Fear not, I am with you; be not dismayed; I am your God. I will strengthen you, and help you, and uphold you with my right hand of justice.

Isaiah 41:10

University of Alberta

Dual Role of Matrix Metalloproteinases-2 in Thoracic Aortic Aneurysm

by

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DEDICATION

to

My parents Jangkyun & Seounghee

&

My brother Youngsang

ABSTRACT

With matrix metalloproteinases-2 (MMP2) deficiency, we observed thoracic aortic aneurysm in 70% of $MMP2^{-/-}$ mice but no abdominal aortic aneurysm following four weeks of angiotensin II (Ang II) infusion. We found markedly suppressed recoil properties in the thoracic aorta of $MMP2^{-/-}$- Ang II mice. mRNA and protein levels of elastin, but not collagen, were significantly reduced in the thoracic aorta of $MMP2^{-/-}$-Ang II compared to WT-Ang II mice. This reduction in elastin levels was due to significantly reduced TGFβ-Smad signaling pathway that mediates synthesis of extracellular components. Thus, the adverse remodeling in the thoracic aorta of MMP2-deficient mice was associated with decreased synthesis of extracellular matrix proteins without concomitant upregulation of proteolytic activities. These findings suggest a protective role of MMP2 in the development and progression of aortic aneurysm, and as such inhibition of MMP2 may exacerbate vascular remodeling and lead to development of thoracic aortic aneurysm.

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LIST OF ABBREVIATIONS

AAA	abdominal aortic aneurysm
ACE	angiotensin converting enzyme
Ang II	angiotensin II
ApoE	Apolipoprotein
ARB	angiotensin II receptor blocker
AT1	angiotensin II receptor, type-1
AT2	angiotensin II receptor, type-2
AV	atrioventricular
BAV	bicuspid aortic valve
BMP	bone morphogenetic proteins
BP	blood pressure
BW	body weight
cDNA	complementary DNA
COL	collagen
DD	diastolic diameter
DNA	deoxyribonucleic acid
ECM	extracellular matrix
Eln	elastin
FBN-1	fibrilin-1
FBS	fetal bovine serum
GT	gomori trichrome
HPRT	hypoxanthine-guanine phosphoribosyltransferase-1
HRP	horse radish peroxidase
HR	heart rate
IL	interleukin
LA	left atrium

LDH	lactate dehydrogenase
LOX	lysyl oxidase
LV	left ventricle
MCP-1	monocyte chemoattractant protein-1
MMP	matrix metalloproteinase
MMPi	matrix metalloproteinase inhibitor (pharmacological)
mRNA	messenger RNA
miRNA	micro RNA
MT1-MMP	membrane type 1-matrix metalloproteinase
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phenylephrine
PV	pulmonary vein
RA	right atrium
RAAS	renin-angiotensin-aldosterone system
RNA	ribonucleic acid
RNA i	RNA interference
miRNA	microRNA
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
RV	right ventricle
SD	systolic diameter
SMC	smooth muscle cells
TAA	thoracic aortic aneurysm
TAD	thoracic aortic diameter
TAV	tricuspid aortic valve

TGF- β	transforming growth factor
TGF R1	Type I transforming growth factor receptor
TGF R2	Type II transforming growth factor receptor
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
VSMC	vascular smooth muscle cells
VVG	verhoeff-van gieson
WT	wild-type

Prefix

c	centi (10^{-2})
m	milli (10^{-3})
µ	micro (10^{-6})
n	nano (10^{-9})

Units

Hz	hertz
L	litre
m	metre
g	gram
Da	dalton
℃	degree Celsius
d	day

Symbols

α	alpha
β	beta

CHAPTER 1

INTRODUCTION

1.1 Introduction overview

Aortic aneurysm is pathological dilations of the aorta. By convention, an enlargement of 50% or greater is considered aneurysmal. As an aortic aneurysm enlarges, the aorta may eventually rupture. Because the aorta is the largest artery that delivers blood from the heart to the body, the mortality due to aortic rupture is extremely high. Collectively, aortic aneurysm represents the 13th most common cause of death in the United States, and the prevalence of the disease is increasing[1]. Gender, race, age, smoking, hypertension, atherosclerosis, and family history are risk factors for aortic aneurysm. Despite various clinical trials, there is insufficient evidence to prove the effectiveness of therapeutic interventions of aortic aneurysm. It is therefore important to identify the underlying mechanism of aortic aneurysm, establish the mechanism of disease progression, and develop more effective therapeutic interventions.

