5 mmol/L $CaCl_2$, 0.5 mmol/L Na_2-EDTA, 11 mmol/L glucose, 1.2 mmol/L $MgSO_4$, pH 7.4). The endothelium was removed by gently rubbing the intimal surface to mitigate any potential endothelial effects on medial contractility.[72] The aorta was divided into 3-mm-long segments (typically 2 segments per mouse) that were each mounted on parallel wires in a water-jacketed tissue bath system (25 mL; Radnoti LLC) maintained at 37°C and connected to an isometric force transducer (Radnoti).[73] The vessels were allowed to equilibrate for 30 minutes in the absence of tension and washed every 15 minutes with warm Krebs-Hanseleit solution aerated with 95% O_2 and 5% CO_2.

Tension was applied by gradually separating the parallel wires, as described previously.[74] The Krebs-Hanseleit solution was supplemented with EGTA (5 mmol/L) to minimize calcium-induced SMC contraction. Stress relaxation was quantified by sequentially stretching vessel segments in 0.1-g increments (from 0.2 to 1.2 g of applied tension) and measuring residual tension after 3 minutes to calculate the percentage of relaxation. Measurements were recorded using Biobench software (National Instruments). Stress relaxation values were normalized to wall thickness for comparison of differently sized segments.

Determination of Aortic Contractile Properties

To determine contractile properties, the aortic rings harvested from young (n=14) and old (n=8) mice were equilibrated with an applied tension of 0.4 g for 30 minutes in standard Krebs-Hanseleit buffer without EGTA. Contraction was stimulated by adding KCl (final concentration of 100 mmol/L in the bath), and the peak contractile force (in grams) generated over the ensuing 8 minutes was recorded. The rings were washed 3 times with fresh Krebs-Hanseleit solution to remove residual KCl. These steps were repeated at 0.1-g increments up to 1.2 g of applied tension, as described previously.[73] To control for differences in ring tissue mass between young and old mice, the peak contractile force was normalized to aortic ring volume, determined from aortic diameter and wall thickness.

Smooth Muscle Cell Isolation

Primary SMCs were isolated using an outgrowth procedure in which minced pieces of aorta from young and old mice (6 young, 6 old) were plated in SMC-specific growth medium (C22062; PromoCell) and allowed to extravasate for 2 weeks. After 2 weeks, the pieces of aorta were removed, and the SMCs were maintained in culture. Cells in passages 3 to 7 were used for all in vitro experiments.

Collagen Gel Contraction Assay

Equal numbers of SMCs (50 000) were embedded in disks of rat tail collagen type I (1 mg/mL in SMC growth medium; Gibco) polymerized in a 24-well nontissue culture-treated plate in the presence of broad-spectrum matrix metalloproteinase inhibitor GM6001 (25 µmol/L final concentration; Millipore). Disk area was measured from digital images collected at 0, 3, 6, 9, 12, 18, and 24 hours. Contraction was expressed as a percentage of baseline area at time 0.

Statistical Analysis

All results are presented as mean±SEM. The Student t test was used to compare measurements of aortic geometry, histology, and peak active tension between young and old groups. Repeated-measures ANOVA was used to compare passive tension and collagen gel contraction between experimental groups. If ANOVA revealed significant differences, post hoc mean separation was performed using Tukey's wholly significant difference test. The relationships between the abundance of ECM components and aortic mechanical properties were established using linear least-squares regression analysis. All statistical analyses were performed using Stata statistical software (version 8, intercooled; StataCorp). P values of <0.05 were considered to be statistically significant.

Chapter 4: A Mouse Model of Aortic Aging and a Novel Contractile Deficit with Age

Introduction

The thoracic aorta is a large elastic artery with functionality beyond a static, passive conduit.[1] Functions of the aorta include regulating systolic blood pressure by accepting the ejection bolus through expansion and maintaining diastolic blood pressure by dynamic, elastic recoil.[75, 76] Consequently, normal functioning of the aorta requires consistent passive and active mechanical properties, which include compliance and contractility.[77] Vessel compliance is generally considered to be related to the composition of the extracellular matrix (ECM).[75] Two ECM proteins in particular, collagen and elastin, provide the aortic wall with tensile strength and elastic recoil and contribute to maintaining compliance properties of the aorta.[78] Aortic contractility is reflected by the contractile force generated primarily by vascular smooth muscle cells (SMCs) and provides the active tension component within the aorta.[79] Together, the extracellular and cellular components regulate mechanical properties within the aorta and, when altered, could contribute to vascular pathophysiology.[80]

The precise roles of these age-related changes in altering aortic mechanical function are not well understood. Accordingly, this study used young and old mice to establish that aging is accompanied by ECM and cellular changes that directly affect the compliance and contractility of the thoracic aorta.

Results

Thoracic Aortic Diameter and Medial Thickness With Aging

Thoracic aortic diameter, measured in vivo, was significantly greater in the old mice than in young mice (Figure 4.1A). The aortic medial layer, measured from histological sections (as shown in representative photomicrographs in Figure 4.1B), was significantly thicker in old mice than in young mice. In addition, lumen perimeter was greater in old versus young mice (Figure 4.1C). Together, these results suggest that an age-dependent increase in aortic diameter was the result of both dilation and increased wall thickness.

Medial Extracellular Matrix and Cellular Changes With Aging

Representative picrosirius red–stained sections in bright-field illumination are shown in Figure 4.2A (top panel). Quantification of the amount of staining from these sections revealed that collagen volume fraction was increased in the old group, as summarized in Figure 4.2A (bottom panel). To assess collagen fiber characteristics, picrosirius red–stained sections were illuminated in polarized light (Figure 4.2B, top panel). The thin fibers show birefringence in the green–yellow spectrum, whereas the thick fibers are birefringent in the orange–red spectrum.[81] The medial volume fraction of thin collagen fibers and thick collagen fibers were both increased in the old group compared with the young group (Figure 4.2B, bottom panel). To examine the elastic architecture, aortic sections from young and old mice were stained with Verhoeff-Van Gieson (Figure 4.2C, top panel). The medial elastin volume fraction was not different between young and old mice (Figure 4.2C, bottom panel). To determine cellular density, hematoxylin and eosin–stained aortas from young and old mice were examined and nuclei were counted (Figure 4.2D, top panel). The density of cells in the medial layer was significantly lower in the aortas from old mice compared with young mice (Figure 4.2D, bottom panel).

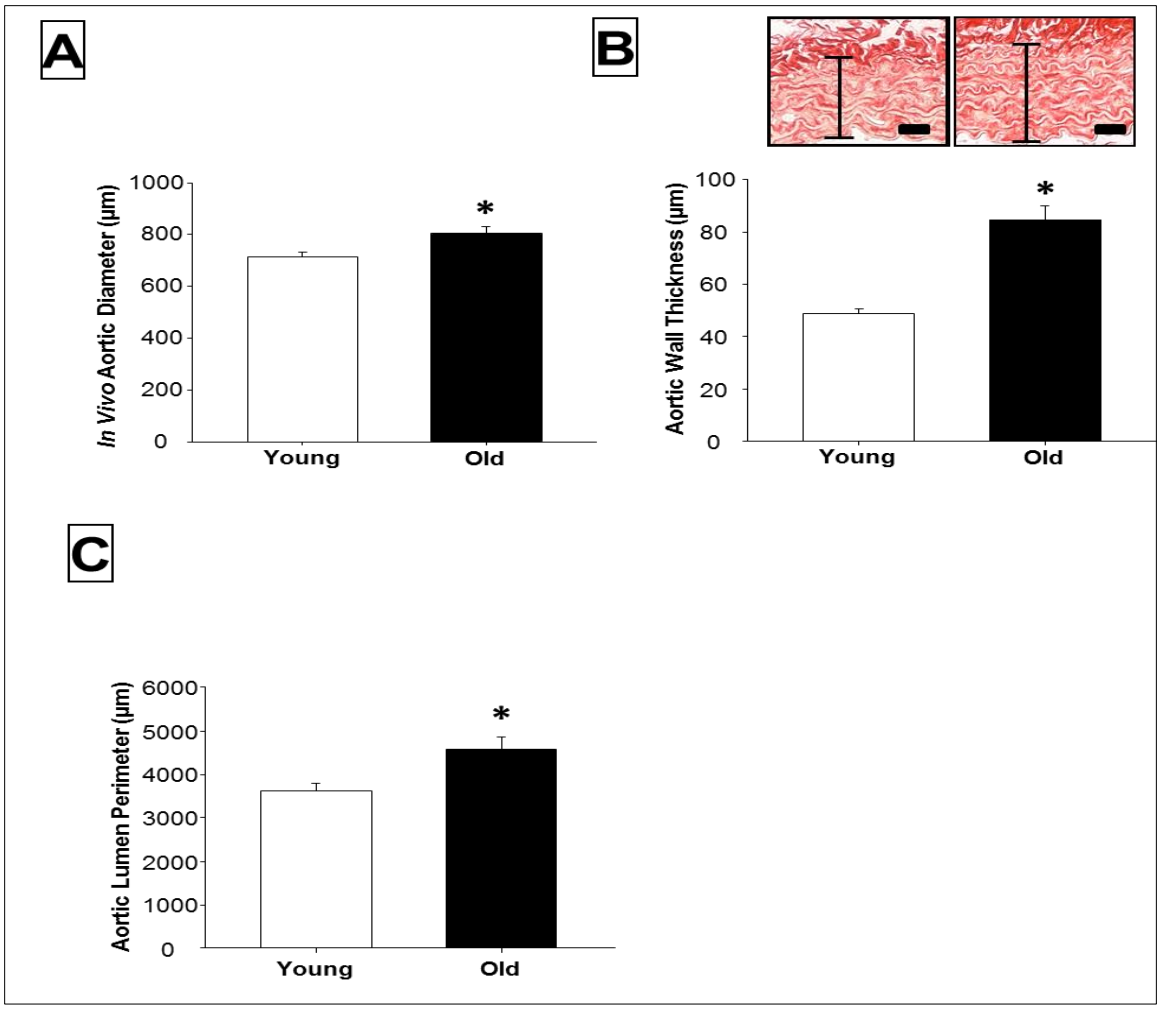

Figure 4.1 *Thoracic aortic diameter, wall thickness, and lumen perimeter with age*. A, *In vivo* aortic diameter (in micrometers) was increased in old mice (n=14) compared with young mice (n=12). B, Representative histology showing full thickness of thoracic aortas from young mice (left) and old mice (right) stained with picrosirius red. Scale bar=20 µm. Aortic wall thickness was greater in old (n=10) versus young (n=10) mice. C, Aortic lumen perimeter was increased in old mice (n=8) relative to young mice (n=8). *P<0.05 vs young.

