CHARACTERIZATION OF THE VASCULAR PATHOLOGY IN THE ACTA2 R258C MOUSE MODEL AND CEREBROVASCULAR CHARACTERIZATION OF THE ACTA2 NULL MOUSE

Carlos A. Villamizar

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CHARACTERIZATION OF THE VASCULAR PATHOLOGY IN THE ACTA2 R258C MOUSE MODEL AND CEREBROVASCULAR CHARACTERIZATION OF THE ACTA2 NULL MOUSE

by

Carlos Andrés Villamizar-Rosales, B.S.

APPROVED:

___________________________________
Dianna M. Milewicz, M.D., Ph.D., Advisor

___________________________________
Ananth Annapragada, Ph.D.

___________________________________
Jaroslaw Aronowski Du, Ph.D.

___________________________________
Siddharth Prakash, M.D., Ph.D.

___________________________________
Yang Xia, Ph.D.

APPROVED:

___________________________________
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston
CHARACTERIZATION OF THE VASCULAR PATHOLOGY IN THE \textit{ACTA2 \textit{R258C}} MOUSE MODEL AND CEREBROVASCULAR CHARACTERIZATION OF THE \textit{ACTA2} NULL MOUSE

A

THESIS

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The University of Texas

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Graduate School of Biomedical Sciences

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Carlos A. Villamizar

Houston, Texas

August 2014
Dedication

First, I would like to dedicate this work to the love of my life, my wife and future Dr. Carolyn Adamski, who really likes science a lot. Like, A LOT. Thank you for putting up with me and helping me along the way.

My work also goes out to all the people who we do this for, the patients and their families. Stay strong, stay hopeful.
Acknowledgements

First and foremost, I would like to thank Dr. Dianna Milewicz mentoring me and allowing me to stay on and pursue a master’s degree in her lab. It has been many years that I have worked in her lab, and I am truly grateful for each one of them.

Next, I would like to thank my committee – Dr. Annapragada, Dr. Aronowski, Dr. Prakash and Dr. Xia - for helping me out along the way, for pushing me to deepen my understanding and develop critical thinking skills, sharing their resources and for giving me constant feedback, and encouragement during my years as part of the GSBS. Special thanks to all the members of the Annapragada Lab, with which I worked closely for over a year.

I would not have accomplished nearly as much as without the help of past and present members of the Milewicz Lab. Jiumei Cao and Shao-Qing Kuang for teaching me everything they knew about mouse care and murine surgeries. To Katerina, Dr. Chen, Callie and all others in the lab who offered their help and friendship over the past few years. A big thank you goes all my other labmates as well for sharing the good and the bad for the past few years.

I would like to thank Ms. Brenda Gaughan in Student Affairs for all her help and understanding during this past year.

Finally, I would not be where I am today if not for my wonderful family, my mother and father, who have all been inspiring figures in my life. Los Amo. Tio, thank you is not enough, you have been the most supportive and special person, both professionally and in life.
CHARACTERIZATION OF THE VASCULAR PATHOLOGY IN THE ACTA2 R258C MOUSE MODEL AND CEREBROVASCULAR CHARACTERIZATION OF THE ACTA2 NULL MOUSE.

Carlos Villamizar B.S

Advisor: Dianna M. Milewicz, M.D., Ph.D.

Mutations in ACTA2, the gene encoding for smooth muscle α-actin, predispose patients to a wide range of vascular diseases and is most commonly associated with thoracic aortic aneurysms (TAAD) and dissections (TAAD) and strokes. TAAD in patients is characterized by aortic media degeneration and loss in contractile force generation followed by aortic enlargement and subsequent rupture. Most stroke cases associated with ACTA2 are described as moyamoya-like cerebrovascular incidents characterized by distal occlusion of the internal carotid artery, stenosis of arteries in the Circle of Willis, and straightening of cerebral arteries. Patient samples are scarcely available for analysis, and the few obtained are from end stages of the disease. For these reason, we decided to study the causative mechanisms of vascular disease using the Acta2R258C transgenic and Acta2+/− mouse models.

The aortic phenotype was studied using the transgenic mouse model bred with Acta2+/− mice to decrease the ACTA2 wildtype to mutant ratio. Acta2+/+ R258C TG mice have decreased aortic contractility and mild medial wall degeneration but no evidence of aortic enlargement was observed. However, Acta2+/− R258C TG mice showed severe aortic wall degeneration, elastic fiber rupture and proteoglycan accumulation along with significant aortic dilation. After carotid artery ligation, Acta2+/− R258C TG mice presented with an excessive proliferative response to injury, leading to increased inflammatory response, unresolved thrombi and severe occlusion of the lumen.
Cerebral imaging and histology were studied in WT, Acta2<sup>+/−</sup>, Acta<sup>−/−</sup> mouse mode. Straightening of the cerebrovascular arteries was observed in Acta2<sup>+/−</sup> and Acta2<sup>−/−</sup> mice when compared with WT mice. Narrowing and/or obstruction of arteries were also present in the Acta2 mutant mice, in particular in the internal carotid arteries. Histopathologic analysis showed thickening of the medial layer in both large and small arteries throughout the vasculature with decreased cell density in the large arteries.

We were able to recapitulate significant characteristics of the aortic and cerebrovascular pathology seen in patients with ACTA2 mutations in our Acta2 mouse models, proving to be a valuable tool to study the underlying mechanisms of vascular disease. Furthermore, both aneurysm formation and SMC proliferative response were exacerbated by decreasing the amount of endogenous Acta2 in our transgenic model. The severity of the cerebrovascular pathology did not vary between Acta2<sup>+/−</sup> and Acta<sup>−/−</sup>, suggesting a dose dependent effect in these mice. Meanwhile, the Acta2 R258C mutation appears to have a dominant negative effect, as its effects are inversely correlated to the amount of endogenous actin in an organism.
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<table>
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<th>Description</th>
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<tr>
<td>AAD</td>
<td>Aortic Aneurysms and Dissections</td>
</tr>
<tr>
<td>Acta2</td>
<td>Smooth-muscle specific alpha-actin</td>
</tr>
<tr>
<td>ATRI</td>
<td>Angiotensin II type 1 receptor</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>Cnn1</td>
<td>Calponin 1</td>
</tr>
<tr>
<td>CW</td>
<td>Circle of Willis</td>
</tr>
<tr>
<td>F actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>fTAAD</td>
<td>Familial thoracic aortic aneurysm an/or aortic dissection</td>
</tr>
<tr>
<td>G actin</td>
<td>Globular (monomeric) actin</td>
</tr>
<tr>
<td>ICA</td>
<td>Internal carotid artery</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing (2D) gel</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>LDS</td>
<td>Loeys-Dietz syndrome</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin Light Chain Kinase</td>
</tr>
<tr>
<td>MMD</td>
<td>Moyamoya Disease</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRTF-A</td>
<td>Myocardin-related transcription factor A</td>
</tr>
<tr>
<td>MRTF-B</td>
<td>Myocardin-related transcription factor B</td>
</tr>
<tr>
<td>Myh11</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCA</td>
<td>Posterior cerebral artery</td>
</tr>
<tr>
<td>SCA</td>
<td>Superior cerebellar artery</td>
</tr>
<tr>
<td>SM22α</td>
<td>Transgelin</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SM-MHC</td>
<td>Smooth muscle- myosin heavy chain</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>sTAAD</td>
<td>Sporadic thoracic aortic aneurysms and/or aortic dissections</td>
</tr>
<tr>
<td>TAAD</td>
<td>Thoracic aortic aneurysms and/or aortic dissections</td>
</tr>
<tr>
<td>TAA</td>
<td>Thoracic aortic aneurysms</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor beta 1</td>
</tr>
<tr>
<td>TGFβR1/2</td>
<td>Transforming growth factor beta 1 receptor type 1/2</td>
</tr>
<tr>
<td>vEDS</td>
<td>vascular (type IV) Ehlers-Danlos Syndrome</td>
</tr>
<tr>
<td>vSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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CHAPTER 1

Introduction
Arterial Wall Structure and Pathology

Arterial walls are comprised of three distinct layers, the adventitia, media and intima\(^1\text{-}^3\). The adventitia is made up of extracellular matrix proteins, myofibroblasts, immune cells and nerve endings (Figure 1.1A). The medial layer is the contractile component of the wall containing elastic fibers established during development and consists mostly of differentiated smooth muscle cells (SMCs). The intima is the innermost layer and is composed of a single cell layer of endothelial cells. A complex network of collagen fibers helps maintain the wall structure and its elastic properties\(^4\text{-}^8\).
Figure 1.1: Structural and Pathologic Characteristics of the Aortic Wall - A. H&E and Movat staining of aortic tissue from control (a,c) and TAA patients (b,d) shows the three-layer organization of the aortic wall. In control patients, cells are neatly arranged in layers (a) in-between elastic fibers (c, elastin shown in black). In TAA patients, cells are disorganized (b), and there is a loss of elastin fibers and accumulation of proteoglycans (c, proteoglycans appear blue). Republished with permission of Annual Reviews, from [Genetic basis of thoracic aortic aneurysms and dissections: Focus on smooth muscle cell contractile dysfunction. Milewicz, D. M., Guo, D. C., Tran-Fadulu, V., Lafont, A. L., Papke, C. L., Inamoto, S., Kwartler, C.S. & Pannu, H. 9, 2008]; permission conveyed through Copyright Clearance Center, Inc. B. Illustration of a lamellar unit, composed of elastin fibers, single layer of smooth muscle cells and microfibrils linking the cells to the fibers. Inset illustrates the smooth muscle contractile unit. Adapted with permission of Annual Reviews, from [Genetic basis of thoracic aortic aneurysms and dissections: Focus on smooth muscle cell contractile dysfunction. Milewicz, D. M., Guo, D. C., Tran-Fadulu, V., Lafont, A. L., Papke, C. L., Inamoto, S., Kwartler, C.S. & Pannu, H. 9, 2008]; permission conveyed through Copyright Clearance Center, Inc.

There are two types of arteries – small, muscular arteries and large, elastic arteries. The aorta and common carotid arteries are examples of elastic arteries. In these vessels, SMCs are organized concentrically around the lumen and encased by layers of elastic lamellae (Figure 1.1B). SMCs are bound to the lamellae through fibrillin-1 microfibrils at focal adhesion points. The elastic recoil properties of the elastic lamellae counteract pulsatile blood flow, a critical property of large arteries. In normal arteries, the
number of elastic layers are determined during development based on the strength of the pulsatile flow. For example, the ascending aorta has more lamellar layers compared to the abdominal aorta and carotids. In contrast, the smaller muscular arteries only have an internal elastic lamina and external elastic lamina separating the intima and adventitia from the media, respectively.

Histological examinations of aortic pathology reveal consistent features common in most diseased aortas. Patients who have suffered dissection, a rupture in the aortic wall, show clear signs of deterioration of the medial layer: SMC disarray, disruption of elastic fibers and extracellular matrix degeneration. SMCs in these aortas lose their concentric organization, leading to regions of both focal loss and gain. Along with SMC disarray, elastic fiber thinning and fragmentation contribute to weakening of the wall which results in the loss of its recoil properties. Previous work has concluded that increased expression of matrix metalloproteinases (MMPs), zinc endopeptidases, responsible for extracellular matrix protein cleavage, plays a significant role in medial degeneration. In the early stages of the disease, cells in the aortic media secrete proteoglycans, highly glycosylated proteins, as a response to fill in the voids left by the disruption of the matrix and cell disorganization. Proteoglycan accumulation progresses along with aneurysm formation (Figure 1.2).
Epidemiology of Thoracic Aortic Aneurysms and Dissections (TAAD)

Thoracic aortic aneurysms and dissections (TAAD) are characterized by a weakening, enlargement and subsequent rupture of the aortic wall, leading to the creation of a false lumen disturbing normal blood flow. TAAD has an incident rate of 10.4 per 100,000 people and cause nearly 15,000 deaths every year in the United States, affecting men and
women equally. Aneurysms are mostly asymptomatic and, if left untreated, it can lead to dissection, causing death in up to 75% of cases. Due to the high mortality rate, dissections are treated as medical emergencies and, in most cases, require surgical repair\textsuperscript{1}, \textsuperscript{7, 8}.

There are two methods used to classify aortic aneurysms and dissections (AAD) based on the location of the enlargement and tear in the wall. The first one, the DeBakey system, is divided into class I, II and III. Class I aneurysms originate at the ascending aorta and can expand through the descending thoracic aorta. Class II only compromises the integrity of the ascending aortic wall while class III is localized only in the descending aorta. Similarly, the Stanford system classifies AAD as Type A or Type B. Type A is a dissection originating in the ascending or aortic arch and can extend to the descending aorta, while type B only involves the descending aorta\textsuperscript{3}. (Figure 1.3)

Figure 1.3: Types Thoracic Aortic Aneurysms and Dissections. A. a. Illustration of the locations along the aorta where aneurysms commonly occur and b. the Stanford and DeBakey aortic dissection classification systems. Republished with permission of Annual
Once a dissection occurs, patients present with a wide array of symptoms including, but not limited to, significant changes in blood pressure - increases and decreases based on the location of the dissection - chest and upper back pains radiating to proximal extremities, numbness, diaphoresis, dyspnea, and loss of consciousness.\textsuperscript{9, 10} Dissections are frequently misdiagnosed, because many of these symptoms overlap with those of myocardial infarctions. When presenting with these symptoms, it is imperative to perform CT scans and transesophageal echocardiograms in patients to safely rule out a dissection. Even if dissections are promptly diagnosed, patients still might not survive the highly invasive procedure required for its repair. Therefore, it is of crucial importance to identify individuals at risk prior to the occurrence of a dissection. Standard protocols for aortic repair are constantly revised due to the genetic and phenotypic heterogeneity of the disease. When possible, a personalized medical approach is encouraged in these patients.\textsuperscript{11-13}

Genetic predisposition is the major risk factor for TAAD, followed by history of smoking, hypertension, high LDL levels and artherosclerosis. Inherited conditions include Marfan Syndrome, Vascular Ehlers-Danlos Syndrome, Loeys-Dietz and bicuspid aortic valves. Patients at risk should be monitored regularly, placed on antihypertensive
drugs (beta-blockers and/or calcium channel blockers) and are discouraged to engage in high intensity physical activities. Even in the absence of symptoms and genetic risk factors, smokers are recommended screening between 60-75 years of age\textsuperscript{14-16}. As we learn more about TAAD, the focus has shifted from repair to early detection, prevention and management of the disease\textsuperscript{15}.