1.2 The cardiovascular system

The cardiovascular system is the unique network of blood vessels (arteries, capillaries and veins) that transport oxygen, nutrients and wastes products in multicellular organisms. The heart pumps the blood throughout the vascular network through systemic and pulmonary circulations.

1.2.1 Structure of the heart

The heart is a four-chamber muscular pump that pumps blood to body. Ventricular wall comprises of three layers: an endocardium (inner lining of the heart), myocardium (thick muscle layer), and epicardium (a connective tissue that

covers the heart). Left side of the heart pumps blood for systemic circulation and right side of the heart pumps blood for pulmonary circulation. Oxygenated blood from the lungs returns to the heart through the pulmonary vein and enters the left atrium (LA), flows to the left ventricle (LV) via the mitral valve, it is then pumped into aorta through the aortic valve, and circulates through the rest of the body. Blood from the body enters the right atrium (RA) through superior and inferior vena cava. Blood from the RA flows to the right ventricle (RV) via the tricuspid valve. The RV pumps the blood into the pulmonary artery through the pulmonary valve to the lungs for oxygenation.

1.2.2 Structure of the vasculature

Blood vessels allow circulation of blood to and from the heart, and to all organs and tissues. Arteries, which serve as a pressure reservoir, carry blood away from the heart to other organs. Arteries then further divided into arterioles and capillaries. Capillaries consist of a single layer of endothelial cells that allow exchange of nutrients and waste products between blood and tissue. Capillaries then merged to venules and veins. Veins, which serve as a blood reservoir, carry blood from other organs to the heart.

1.3 Structure of the aortic wall in normal physiology

The aorta is the largest artery that delivers blood from the LV to the rest of body. The aortic wall is made up of three layers: the tunica intima, the tunica media, and the tunica adventitia (Figure 1.1). The innermost layer, the tunica intima, is made of endothelial cells supported by a basement membrane. Endothelial cells

form a physical barrier between blood and tissue, regulate platelet function, maintain the vascular wall by regulating smooth muscle proliferation, and provide a smooth surface that allows blood to flow with low resistance[2, 3]. The tunica intima contributes little to the mechanical properties of the aorta. The tunica media is composed of smooth muscle cells (SMCs), elastin and collagen fibers. SMCs, circumferentially and spirally oriented, regulate the diameter of the blood vessel upon contraction and relaxation. Elastin fibers work as a pressure reservoir and provide elasticity which allows the arterial wall to change diameter in response to pressure changes to regulate blood flow. Collagen fibers provide strength against pressure to prevent the arterial wall from rupturing. The tunica adventitia, the outermost layer of the aortic wall, is composed mainly of longitudinally arranged collagen fibers and fibroblasts. The tunica adventitia also contains small blood vessels that provide nutrients to the aortic wall, called the vasa vasorum.

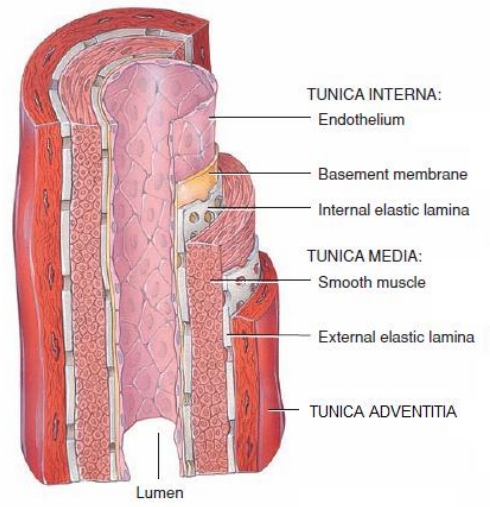

Figure 1.1 Structure of aortic wall.

The aortic wall is made up of three layers: the tunica intima, the tunica media, and the tunica adventitia. Adapted from Tortora & Derrickson[4].