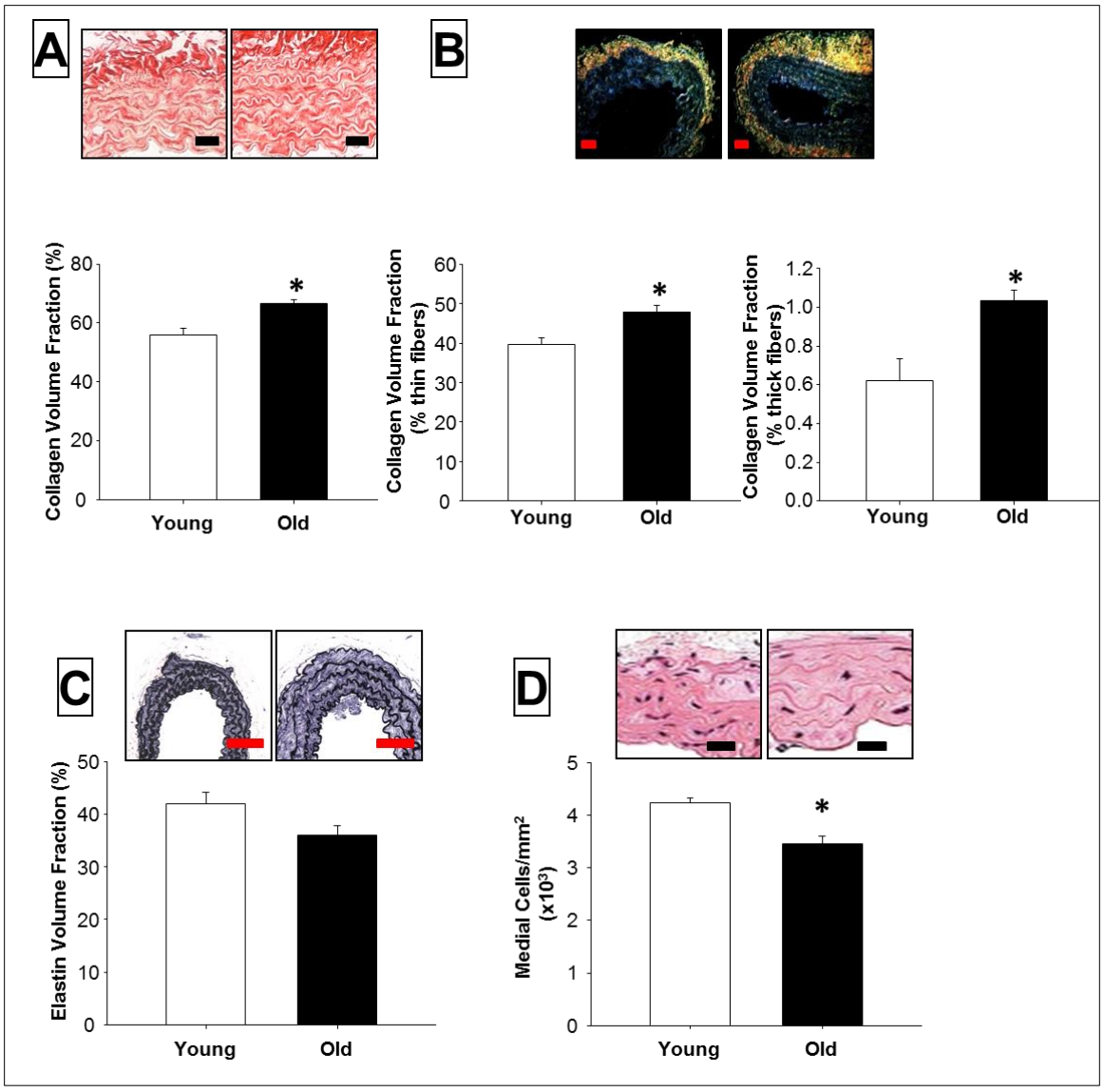

Figure 4.2 *Thoracic aortic structure and composition changes with age*. The structure and composition of the aortic medial layer were compared between young (n=10) and old (n=10) mice using histological staining. A, Representative images of collagen stained with picrosirius red viewed under bright-field light in young (left) and old (right) mice. Scale bar=25 µm. Collagen volume fraction was increased in old mice relative to young mice. B, Representative images of collagen stained with picrosirius red viewed under polarized light in young (left) and old (right) mice. Scale bar=25 µm. The volume fraction of thin collagen fibers (green–yellow birefringence) was increased in old mice relative to young mice (left graph). Similarly, the volume fraction of thick collagen fibers (red–orange birefringence) was increased in old mice relative to young mice (right graph). C, Representative images of elastin stained with Verhoeff-Van Gieson in young (left) and old (right) mice. Scale bar=75 µm. Elastin volume fraction was not different between young and old mice. D, Representative images of aortic sections stained with hematoxylin and eosin (red/pink indicates cytoplasmic and extracellular proteins, black/blue indicates nuclei) in young (left) and old (right) mice. Scale bar=15 µm. The total number of nuclei, equivalent to medial cellularity, was reduced in old mice relative to young mice. *P<0.05 vs young.

Thoracic Aortic Compliance Declines With Age, Correlates With Medial Collagen Volume Fraction but Not With Medial Elastin Volume Fraction

Alterations in the relative abundance of medial ECM proteins may be expected to alter the passive mechanical properties of the thoracic aorta. Accordingly, stress relaxation was measured in aortic rings of young and old mice as a direct indicator of aortic compliance. The results demonstrated that aortic rings from old mice were less compliant than rings from young mice, indicated by the downward shift in the relationship between applied tension and stress relaxation (Figure 4.3A). As one may expect, stress relaxation was negatively correlated with collagen volume fraction ($r=-0.6206$, $P<0.05$) (Figure 4.3B). There was no correlation between stress relaxation and elastin volume fraction ($r=0.2824$, P was not significant) (Figure 4.3C).

Thoracic Aortic Contractility With Aging

Peak contractile force was used to compare thoracic aortic contractility between young and old mice.[73] Peak contractile force normalized to aortic tissue volume (Figure 4.4) was reduced in aortic segments from old mice compared with young mice.

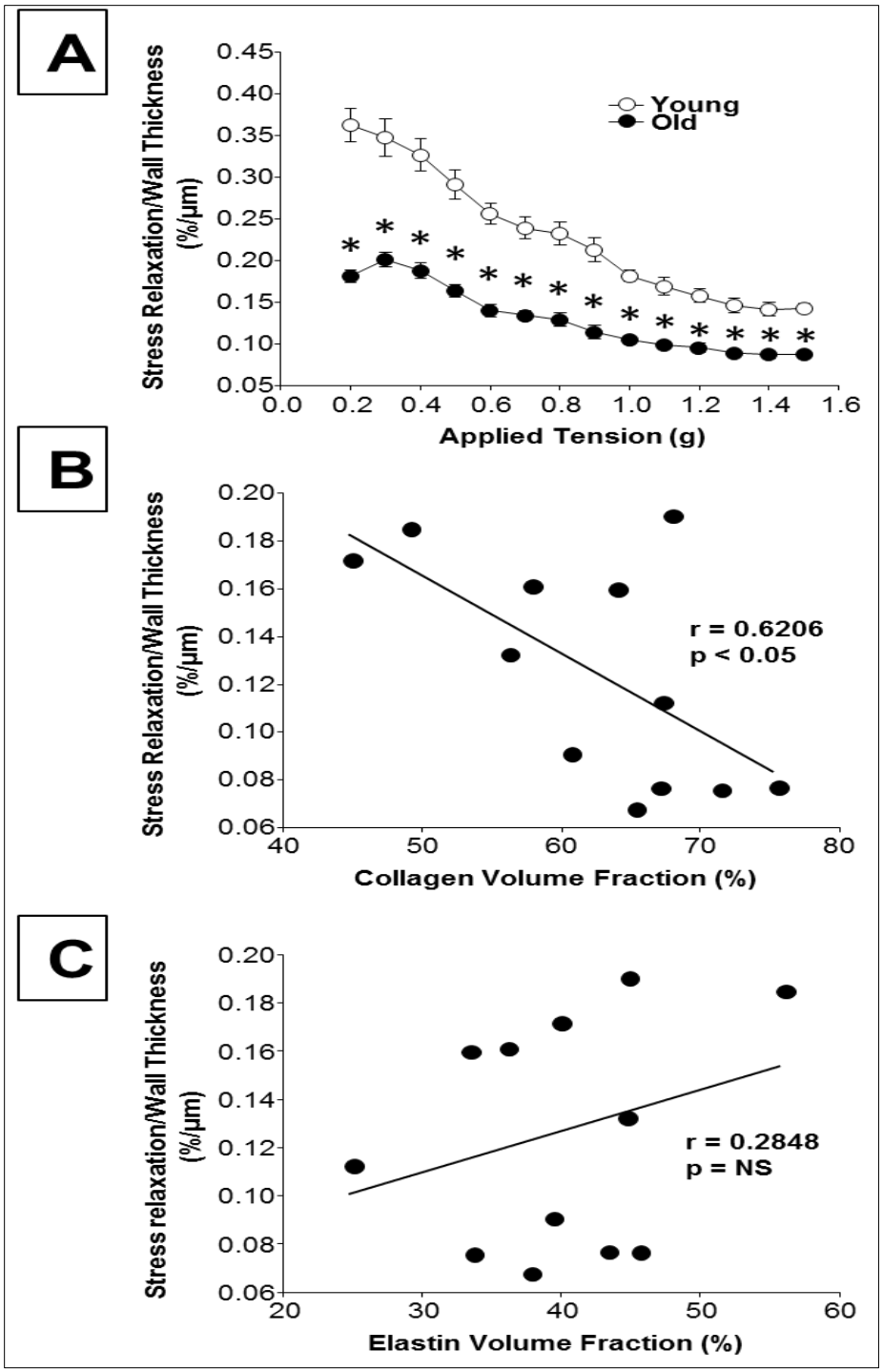

Figure 4.3 *Mechanical properties of young and old aortas*. A, Stress relaxation normalized to wall thickness was decreased in old mice (n=24) relative to young mice (n=24). B, Stress relaxation normalized to wall thickness is negatively correlated with collagen volume fraction; correlation coefficient, R, and P value of linear regression are shown. C, Stress relaxation normalized to wall thickness was not related to elastin volume fraction; correlation coefficient, R, and P value of linear regression are shown. *P<0.05 vs young.