**Genetic Basis of TAAD**

Initially, TAAD was associated with various connective tissue disorders, mainly Marfan Syndrome and vascular Ehlers Danlos Syndrome. Additional syndromes, such as Loeys-Dietz, were later identified by mutations in key players of the TGF-β pathway. These account for roughly 5% of all TAAD cases. More recently, mutations causing non-syndromic TAAD have been discovered. Inherited mutations, termed familial TAAD (fTAAD) account for 15% of cases, while the other 80% is attributed to sporadic occurrences (sTAAD)\textsuperscript{16-18}.

*Connective Tissue Disorders Causing Aortic Aneurysms and Dissections*

Marfan Syndrome is the most common connective tissue disorder associated with TAAD, affecting one out of five thousand people in the United States\textsuperscript{19, 20}. Diagnosis criteria for this disease include a wide range of abnormal skeletal features (long elastic limbs, tall stature, scoliosis, high arched palate, pectus excavatum or carinatum) along with ectopia lentis (lens dislocation) and aortic enlargement. Dominant mutations in FBN1, a gene coding for ECM protein fibrillin, were identified to be the cause of the disease\textsuperscript{3, 21, 22}.
Microfibrils connecting the SMCs to elastic fibers in the arterial wall are constituted mainly of fibrillin. In normal conditions, fibrillin sequesters TGF-B1, rendering it inactive. When there is a defect in the protein, TGF-β1 is released and signaling enhanced. Several studies have suggested increased TGF-B1 signaling in SMCs and myofibroblasts to be one of the driving forces behind aneurysm formation\textsuperscript{23-25}. Promising studies in Marfan mouse models have indicated that treatment with angiotensin II type I receptor (ATRI) blockers, commercially known as losartan and pravastatin, are able to prevent aneurysm formation. Further studies are required to establish the connection between ATRI and regulation of the TGF-B pathway\textsuperscript{23, 26}.

A less common disorder, vascular Ehlers Danlos Syndrome (vEDS) is caused by mutations in COL3A1, a gene encoding for procollagen\textsuperscript{3}. Patients present with impaired wound healing, clear translucent and elastic skin, increased joint flexibility and various other skeletal features. Arterial dissection is the only life-threatening symptom of the disease and, contrary to the other syndromes, dissection may occur without prior dilatation. Environmental risk factors such as smoking, extreme physical activity and hypertension, have been suggested as triggers of spontaneous dissections in these patients. Therefore, early detection of mutations in COL3A1 is imperative for proper disease management\textsuperscript{27-29}.

Even though Loeys-Dietz Syndrome is not caused by alterations in connective tissue proteins, it does present overlapping features with Marfan Syndrome. Mutations in TGFBR1 and TGFBR2, major players in the TGF-B pathway, have been linked to the disease. Patients suffering from this syndrome present with similar skeletal and cardiovascular abnormalities as those with Marfan Syndrome along with cleft palate,
thinned skin, and bifid uvula. The overlap of features is expected based both being caused by dysregulation of the TGF-B signaling pathway\textsuperscript{3, 30}.

\textit{Non-Syndromic TAAD}

Patients with early onset non-syndromic TAAD without prior family history are described as sporadic cases. Accounting for nearly 80\% of all incidents, these patients are probably victims of old age, environmental risk factors (i.e. obesity and smoking), rare genetic variants and/or \textit{de novo} mutations\textsuperscript{3, 31}. Because of the asymptomatic nature of aneurysms and lack of established methods for early detection, these patients suffer a greater risk of dissection.

Recently, focus has shifted to genetic risk factors known as “rare variants.” These non-synonymous variants do not segregate in a Mendelian manner but are more frequent than single nucleotide polymorphisms (SNPs) in patients with TAAD. It is believed these variants predispose patients to aortic disease but not without the presence of additional risk factors. Our lab has identified a missense R247C rare variant in MYH11, a gene coding for smooth muscle myosin, which is a key component of the contractile apparatus in SMCs. Studies suggest this alteration causes a 10-12 fold increased risk of aortic disease. Additionally, kinetics studies of the myosin motor showed a decrease in ATPase activity in R247C mutants when compared to controls. A knock-in mouse model of MYH11 R247C was created to further understand the role this variant plays in aortic pathogenesis. Although there seemed to be no significant enlargement of the aorta, \textit{ex vivo} contractility studies confirmed decreased force generation in mutant aortic rings. Explanted murine aortic SMCs were dedifferentiated and more proliferative when
compared to controls. To corroborate these findings *in vivo*, left carotid artery ligations were performed in both MYH11 R247C and WT mice. Analysis of the vascular injury showed increased medial thickening and enhanced neointimal formation. These results suggest that even in the absence of aortic disease, R247C mutations do have an impact in the contractile apparatus as well as in cellular response to injury\textsuperscript{31, 32}.

Genome-wide association studies have been used to identify genetic variants responsible for sTAAD. A total of 765 sporadic cases and 874 controls were compared identifying SNPs at a 15q21.1 locus associated with sTAAD. These SNPs fall into the region including the FBN1 gene. Interestingly, mutations in the FBN1 gene cause Marfan syndrome. The study suggests that variants in this region act via FBN1, leading to similar aortic pathogenesis between Marfan syndrome and sTAAD\textsuperscript{33}.

*Familial Thoracic Aortic Aneurysms and Dissections (fTAAD)*

Much of our effort has been focused on identifying single gene mutations inherited in a Mendelian manner responsible for familial cases of TAAD. Accounting for 15-20\% of all cases, these mutations are heterozygous, dominant negative and have variable expression with decreased penetrance\textsuperscript{3, 16, 34}. Earlier studies in syndromic TAAD have identified mutations in the TGF-B1 pathway as key culprits of aortic disease. Not surprisingly, genes in this pathway (TGFBR1/2, SMAD3, TGF-B1,) were also found to be the cause non-syndromic fTAAD\textsuperscript{35-39}. Using a similar approach, genes of the contractile SMC apparatus were screened in the patient population. So far, deficiencies in three contractile genes (MYH11, MLCK and ACTA2) have been identified to cause fTAAD\textsuperscript{3, 40, 41}.
**Mutations in TGF-B1 Pathway associated with TAAD**

The TGF-B superfamily includes three distinct ligands crucial for a range of cellular pathways. In SMCs, it is a key component in several processes including proliferation, migration and differentiation. When TGF-β1 binds to a heterodimer of TGF-β receptors, TGFBR1/2, these tyrosine kinase receptors phosphorylate each other and downstream targets. The canonical pathway involves the activation of the SMAD family in response to TGF-β signaling. SMAD2/3 are activated and subsequently bind to SMAD4, which translocates the complex to the nucleus, activating a large number of downstream target genes. Simultaneously, TGFBR1/2 can also activate a “non-canonical” pathway independent of SMAD signaling, having mitogen-associated protein kinases (MAPKs) as the primary targets\(^{42,43}\).

In normal cells, TGF-β signaling induces contractile gene expression, mainly ACTA2 and MYH11, driving SMC differentiation. In patients with TGFBR1/2 mutation, isolated SMCs *in vitro* are characterized by poor contractile filament formation and appear to be in a constant de-differentiated state. Alterations in TGFBR2 appear to have no effect in the canonical signaling, but there is a clear disruption of the MAPK pathway\(^{39,44}\). Clearly, *in vitro studies* indicate a loss of function in patients with TGFBR1/2 mutations. Paradoxically, immunohistochemical analysis of diseased aortas shows an increase in phosphorylated SMAD2/3. The contradicting results suggest that mutations in these receptors do cause a loss of function, but *in vivo*, through a yet
unknown mechanism, TGF-β signaling is increased leading to increased SMAD activation. Therefore, some scholars consider these alterations as “gain-of-function” mutations\textsuperscript{39,45}.

\textit{Contractile Gene Mutations}

The contractile apparatus is a paramount constituent of SMC function. Therefore, a disruption of any of its major components has the ability to hamper SMC contractility and subsequently weaken the aortic wall. Thus far, mutations have been identified in three contractile genes: MLCK, MYH11 and \textit{ACTA2}\textsuperscript{3,41}. We will focus on the latter two.

MYH11 and \textit{ACTA2} code for smooth muscle myosin and α-actin, respectively. During contraction, calcium-activated phosphorylation of myosin filaments causes binding to actin through rapidly cycling cross-bridges, leading to contraction and force generation. Therefore, any disruption in the cyclic interaction between these two molecules can lead to detrimental effects in arterial function. Mutations in MYH11 have been associated with fTAAD and vascular occlusive diseases along with patent ductus arteriosis (PDA), where the fetal connection between the aorta and pulmonary arteries remains open after birth. Analyses of patient’s aortas show classic medial degeneration including proteoglycan accumulation, SMC hyperplasia and disarray, and elastic fiber deterioration\textsuperscript{40,46}. Occlusion of the vasa vasorum in the aortic wall suggests a proliferative pathology caused by SMC hyperplasia in smaller vessels\textsuperscript{47}.

Mutations in \textit{ACTA2} are the most common cause of fTAAD, accounting for 13\% of all familial cases\textsuperscript{47,48}. Patients with \textit{ACTA2} mutations present with similar aortic and vascular occlusive pathology (strokes), but no association with PDA was found. Unique to these families, is the segregation of coronary artery disease (CAD), livedo reticularis
(occlusion of dermal vessels), early-onset stroke and Moyamoya-like disease. A total 16 mutations have been identified in ACTA2, each with distinct penetrance and vascular occlusive presentations. For example, 30% of individuals with R118Q mutations were associated with early on-set CAD, but low frequency of strokes. In contrast, patients with R258C/H had a 6.51-fold increase risk of stroke when compared to other ACTA2 mutations\textsuperscript{47,49}.

**Mutations in ACTA2 Cause Global Vascular Dysfunction.**

As previously described, mutations in ACTA2 cause the most severe aortic and vascular occlusive phenotypes in patients with fTAAD. For that reason, our lab has focused on identifying the molecular mechanisms of vascular pathogenesis using patient samples, *in vitro* analysis and murine disease models of ACTA2 mutations.
In individuals with ACTA2 mutations, we see classic markers of aortic disease. At the molecular level, explanted SMCs from affected aortas show different degrees of disruption of α-actin polymerization based on the location of the mutation (Figure 1.4A). To study a wider array of mutations, dermal fibroblasts were explanted from ACTA2 mutation carriers. Even after treatment with TGF-B1, which induces expression of contractile genes and differentiation, these cells were unable to produce healthy actin.
filaments. Proliferation assays were then performed to corroborate the proliferative phenotype observed in patient aortas. As expected, both SMC and dermal fibroblasts showed an increased proliferative response when compared to controls. (Figure 1.4B).49

These observations suggest that in smaller contractile vessels (i.e. distal end of internal carotids), disruption of actin filaments leads to increased SMC proliferation in vivo as a compensatory mechanism; supporting our hypothesis of SMC hyperplasia as the main cause of the stenotic lesions seen in patients with livedo reticularis, CAD, stroke and/or MMD48,49.

ACTA2 and Moyamoya Disease

Moyamoya disease (MMD) affects 1 in 119,000 people in the United States alone. Diagnosis is based on cerebrovascular imaging of bilateral stenosis of the distal internal carotid arteries with an abnormal vascular network surrounding the lesions50. Occlusion of the ICAs and weakness of the collateral circulation can lead to ischemic and hemorrhagic strokes as early as the first decade of life51. There are two different post-ischemic surgical methods to treat for MMD: direct and indirect revascularization. The most common direct procedure is a superficial temporal artery to middle cerebral artery anastomosis which induces intracranial neovascularization and helps prevent future ischemic attacks. Indirect revascularization methods use richly vascularized tissue and attach it to areas of lesion in the cortex, such as inversion of the dura (encephalodurosyangiosis). Proponents of this method claim low post-operative morbidity, mortality and decreased incidences of recurrent strokes52-54.
Given the severity of MMD, much emphasis has been placed on finding the genetic contributors of the disease. MMD has been associated with numerous genetic disorders including: Marfan Syndrome, neurofibromatosis, Ehler-Danlos Syndrome and Down Syndrome among others. Non-syndromic familial cases have been identified in patients with mutations in ACTA2, RNF213 and Xq28 deletions.

Initial screenings identified two mutations in ACTA2 at the SM α-actin SD4 domain, R258C/H and R212Q, responsible for TAAD, stroke and MMD. Recently, a third, de novo mutation in ACTA2, R179H, was associated with an early onset severe phenotype caused by global smooth muscle dysfunction. Patients with this mutation present with persistent ductus arteriosis, congenital mydriasis, bladder and gastrointestinal deficiencies, and pulmonary hypertension. Interestingly, these patients have a Moyamoya-like cerebrovascular presentation that includes occlusion of the distal ICAs and abnormally straight course intracranial arteries, but without the typical “moyamoya” collaterals. Stenosis in ICAs occurs at the level in which the arterial wall changes from elastic to muscular, increasing the susceptibility of this area to changes in vascular pressure and fluid dynamics leading to proliferative vascular occlusive lesions. Interestingly, similar pathology is thought to cause stroke in other patients with ACTA2 mutations that did not meet the criteria for MMD.