1.4 Regional heterogeneity within the aorta

Thoracic and abdominal regions of the aorta have different developmental origins. Vascular smooth muscles cells (VSMCs) in thoracic aorta have neural crest origin whereas VSMCs in abdominal aorta are derived from the mesoderm[5]. Due to this developmental heterogeneity, thoracic and abdominal regions of the aorta respond differently to growth factors[6]. In addition, composition of the aortic wall is different between thoracic and abdominal aortas. Although the total amount of elastin plus collagen is similar, the proportional amount of elastin and collagen are different between the thoracic and abdominal aorta as thoracic aorta contains more elastin than collagen fibers and opposite compositions are observed in the abdominal aorta[7, 8]. Moreover, abdominal and thoracic aortas show different expression patterns for over 100 genes in the normal and disease state[9], as such different genes may play different role in thoracic versus the abdominal aorta. These structural and molecular heterogeneities lead to different histopathologies between abdominal and thoracic regions of the aorta.

1.5 The extracellular matrix (ECM)

1.5.1 ECM remodeling

The extracellular matrix (ECM) is a network structure which holds the cellular components together to provide structural integrity of the vessel wall. Collagen and elastin fibers are the predominant structural components of the aortic ECM. Collagen fibers, which comprise 20% of total protein in normal aorta, work as a blood reservoir to protect the aortic wall from rupturing, and elastin fibers provide elasticity which allow the aortic wall to recoil during cardiac relaxation[10,

[11]. Collagen and elastin fibers are synthesized by medial SMCs and fibroblasts in the adventitia[12]. Excessive degradation or deposition of these structural proteins leads to impairment of mechanical strength and integrity of the aortic wall[7]. Matrix metalloproteinases (MMPs) degrade these ECM proteins and their activities are tightly regulated by their physiological inhibitors, tissue inhibitor of metalloproteinases (TIMPs). The ECM constantly undergoes turnover and its integrity is maintained by homeostasis of MMPs and TIMPs levels. Overall imbalance in its turnover leads to adverse pathological remodeling of ECM.

1.5.2 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are members of zinc-dependent endopeptidases which degrade a wide range of extracellular components. By actively modulating in ECM remodeling and turnover, MMPs play an important role in regulating physiological and pathological processes including cell apoptosis, embryogenesis, tissue morphogenesis, would healing, bone development[13-15]. There are 26 MMPs discovered to date[16, 17]. MMPs have been traditionally classified according to their preferential substrates: collagenases (MMP1, MMP8, MMP13 and MT1-MMP), stromelysins (MMP3, MMP10, and MMP11), membrane-type MMPs (MT1-MMP, MT2-MMP, MT3-MMP, and MT4-MMP), gelatinases (MMP-2 and -9), elastase (MMP12), epilysin (MMP28), and matrilysin (MMP7 and MMP26)[18-21]. Although MMPs have substrate specificity, most of MMPs are not specific to a single substrate such that a number of MMPs degrade a wide range of ECM proteins.

MMPs generally contain five conserved domains: amino (N)-terminus signal peptide, pro-domain, catalytic domain, hinge region and carboxyl (C)-

terminus hemopexin domain (Figure 1.2). The signal peptide directs translocation of MMPs to the endoplasmic reticulum for secretion[22]. The pro-domain containing cysteine sequences forms a bond with a zinc ion located at the catalytic domain to maintain MMPs as a form of inactive zymogen[23]. The hinge region regulates autoproteolysis and collagenolytic activities of MMPs[24]. The hemopexin domain contributes to recognition and cleavage of substrates[25]. Tissue inhibitors of metalloproteinases (TIMPs) are physiological inhibitors of MMPs that bind to the catalytic domain to regulate activity of MMPs[22, 26].

MMPs are regulated at three levels: transcription, activation and inhibition. MMPs are induced at transcriptional level by bioactive molecules (Angiotensin II or Endothelin-1), inflammatory cytokines (Tumor necrosis factors, or interleukin-1β), growth factors (transforming growth factors), oxidative stress, or mechanical stimuli[27-30]. Catalytic domain of inactive zymogens is blocked by the pro-domain. Proteolytic degradation or reactive oxygen species expose the catalytic domain of MMPs by unfolding or cleaving the pro-domain[31]. Activated MMPs are inhibited by their predominant inhibitors TIMPs.