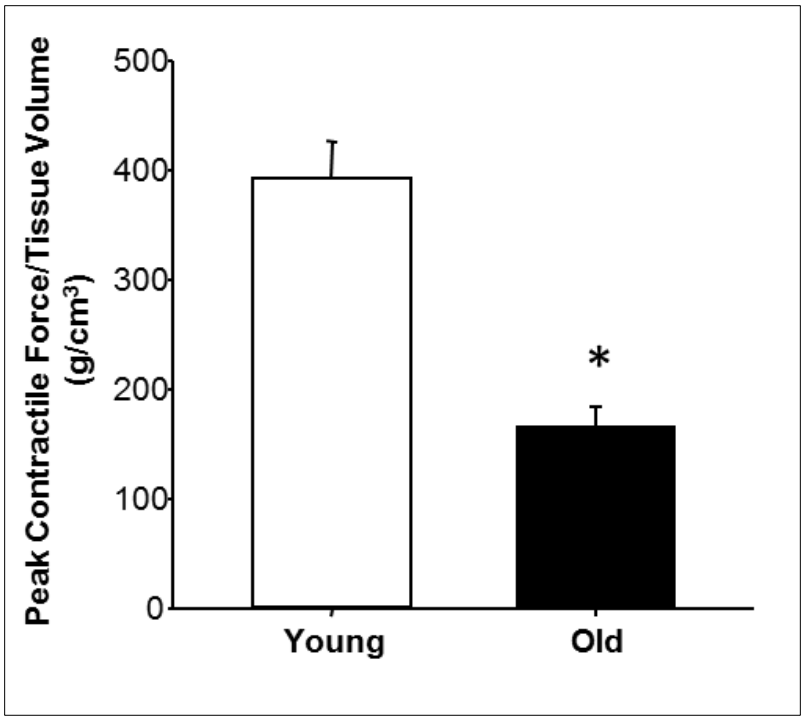

Figure 4.4 *Aortic contractility decreases with age*. Peak contractile force normalized to tissue volume was reduced in old mice (n=8) relative to young mice (n=14). *P<0.05 vs young.

Smooth Muscle Cell Contraction of Collagen With Aging

The collagen gel contraction assay was used to determine whether the reduced peak contractile force observed in aortas from old mice was due to age-related changes in the contractile phenotype of aortic SMCs (ASMCs) or just due to a decrease in overall medial cellularity. Isolated primary SMCs from young and old mice were examined in the presence of a global matrix metalloproteinase inhibitor. Collagen disks seeded with AMSCs isolated from young and old mice displayed a time-dependent reduction in area over the 24-hour duration of the assay (Figure 4.5). Gel contraction mediated by ASMCs from old mice was significantly reduced compared with ASMCs from young mice (repeated-measures ANOVA, F=133.875, P<0.05). In comparison, the reduction in disk area at and after the 6-hour time point was significantly attenuated in ASMCs isolated from old versus young mice (Figure 4.5).

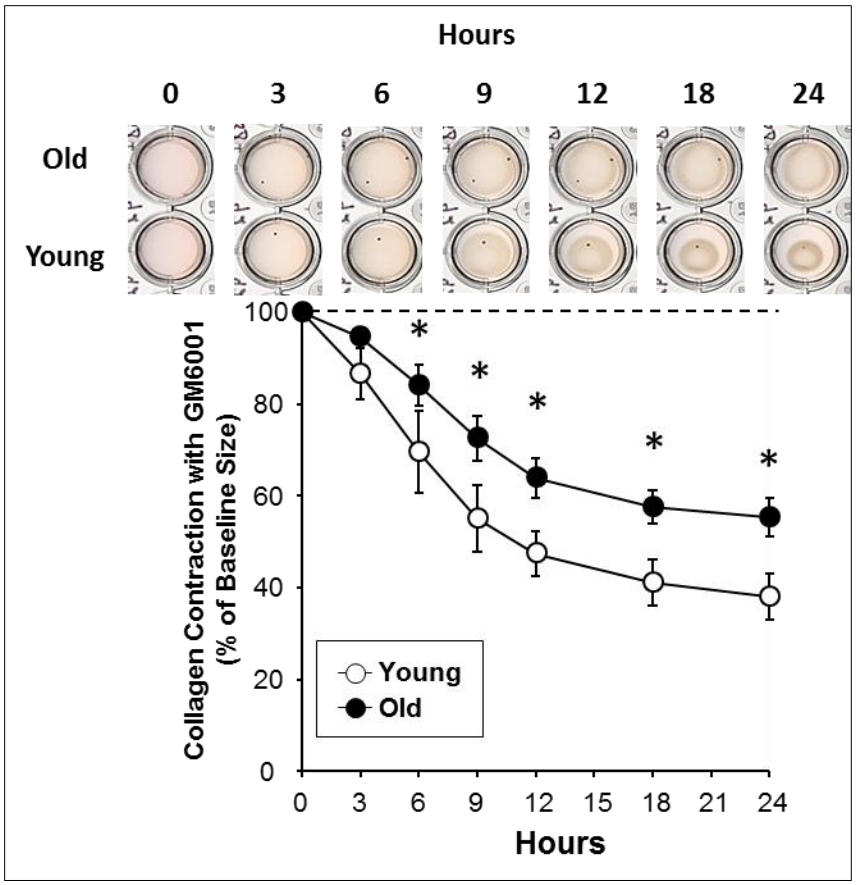

Figure 4.5 *Collagen gel contraction by aortic smooth muscle cells decreases with age*. Collagen gel contraction in the presence of the matrix metalloproteinase inhibitor GM6001 by aortic smooth muscle cells from old mice (n=6) is reduced over 24 hours relative to those from young mice (n=6). *P<0.05 vs young.

Discussion

Age-related structural and cellular alterations within the aorta occur concomitantly with biomechanical dysfunction. Accordingly, this study tested the hypothesis that alterations within the ECM and cellular composition that occur with age directly affect the compliance and contractility of the thoracic aorta. The findings of the present study demonstrate that aortic diameter, thickness of the medial layer of the aortic wall, and collagen volume fraction were higher in the older mice. Moreover, there was an age-related decrease in cell density, aortic compliance, and aortic contractility. Interestingly, changes in aortic compliance were correlated

with collagen content but not with elastin content. This report demonstrates that the age-dependent changes in the material properties of the thoracic aorta in a murine model contribute, at least in part, to reduced compliance and contractility of the thoracic aorta. Taken together, these age-related changes in aortic material properties and mechanical characteristics may yield an aortic substrate that is more conducive to the development of aortopathies, such as aneurysm of the thoracic aorta.

Changes in the geometry of the thoracic aorta that occur with age include aortic dilation and increased aortic wall thickness.[53, 61] Mao et al reported that dilation of the thoracic aorta occurred in older but healthy humans.[82] Similarly, Sonesson et al reported that there was dilation of the abdominal aorta with increasing age.[83] Taken together, these previous studies suggest that age-related dilation may occur throughout the aorta. The thickness of the aortic wall has also been reported to increase with age.[84-86] Pearson et al reported that age was positively correlated with thoracic aortic wall thickness.[57] Consistent with these past findings, the present study demonstrated that diameter, luminal perimeter, and wall thickness of the thoracic aorta were increased as a function of age. Because wall stress (Laplace's law) is directly proportional to vessel diameter, a physiological consequence of an increase in lumen diameter is increased wall stress; therefore, wall thickness, which is inversely related to wall stress, may have increased to normalize wall stress, as described previously in humans.[87, 88] Consequently, this murine aging model recapitulates the morphological hallmarks of human thoracic aortic aging and may serve as a valid model for human aortic disease.

The structure and composition of the aortic wall were examined to explore whether age-related morphological changes occurred concomitantly with biochemical alterations to the aortic wall. In the present study, aortic wall collagen content increased in old mice, whereas elastin content

remained the same between young and old mice. Moreover, both thin and thick collagen fibers were increased in the aortas from old animals. These findings are consistent with previous studies that reported increased collagen and collagen fiber size in the aortic walls of older rats and humans.[61, 89] Also similar, aortic elastin content has been reported to remain unchanged or to decrease with increasing age.[90, 91] The current study builds on these past reports by demonstrating that an age-dependent change in aortic collagen content was correlated with a decrease in aortic stress relaxation. The stress relaxation of a vessel is related to its passive compliance, and an age-dependent decline in stress relaxation suggests that the aorta becomes stiffer with age.[92] Moreover, the negative correlation between aortic stress relaxation and collagen content suggests that collagen, but not elastin, is a key factor in determining the passive compliance of the thoracic aorta. Taken together, the findings from these past reports and the present study suggest that remodeling of the ECM within the thoracic aorta occurs as a function of age and that this remodeling appears to be driven by selective deposition of collagen. Importantly, increased aortic collagen in the setting of aortic dilation with age may initially compensate for increased wall stress; however, when these compensation mechanisms are exhausted, increases in wall stress in combination with a less compliant aorta may result in increased susceptibility to rupture.

In addition to changes in aortic structure and ECM composition, changes in cellular composition also occur. In the present study, the density of cells in the medial layer of the aortic wall was decreased in old mice; this finding is consistent with past studies.[93, 94] Given that SMCs make up most of the cellular volume of the aortic media, a functional consequence of a decrease in cell number may be reflected as a reduction in contractility of the aortic wall.[94] Indeed, peak contractile force in aortic rings from old mice was reduced compared with that of young mice. A similar decline in contractility of aortic rings from old animals has been reported in rats.[72]

Nonetheless, these ex-vivo contractility determinations do not directly delineate the contribution of SMCs to generate contractile force. To address this issue, the present study examined the effects of isolated SMCs on the contraction of collagen disks. In the collagen gel contraction assay, both cellular contraction and protease degradation can act together to reduce collagen disk area. To determine the contribution of only the contractile component, collagen gel compaction was measured in the presence of a broad-spectrum matrix metalloproteinase inhibitor. Contraction of disks seeded with primary SMCs isolated from aortas of old mice was reduced compared with that of SMCs isolated from young mice. These results suggest an age-dependent decline in contractile function of primary ASMCs.