Acta2 Mouse Models

ACTA2 has become a prime candidate gene to study vascular diseases because of its early phenotypic presentation and widespread SMC dysfunction. Challenges arise when studying the vascular pathogenesis of ACTA2 mutations using patient samples.
First, patient samples are not readily accessible. Also, these samples are only obtained in the end-stages of disease, rendering it impossible to observe the causative mechanisms of the disease. For these reasons, we decided to look at vascular pathogenesis in two mouse models, Acta-/- and a transgenic Acta2 R258C model. We started working on developing an R179H knock-in mouse model as well, the most severe ACTA2 mutation, but it has been technically challenging.

Analysis of the Acta2 null mouse model determined that α-actin is not necessary for cardiovascular development and has minor effects in the digestive and reproductive systems. Even though SM α-actin is the most abundant protein in mature SMCs, increased levels of skeletal α-actin, usually not expressed in SMCs, might explain the viability of this model. However, impaired vascular contractility and tone and decreased blood pressure were observed in these mice. Furthermore, evaluation of Acta2-/- aortas found an abnormal aortic wall structure starting at one month of age. The average number of elastic lamellae increases from 7.5, in WT aortas, to 11, in null aortas. Quantitation of cells in the aortic wall showed increased density of SMCs in the Acta-/- mice (Figure 1.5A). The increased number of elastic fibers and cell density suggests a compensatory mechanism for the loss in contractility. By three months of age, significant enlargement of the ascending aorta is observed in the absence of Acta2 and by 12 months of age the wall presents with the classic markers of aortic disease. Medial thickening with increased expression of Mmp2 leading to elastic fiber disruption and proteoglycan accumulation was observed (Figure 1.5B). These results mirror the pathology present in the aortic wall of patients with ACTA2 mutations.
A transgenic mouse model of R258C was generated in the Milewicz lab to further understand and characterize one of the most severe dominant negative mutations in Acta2. A cDNA construct of the Acta2 gene, including the R258C mutation in exon 7 and the endogenous Acta2 promoter, was generated and inserted into a C57Bl/6 background. In parallel, a second transgenic line using the same construct was generated to assure that

**Figure 1.5: Aortic Pathology in the Acta2<sup>-/-</sup> Mice** - A. Thickening of the media, increased number and fragmentation of the elastic lamellae are observed in Movat stains of aortic cross-sections. B. Acta2<sup>-/-</sup> mice present with enlarged aortas at 2 months of age, increased SMC density at birth and thicker media after 12 months of age<sup>60</sup>.
the observed phenotype was caused by the mutation itself and not by the site of insertion. Confirmation of the mutant gene transcription was achieved through quantitative real-time PCR (Figure 1.6A). Construct was made by Dr. Kuang in the Milewicz Lab.

Furthermore, confirmation of protein expression was confirmed by isoelectric focus (IEF) blot using aortic protein lysates.\textsuperscript{60} (Figure 1.6B).

**Figure 1.6: Generation of the Acta\textsuperscript{2R258C} Transgenic Mouse Model - A. Construct design for mutant transgene, containing a wildtype $\alpha$-actin promoter up to intron $^1$. B. IEF blot confirming mutant (R258C) $\alpha$-actin expression in the aorta.\textsuperscript{66}**

The focus of the thesis will be to characterize the dominant negative effect of the Acta\textsuperscript{2} R258C mutation in the transgenic mouse model and study the cerebral vascular occlusive pathogenesis in the Acta\textsuperscript{2} null model. Furthermore, we believe that decreasing the wildtype:mutant actin ratio increases phenotypic presentation of the disease.
CHAPTER 2

Materials and Methods
Mouse Breeding Scheme

Pure C57Bl/6 background male Acta2\(^{-/-}\) mice were crossed with Acta2\(^{+/+}\) R258C female mice. The resulting pups were Acta2\(^{+/-}\) and Acta2\(^{+/-}\) R258C. The resulting Acta2\(^{+/-}\) R258C females were mated with Acta2\(^{-/-}\) males (Figure 3.2). No Acta2\(^{-/-}\) R258C mice reached weaning age. (Table 1 Supplement)

Murine Echocardiography

Echocardiography of the aortic root and ascending aorta were performed on gender and age matched Acta2\(^{+/+}\) R258C, Acta2\(^{+/-}\) R258C, Acta2\(^{-/-}\) and wildtype control mice to determine aortic diameter. We use the Vevo 770 40MHz ultrasonic probe (VisualSonics Inc., Toronto, Ontario, Canada) to follow aortic diameter at 1 month, 3 months, 6 month and 1 year of age. Measurements of the aortic root and ascending aorta were obtained in late diastole at the parasternal long-axis view. Analysis was performed using the Vevo 770 Software.

Aortic histology and analysis

Mice were anesthetized using 0.7cc of 2.5% avertin. The chest cavity was then exposed and a 27\(^{1/2}\) gauge needle inserted into the left ventricle. Using the Masterflex C/L Pump (Cole-Palmer, 625 East Bunker Court, Vernon Hills, IL 60061) to control for rate (5ml/min), the mouse was perfused using 10ml Dulbecco’s PBS, followed by 10ml of 10% neutral buffered formalin. The lungs were extracted and the aorta was carefully dissected in situ. Once clean, the aorta was explanted, cut at the root and arch distal to the left carotid (2mm of each carotid were kept for orientation purposes), and stored in 10%
formalin overnight. The tissue was then subjected to a series of dehydrating washes (20 minutes each) using 50%, 70%, 85%, 95%, 100% ethanol and 100% histoclear. Aortas were then incubated in melted paraffin at 60°C for 1 hour before being embedded in paraffin blocks.

Sectioning of the paraffin blocks was done at 5um per section using the Olympus 4055 Microtome (Olympus America Inc. Center Valley, PA 18034). Standard protocols were used for MOVAT pentachrome and hematoxylin and eosin staining. Images were taken using Olympus DP-71 Digital Camera and BX-60 Microscope (Olympus America Inc. Center Valley, PA 18034) at 40x, 100x, 200x and 400x magnification. All histological data collection and analysis was done using Image J software (NIH). The color threshold tool was used to count pixels for elastin (black), proteoglycans (blue) and SMCs (red). Quantification of the elastin breaks were done by visual inspection.

**Western blot assays**

Mice were anesthetized using 0.7mm3 of 2.5% avertin. The chest cavity was exposed, the thoracic organs extracted and the aorta was carefully dissected. Once explanted, the aorta was cleaned in Dulbecco’s PBS and snap frozen in liquid nitrogen. The tissue was homogenized using a Fisherbrand handheld homogenizer in RIPA buffer, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). 5 μg of protein isolate for each sample were separated by SDS-PAGE on Tris-HCL gel (Bio-Rad, Hercules, CA) and transferred on polyvinylidene difluoride membranes at 80V for 90 minutes. (Milliford, Bedford, MA).
Immunoblots were achieved using primary antibody (incubated 3 hours at 25°C). The membrane was then incubated for 1 hour in the corresponding horseradish peroxidase secondary antibody (Jackson Laboratories, West Grove, PA). Chemiluminescence (GE Healthcare, Piscataway, NJ) was used to visualize immunoblots.

**Contractility assays**

Fourteen week old mice were anesthetized using 2.5% avertin. Aortas were dissected (see method above), cut into 3mm rings, weighed and mounted on wire triangles. The tissue was placed on an isometric force apparatus, equilibrated and stretched to 1.8g. After the initial stretch, the tissue was allowed to recover for 45 minutes. A buffer imitating physiological conditions (118.5mM NaCl, 4.75mM KCl, 1.2mM MgSO4, 1.2mM KH2PO4, 24.9mM NaHCO3, 1.6mM CaCl2, 10.0mM D-glucose, pre-gassed with 95% O2/5% CO2 at 37°C) was used during the recovery periods. Aortic rings were subjected to four rounds of stimulation with KCl (90mM), 5 minutes per round. A 5-minute recovery period was allowed after each round. After the final round, a second 45-minute recovery period was allowed and then a final stimulation with 10mM phenylephrine for 5 minutes was performed.

**Carotid artery ligation**

Left carotid artery ligation flow-cessation model was performed on 8-week-old WT, Acta2+/−, Acta2−/−, Acta2+/+ R258C and Acta2+/− R258C mice. Mice were anesthetized using 2% isoflurane through nosecone inhalation. The left common carotid artery was ligated 1-2mm from its bifurcation using 5-0 silk suture. Animal survival rate was > 95%.
Mice were sacrificed 21 days post ligation and both carotids harvested. The entire length of the left and right carotid arteries was sectioned (at 5um per section) and examined for identification of the site with most significant thrombus formation and stenosis. Carotid artery tissues were stained using standard protocols. Specimens were imaged and photographed using an Olympus microscope. For morphometric analyses, images of H&E stained cross-sections of injured and control arteries were analyzed using Image J (NIH). Perimeters of the lumen, internal elastic lamina and external elastic lamina were obtained by tracing the contours on digitized images using ImageJ software. Neointimal/thrombus and medial areas were calculated. Percent lumen stenosis was calculated using the formula: thrombus area/lumen area × 100.

**Irradiation and Bone Marrow Transplant**

At 6 weeks of age, wildtype and Acta2\(^{-/-}\) mice were irradiated using the RS2000 Biological Research Irradiator (RAD Source Technologies, Suwanee, GA). Mice were irradiated in two separate sessions, 3 hours apart at 500 rads each, for a total of 1000 rads. Five million bone marrow cells from C57Bl/6J UBC-GFP (Jackson Laboratory, Bar Harbor, ME) were transplanted through intraocular injection using a 28\(\frac{1}{2}\)-gauge needle 4 hours post irradiation. Mice were placed in a sterile room and fed sterile food and water. Baytril (0.5mg/ml) was administrated in the drinking water to prevent bacterial infections. Mice were monitored twice daily, weighed and assessed for irradiation poisoning for 14 days. Three weeks post irradiation, carotid ligation was performed and carotids harvested after 21 days for analysis (as indicated above).
Bone Marrow Stem Cell Isolation

C57Bl/6J UBC-GFP (Jackson Laboratory, Bar Harbor, ME) male mice 8-10 weeks of age were anesthetized using 0.7mm3 of 2.5% Avertin. The mice were sacrificed by cervical dislocation and the lower limbs removed at the hip joint. The femur and tibia were carefully dissected removing skin and muscle tissue and placed in a culture dish with RPMI-1640. The ends of the bones were cut and bone marrow flushed with a 27-gauge needle containing 10mls of RPMI-1640. The media with the bone marrow was then passed through a 22-gauge needle to break tissue clumps. Cells were separated from the remaining solution using BD-40um strainers. Cells were then centrifuged at 100g for 10 minutes and rediluted in PBS with a final concentration of 50 million cells/ml.

Brain Imaging

All in vivo procedures were approved by the Baylor College of Medicine IACUC and Imaging was provided by Dr. Annapragadas Lab. A novel liposomal CT contrast agent (~120nm particle size, PEG surface coating, total iodine ~150 mg/ml, dose~6uL/g) was injected via tail vein into test animals. Animals were anesthetized by isoflurane inhalation and maintained on 0.2-0.4% isoflurane delivered by face-cone. A low resolution pre-contrast scan was conducted to verify and record the presence of any radiopaque structures. The contrast agent was then injected (~8µL/g) via tail vein. For low resolution (38µ isotropic voxels) the following parameters were used: 60kVp, 400µA, 360 projections, 850ms exposure. For high resolution (19µ isotropic voxels) the
following parameters were used: 60kVp, 500µA, 1440 projections, 850ms exposure (Figure 2.1).

Figure 2.1 Diagram of the Circle of Willis. (Left) Diagram showing all vessels analyzed around and within the Circle of Willis. (Right) Dimensional Parameters of the Circle of Willis. With permission from60.

Automated scripts were written in Matlab (Version 7, Mathworks Inc., Cambridge MA) for these calculations. Vessels were segmented using the intensity-gradient-maximum technique to identify the vessel margin in the radial direction. Centroids of the vessel boundary shape in each section were connected to approximate the centerline of the vessel, from which all distance and area measurements were made. The following data was collected:
1. Vessel diameters: were approximated as the diameter of the circle of equal area to each section, from which a mean diameter was calculated.

2. Vessel (Arc) length: approximated as the sum of the linear distances between centroids of each section.

3. Vessel curvature was measured by 3 parameters: (a) The Cauchy curvature for a concave segment was estimated by drawing normals to the centerline curve at its end points, and calculating the radius of curvature by calculating the intersection of the normals. (b) The Arc-to-length ratio of a segment and (c) the Inflexion count, measuring the number of locations where the concavity of the vessel changed direction. Thus, the Cauchy curvature assumed the segment was singly concave, the arc-to-length ratio permitted multiple concavities, and the Inflection count measured the number of concavities. Since some vessels exhibited straightening at the ends of the segment while curvature persisted at the center, we defined the “middle curvature” as the Cauchy curvature of the central third of the segment, and reported it as a separate parameter.

4. The intercarotid distance and the width of the Circle of Willis were measured as the Euclidean distance between the proximal ends and the distal ends of the internal carotids, respectively.

5. The area of the Circle of Willis was measured by the area of its projection on the coronal plane. The Circle of Willis was then divided into an upper-half, and a lower-half, using the line that connected the proximal ends of the internal carotids as the divider. The areas of the two halves were measured in the coronal plane.
6. Basal Triangle Area: An additional measure of the area was based on the area of the triangle formed by the distal end of the basilar and the proximal ends of the two internal carotids, in its own plane.

7. Narrowing Count: As an indication of the number of incidences of a narrowed vessel, the fraction of segments with diameter lower than 85% of the mean diameter was calculated.

8. Mean circularity: In the cross-sectional plane normal to the centerline, the circularity was calculated as the ratio between the contour area and area with a circle with radius R, where R is calculated based on circumference of contour. The mean was calculated over the set of individual circularities.