Importantly, the mechanical findings of this study are aligned with previous work examining the effects of aging on the human aorta. O'Rourke et al found that aortic stiffness, when measured in vivo as pulse wave velocity, increased by ≈100% between the ages of 20 and 80 years.[95] Using an intra-aortic ultrasonic catheter, Stefanadis et al found a positive correlation between age and the slope of the aortic pressure–diameter loop (aortic elasticity).[96] Increasing aortic stiffness with age has also been demonstrated by Sun et al with multiphase computed tomography imaging and through ex vivo biaxial mechanical testing of aortic specimens.[97, 98] Results presented in this report examining aortic aging in a murine model agree with these previous studies demonstrating a direct relationship between increased chronological age and increased aortic stiffness such that as age increased, so did aortic stiffness.

Increased aortic stiffness has a clinically significant impact on adjacent organ systems as well as on the aorta itself. Increased aortic stiffness, for example, is an independent risk factor for nonfatal cardiovascular events, ischemic stroke, and renal failure.[99-101] Incorporation of aortic compliance along with other risk factors in selected patient populations has been

suggested as a screening tool.[39] Furthermore, reduced aortic compliance is a common feature in aortic dissection, aneurysm, and rupture that has been pathogenetically implicated because of its deleterious effects on wall integrity.[98, 102, 103] Guidelines on addressing aortic mechanical function clinically have been established and include pharmacological approaches to reduce the effects of arterial stiffness, such as antihypertensive, hypolipidemic, and antidiabetic agents.[104-107] Likewise, SMC dysfunction, which can result in reduced contractility, has been associated with aortic aneurysm and dissection.[108] Novel approaches to ameliorate the mechanical effects of aging on the aorta, such as microRNA therapies to modulate the aortic ECM and smooth muscle function, have also been proposed.[109, 110] Long-term therapeutic trials of these pharmacological approaches should be conducted to assess the value of treating these age-related mechanical deficits in terms of cardiovascular risk reduction.

This study is not without limitations. First, other ECM components, including proteoglycans and matricellular proteins, may also change with age and affect aortic mechanical properties.[90] In addition, although the correlation between elastin content and aortic stress relaxation was not statistically significant, a correlation of 0.28 could be meaningful in other settings and perhaps did not attain significance because of the sample size. Second, although a reduction in cell density with age was identified, the types of cells that were reduced were not identified. A shift in populations of noncontractile fibroblasts or contractile myofibroblasts relative to ASMCs may also affect aortic mechanical properties. Future studies could examine the relative abundance of cell types using cell type–specific markers. Finally, an index of SMC contractility was assessed using type 1 collagen disks. Care should be taken in extrapolating these results to more complex ECM environments like those found in vivo, which are difficult to replicate with in vitro assays. Nevertheless, the unique findings of the present study provide potential explanations for reduced compliance and contractility with aging. In conclusion, these results have identified

age-related changes in geometry, ECM collagen content, and medial cellular content within the thoracic aorta that contribute to alterations in aortic mechanical properties. Importantly, both aortic compliance and contractility were shown to decrease with age, and this decrease may have significant clinical implications for the risk of developing aortic disease with advanced age.

Chapter 5: Methods for Measuring SMC Phenotype Parameters

Overview

The goal of this study was to determine the effects of age on thoracic aortic SMC phenotype using defined phenotypic parameters.

Animals

All procedures were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (2012) and approved by the institutional animal care and use committee of the Medical University of South Carolina. C57BL6 wild-type male and female aged 6 and 21 months were obtained from the National Institute on Aging mouse colony at Charles River Laboratories International (Wilmington, MA, USA). Equal numbers of male and female mice were used in each study.

Smooth Muscle Cell Isolation

Primary SMCs were isolated using an outgrowth procedure in which minced pieces of thoracic aorta from young and old mice (6 young, 6 old) were plated in SMC-specific growth medium (C22062; PromoCell) and allowed to extravasate for 2 weeks. After 2 weeks, the pieces of aorta were removed, and the SMCs were maintained in culture. Cells in passages 3 to 7 were used for all *in vitro* experiments.

Proliferation Assay

An *in vitro* proliferation assay was performed using the Cyquant Direct Cell Proliferation Assay Kit and manual (C35011, ThermoFisher). Briefly, cultured SMCs were seeded on to a poly-D-lysine coated, 96-well plate (354461, Corning) at 1,000 cells per well in 100ul of culture media, in triplicate for each assay day, and allowed to adhere overnight. In addition, a standard curve of cells was plated in duplicate to determine cell number during the proliferation period of 7 days. After incubating overnight, 100ul of 2X detection reagent was added to the three wells corresponding to Day 0 and standard curve wells, and then incubated for 60 minutes at 37 degrees Celsius. These wells were read from the bottom with a spectrophotometer (Spectramax M3, Molecular Devices) at an excitation/emission of 480/535 nm and analyzed using SoftMax Pro (version 5, Molecular Devices). In the same manner, proliferation was measured at 24 hour intervals over the next 7 days. Results were expressed as percent increase in cells versus Day 0.

Migration Assay

An *in vitro* migration assay was performed using tissue-culture treated Costar Transwell 6-well plates (3428, Corning) with polycarbonate membranes containing 8um pores. Cultured SMCs were seeded at 50,000 cells per upper insert and allowed to adhere overnight. Afterward, cells were incubated with 8ug/ml Mitomycin C (M4287, Sigma) in culture medium to halt proliferation, allowing for only measurement of migrated cells. After 3 hours, the mitomycin containing medium was removed, regular culture medium added to the upper insert, culture medium with 10% Fetal Bovine Serum (FBS) was added to the lower well, and the cells were allowed to migrate for 3 days. Non-migrated cells were removed from the membrane with a sterile swab. The inserts were then placed in methanol for 5 minutes to fix the migrated cells on the underside of the filter. The inserts were air-dried for 15 minutes and then stained with a

1:20 solution of Giemsa stain to deionized water. The membranes were rinsed in deionized water, allowed to dry, and then observed using a Zeiss microscope. The total number of migrated cells in 4 high-power fields was counted per insert. Results were expressed as number of migrated cells.

Adhesion Assay

An *in vitro* adhesion assay was performed using the Cyquant detection reagent used in the proliferation assay. Cultured SMCs were seeded on to a poly-D-lysine coated, 96-well plate (354461, Corning) at 2,000 cells per well in 100ul of culture media in triplicate and allowed to adhere for 2 hours. The cells were washed 3 times with PBS containing calcium and magnesium using the Bio-Plex Pro II Wash Station (Bio-Rad) for consistency. Afterward, washed wells were labeled with 100ul of Cyquant detection reagent and culture media in a 1:1 ratio and incubated for 60 minutes at 37 degrees Celsius. These wells were read from the bottom with a spectrophotometer (Spectramax M3, Molecular Devices) at an excitation/emission of 480/535 nm and analyzed using SoftMax Pro (version 5, Molecular Devices). The washed wells were compared to corresponding unwashed wells, also seeded in triplicate using the same fluorescent labeling method. Results were presented as a percent of adhered cells relative to unwashed wells.

Gene Expression by PCR Array and Radar Plot Profile

RNA was isolated from cultured thoracic aortic SMCs using the RNeasy Mini Kit (74106, Qiagen) according to the Fourth Edition of the RNeasy Mini Handbook. RNA quality and concentration was measured using the Agilent RNA 6000 Nano Kit (5067-1511) and Agilent 2100 Bioanalyzer. For each sample, 1ug RNA was converted to cDNA with iScript (170-8891, Bio-Rad) and then loaded onto custom, murine-specific RT2-Profiler PCR Array (CAPM09716D, SABiosciences) per

the array user manual. The arrays were read using a Bio-Rad CFX96 plate-reader according to the protocol described in the SABiosciences Technical Note for Software version 1.6. Expression was calculated using the delta-CT method and results were expressed in a radar plot profile as the relative fold increase in expression of three groups of genes: ECM proteins, matrix metalloproteinases (MMPs), and tissue inhibitors of MMPs (TIMPs). Primer specificity was confirmed by performing a melt curve with each array.

Statistical Analysis

All results are presented as mean ± SEM. The Student t-test was used to compare measurements of aortic SMC migration and adhesion between young and old groups. Pairwise comparison was used to compare proliferation over 7 days between experimental groups. All statistical analyses were performed using Stata statistical software (version 8, intercooled; StataCorp). P values of <0.05 were considered to be statistically significant.

Chapter 6: Aortic SMC Phenotype is Not Stable with Age

Introduction

The thoracic aorta undergoes geometric and mechanical changes with age, including dilation and stiffening in association with increased collagen in the aortic wall.[61, 111] These age-related alterations are not benign. Indeed, increased aortic stiffness is now recognized as a clinically relevant risk factor for cardiovascular disease.[112] In the setting of normal aging, resident cells within the aortic wall likely mediate these age-related changes. Whether aged resident cells initiate, or react to, the aortic aging process is not clear. In either case, aortic cell phenotype would be expected to be altered with age. While age-associated alterations in aortic geometry and mechanical function at the tissue-level have been well-described, changes in aortic cell phenotype with age have received relatively little attention.

Smooth muscle cells (SMCs) are the most abundant cell type in the aortic media, the vessel layer which bears the largest share of hemodynamic stress.[113] These cells perform several functions that mediate aortic geometry and function, including contraction and extracellular matrix (ECM) remodeling, and are critical mediators of aortic integrity. Thus, an age-associated shift in the phenotype of aortic SMCs could impact both passive and active mechanical function.[114] Indeed, alteration in SMC phenotype has been reported in several aortopathies.

Changes in aortic SMC phenotype have been described in association with mutations in transforming growth factor – beta receptors (TGFBR) and fibrillin-1, which cause Loeys-Dietz syndrome and Marfan syndrome, respectively. In aortic SMCs from patients with TGFBR

mutations, decreased expression of contractile genes has been asserted to cause defective contractile function and contribute to aneurysm pathogenesis.[115] While SMCs from dilated aortas of Marfan syndrome patients, demonstrated increased expression of contractile genes that was believed to contribute to aneurysm pathogenesis by increasing aortic stiffening .[116] In both syndromes, an alteration in SMC phenotype was pathologically implicated.

Cellular phenotype can be defined by multiple parameters of cell behavior and structure. In the context of aging, several phenotype parameters may contribute to structural and functional changes within the aorta, including proliferation, migration, adhesion, and gene expression of ECM remodeling genes. A better understanding of how these parameters are altered with age may provide insight into cell-mediated mechanisms of aortic aging, as well as potentially inform our understanding of the role of aging in aortopathies. Therefore, our study compared these specific phenotype parameters in aortic SMCs isolated from young and old mice to test the hypothesis that aging results in an altered aortic SMC phenotype.

Results

Thoracic Aortic SMC Proliferation is Decreased with Aging

Proliferation over a 7 day period was assessed using a fluorescent DNA-binding dye and found to be significantly decreased in aortic SMCs from the old group compared to those from the young group (Figure 6.1). This difference became statistically significant on Day 5 and persisted through Day 7. The typical sigmoidal appearance of the young aortic SMC proliferation curve is in contrast to the old aortic SMC proliferation curve, which has a flattened sigmoid appearance.

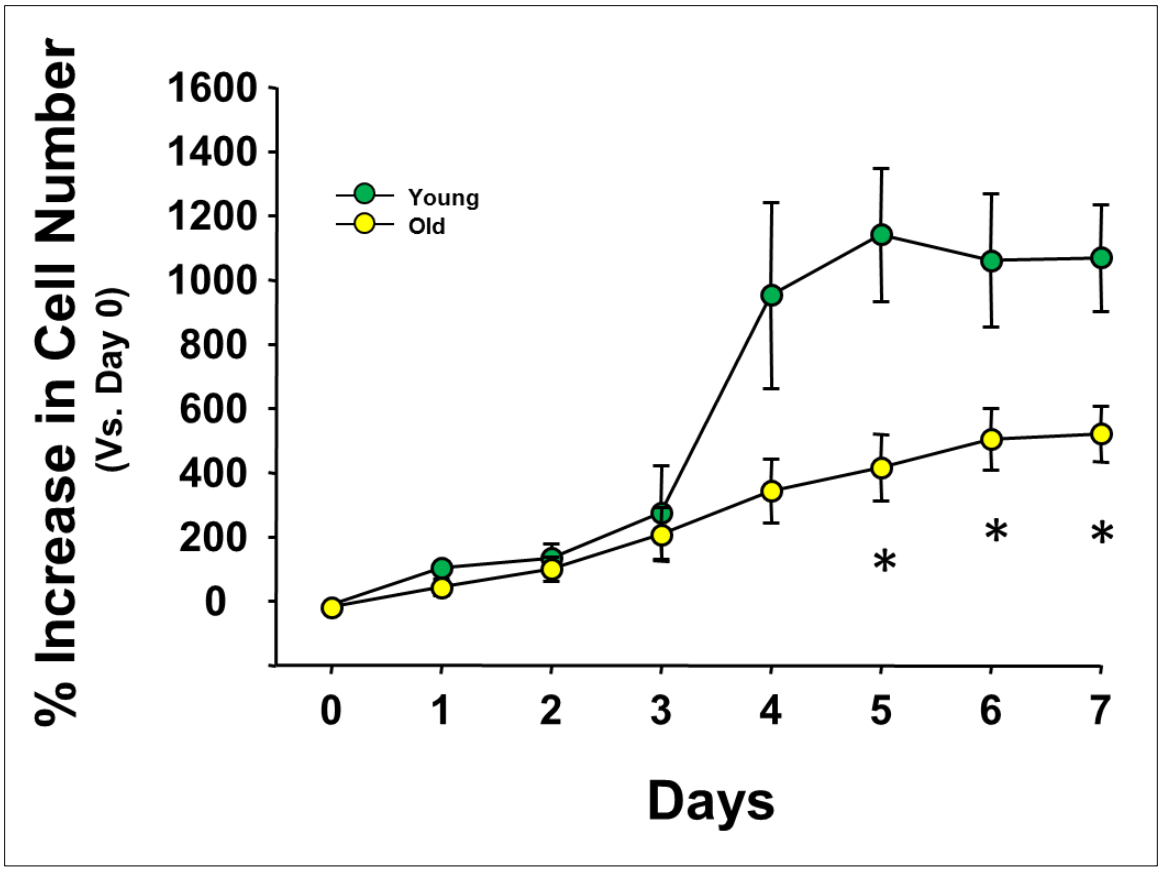

Figure 6.1 *Thoracic aortic SMC proliferation is decreased with aging.* Proliferation was decreased in aortic SMCs from old mice (n=6, yellow circles) compared to those from young (n=6, green circles). *P<0.05 vs. Young.

Thoracic Aortic SMC Migration is Decreased with Aging

Representative high-power fields of fixed, Giemsa-stained SMCs (blue) from young and old mice that have migrated to the side of the membrane containing culture media with 10% FBS added as a chemoattractant are shown (Figure 6.2, Top Panel). After mitotic-inhibition with mitomycin C and a 3 day incubation period, quantification of the number of aortic SMCs that had migrated through a porous (8 micron diameter) membrane revealed that migration was significantly decreased in old SMCs relative to young SMCs (Figure 6.2, Bottom Panel).

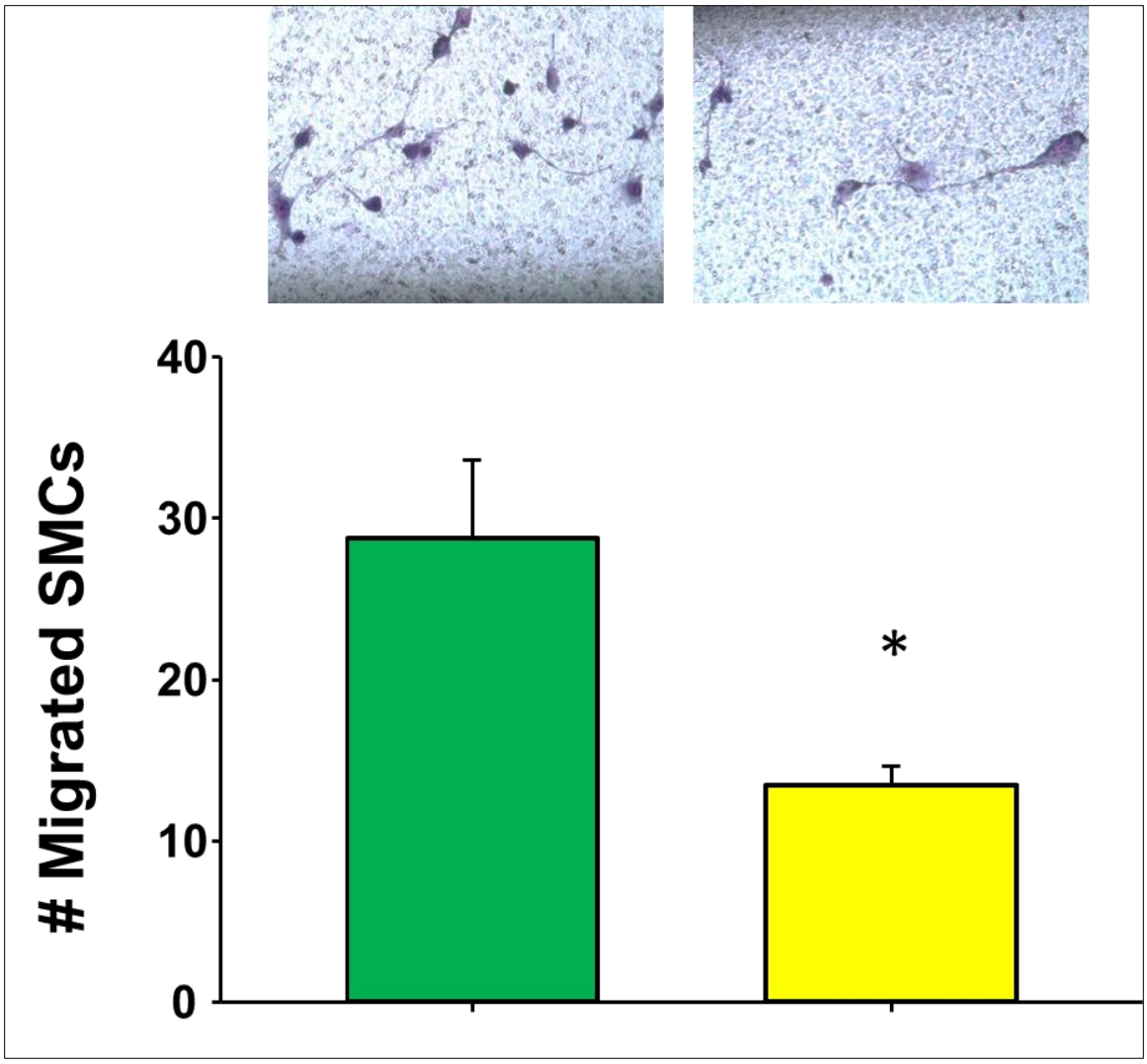

Figure 6.2 *Thoracic aortic SMC migration is decreased with aging.* Representative high-power fields of Giemsa-stained, young and old aortic SMCs that have migrated through a porous membrane. Migration was decreased in aortic SMCs from old mice (n=6, yellow) compared to those from young (n=6, green). *P<0.05 vs. Young.

Thoracic Aortic SMC Adhesion is Decreased with Aging

Adhesion of aortic SMCs to a tissue-culture treated, poly-D-lysine coated 96-well plate was assayed using a fluorescent DNA-binding dye. After automated washing, the percent adherence, as measured by remaining fluorescence relative to unwashed wells, was significantly reduced in aortic SMCs isolated from old mice relative to those from young mice (Figure 6.3).