9. Mean aspect ratio: The aspect-ratio was defined as the ratio of first and second eigenvalues calculated on the contour at a point on centerline. The mean was calculated for a set of individual aspect ratios.

Cerebrovascular histology and analysis

At 16 weeks, female WT, Acta2+/-, Acta2-/- mice were anesthesized using 0.7mm³ of 2.5% avertin. The chest cavity was then exposed and a 27½ gauge needle inserted into the left ventricle. Using the Masterflex C/L Pump (Cole-Palmer, 625 East Bunker Court, Vernon Hills, IL 60061) to control for rate (5ml/min), the mouse was perfused using 20ml Dulbecco’s PBS, followed by 20ml of 10% neutral buffered formalin. Following perfusion, the head was severed, the brain carefully extracted and stored in 10% formalin at 4°C overnight. The tissue was then subjected to a series of dehydrating washes (45 minutes each) using 50%, 70%, 85%, 95%, 100% ethanol, 50/50
histoclear ethanol mixture, 100% histoclear. Brains were then incubated in a melted mixture of 50/50 histoclear and paraffin at 60°C for 30 and finally in pure paraffin for 1.5 hours before being embedded in paraffin blocks.

Brain sections (5µm) were collected every 120µm using the Olympus 4055 Microtome (Olympus America Inc. 3500 Corporate Pkwy, Center Valley, PA 18034). Standard protocol was used hematoxylin and eosin staining. Images were taken using Olympus DP-71 Digital Camera and BX-60 Microscope (Olympus America Inc. Center Valley, PA 18034) at increasing magnifications - 40x, 100x, 200x and 400x. Vessel measurements obtained with ImageJ v1.47 (NIH, Bethesda, MD) were used to calculate wall-to-lumen ratio (WLR=wall thickness/(diameter-2 x wall thickness)) and percentage wall thickness (%TH=[(2 x average medial thickness)/external diameter] x 100) 62 63, 64. Vessels in all regions of the brain of the mice were divided into groups by the lengths of their short diameter (100-200µm and >200µm). Smaller arteries in the cerebral parenchyma (diameter < 100µm) could not be accurately assessed using %TH and WLR and were classified as “normal” or “thickened”.

To assess SMC hypertrophy of the medial layer, measurements were made on cross sections of arteries (Diameter > 100 µm) for the WT and Acta2-/- mice. Density of cells in the media was calculated by counting the SMC nuclei in the media and dividing by the vessel wall area.

**Statistical analysis**

For CT based vascular morphometry the Kolmogorov-Smirnov test was used to assess normality and all distributions failed the test. Therefore, the Kruskal-Wallis test was used to compare groups. Statistical significance in inter-group differences was
assessed at the 95% confidence level (p<0.05). All statistical calculations were performed in Matlab (Version 7, Mathworks Inc., Cambridge MA).

Data from pathological measurements were tested for normality using the Shapiro-Wilk test. When data were normal, one-way analysis of variance (ANOVA) was performed, and pairwise multiple comparisons were performed using Bonferroni multiple comparison procedures. When data failed the equal variance test, Kruskal–Wallis ANOVA on Ranks was performed and pairwise multiple comparisons were made using the Dunn’s method. Cell density data were both analyzed non-parametrically by the Mann-Whitney U test, followed by independent t test using SPSS 22.0.0 (Statistical Package for the Social Sciences, Chicago, IL). Analysis provided by Dr. Annapragada’s Laboratory, Texas Children’s Hospital and Baylor College of Medicine.
CHAPTER 3

Characterization of the Acta$_2^{R258C}$ Mouse Model Aortic Phenotype
Introduction

Analysis of the Acta2-/- mouse model gave us an insight into the effects of the loss of α-actin in the vascular system. Mutation in patients with ACTA2 mutations are expected to have a dominant negative effect; therefore, we predict that a mouse model harboring an Acta2 mutation will have similar vascular pathogenesis. As a result, we chose to study the vascular effects of ACTA2 R258C mutation. Patient families that were originally identified to carry the ACTA2 R258C alteration, showed that 70% of mutation carriers developed TAAD and 50% of cases suffered early onset strokes\(^6\) (Figure 3.1)

Figure 3.1: ACTA2 R258C mutation causes TAAD and Stroke. Reprinted from The American Journal of Human Genetics, Dong-Chuan Guo, Christina L. Papke, Van Tran-Fadulu, Ellen S. Regalado, Nili Avidan, Ralph Jay Johnson, Dong H. Kim,

Due to the severity of the disease, a transgenic mouse model with an Acta2 R258C mutation was generated to deepen the understanding of its role in aortic pathogenesis, both in vitro and in vivo\textsuperscript{60}. Furthermore, we hypothesize that a decrease of endogenous Acta2 expression would increase the severity of the phenotype in these mice. Therefore, we developed a breeding scheme by crossing our transgenic mouse with the Acta2\textsuperscript{−/−} null model, that yielded 4 genotypes Acta2\textsuperscript{+/−}, Acta2\textsuperscript{+/−} R258C, Acta2\textsuperscript{+/-} and Acta2\textsuperscript{+/-} R258C. (Figure 3.2A). Furthermore, we used aortic explants from Acta2\textsuperscript{−/−} R258C embryos – these mice were not viable postnatally – to test the expression of mutant smooth muscle a-actin (Figure 3.2B).
Figure 3.2 Acta2<sup>+/−</sup> R258C Mice Breeding Scheme - A. Breeding scheme to decrease endogenous Acta2 expression. B. Immunoblot showing α-actin expression in aortic tissue from Acta2<sup>−/−</sup> R258C TG mice.

Aortic lysates from all four genotypes were used for in vitro studies. cDNA from SMC generated for Quantitative PCR show a significant increase in all smooth muscle contractile gene expression in Acta2<sup>+/+</sup> R258C cells when compared to wild type. Similarly, Acta2<sup>+/−</sup> R258C show significant increases in calponin 1 (Cnn1) and MYH11, but no difference in Acta2 and SM22a expression (Figure 3.3A). Acta2<sup>+/−</sup> cells did not seem to have differences in contractile gene expression when compared to controls (Figure 3.3A). Analysis of contractile protein expression showed similar increases in Acta2<sup>+/+</sup> R258C. Meanwhile, both α-actin and SM22a protein expression was decreased in Acta2<sup>+/−</sup> R258C and Acta2<sup>+/−</sup> cells when compared to wild type controls, even though there were no
changes observed in their respective RNA expression. These results suggest the decrease of endogenous expression of Acta2 alter the pathways involved in pathogenesis\(^6\). (Figure 3.3B)

**Figure 3.3 Contractile Gene and Protein Expression in Wildtype (WT), Acta2\(^{+/+}\) R258C \(^T\)G, Acta2\(^{+/-}\) and Acta2\(^{-/-}\) R258C \(^T\)G SMCs. A.** Quantitative real-time PCR data for the expression of the contractile genes Acta2, Myh11, Cnn1 and SM22\(\alpha\) in all four genotypes. Wildtype is set to 1 for each assay and gene expression in the other genotypes is presented as a fold difference from the wildtype. All results are normalized to Gapdh. * p<0.05, ** p<0.01. Error bars are ±standard deviation (s.d.) B. Contractile protein expression, as shown by Western blot. SM-MHC = smooth muscle myosin heavy chain.
Formation and stability of α-actin filament in SMC showed interesting differences between all four genotypes. When treated with TGF-B1, immunofluorescence studies showed Acta2+/+ R258C cells were able to form actin filaments, while Acta2+/- R258C showed little filament formation and large pools of unpolymerized actin (Figure 3.4A). Actin filament stability was then determined using an F/G actin assay. Results showed that both transgenic cell lines lacked pelleted actin filaments. This suggests that, even though Acta2+/+ R258C cells are able to produce actin filaments, they are not as stable as those in wildtype cells and are disrupted by the stress and agitation required to perform the assay60. (Figure 3.4B) Furthermore, assays done in purified wildtype and R258C actin showed that the mutant actin had decreased motility and increased flexural rigidity, the force required to bend a non-rigid structure to a certain curvature, which indicates the presence of frail filaments65. Together, these results suggest a significant impact of R258C mutation in filament formation and stability in vitro, leading to the hypothesis that these changes might lead to aortic pathogenesis in vivo.

SM α-actin = smooth muscle-specific α-actin. With permission60.
Figure 3.4 SM α-actin Polymerization 72 Hours post TGF-β1 Treatment *in vitro* - A.

Immunofluorescent staining after 72 hours of TGF-β1 treatment for α-SMA (green) and total filamentous actin (phalloidin, red). Nuclei were stained with DAPI (blue). B. F/G actin assay after 72 hours of TGF-β1 treatment. Positive ctrl= wildtype cells treated with phalloidin. U=unpolymerized. P=polymerized. With permission®
Results

Aortic Pathology

Aortic contractility assays were performed on $\text{Acta2}^{+/+} \text{ R258C}$ and wildtype mice in collaboration with the Stull lab at University of Texas Southwestern Medical Center. Aortas from three-month-old male mice were dissected and aortic rings of the thoracic ascending, thoracic descending and abdominal aortas were mounted on an isometric force device. $\text{Acta2}^{+/+} \text{ R258C}$ aortas showed significantly lower contractile force generation across the three sections compared to wildtype mice ($P<0.05$). Therefore, it is safe to assume that the unstable $\alpha$-actin filaments observed in $\text{Acta2}^{+/+} \text{ R258C}$ SMCs disrupt the proper functioning of the contractile apparatus in the aortic tissue (Figure 3.5A).
Aortic histology and echocardiograms were used to assess disease progression in all four genotypes (Acta2\textsuperscript{+/+}, Acta2\textsuperscript{+/-}, Acta2\textsuperscript{+/+} R258C and Acta2\textsuperscript{+/-} R258C) at 1 month, 3 months, 6 months and 1 year of age (Figure 3.5B). At the first month of age, all mice showed normal lumen diameter and no evidence of elastic fiber disruption or proteoglycan deposition (Figure 3.6A). A few abnormalities were observed in the aortic wall structure, characterized by increased cell density in Acta2\textsuperscript{+/+} R258C and Acta2\textsuperscript{+/-} R258C and increased numbers of elastic lamellar layers in Acta2\textsuperscript{+/-} R258C when compared with both Acta2\textsuperscript{+/-} and wild-type aortas. Acta2\textsuperscript{+/-} R258C aortas had an average of 10.2 elastic lamellae compared with 9.2 and 9.4 lamellae in wildtype and Acta2\textsuperscript{+/-}, respectively (P<0.05) (Figure 3.6B). Quantitation of SMC cells per mm\textsuperscript{2} in the aortic media, show a significant increase in cell density with 2,122 cells/mm\textsuperscript{2} and 1,758 in Acta2\textsuperscript{+/-} R258C and Acta2\textsuperscript{+/+} R258C aortas, respectively. In contrast, cell density in wildtype aortas was only 1,431 cells/mm\textsuperscript{2} (P<0.01). No significant difference between wildtype and Acta2\textsuperscript{+/-} was observed (Figure 3.6C). Contrary to the recorded medial thickening in the Acta2 null model, we measured decreased medial area in both Acta2\textsuperscript{+/-} R258C and Acta2\textsuperscript{+/+} R258C aortas during the first month of age, but no difference at 3, 6 and 12 months (Figure 3.6D).
Positive staining of calponin 1, a SMC-specific contractile protein, confirmed that these were indeed SMCs. There was no evidence of proteoglycan deposition or elastic fiber disruption, two classic markers of aortic disease, at this age.

**Figure 3.6 Aortic Pathology in Acta2<sup>R258C</sup> Mice - A.** Aortic cross-sections of one month old mice. Top panels (H&E stain) and bottom panels (Movat Stain) of the aortic wall show no evidence of elastin disruption and proteoglycan accumulation. Increased
number of elastic lamellae can be appreciated in the Acta2+/R258C mice. B. Quantification of elastic lamellae at month of age confirms a significant increase in the number of lamellae in Acta2+/R258C aortas. C. Histological analysis of the progression of cell density during the first year of life in mice. After the first month, both Acta2+/+R258C and Acta2+/−R258C aortas increased cell density compared to WT, but the difference dissipates at 3 months of age in Acta2+/+R258C and after 6 months in Acta2+/−R258C mice. Suggesting excessive SMC proliferation during development but not after birth, hence the growth of existing cells decreases cell density over time. D. Histological quantification of aortic medial area during the first year of life. The media is significantly smaller only at the first month time-point in both transgenic genotypes. Starting at 3 months of age, no significant differences in the area of the aortic media are observed. E. Aortic diameters during the first year of life measured in vivo by echocardiogram. Mean aortic enlargement is observed in Acta2+/+R258C mice starting at 3 months of age compared to WT. At 6 and 12 months of age, Acta2+/+R258C aortas are significantly larger than both WT and Acta2+/− aortas. No significance was observed in Acta2+/− or Acta2+/+R258C mice, which indicates that decreased endogenous Acta2 expression does have a deleterious effect in aortic pathology.

Interestingly, by 3 months of age, only Acta2+/−R258C mice had enlarged thoracic aortas by echocardiogram analysis when compared to both wildtype and Acta2+/− aortas (P<0.05) but no significant difference was observed with Acta2+/+R258C mice. The average aortic diameter during late diastole of Acta2+/+R258C was 1.51 mm compared to 1.38 mm, 1.45 mm and 1.46 mm in wildtype, Acta2+/+ R258C and Acta2+/−, respectively.
The lack of a significant difference between $\text{Acta2}^{+/+}$ R258C and $\text{Acta2}^{+/+}$ R258C aortas is due to the high variability of aortic diameter observed within the former cohort most likely due to epigenetic differences in the expression of the transgene. The significant increase in cell density observed at 1 month of age was not evident in the $\text{Acta2}^{+/+}$ R258C but still present in the $\text{Acta2}^{+/+}$ R258C aortas by three months. Quantification of the elastic lamellae showed no difference from the previous time point. Again, elastic fragmentation and proteoglycan deposition was not evident in these aortas.