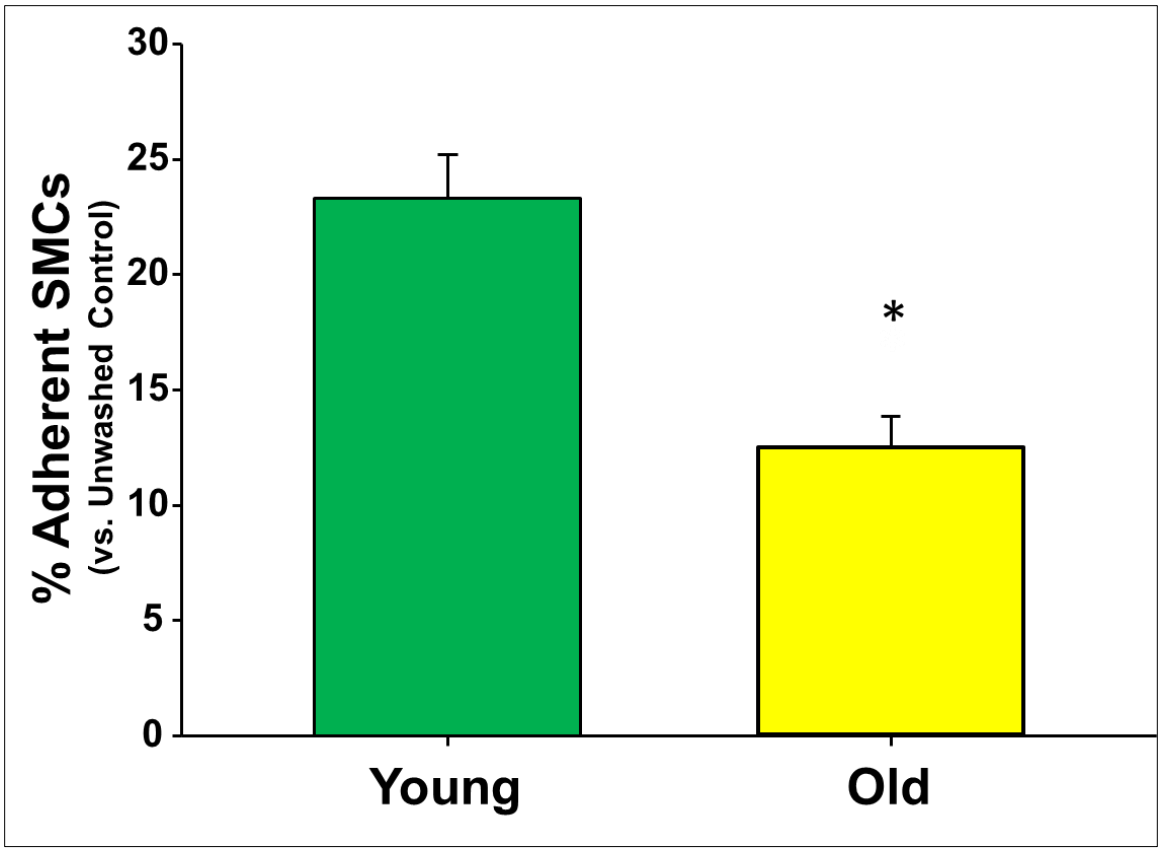

Figure 6.3 *Thoracic aortic SMC adhesion is decreased with aging.* Adhesion to a tissue-culture treated surface after automated washing was decreased in aortic SMCs from old mice (n=6, yellow) compared to those from young (n=6, green). *P<0.05 vs. Young.

Thoracic Aortic SMC Gene Expression Profile with Aging

If an SMC phenotype shift occurs with age, a change in gene expression would be expected. Given that our previously published data indicated an increase in aortic wall collagen content with age that correlated with vessel compliance, the expression of ECM remodeling genes was examined using a PCR array to determine whether the aortic SMC population may contribute to these age-related ECM and mechanical changes (Table 6.1). A gene expression profile was generated incorporating ECM remodeling genes on the array into three categories: ECM proteins, matrix metalloproteinases (MMPs), and tissue inhibitors of MMPs (TIMPs). These categories were graphed in a radar plot to show fold changes in gene expression relative to the young group and demonstrated a distinct expression profile in old aortic SMCs (Figure 6.4),

suggesting that a shift in SMC phenotype contributes to aortic ECM and mechanical changes with age. Interestingly, the stromelysin MMPs (MMP-3 and MMP-11) were increased significantly with age.

Table 6.1 *Table of ECM Remodeling Genes Examined by PCR Array*.

Extracellular Matrix Proteins (ECM)	Fold	p	Matrix Metallo-proteinases (MMPs)	Fold	p	Tissue Inhibitors of MMPs (TIMPs)	Fold	p
Collagen 1a1	1.58	<0.05	MMP-2	1.58	<0.05	Timp-1	1.09	NS
Collagen 1a2	1.44	<0.05	MMP-3	1.86	<0.05	Timp-2	1.34	NS
Collagen 3a1	2.78	<0.05	MMP-7	1.10	NS	Timp-3	2.69	<0.05
Collagen 4a1	1.87	<0.05	MMP-8	1.54	NS	Timp-4	1.68	NS
Collagen5a1	1.51	NS	MMP-9	0.45	<0.05			
Collagen 6a1	1.99	<0.05	MMP-11	1.33	<0.05			
Elastin	1.52	NS	MMP-13	0.75	NS			
Lumican	1.51	NS	MMP-14	1.14	NS			
Fibronectin-1	1.55	NS	MMP-15	0.81	NS			
Fibulin-1	1.07	NS	MMP-16	2.17	<0.05			
Fibrillin-1	1.39	<0.05	MMP-17	1.38	NS			
			MMP-24	1.31	<0.05			

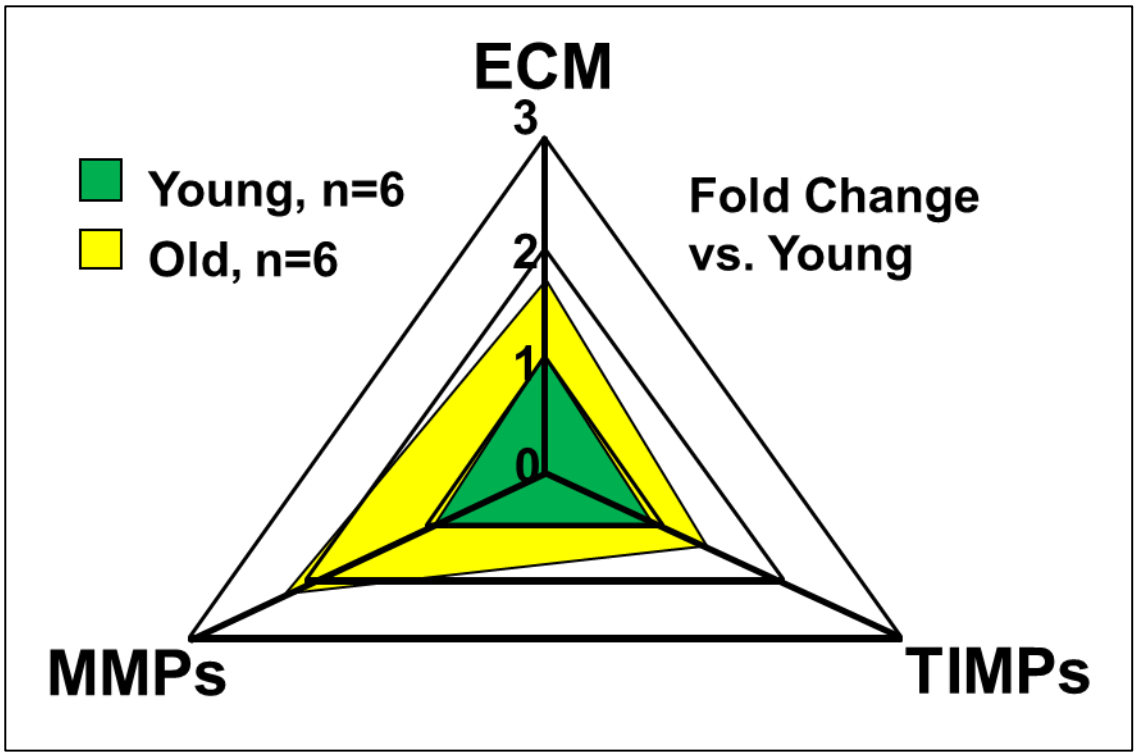

Figure 6.4 *Gene expression profile of ECM remodeling genes with aging*. Radar plot of the relative fold increase in expression of three groups of ECM remodeling genes: ECM proteins (ECM), matrix metalloproteinases (MMPs), and tissue inhibitors of MMPs (TIMPs), in old aortic SMCs (n=6, yellow triangle) compared to young aortic SMCs (n=6, green triangle).

Collagen Isoform Expression Increases with Age

Several collagen alpha chain isoforms were included within the "ECM" group of the PCR array, including collagen 1a1, 1a2, 3a1, 4a1, 5a1 and 6a1. The gene expression levels of all of these collagen alpha chains, except 5a1, were increased in old aortic SMCs compared to young (Figure 6.5).

Expression of TGF- β Signaling Pathway Components Increases with Age

Increased fibrotic signaling in aortic SMCs with age could be responsible for increased ECM protein expression. To evaluate whether this was the case, the expression levels of several mediators of TGF- β signaling, which has been reported to increase in whole aortas with age,

were compared between young and old aortic SMCs. Expression of TGF-β Receptor Type 1, TGF-β Receptor Type 2, Smad2, and Smad4 was increased in old aortic SMCs (Figure 6.6).

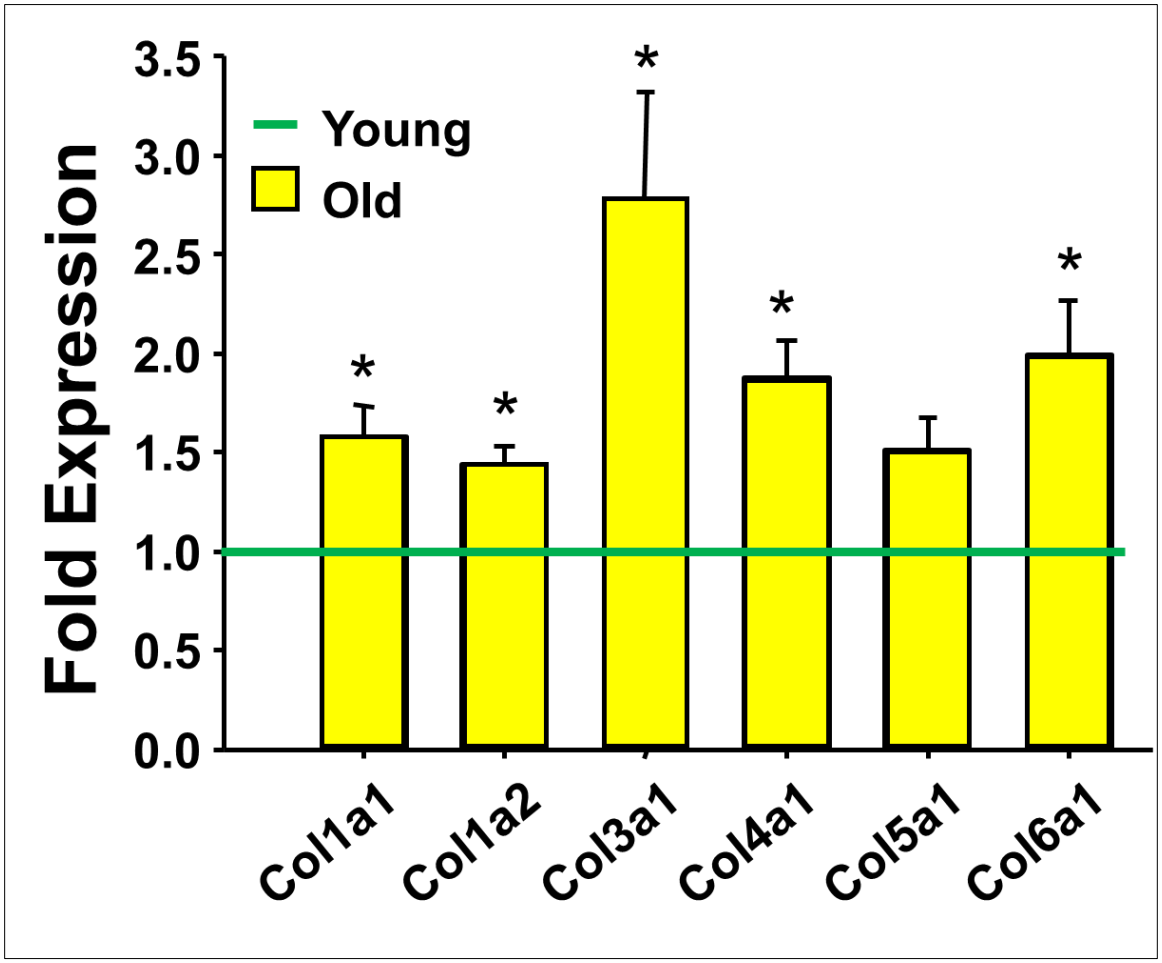

Figure 6.5 Aortic SMC collagen isoform expression with aging. Fold expression of multiple collagen isoforms increased in aortic SMCs with age (n=6, yellow bars) relative to young SMCs (n=6, green line). *P<0.05 vs. Young.