Aortic tissues from $\text{Acta2}^{+/+}$ R258C mice begin to show signs of medial degeneration by 6 months of age, as the elastic lamina is thinner in these mice when compared with both wildtype and $\text{Acta2}^{+/+}$. This can be observed by a decrease in the percent medial area composed by the elastic fibers; these fibers account for 43% of the total medial area in $\text{Acta2}^{+/+}$ R258C aortas compared to 47% and 48% in both wildtype and $\text{Acta2}^{+/+}$ aortas (P<0.05), respectively (Figure 3.7A). The fact that there is not difference in total medial area suggests that, indeed, the difference is due to thinning of the elastic fibers and not an increase in cellular area. It is important to notice that no changes in the number of elastin fibers occurs after birth. Still, no significance in proteoglycan accumulation and elastin breaks is observed in aortic histology. Interestingly, the increased cell density persists between $\text{Acta2}^{+/+}$ R258C and wildtype aortas (P<0.05) even though the medial areas show no significant difference (Figure 3.6C). No difference in cell density is observed between the other genotypes. By this time point, we observe a greater increase in aortic diameter of $\text{Acta2}^{+/+}$ R258C mice (P<0.01) suggesting there is, indeed, a dose dependent effect seen by the decrease in endogenous $\text{Acta2}$ (Figure 3.6E).
Analysis of elastic fibers integrity at 12 months of age shows an increase in elastin breaks in $Acta2^{+/-}\, R258C$ and $Acta2^{+/-}\, R258C$ aortas when compared to wildtype aortas (P<0.05 and P<0.01). An increase in elastin breaks was only significant in $Acta2^{+/-}\, R258C$ when compared to $Acta2^{+/-}$ aortas (P<0.05) (Figure 3.7B). Thinning of the elastic fibers was also evident at this time. Another important marker of aortic disease, increased deposition of proteoglycans in the aortic media, was observed in the $Acta2^{+/-}\, R258C$ aortic by the first year of age when compared to both wildtype and $Acta2^{+/-}$ mice. There smaller pools of proteoglycan accumulation in the $Acta2^{+/-}\, R258C$ aortic media, but not enough to reach a significant difference (Figure 3.7C). We should point out that a pathogenic marker in the $Acta2^{-/-}$ model, medial thickening, was not observed in our mutant mice (Figure 3.6D). By now, cell density has leveled between the 4 genotypes, suggesting that the hyperplastic remodeling observed during development ceases in the early stages of mice’s life and increases in medial area are mainly due to cell growth as opposed to proliferation.
Figure 3.7 Elastic Fiber Integrity in the Tunic Media at 12 months of age – A. Elastic fiber thinning and degeneration is evident in Acta2+/− R258C mice compared to all other genotype is demonstrated by a decrease in the percent area of the media comprised by elastin fibers in these mice (while the total medial area is statistically insignificant across all four genotypes). B. Both, Acta2+/+ R258C and Acta2+/− R258C have more elastic fiber breaks, and hence more severe medial degeneration, when compared to WT. Only Acta2+/− R258C mice have significantly more breaks than Acta2+/− mice, again supporting our theory that decreased endogenous Acta2 leads to increased pathologic severity. C. Movat stains of aortic cross sections elucidate three clear markers of aortic disease in the medial wall in Acta2+/− R258C mice: Elastic fiber thinning, elastic fiber breaks (yellow arrows) and proteoglycan accumulation (stains blue, focal accumulation depicted by black arrows)
Analysis of the aortic diameter by echocardiography shows interesting results in our last time point. As expected, at 12 months Acta2\textsuperscript{+/−} R258C aortas have a significantly larger diameter at an average of 1.67 mm, compared to 1.52 mm in wildtype and 1.57 mm in Acta2\textsuperscript{+/−} mice (Figure 3.6E). The interesting observation comes when analyzing the aortic diameter of Acta2\textsuperscript{+/+} R258C mice. These aortas average 1.62 mm, which, by itself, would be considered a slightly “enlarged” aorta. The lack of significance is due to the great variability observed in this genotype, with the smallest measurement being 1.47 mm and the largest 1.75 mm (clearly enlarged). By comparison, the largest aortas observed in wildtype and Acta2\textsuperscript{+/−} were 1.55 and 1.59, respectively. These results suggest that the transgene does have a pathogenic effect in the aortic wall, but the increased expression of endogenous Acta2 in the Acta2\textsuperscript{+/+}R258C mice offsets the dominant negative effect of the mutation and induces a greater variability between mice of this same genotype. Future experiments could focus on studying the expression of endogenous Acta2 in all four genotypes.

Loss of Transgene Expression

Unfortunately, after the sixth generation of the Acta2 R258C transgenic mice we discovered that the transgene was no longer expressed at significant levels. The loss of expression was first seen in IEF blots, which only showed traces of the transgenic α-actin were being expressed from aortic lysates\textsuperscript{66} (Figure 3.8A).
Figure 3.8 Loss of Transgene Expression - A. IEF blots of aortic lysates show insignificant amounts of mutant SM α-actin. B. cDNA quantification PCR shows insignificant presence of the mutant mRNA (red circle), compared to the endogenous Acta2 mRNA.

To confirm the actual loss of the transgene expression, we synthesized cDNA from RNA (obtained from aortic lysates). Amplification of the cDNA corroborated the results seen at the protein level - insignificant expression of mutant Acta2. (Figure 3.8B) A new transgenic mouse model of the Acta2 R258C mutation is currently being generated using the novel TARGATT gene insertion system to complete our studies. This method guarantees single gene insertion into a defined, transcriptionally-active locus with high levels of gene expression."
CHAPTER 4

SMC Proliferation and Vascular Response to Injury *In Vivo* in Acta2 Mouse Models
**Introduction**

Initially, mutations in *ACTA2* were associated as a major cause of fTAAD, but after studying the clinical history of patient families, an unexpected link was made between aortic disease and vascular occlusive disorders such as CAD and ischemic stroke (68). Unlike atherosclerosis, in which the occlusion is due to accumulation of plasma lipids and inflammatory cell response, (133C) excessive SMC proliferation and migration into the lumen of small vessels are the most likely causes of the occlusive vascular pathology in patients with *ACTA2* mutations. The hypothesis is strengthened by positive SM α-actin staining in cells occluding the lumen of epicardial vessels in a patient carrying an *ACTA2* R118Q mutation (Figure 4.1A) and the vasa vasorum in patients with R149C mutations (Figure 4.1B)

![Figure 4.1 Vascular Occlusive Pathology in Patients with ACTA2 Mutations - A.](image)

Epicardial vessels stenosis in *ACTA2* R118Q caused by excessive SMC proliferation.
Cells stain positive for SM alpha-actin stain in vessel lumen. B. The *vasa vasorum* of patients with *ACTA2* mutations is completely occluded (top panel) with cells that stain positive for α-actin (bottom panel), indicating these cells are smooth muscle-like. Reprinted by permission from Copyright Clearance Center: Nature Genetics, copyright (2007).

One of the most severe mutations in *ACTA2* is R258C, which causes early-onset stroke in nearly 50% of carriers\(^47\)(Figure 3.1). Therefore, the transgenic *Acta2* R258C mouse model became an invaluable tool for studying the effects of this mutation in SMC proliferation. As expected, explanted aortic SMCs from *Acta2\(^{+/+}\)* R258 and *Acta2\(^{+-}\)* R258 mice proliferated more rapidly than wildtype SMCs. When comparing the first two cell lines, *Acta2\(^{+-}\)* R258 SMC proliferated at a significantly higher rate than *Acta2\(^{+/+}\)* R258 cells. Suggesting a link between α-actin filament disruption and proliferation in SMCs\(^60\).

**Figure 4.2 Phenotypic Plasticity in SMC.** SMC dynamic phenotypic changes from proliferative, migratory, secretory and non-contractile (left) to nonproliferative,
nonmigrating, and contractile with mature focal adhesions (right)\textsuperscript{34}. Permission from
Copyright Clearance Center: NPG \emph{Genet Med} (2010)

In mature vessels, SMCs are known to retain their phenotypic plasticity, ranging from a synthetic proliferative phenotype to a quiescent contractile one\textsuperscript{68} (\textbf{Figure 4.2}). In healthy vessels, most SMCs are found in the contractile state and are characterized by increased expression of contractile proteins organized into lattice-like filament structures. However, SMCs can revert to a more proliferative phenotype when, in response to endogenous and environmental factors, contractile gene expression is switched to expression of growth and matrix generating genes\textsuperscript{69}. The serum response factor (SRF) – myocardin related transcription factor (MRTF) axis is widely accepted as the canonical pathway driving the switch between SMC phenotypes\textsuperscript{70}. SRF is a ubiquitously expressed transcription factor responsible for the transcription of more than 200 genes. In SMCs, MRTF-A and MRTF-B are able to move intracellularly between the nucleus and the cytoplasm, binding to SRF while in the nucleus and preventing growth gene expression\textsuperscript{68, 71}. Globular actin is responsible for sequestering MRTF-A in the cytoplasm - freeing nuclear SRF to bind to other growth gene coactivators\textsuperscript{72}. As previously stated, \textit{Acta2}\textsuperscript{+/- - R258C} aortic SMCs had large pools of globular $\alpha$-actin in the cytoplasm \textit{in vitro} compared to both \textit{Acta2}\textsuperscript{+/+ - R258C} and wildtype cells (\textbf{Figure 3.4A}). Unsurprisingly, \textit{Acta2}\textsuperscript{+/- - R258C} SMCs showed decreased nuclear localization of MRTF-A, which correlates with the increased levels of cytoplasmic globular actin and lower expression of contractile proteins. Together, these findings explain the increased proliferative response in the \textit{Acta2}\textsuperscript{+/- - R258C} SMCs\textsuperscript{60}. 
Even though increased proliferation is seen at the cellular level in both Acta2\textsuperscript{+/+} R258C and Acta2\textsuperscript{+/-} R258C SMCs, these mice did not present with spontaneous strokes in vivo. Therefore, a different approach was required to study SMC proliferation in these mice. Previous studies in the Acta2 null mouse used a carotid artery injury model as a catalyst to promote SMC proliferation. This model is commonly used to study SMC proliferation and migration in response to injury\textsuperscript{73}. Three weeks post-ligation, Acta2\textsuperscript{-/-} mice showed evident increase in SMC proliferation represented by an exaggerated
neointimal formation and intima/media ratio compared to wildtype mice (Figure 4.3A). Furthermore, treatment with Imatinib, a tyrosine kinase inhibitor use for treatment of various cancers, prevented excessive SMC proliferation after injury in Acta2 null mice (Figure 4.3B). Based on these studies, we decided to adopt this same injury model to study SMC proliferation in the Acta2 transgenic mice.

**Role of Bone Marrow Derived Stem Cells in Vascular Wound Healing**

Following vascular injury, bone marrow derived stem cells are recruited and take residence in the neointima to assist in the healing process. These cells differentiate into SMC-like cells, expressing copious amounts of SM α-actin, but are missing other key markers of fully differentiated SMCs. It is thought that these newly formed myofibroblasts aid in constricting of the elements in the thrombus formed by the inflammatory response. Without a functioning contractile apparatus, i.e Acta2 mutations, healing of the thrombus might be delayed or even fail, leading excessive immunological and proliferative response, running the risk of completely occluding the vessel lumen.

**Results**

**In vivo SMC Proliferation in Acta2 R258C Transgenic Mouse Models**

Left carotid artery ligations were performed in 8-week-old wildtype, Acta2+/+ R258C and Acta2+/− R258C mice to determine if the disruption of actin filaments seen in SMCs also affects the cellular proliferative response *in vivo*. Three weeks post ligation, histopathological analysis of the left carotids showed excessive proliferation in the
Acta2\textsuperscript{+/-} R258C mice when compared to the other two genotypes (Figure 4.4A). The assessment was based on total area of the neo intima, media and neointima/media ratio. At site of with most significant occlusion, Acta2\textsuperscript{+/-} R258C carotids had an increased medial area, compared to both Acta2\textsuperscript{+/+} R258C and wildtype carotids. Total neointima area and neointimal/media ratio were also significantly increased in Acta2\textsuperscript{+/-} R258C mice (P<0.01 and P<0.05, respectively compared to wildtype), even when the medial area was significantly increased in these mice as well (Figure 4.4B). There was no significance found between Acta2\textsuperscript{+/+} R258C and wildtype mice, probably due to epigenetic differences in the transgene expression. Mutant gene expression has not yet been quantified to address this hypothesis.

Figure 4.4 Neointimal Proliferation after Carotid Ligation Injury - A.
Representative images of carotid cross-sections 10µm from the ligation site. The excessive proliferative response forming the neointima and occluding the lumen can be appreciated in the $Acta2^{+/−}$ R258C ligation. B. Quantitative analysis of the carotid cross-sections 21 days post-injury. Increased area of the media is observed in $Acta2^{+/−}$ R258C mice compared to WT. More importantly, neointimal area and media:intima ratio is significantly larger in $Acta2^{+/−}$ R258C than the other two genotypes. No significance was observed between $Acta2^{+/−}$ R258C and WT mice, mainly due to the large variation seen in the $Acta2^{+/−}$ R258C cross-sections.

Serial sectioning from the site of ligature to the branchpoint of the ascending aorta, subjecting every 150 µm to H&E staining, allowed us to construct a map of the varied proliferative response along the length of the carotid. We found that in wildtype, $Acta2^{+/+}$ R258C and $Acta2^{+/−}$ mice, most of the thrombotic and proliferative response to the lesion had resolved. In contrast, the $Acta2^{+/−}$ R258C carotids presented an exaggerated poor thrombus resolution, characterized by a dense population of proliferative cells in the luminal area, in the first 1.9 mm proximal to the ligation site ($p<0.01$) (Figure 4.5). These results complement the increased proliferative nature of $Acta2^{+/−}$ R258C SMCs observed in vitro. Furthermore, the results suggest the decrease in endogenous $Acta2$ along with the dominant negative effect of the R258C mutation cause an increase in cell proliferation and disrupt mechanisms involved in post-injury thrombus resolution.
Figure 4.5 Representative Images of Serial Sectioning Starting at the Carotid Ligation Site 21 Days After Surgery. From left to right, each panel represents a cross-section 150μm away from the previous one. The unresolved, and still active thrombus (neointima) can be observed in the Acta2^{+/−} R258C ligation, corroborates the critical role Acta2 plays in wound healing. The uncontrolled proliferative response and poor thrombus resolution may lead to arterial stenosis and strokes in patients with ACTA2 mutations.

**Bone Marrow Stem Cell Replacement Therapy in vascular injury model of Acta2^{−/−} mice**

*Originally, we planned to perform the following studies in our Acta2 transgenic mouse model, but due to the loss of transgenic α-actin expression we decided to use the Acta2 null model instead. Published work done in this model shows a similar response to carotid injury ligations.*

We believed it was necessary to repeat the carotid ligation studies in the Acta2^{−/−} mice to establish a reliable baseline for the bone marrow stem cell treatment. To confirm the absence of α-actin alters vascular healing and induces an excessive proliferative response after injury by carotid ligation, we ligated left common carotid arteries from 10-week-old mice and harvested the arteries at post-operative day 21. We serially sectioned the entire
length of the vessels from the ligature site down to the proximal branchpoint from the ascending aorta and stained the cross-sections every 150 μm with H&E. As expected, our results replicated our previous observations, in which Acta2−/− mice display signs of active and proliferative thrombus organization invading the luminal area. In contrast, wildtype carotids present a more mature neointimal lesion, occupying a significantly smaller area of the lumen73 (Figure 4.6).

![Figure 4.6 Increased Proliferative Response in Acta2+/− Carotids Post Ligation. We were able to replicate previous results by C. Papke in the Milewicz Lab, and showed increased neointimal formation in Acta2+/− mice in order to establish a baseline for the bone marrow transplant experiments.](image)

Following carotid artery injury, bone marrow derived stem cells are recruited and take residence in the neointima to assist in the healing process. Most of these cells stained positive for α-actin, but not other markers of fully differentiated SMCs74. To test the role of α-actin in the vascular healing process, we reconstituted the bone marrow of wildtype and Acta2−/− mice with GFP+ wildtype cells. Carotid ligations were performed 4 weeks after bone marrow transplant. Cells in the neointima stained positive for GFP in
both wildtype and Acta2\(^{-/-}\) carotids. Some of the GFP\(^+\) cells also expressed \(\alpha\)-actin in Acta2\(^{-/-}\) mice, proving the active role of bone marrow derived cells in thrombus resolution (Figure 4.7A). Surprisingly, there was still a significantly larger thrombus (neointima) and percent stenosis in the Acta2\(^{-/-}\) compared to wildtype vessels. Comparison between control Acta2\(^{-/-}\) and Acta2\(^{-/-}\) mice with the reconstituted bone marrow showed no significant difference in thrombus resolution. (Figure 4.7B)
Figure 4.7 Vascular Response to Injury After Bone Marrow Transplant – A. GFP-tagged exogenous WT bone marrow stem cells are present in the neointima of irradiated WT and Acta2−/− mice (brown stain), confirming successful bone marrow transplant. B. Representative images of carotid cross sections after bone marrow transplant and carotid ligation. The wildtype phenotype was not rescued by the presence of exogenous WT stem cells in the neointima. C. Quantitative analysis of carotid stenosis and neointimal area in control (non-irradiated) and irradiated (bone marrow transplant) mice. Increased neointimal area is evident in the Acta2−/− mice (top panels) when compared to WT mice. There was no significant decrease in the neointimal area of the Acta2−/− mice that underwent bone marrow transplant (irradiated) compared to control Acta2−/− mice, indicating that the role of Acta2 in bone marrow stem cells is not crucial for vascular repair. Similar results are seen for percent stenosis (bottom panels), in which the WT was not recovered after bone marrow transplant.

Infiltration of inflammatory cells is a crucial component of the wound healing process. During the initial inflammatory phase, the presence of neutrophils and macrophages initiates the recruitment of fibroblasts. Therefore, lesser activity of inflammatory cells is expected in latter stages of the healing process. Segments of the carotids containing thrombi were immunoprobed with Mac-2, a marker for murine macrophages. As expected, wildtype carotids contained a low number of macrophages in the residual thrombi, while high macrophage activity was observed in Acta2−/− carotids (Figure 4.8). Interestingly, irradiated carotids showed increased macrophage activity in both WT and Acta2−/− compared to non-irradiated controls. No difference was observed
between between irradiated WT and Acta2²⁻, which suggests increased stress in the vasculature due to radiation exposure. Our results suggest the absence of α-actin has a negative effect in the healing process after injury.

**Figure 4.8 Inflammatory Response After Carotid Ligation.** Few macrophages are observed in the control (non-irradiated – top left) WT, indicating the thrombus is in its final stages of healing. In contrast, the abundant macrophages (brown stain) in the control (non-irradiated – bottom left) Acta2²⁻ mice advocates the presence of an active, unresolved thrombus. Similar results were expected in the irradiated (bone marrow transplant – right) mice, but the control WT phenotype was not rescued. In fact, a dense concentration of macrophages is present in the irradiated WT type, possibly due to lingering inflammatory effects of radiation in the vasculature.
Overall, these results indicate that, even though SM α-actin positive bone marrow derived cells play an active role in the neointima, the recovery of α-actin in these cells was not enough to attenuate the pathological response to injury. Most likely, Acta2 deficient cells (fibroblasts, SMC) migrating for the vascular wall play a more significant role in the wound healing process.
CHAPTER 5
Cerebrovascular Characterization of the Acta2−/− Mouse Model
Introduction

Moyamoya disease (MMD) is characterized by stenosis of the distal internal carotid artery and arteries of the Circle of Willis along with growth of a weak vascular network providing collateral flow. In angiography, these neovessels have the “puff of smoke” appearance, which translates to moyamoya in Japanese. There are two peaks in incidence, during the first decade of life and a second one in patients during their third and fourth decades. Patients with MMD have a high risk of ischemic stroke recurrence, thus its characterization and accurate diagnosis is vital for adequate treatment. As many as 10% of non-syndromic MMD cases involve a family history, with increased incidence in Asian Americans compared to Americans of Europeans decent, suggesting a strong genetic predisposition to the disease.

Mutations in \textit{ACTA2} have been linked to MMD, with 50% of carriers of the R258C mutation presenting classic markers of the disease. Furthermore, a \textit{de novo} mutation in \textit{ACTA2}, R179H, was initially was associated MMD in children, but after examination of patient histories found some features distinguishing from the classic disease presentation. Mainly, vessels with R179H mutation had a combination of ectasia and stenosis, absence of collaterals and strait arterial course by angiograph examination (Figure 5.1A). Histological analysis of dural vessels showed abnormalities, characterized by intimal thickening and elastin deposition (Figure 5.1B). Interestingly, R258C/H mutation carriers also lacked collateral vessel formation, but no evidence of straight arterial course was observed.
Figure 5.1 Cerebrovascular Abnormalities in Patients with ACTA2 Mutations – A.

Internal carotid artery injections of a cerebral angiogram of (Left) an unaffected 3-year-old child, (Right) a child with ACTA2 Arg179H is and (Bottom) a child with idiopathic moyamoya disease. Distinctive cerebrovascular features of ACTA2 mutations with dilatation of the proximal internal carotid artery, occlusive disease of distal intracranial circulation, an abnormally straight course of intracranial arteries and absence of ‘moyamoya’, other basal and leptomeningeal collaterals, which are prolific in the child with moyamoya disease B. The images show representative small arterial dural vessels stained with H&E, elastin Van Gieson and smooth muscle actin in patients with ACTA2 R179 mutations. There is thickening of the intima, which is most prominent in Patient 1 but subtle in Patients 7 and 8. There is focal intimal thickening with deposition of elastin (black on the elastin Van Gieson). There is focal reduplication of the internal elastic lamina in Patient 7. Permission Copyright Clearance Center: Oxford Journals *Brain*58

Many challenges arise when studying MMD pathogenesis in human, including the limited amount of patient samples and the fact that most are acquired at the end-stages of
the disease. Based on the mutations described above, disruption in α-actin plays a key role in MMD pathogenesis. Therefore, we opted to study the cerebral vasculature in the $\text{Acta2}^{-/-}$ mouse, which provides an accurate model on how deficiencies in Acta2 may lead to cerebral vascular pathogenesis.

**Results**

*Cerebrovascular imaging and Histopathological Analysis of Acta2$^{+/+}$ and Acta2$^{-/-}$ mice*

Volume-rendered images of the Circle of Willis (CW) and nearby vessels in three mice from all three genotypes, WT, Acta2$^{+/+}$, Acta2$^{-/+}$ are illustrated in (Figure 2A). While the wild-type (WT) mice exhibit essentially normal vasculature for this stage of development, both the Acta2$^{+/+}$ and Acta2$^{-/-}$ mice exhibit distinct morphological differences in the vessels in and around the CW. First, there is evidence for progressive straightening of the vessels with change in genotype from WT to Acta2$^{+/+}$ to Acta2$^{-/-}$, as observed in the ICA (Figure 5.2A, labelled (a, d and g)). Similarly, there is evidence for narrowing and/or obstruction of vessels (shown by label b in WT), illustrated in the basilar artery in the Acta2$^{+/+}$ mice (e) and at the branching of the basilar into the left and right superior cerebellar artery (SCA) in the Acta2$^{-/-}$ mice (h). Additionally, the overall width of the CW narrows as one progresses from the WT to Acta2$^{+/+}$ to Acta2$^{-/-}$ (labelled c, f, and J) CT images of a curved multiplanar reconstruction of an occluded region of the SCA in an Acta2$^{-/-}$ mouse shows decreased flow in the artery and a crescent shaped lumen on cross sectional imaging, while other arteries nearby without evidence of decreased flow show a round lumen on cross section (Figure 2B).
Figure 5.2 Volume Rendered Images of the Circle of Willis - A. Volume rendered images of the CW in three representative mice: WT, Acta2+/−, and Acta2−/−. Characteristic phenotypic features of the heterozygous and knockout animals are evident and marked by arrows: (a,d,g) straightened vessels in the Acta2 mutant mice compared to WT; (b,e,h) narrowed or obstructed vessels in the Acta2 mutant mice compared to patent vessels in WT; (c,f,j) narrowed CW in the Acta2 mutant mice compared WT. (B) CT images illustrate a curved multiplanar reconstruction of an occluded region of the SCA in an Acta2−/− mouse (Right panel). Cross sections at locations p1, p2 and p3 are shown in the three panels to the right. Panel p2 shows a partially occluded SCA while p1 and p3 show
normal open lumens of other arteries. In the left panel, a curved multiplanar reconstruction of an occluded region of the Basilar artery in an Acta2^{+/−} mouse. Panel p1 and p2 show a crescent shaped occlusion of the arterial lumen, while p3 shows a completely unobstructed lumen. Images provided by Dr. Annapragada’s Laboratory.

Morphometric analyses for each of the arterial narrowing, curvature measurements and CW width parameters were performed and the results are shown in Tables 1 and 2. For each measured parameter, three comparisons were made between the following: WT vs. Acta2^{+/−}, WT vs. Acta2^{−/−} and Acta2^{+/−} vs. Acta2^{−/−}. The vessel radii are significantly decreased in the right and left ICAs of the Acta2^{−/−} mice compared to both Acta2^{+/−} and WT mice. Arterial straightening and decreased tortuosity is evident in the right superior cerebellar artery (SCA) and left ICA of Acta2^{+/−} and Acta2^{−/−} mice compared with WT, as determined by decreases in the Middle Curvature and Arc-length ratio, respectively (Table 1 – for clarification on the parameters mentioned please refer to methods section). Interestingly, these same parameters in the left posterior cerebral artery (PCA) are only different between the WT and the Acta2^{+/−} mice, but not the Acta2^{−/−} mice. The right PCA demonstrates a significant narrowing in the Acta2^{−/−} mice. Sporadic differences in length are noted, with the most significant being in the left ICA. The circularity, and aspect ratio, measures of how circular and symmetric the cross sections of the vessels are, do not show statistically significant variation with genotype, suggesting that in spite of the other morphological changes, the vessels are retaining their intrinsically circular cross sectional shape. Finally, the lower-half area and the basal
triangle area of the CW are significantly smaller in the WT than the Acta2+/- and Acta2-/- mice (Table 2).

### Table I. Statistical differences in the dimensions of cerebrovascular arteries on CT imaging in the WT, Acta2+/- and Acta2-/- mice.