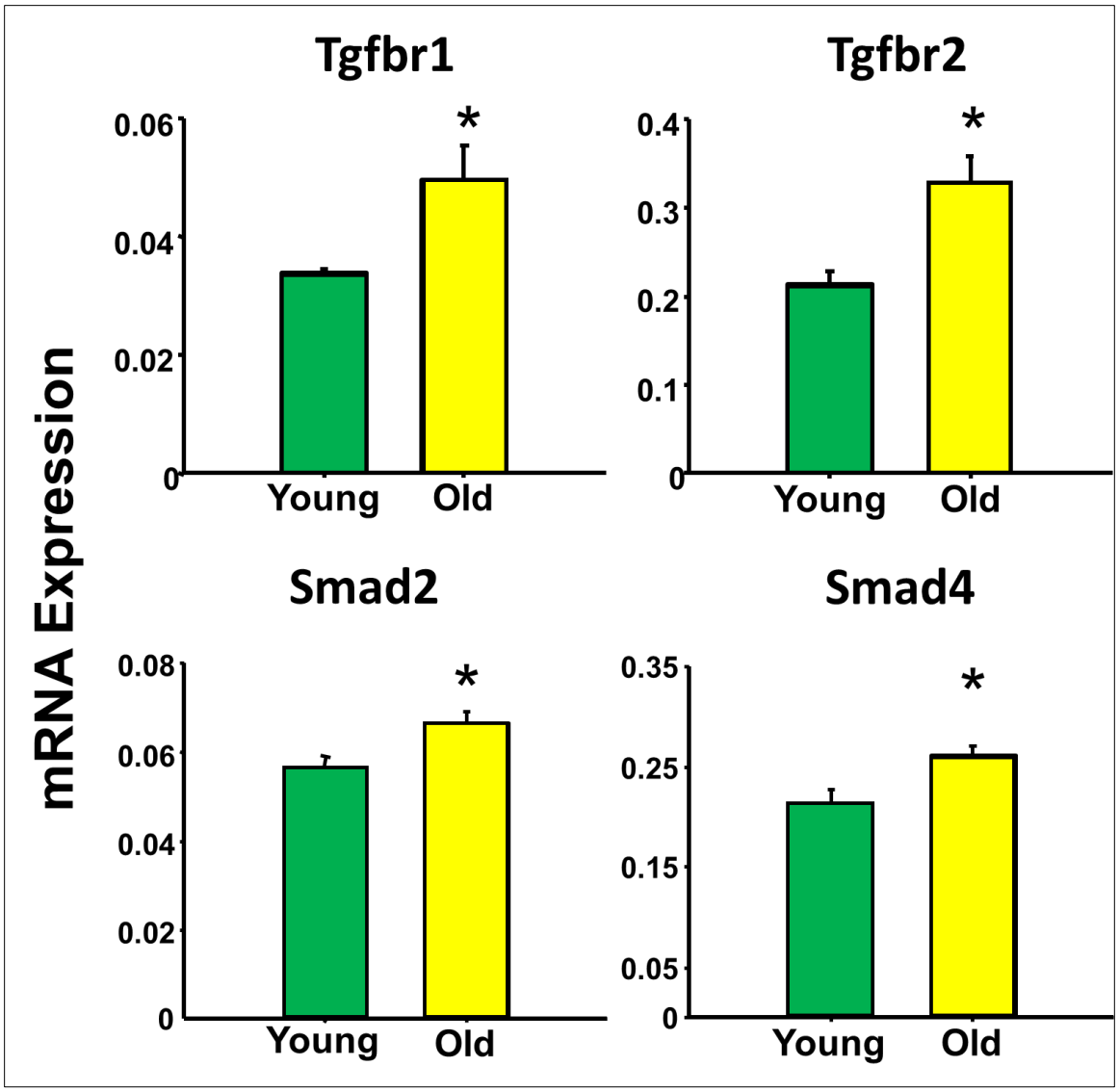

Figure 6.6 Expression of TGF-β signaling pathway components with aging. Gene expression levels of TGF-β Receptor Type 1/Tgfbr1 (Panel A), TGF-β Receptor Type 2/Tgfbr2 (Panel B), receptor Smad2 (Panel C), and common Smad4 (Panel D) were increased in old aortic SMCs (n=6, yellow bar) compared to young aortic SMCs (n=6, green bar). *P<0.05 vs. Young

Discussion

This set of studies tested whether thoracic aortic SMC phenotype is altered by aging. Phenotype was defined by the following parameters: proliferation, migration, adhesion, and gene expression. Using in vitro assays to assess these parameters, isolated aortic SMCs from old mice demonstrated reduced proliferation, migration, and adhesion, relative to those from young

mice. In accordance with a change in phenotype, isolated aortic SMCs from old mice also exhibited a distinct gene expression profile consistent with increased ECM remodeling. Together, these studies support the conclusion that aortic SMC phenotype is not stable with age. Furthermore, the age-related changes in SMC phenotype parameters are consistent with the aortic structural and mechanical alterations previously described with age, suggesting this cell population contributes, in part, to aortic aging.[114] Overall, these results represent one of the first literature reports of thoracic aortic aging at the cellular level.

While previous reports on aortic aging largely describe a decline in compliance in association with increased collagen, diameter, and wall thickness, little has been published on which resident cell population may contribute to this age-related remodeling.[61, 111] For example, though publications describe the decline in aortic medial cell content with age, the means by which this occurs has not been well-studied. In this study, we found that *in vitro* proliferation of SMCs from old mice exhibited decreased proliferation relative to those from young mice. This finding is in contrast to studies that used aortic SMCs isolated from aged rat models to examine SMC proliferation with age.[117, 118] However, other studies using primary SMCs from rats and other species have produced contrasting data suggesting proliferation is reduced with age.[119] This conflicting data may be due to variation in model species, models of vascular injury, isolation methods, and methodology.[120] Importantly, our findings are consistent with studies that used aortic SMCs isolated from C57BL/6 mice and humans, which demonstrated decreased proliferation with age.[121-124] Furthermore, in patients over 70, restenosis due to SMC proliferation after carotid endarterectomy was less common than in younger patients, suggesting that aged SMCs may have a reduced capacity to proliferate and completely restore their population after injury or death.[125] Our result, together with previous murine and

human data from other investigators, suggests that aortic SMC proliferation is reduced with age and may contribute to the decline in aortic medial cell content with age.

After proliferation, migration is the most commonly reported SMC phenotype parameter. Previously, we published that aortic SMCs from aged mice have reduced contractility.[114] Given that contractility is a necessary component of motility, SMC migration was expected to be reduced with age. Our study examined migration using a Boyden chamber with serum as a chemoattractant, and indeed, migration was reduced in aortic SMCs from aged mice. Like proliferation, a majority of migration studies have been performed using primary cells from rats, which report increased migration with age.[120, 126, 127] However, a study using aged human SMCs in a Boyden chamber demonstrated decreased migration with age, in agreement with our findings. Such contradicting proliferation and migration results in humans emphasize the need for caution when extrapolating from rat SMC studies. Further complicating interpretation of these studies is that the methods to study migration are quite varied and include gel invasion assays, scratch/wound assays, and Boyden chamber assays. Each of these methods examine distinct aspects of migration and care should be employed when comparing them.[128] Overall, our finding of reduced SMC migration with age further supports our previously published findings on SMC contractility with age and suggests aged SMCs may be less able to migrate within the aorta if needed.

The effect of aging on SMC adhesion has not been examined to the extent that proliferation and migration have been in the literature. The majority of articles on adhesion and the aging vasculature report an increase in the expression and abundance of adhesion molecules, such as vascular cell adhesion molecule-1 VCAM-1. Most of these studies refer to these adhesion molecules in the context of inflammatory cell adhesion and infiltration. Few studies have

focused on the ability of SMCs to remain attached to its substrate under stress. In this study, fewer old SMCs remained adherent to their underlying substrate after washing relative to young SMCs. In a similar study, atomic force microscopy was used to examine the adhesion force of rat aortic SMCs to fibronectin coated microbeads. Adhesion force per cell increased with age.[129] However, this method employed a single, specific substrate to assess adhesion, whereas the washing method evaluates adhesion strength onto a matrix produced by SMCs. Both results may reflect a shift in integrin subtype abundance on the SMC surface with age, which has been previously suggested.[117, 130] Such a shift may have significant consequences on cell phenotype and contractile function.[117, 130, 131]

Lastly, if an age-related shift in SMC phenotype occurs, a change in the profile of genes expressed would be expected. Because of the increase in aortic wall thickness and collagen content with age, we chose to examine three groups of ECM remodeling genes - matrix metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs), and ECM proteins. Aged aortic SMCs exhibited a distinct gene expression profile consistent with a greater role for SMCs in vascular remodeling. Increased collagen expression, which may be due to elevated expression levels of multiple TGF-β signaling pathway components, by old aortic SMCs suggests that this cell type contributes to increased aortic wall thickness and decreased compliance with age. This finding, in combination with decreased contractility, suggests that SMCs undergo a functional shift with age and contribute to passive and active mechanical dysfunction of the aorta. Importantly, increased aortic stiffness due to elevated collagen content has been reported to raise arterial pulse pressure, which in turn increases cardiac work and decreases downstream organ perfusion. Thus, a shift in aortic SMC phenotype with age may also impact cardiovascular disease risk. However, this has yet to be demonstrated experimentally.

While this study provides evidence that aortic SMCs undergo a phenotype shift with age and suggests this cell population participates in the hallmarks of aortic aging, it is not without its limitations. This study focused on the impact of age on aortic SMCs. While this is the most abundant cell type within the aorta, other resident cell types, such as fibroblasts and endothelial cells, could play significant roles in aortic aging and were not evaluated in this study. Furthermore, these cell populations likely interact and influence the other's behavior. Therefore, future studies examining the impact of aging on these cell types and their interactions are necessary to better understand the cellular mechanisms of aortic aging. Moreover, mechanical and environmental inputs from the ECM also influence SMC phenotype.[132-134] The *in vitro* studies used here serve to illustrate phenotypic differences due to aging but do not necessarily reflect *in vivo* behavior, as the complex *in vivo* environment (wide variety of ECM proteins, mechanical cues, etc) is not easily replicated. Thus, caution should be employed when generalizing these results to *in vivo* SMC phenotype. In addition, the influence of other age-related factors on the results of our phenotype studies cannot be excluded. For example, it has been reported that SMCs differentially respond to certain growth factors with age.[135, 136] Decreased SMC proliferation and migration with age may be the result of an attenuated response to the serum growth factors used in these studies. Thus, an increase or decrease in these phenotype parameters with age may be dependent on which specific growth factors are used in these assays and may perhaps explain some of the conflicting results reported by other investigators. Nevertheless, this data provides evidence that aortic SMC phenotype is not stable with age and such a phenotype shift likely contributes to changes in aortic structure and mechanical function.

Future studies will investigate the age-related mechanisms regulating SMC phenotype and whether manipulation of SMC phenotype translates to alterations in aortic structure and

mechanical function *in vivo*. If aortic mechanical dysfunction with age can be attenuated by inhibiting the age-associated phenotype shift, subsequent studies performed in animal models of aortic disease, such as aneurysm, could examine the contributions of an aged aortic substrate to its pathogenesis.

Chapter 7: Summary and Future Directions

The studies described by this thesis provide supporting results for a non-transgenic mouse model of thoracic aortic aging. Importantly, mechanical studies in this model identified a novel age-related contractile deficit in *ex vivo* aortic segments after depolarization with potassium, which had not been previously described in the literature. Further, an innovative variation of the collagen gel contraction assay in the presence of a broad MMP inhibitor indicated that the contractile deficit was present at the level of the individual smooth muscle cell, suggesting that decreased aortic contractility with age may be due to both a decrease in cellular density within the aortic wall and a decrease in smooth muscle cellular contractility. This decrease in contractility suggested that SMC phenotype may have been affected with age. Subsequent studies of phenotype parameters implicated a phenotype shift in aortic SMCs with age. Moreover, the results of the phenotype studies were congruent with age-related structural and mechanical changes observed histologically and with *ex-vivo* studies in the aged mouse model. For example, decreased aortic SMC proliferation was congruent with decreased cell density within the aortic wall histologically. Likewise, decreased adhesion and migration with age was consistent with the finding of decreased cellular contractility. Additionally, increased expression of collagen alpha chains and an altered gene expression profile are consistent with the observed increase in aortic collagen, wall thickness and stiffness with age. Together, these studies collectively provide support that aortic smooth muscle cells mediate age-related aortic structural and mechanical alterations, in part, through a shift in phenotype. Whether this phenotype shift initiates age-related changes within the aorta or develops as a consequence of

these changes, it not yet known and requires further study. Regardless, these results support that aged aortic smooth muscle cells contribute to aortic aging through increased collagen within a thickened aortic wall leading to decreased compliance, as well as decreased cell density and contractility that may result in dilation.

A phenotype shift in aortic SMCs with age offers an intriguing mechanism with the potential to unify several age-associated aortic changes. It is interesting to speculate that an aortic SMC phenotype shift could explain both aortic stiffening and dilation with age. Importantly, while aortic dilation with age has been described, the mechanism behind this dilation has not been well studied. It may be that a phenotype shift results in both decreased cell density, through decreased proliferation, and reduced contractility. Together, this reduction in the number of SMCs and their ability to contract to regulate aortic diameter, may explain aortic dilation with age. Furthermore, as a compensatory mechanism in response to increased wall stress, the aged SMCs may produce more collagen in order to normalize wall stress through increased wall thickness. Thus, a phenotype shift in aortic SMCs could be a potential mechanism for both increased aortic dilation and stiffness with age (Figure 7.1). However, further studies are needed to support this mechanism.

Interestingly, altered ECM composition itself as a result of a phenotype shift may induce or maintain cellular phenotype shifts. Indeed, a change in basement membrane proteins with age could produce an altered SMC phenotype. It has been reported that a basement membrane composed of laminin aids in maintaining a “contractile” SMC phenotype, while a basement membrane composed primarily of fibronectin results in a “synthetic” SMC phenotype. The role of these basement membranes in aortic SMC phenotype shift has not been fully examined but remains a valid avenue of investigation. Additionally, SMC phenotype may also be regulated not

only by the proteins to which the SMCs adhere but also by the adhesion proteins linking the ECM to the SMC membrane, e.g. integrins. Together, SMCs may interpret extracellular cues from both specific ECM proteins and integrins that mediate attachment to mechanically sense their environment. Indeed, it is intriguing to speculate that with age the abundance of both ECM proteins and integrins are altered in a manner that results in altered SMC phenotype. If so, mechanotransduction – the signaling process by which extracellular mechanical stimuli are transduced into the cell and incorporated into a cellular response – may be altered with age as well. Of note, dysregulated mechanotransduction has been proposed as a potential unifying mechanism for aortic aneurysm development based on associations with mutations in several mechanotransduction proteins, including smooth muscle actin. Smooth muscle actin is a component necessary for contractility and has also been used as a marker for phenotype shifts. Thus, whether through altered contractility, ECM proteins, or integrin abundance with age, aortic mechanotransduction may be disturbed in a such a way that predisposes to aneurysm development.

Future experiments may examine the role of TGF-β signaling in regulating the phenotype parameters examined, including proliferation, migration, adhesion, gel contraction, and gene expression. It may be that TGF-β signaling regulates some, but not all, of the parameters studied, implying that age-related SMC phenotype modulation is complex. Given that the aged phenotype was observed in association with increased expression of canonical TGF-β signaling receptors and mediators, methods to inhibit this pathway, such as TGF-β neutralizing antibody, could be used *in vitro* in an attempt to modulate parameters. It would be interesting to speculate that a "young" phenotype could be restored with TGF-β inhibition in SMCs from old aortas. Of special interest would be the role of TGF-β signaling in mediating gel contraction. A previous report demonstrated that TGF-β stimulation in VSMCs increases expression of

contractile proteins, which would be expected to increase gel contraction. Perhaps, TGF-β signaling has a differential influence with age, such that increased stimulation in old SMCs results in reduced contractile protein expression and reduced gel contraction. Alternatively, increased TGF-β signaling in old SMCs may impair contractility via pathways independent of contractile protein expression.

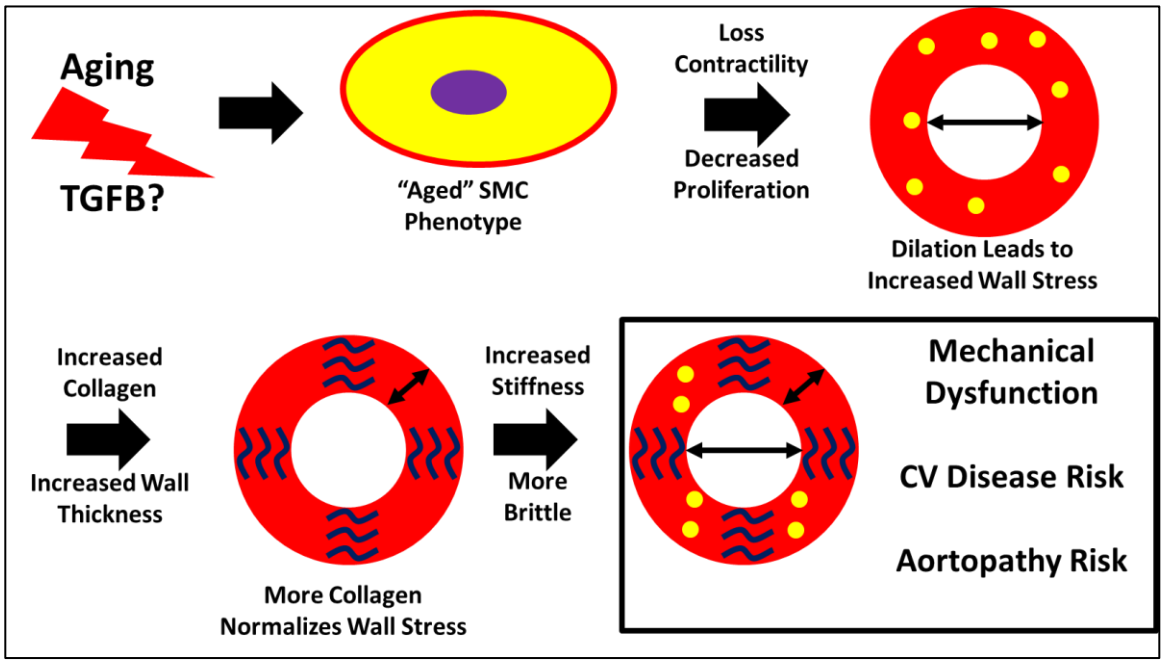

Figure 7.1 Summary: A New Understanding of Aortic Aging. An SMC phenotype shift with age, potentially due to increased TGFB signaling, results in both decreased cell density, through decreased proliferation, and reduced contractility. Together, this reduction in the number of SMCs and their ability to contract to regulate aortic diameter, may explain aortic dilation with age. As a compensatory mechanism in response to increased wall stress, the aged SMCs may produce more collagen in order to normalize wall stress through increased wall thickness, resulting in a more stiff and brittle aorta. Thus, a phenotype shift in aortic SMCs could be a potential mechanism for both increased aortic dilation and stiffness with age., in addition to resulting in mechanical dysfunction, cardiovascular and aortopathy risk. TGFB, Transforming Growth Factor-Beta; SMC, Smooth Muscle Cell; Yellow circles, Aged Aortic Smooth Muscle Cells; Curved Black Lines, Collagen.

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