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Radius</th>
<th>Length</th>
<th>Middle</th>
<th>Arc/Length</th>
<th>Narrowing</th>
<th>Mean</th>
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<tr>
<td>WT vs. Acta2+/−</td>
<td>0.54</td>
<td>0.61</td>
<td>0.84</td>
<td>0.47</td>
<td>0.72</td>
<td>0.17</td>
</tr>
<tr>
<td>Acta2+/− vs. Acta2+/−</td>
<td><strong>0.00</strong></td>
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<td>0.69</td>
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<td>0.54</td>
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<td>Acta2+/− vs. Acta2+/−</td>
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<td>0.86</td>
<td><strong>0.04</strong></td>
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<td>0.37</td>
<td>0.73</td>
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<td>0.37</td>
<td><strong>0.00</strong></td>
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<tr>
<td>WT vs. Acta2+/−</td>
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<td>0.68</td>
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<td><strong>0.01</strong></td>
<td>0.46</td>
<td>0.73</td>
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<tr>
<td>WT vs. Acta2+/−</td>
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<tr>
<td>Acta2+/− vs. Acta2+/−</td>
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<td>0.39</td>
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<tr>
<td>WT vs. Acta2+/−</td>
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<td><strong>0.02</strong></td>
<td><strong>0.00</strong></td>
<td><strong>0.01</strong></td>
<td>0.35</td>
<td>0.14</td>
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<td><strong>Left Posterior Cerebral</strong></td>
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<tr>
<td>WT vs. Acta2+/−</td>
<td>0.07</td>
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<td><strong>0.01</strong></td>
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<td>0.10</td>
<td>0.84</td>
<td>0.98</td>
<td>0.45</td>
</tr>
<tr>
<td><strong>Left Superior Cerebellar</strong></td>
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<tr>
<td>WT vs. Acta2+/−</td>
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<td>0.54</td>
<td>0.91</td>
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<td>Acta2+/− vs. Acta2+/−</td>
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<td>0.61</td>
<td>0.95</td>
<td>0.39</td>
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<td>0.78</td>
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<tr>
<td>WT vs. Acta2+/−</td>
<td>0.63</td>
<td>0.95</td>
<td>0.95</td>
<td>0.10</td>
<td>0.38</td>
<td>0.53</td>
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<tr>
<td><strong>Left Anterior Cerebral</strong></td>
<td></td>
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</tr>
<tr>
<td>WT vs. Acta2+/−</td>
<td>1.00</td>
<td>0.30</td>
<td>0.41</td>
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<td>1.00</td>
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<td>Acta2+/− vs. Acta2+/−</td>
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<td>0.46</td>
<td>0.95</td>
<td>0.86</td>
<td>0.64</td>
<td>0.22</td>
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<tr>
<td>WT vs. Acta2+/−</td>
<td>0.23</td>
<td>1.00</td>
<td>0.29</td>
<td>0.73</td>
<td>0.92</td>
<td>0.45</td>
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</table>

In all cases, null hypotheses H0: WT=Acta2+/-, H0: WT=Acta2+/, H0: Acta2+/-=Acta2+/- were tested. For the radius, length, and mean aspect ratio measures, the alternate hypotheses were H1: WT>Acta2+/-, H1: WT>Acta2+/, H1: Acta2+/->Acta2+/- were tested. For the middle curvature, arc/length ratio and narrowing count measures, the alternate hypotheses were: H1: WT<Acta2+/-, H1: WT<Acta2+, H1: Acta2+/-<Acta2+. The Wilcoxon Rank-Sum test was used to assess statistical
significance. Bolded entries indicate measures where the null hypothesis was disproven at the 95% confidence level. Entries in italics were non-significant. The complete analysis of variance is presented in the supplemental data section. Analysis provided by Dr. Annapragada’s Laboratory at Texas Children’s Hospital.

Table II. Statistical differences of the dimensions in the Circle of Willis by imaging in the WT, Acta2<sup>+/−</sup> and Acta2<sup>−/−</sup> mice.*

<table>
<thead>
<tr>
<th>Circle of Willis</th>
<th>Total Area</th>
<th>Upper Half Area</th>
<th>Lower Half Area</th>
<th>Half Basal Triangle Area</th>
<th>Width</th>
<th>Narrowing % of all COW vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT vs. Acta2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.02</td>
<td>0.92</td>
<td>0.01</td>
<td>0.01</td>
<td>0.35</td>
<td>0.27</td>
</tr>
<tr>
<td>Acta2&lt;sup&gt;+/−&lt;/sup&gt; vs. Acta2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.46</td>
<td>0.09</td>
<td>0.53</td>
<td>0.61</td>
<td>0.39</td>
<td>0.47</td>
</tr>
<tr>
<td>WT vs. Acta2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>0.45</td>
<td>0.10</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
</tr>
</tbody>
</table>

For the total area, upper half area and width, the null hypotheses H<sub>0</sub>: WT=Acta2<sup>+/−</sup>, H<sub>0</sub>: WT=Acta2<sup>−/−</sup>, H<sub>0</sub>: Acta2<sup>+/−</sup>=Acta2<sup>−/−</sup> were tested while the alternate hypotheses were H<sub>1</sub>: Acta2<sup>+/−</sup>, H<sub>1</sub>: WT=Acta2<sup>−/−</sup>, H<sub>1</sub>: Acta2<sup>+/−</sup>=Acta2<sup>−/−</sup> In all cases, the Wilcoxon Rank-Sum test was used to assess statistical significance. Bolded entries indicate measures where the null hypothesis was disproven at the 95% confidence level. Entries in italics indicate measurements where the null hypothesis was not disproven. The complete analysis of variance is presented in the supplemental data section. Analysis provided by Dr. Annapragada’s Laboratory at Texas Children’s Hospital.

Taken as a whole, the data suggest that narrowing and straightening of the cerebral vessels, and a distinct narrowing of the diameter of the CW, occur with loss of SM α-actin. The ICAs differ in more parameters than any of the other vessels, exhibiting narrowing, straightening, and lengthening.

Histologic analyses of Acta2<sup>+/−</sup> and Acta2<sup>−/−</sup> brains show thickening of the walls of the arteries independent of size when compared to the WT brains (Figure. 5.3A). Immunostaining of a SMC-specific marker (calponin-1) indicated that the wall thickening involved SMCs in the medial layer of the artery. Similarly, immunostaining for CD31 (endothelial cell marker) and Movat pentachrome staining of elastin (stains black) show the thickening of the wall does not involve the endothelial layer and is outside the internal elastic lamellae. To confirm the thickening of the medial layer of the arteries in the Acta2 mutant mice, the wall-to-lumen ratio and percent wall thickness was calculated and found to be significantly greater in Acta2<sup>+/−</sup> and Acta2<sup>−/−</sup> compared to WT for
cerebrovascular arteries of different sizes (Figure 5.3B; \( p < 0.05 \) for all analyses between the WT and Acta2 mutant mice for all arteries > 100\( \mu \)m in diameter). Both vessel groups showed significant difference nonparametric testing. In this pairwise multiple comparisons, there was no difference between the Acta2\(^{+/-}\) and Acta2\(^{-/-}\) wall-to-lumen ratio or percent wall thickness of arteries.

**Figure 5.3** Histological comparison of cerebral arteries (diameters 100-200\( \mu \)m)
and >200 μm) from WT, Acta2+/−, and Acta2−/− mice - A H&E staining of arterial cross-sections. Arteries of similar diameter were analyzed according to their genotypes. Regardless of vessel size, the walls of intracerebral arteries from the Acta2 mutant mice appear markedly thickened compared with WT arteries. B. Morphologic analysis of cerebrovascular pathology in WT, Acta2+/− and Acta2−/− mice. Left column: wall-to-lumen ratio. Right Column: percent wall thickness. In both medium (diameter 100-200μm) and large (diameter >200μm) vessels, wall-to-lumen ratio and percent wall thickness of Acta2 mutant mice was significantly greater than that of WT group (* = p<0.05). C. Smooth muscle cell density in the medial layer of circular vessels in WT and Acta2−/− mice. (Top Panel) EC: Endothelial cells. SMC density was calculated by measuring the number of SMC nuclei in the media layer of the arterial cross-sections. (Bottom panel) WT group had a significantly lower cell density than Acta2−/− group. This finding is highly suggestive of medial SMC undergoing hypertrophy rather than hyperplasia.

Analysis of wall thickness in smaller arterioles (short diameter <100μm) in the cerebral parenchyma showed a significantly higher percentage of thickened vessels in both Acta2+/− and Acta2−/−, compared to WT (Figure 5.4B; p<0.05). No significant difference in number of thickened vessels was observed between Acta2+/− and Acta2−/− mice.
To determine whether SMC proliferation or hypertrophy was driving medial layer thickening, the cell density of the medial layer of circular vessels from WT and Acta2\(^{-/-}\) mice was assessed. This analysis showed significantly increased cell density in the WT compared to the Acta2\(^{-/-}\) mice (p<0.001; Figure 5.3C). This finding suggests that the
medial SMCs in the cerebrovascular system in the Acta2<sup>−/−</sup> mice have undergone hypertrophy rather than hyperplasia.
CHAPTER 6

Discussion
Discussion

Aortic Pathology in Acta^{R258C} Mouse Model

Previous studies linked TAAD, stroke and other vascular diseases to mutations in a single gene, ACTA2. Analysis of aortas from these patients revealed classic markers of end-stage aortic disease including contractile fiber degradation, SMC disarray, proteoglycan accumulation and increased medial thickness. Furthermore, these patients presented occlusive vascular lesions in smaller vessels, which we hypothesize to be the cause of strokes\textsuperscript{47-49}. Due to the challenges involved in studying disease progression in humans, we engineered a mouse model with an Acta2^{+/+} R258C mutation\textsuperscript{60}. We chose this alteration based on the severe phenotypic presentation of vascular disease in patients, primarily early onset strokes (<20 yoa) and TAAD\textsuperscript{49}. We expected this mutation to have a dominant negative effect and therefore mimic the human pathogenic phenotype in our mouse model. Additionally, we hypothesized that a decrease in endogenous expression of Acta2 would increase the severity of the observed phenotype. Initial studies of ex-vivo aortic rings confirmed decreased contractile force development in Acta2^{+/+} R258C aortas compared to wildtype\textsuperscript{66}. The mutation lies within the SD4 domain, which plays an important role in opening/closing of the nucleotide binding cleft. When ATP/ADP is bound to actin, the nucleotide binding cleft interacts allosterically with the DNase binding loop causing conformational changes during contraction. A disruption of the cleft might explain the significant decrease in contractile force generation\textsuperscript{80}. To further support our claim, we assessed the capability of Acta2^{+/+} R258C TG and Acta2^{+/−} R258C TG SMCs to form functional α-actin filaments. Immunofluorescent imaging showed
monomeric pools of α-actin Acta2^{+/−} R258C TG SMCs with little to no filament formation even after treating with TGFB1. Acta2^{+/+} R258C TG SMCs did form α-actin filaments. An F/G actin assay was then performed and results showed both cell lines lacked pelleted filaments. This suggested that, even though Acta2^{+/+} R258C TG SMCs are able to produce filaments, they are weaker than those in wildtype SMCs and the shear stress of the assay was enough to cause significant disruption. Therefore, we can safely conclude that the R258C mutation not only causes structural instability, but also affects interactions between actin monomers leading to severe disruptions in the contractile apparatus. Moreover, these results may translate in vivo where mechanical stress and other differentiating factors may lead to filament formation and stabilization, decreasing the potential deleterious effects of the mutation on the aortic wall.

The aortic phenotype in the mouse model did not present with the same degree of severity as observed in SMCs in vitro, but the results gave us valuable insight into the underlying mechanisms and factors involved in aortic wall degeneration and aneurysm formation. As early as 4 weeks of age, we can observe significant developmental differences between mutant and wildtype mice. There was an increase in the number of elastin layers laid down during development and density of SMCs in both Acta2^{+/−} R258C and Acta2^{−/−} R258C mice. Elastin plays a crucial role in aortic capacitance in response to flow, which is also regulated by the contractile properties of the SMCs in the aortic wall. Interestingly, similar aortic pathology was observed in the hemiygous ELN mice, the gene that encodes for elastin protein. These mice had a 50% decrease in elastin protein expression as well as increased SMC proliferation and an increased number of thinner elastic lamellae at birth. The aortic extensibility at physiological pressures is normal,
suggesting that the increase in elastic lamellae is due to decreased elastin protein expression and helps maintain normal levels of aortic extensibility. Therefore, an increase in the number of elastic fibers in the Acta2 mutant mice may be associated with a physiological response to compensate for the decreased contractile force generation of SMCs and the aortic wall contractile apparatus as a whole. The increased number of SMCs may also be due to a compensatory proliferative response, which will be discussed later in this chapter. The fact that the number of elastin layers remained constant during the mice’s lifespan, and that there is no evidence of continued proliferation after birth, indicates that modifications in the elastin lamellae occur during development.

The compensatory mechanisms described above proved to be insufficient in maintaining the aortic wall integrity and by 3 months of age, we observe a 9% increase in the average aortic diameter in Acta2+/− R258C mice when compared to both wildtype. By 12 months of age there is an average increase of 10% in aortic diameter compared to wildtype and a 6% increase compared to Acta2+/−. Even though the average increased only 1% over that period of time, a more significant difference is observed in the range of aortic diameters. The Acta2+/− R258C did not show a significant increase in the average aortic diameter, mainly due to the wide range of diameters observed within the cohort. Of notice is the fact that the largest diameter observed in an Acta2+/− R258C mouse and an Acta2+/− R258C were 1.81mm and 1.75mm, respectively. These are clearly enlarged aortas when compared to the maximum diameter observed in Acta2+/− (1.59mm) and wildtype (1.55mm) mice. The variability in Acta2+/− R258C is to be expected, based on the variable penetrance observed in patients with the same ACTA2 mutation. The variability in the phenotype can also be attributed to epigenetic changes in transgene expression, which we
were planning to test for before we lost expression of the mutant gene in the mice. Nevertheless, these results confirm that the mutant Acta2 plays a critical role in the aortic enlargement observed in our mouse models. We believe one of the possible triggers of the pathogenic enlargement relates to the results of the F/G actin assay in vitro, in which the shear stress of the assay disrupted the filaments formed in Acta2+/R258C SMCs. Correspondingly, the stress caused by the force of pulsatile flow in the ascending aorta has a similar effect on actin filament integrity in the SMC in the aortic wall. In fact, increased blood pressure caused surgical constriction of the aorta in mice can lead to increased root diameter and alterations in AT1 signaling; a similar pathway to the one observed in the Marfan mouse model82-84. We began testing this hypothesis by inducing hypertension in our mutant mice, an important risk factor associated with aortic disease. Unfortunately we were unable to complete these studies because we lost expression of the transgene in these mice.

Histological analysis of aortic tissue showed elastic fiber straightening, thinning and fragmentation along with proteoglycan accumulation in Acta2+/R258C mice compared to both Acta2+/− and wildtype mice. We first observed elastic fiber thinning at the 6month time point without significant number of elastin breaks or proteoglycan accumulation. The weakening of these fibers progressed to increased breaks and accumulation of proteoglycans by 12 months of age. These features resemble the end-stage of aortic disease in tissue from individuals with ACTA2 mutations and are considered classical markers of aortic wall degeneration (Figure 1.2) Atypical features in the aortic wall of these patients, such as focal areas of increased numbers of SMCs, were also present in Acta2+/R258C mice47. A recent study linked pregnancy in women with ACTA2 mutation to
increased risk of acute aortic dissections with minimal aortic degeneration. Accumulation of proteoglycans in the aortic wall was the common pathologic feature found in these cases of acute dissection. Previous studies have suggested proteoglycan accumulation decreases tensile strength of the aortic wall, which could lead to aneurysm formation and rupture. Interestingly, this study showed proteoglycan accumulation in pregnant women with Acta2 mutation happens independent to medial degeneration, while in our mouse model we observed this phenomenon was preceded by elastin degeneration. Leading us to conclude that, even though these are both markers of aortic disease, they most likely develop through separate mechanisms. Future studies could determine if the presence of both defects have a synergistic effect on aortic wall debilitation. It would be of interest to determine if proteoglycan accumulation alone is enough to increase the risk of aortic dissections in all patients with ACTA2 mutations. In addition, 63% of the women who suffered acute aortic dissection also had a history of hypertension, providing further evidence that ACTA2 mutations decrease the aortic wall’s ability to effectively withstand the stress of pulsatile flow.

Fibrotic and Proliferative Response to Injury in Acta2 mutant Mice

ACTA2 mutations cause a shift in SMC phenotype from a quiescent/contractile to a more proliferative state. This was first seen in SMCs from patient explants that presented with decreased SM α-actin filament formation and increased proliferation. Some patients with ACTA2 mutations suffered from vascular occlusive diseases characterized by increased number of SMCs invading the vessel lumen. Mutated α-actin promotes excessive proliferation and occlusion of smaller arteries, contrary to its effect
on larger vessels in which the mutant protein compromises aortic wall integrity and leads to aneurysm formation. Uncontrolled proliferation, and not atherosclerotic lesions, is believed to be the main cause of stroke and CAD in patients with ACTA2 mutations. Complete proliferative occlusions can be seen in the vasa vasorum in the aortic wall of these patients supporting our claim that the invasion of SMCs into the lumen can lead to widespread vascular occlusive diseases.\textsuperscript{48, 49, 58, 87, 88}

Although great advances have been made recently to understand the underlying mechanisms of SMC proliferation \textit{in vitro}, the triggering factors promoting a shift to the proliferative state \textit{in vivo} are poorly understood. The Acta2\textsuperscript{R258C} transgenic mice do not stroke spontaneously, limiting our ability to study SMCs \textit{in vivo} under physiological conditions. To overcome this limitation, we adopted the carotid injury model as a mechanism to study the proliferative response of SMC in vivo. Previous studies on Acta2 knockout mice used the same model and observed increased proliferation of SMCs invading the lumen causing significant stenosis.\textsuperscript{73} Similar pathology was observed in Acta2\textsuperscript{+/-R258C} mice after injury. In most of these mutant vessels, the carotid lumen was occluded by a large fibroproliferative thrombus that failed to resolve 3 weeks after surgery, which we will refer to as neointima. Our results show no indication that the initial inflammatory response - characterized by macrophage infiltration, stem cell migration and increased number of recruited myofibroblasts to the injury site - was altered in the Acta2\textsuperscript{+/-R258C} mouse model. This is followed by a proliferative stage, in which myofibroblasts and SMCs secrete large quantities of collagen and other matrix proteins forming granulation tissue. At the time of sample collection, most of the granulation tissue has resolved in both Acta+/- and wildtype carotids but it is still present...
in most the Acta2\textsuperscript{+/R258C} carotids. Therefore, we believe alterations in SM \(\alpha\)-actin severely affect the transition to the final contractile stage\textsuperscript{77, 78, 89}. Normally, SMCs and myofibroblasts play an important role in organizing the thrombus by contracting and promoting its resolution\textsuperscript{89}. Mutations in Acta2, might then decrease the effectiveness of the contractile apparatus, causing disorganized and decreased contraction of the thrombus. Without the ability to contract properly, the tissue enters an uncontrolled proliferative phase promoting the observed fibroproliferative neointimal lesion.

Supporting evidence is observed in vitro, where Acta2\textsuperscript{+/R258C} SMCs were unable to form SM \(\alpha\)-actin filaments, differentiate, and had an increased proliferative profile compared to wildtype cells. We believe the decreased ratio of endogenous to mutant actin in Acta2\textsuperscript{+/R258C} SMCs is primarily responsible for this phenomenon, which eventually leads to the uncontrolled proliferation observed in these mice\textsuperscript{60}. Even though the carotid ligation model does not mimic physiological conditions, it is still a valuable model to study the proliferative and contractile mechanisms of SMCs, allowing us to understand the effects of Acta2 mutations in vivo in a controlled environment.

**Bone Marrow Replacement Therapy in Vascular Repair**

Stem cell replacement therapy to treat genetic disorders has been gaining traction in the medical field over the past few years. Studies show stem cells migrate to vascular tissue after injury and differentiate into SMC-like cells, expressing common SMC markers including SM \(\alpha\)-actin. These differentiated cells aid in the contractile phase of wound healing similar to the action of myofibroblasts and SMCs\textsuperscript{74, 76}. We hypothesized that if the bone marrow cells in Acta2\textsuperscript{+/} mice were reconstituted with wildtype bone
marrow, the migration of wildtype stem cells to the injury site after carotid ligation would contribute to the stabilization of the contractile phase and prevent uncontrolled proliferation and formation of a disorganized thrombus. According to our results, thrombus resolution was not affected by the introduction of wildtype SMC-like cells into the lesion. There was no significant difference between sizes of the fibroproliferative lesions between Acta2−/− mice with reconstituted bone marrow and those that did not receive stem cell treatment. Furthermore, we were able to confirm that wildtype stem cells migrated to the lesion site and differentiated into SMC-like cells, evidenced by the presence of SM α-actin in the thrombus.

One possible explanation is that, even though important, the role of bone marrow stem cells in vascular injury is not critical for vascular repair. It is possible that the function of resident SMCs and fibroblasts migrating to the injury site, which still lacked SM α-actin, is far more important than the migration and differentiation of stem cells. The activity of these stem cells in the injury site was not assessed, but it is possible that they had decreased functionality. One way to prove that is to perform ligations in the GFP-tagged WT mouse and look at stem cells protein expression in the affected tissues. Another possibility has to do with the type of injury inflicted in the vessel. One study detailed the migration of stem cells to different types of vascular injury, including carotid ligations. The author claims endoluminal denudation injuries elicit the highest migration and differentiation response from stem cells. In comparison, carotid ligations present relatively low numbers of bone marrow stem cells in the injury site. In fact, endoluminal denudation is a more physiologically relevant model - maintains flow and endothelial injury and endothelial injury occurs frequently in vivo - and should be
considered for future studies. Before completely discarding stem cell therapy as a reliable treatment option for proliferative vascular diseases in patients with Acta2 mutations, it would be interesting to first establish the role of stem cells in thrombus resolution in endothelial lesions.

*Cerebrovascular Pathology in Acta2+/− and Acta2−/− Mice*

Acta2 mutant mice exhibit cerebrovascular disease that mimics the arterial disease found in children and young adults with ACTA2 missense mutations 48, 58. Certain arteries (left ICA, PCA and SCA) in the mutant mice are straighter than corresponding arteries in WT mice, mirroring the straightening of the cerebral arteries observed in children with ACTA2 mutations. The occlusion of the arteries in ACTA2 mutation patients is similar to the pattern observed in MMD and primarily involves the distal ICA but often extends to the middle and anterior cerebral arteries and can involve the posterior arteries 91. Both the Acta2+/− and Acta2−/− mice exhibit distinct morphological differences in the vessels in and around the CW, including reduction in arterial diameter. Some Acta2−/− mice also exhibit complete occlusion of some vessels. Finally, as observed in individuals with ACTA2 mutations, the mouse model shows no evidence of compensatory collateral blood vessel formation like that observed in typical MMD. Therefore, the Acta2 mutant mouse models recapitulate the clinical features of the cerebrovascular disease observed in children with cerebrovascular disease due to ACTA2 missense mutations that disrupt R179 or R258.

Vascular remodeling results from interplay between arterial structure and luminal pressures. In cerebral arteries, the increased intraluminal pressures from hypertension
leads to thickening of the wall and an increase in wall thickness and wall/lumen ratio but without growth of the artery, which generally leads to a smaller lumen and increased wall to lumen ratio. Studies in the spontaneously hypertensive rats show increased thickness of the arterial walls in a large part due to increased SMC mass, with additional studies confirming that the increased SMC mass is due to growth of SMC’s (i.e. hypertrophy) and not increased number of these cells (i.e., not hyperplasia). The histologic changes in the cerebral arteries in the Acta2 mutant mice are similar to the changes observed with hypertension in rat and mouse models, specifically the cerebrovascular arterial walls are diffusely thicker due to hypertrophy of the SMCs. These histologic changes are present despite the fact that the Acta2+/− mice have blood pressure similar to WT mice and the Acta2−/− mice are hypotensive with a systolic blood pressure 26% lower compared to WT mice. Thus, the loss of SM α-actin in the absence of hypertension triggers vascular remodeling similar to the remodeling associated with increased luminal pressures due to hypertension.

The cerebral arteries are straighter in the Acta2 mutant mice, which raises the questions as to why there are no reports of straightened arteries in the hypertensive animal models if the cerebrovascular pathology is similar. The most extensively studied animal models of hypertension are the spontaneously hypertensive rat (SHR), which develop hypertension at 5 to 6 weeks of age, and stroke-prone spontaneously hypertensive rat (SPSHR), which develops hypertension at 3 to 4 weeks of age. However, the apparent absence of arterial straightening in SHR and SPSHR could be explained by the lack of detailed cerebrovascular imaging and assessment of arterial curvature of hypertensive rats to date. Another possible explanation is that the
straightening may be due to thickening of the arteries during development. The deficiency in SM α-actin is present in the vasculature during development in the Acta2 mutant mice but both hypertensive rat models develop hypertension after birth.

It is notable that the included pathologic analysis of randomly selected arteries throughout the cerebrovascular circulation and did not limit the analyses to the large arteries at the base of the brain. These analyses confirmed that the thickening of the medial layer occurred in all sized arteries throughout the brain, including the small arteries in the parenchyma. Children with the ACTA2 R179 mutations, the pattern of brain injury is indicative of both large and small artery involvement. Magnetic resonance imaging shows ischemic lesions, consistent with large arterial occlusions, but all these children show perivascular white matter pathology. The degree of changes in the white matter suggests the presence of small vessel disease as well. Our data confirm that the Acta2 mice do have diffuse involvement of all sized arteries in the brain, including the small vessels in the parenchyma of the brain.

Children and young adults with ACTA2 mutations that lead to cerebrovascular disease can experience transient ischemic attacks and ischemic strokes. Since the Acta2−/− mice have similar pathology and imaging findings, this animal model can be used to test treatments to decrease the narrowing of the arteries and determine the pathology associated with total occlusion of the arteries, with the ultimate goal of providing better treatments and preventing strokes in children with ACTA2 mutations.

Conclusion (Table 2 Supplement)
We were able to recapitulate significant characteristics of the aortic and cerebrovascular pathology seen in patients with ACTA2 mutations in our Acta2 mouse models, proving to be a valuable tool to study the underlying mechanisms of vascular disease. Furthermore, both aneurysm formation and SMC proliferative response were exacerbated by decreasing the amount of endogenous Acta2 in our transgenic model. The severity of the cerebrovascular pathology did not vary between Acta2+/- and Acta-/-, suggesting there is a minimum threshold of expressed α-actin required for proper filament formation. As observed in vitro, Acta2+/- did not reach such threshold and very little actin filament formation was observed. Meanwhile, the Acta2 R258C mutation appears to have a dominant negative effect, as its effects are inversely correlated to the amount of endogenous actin in an organism. And, the inability of the Acta2-/-R258C mice to live post-partum further proves the deleterious effect of the mutation. These studies set part of the basis for the development of better care and potential therapeutic treatments in patients with ACTA2 mutations.
Supplement
### Table 1 Supplement Number of Mice in Each Study

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<th>WT</th>
<th>Acta2&lt;sup&gt;+&lt;/sup&gt; R258C</th>
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### Table 2 Supplement Summary of Results

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*<sup>n/a</sup> = not available
Reference List


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**Vita**

Carlos Villamizar-Rosales was born in Barranquilla, Colombia on September 22, 1986 and is the son of Carlos Villamizar Valdivieso and Rocio Rosales Cepeda. After attending and graduating from an American accredited High School in his native city, Karl C. Parrish School (2005), he went on to attend The College of St. Scholastica in Duluth, MN, from which he earned the degree of Bachelor of Science in Biochemistry *summa cum laude* in May 2009. Carlos enrolled at the University of Texas Graduate School of Biomedical Sciences in August 2010 and in the University of Texas at Houston Medical School in August 2013.

**Permanent address:**

7510 Brompton St. Apt 547

Houston, TX 77025

Email: cv8622@gmail.com