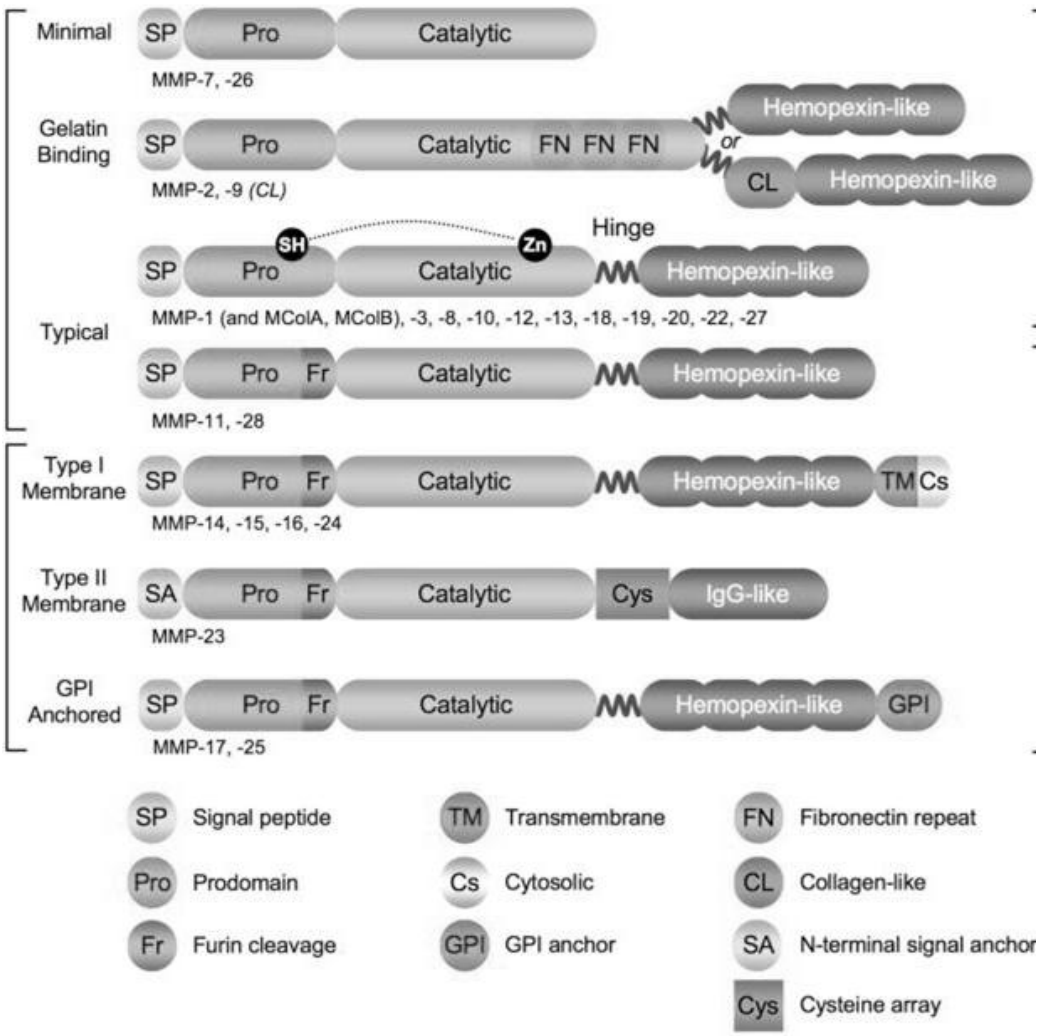

Figure 1.2 Structure of MMPs.

The MMPs generally contain five conserved domains: amino (N)-terminus signal peptide, pro-domain, catalytic domain, hinge region and carboxyl (C)-terminus hemopexin domain. Extracted from Ra & Parks [32].

1.5.3 Tissue inhibitors of metalloproteinases (TIMPs)

TIMPs are a family of four endogenous MMP inhibitors with 37-51% homology[33] (Figures 1.3 and 1.4). TIMPs consist of two domains, amino (N) terminal and carboxyl (C) terminal domains and cysteine-rich proteins with three

disulfide bonds stabilize these domains[34]. TIMPs work as competitive and reversible inhibitors of activated MMPs by forming a non-covalent bond between N-terminus of the TIMPs and the catalytic domain of MMPs in a 1:1 stoichiometric ratio[35, 36]. Among four TIMPs, TIMP2 forms a trimolecular complex with proMMP2 and MT1-MMP[37, 38]. TIMP2 inhibits the activity of MMP2 at higher concentrations while is required for cell surface activation of proMMP2 at lower concentrations[39]. In addition to MMP inhibitory functions, TIMPs have other functions. TIMP2 has been shown to stimulates cell growth[40]. TIMP3 has been found to inhibit angiogenesis and to stimulate VSMCs apoptosis[41, 42].

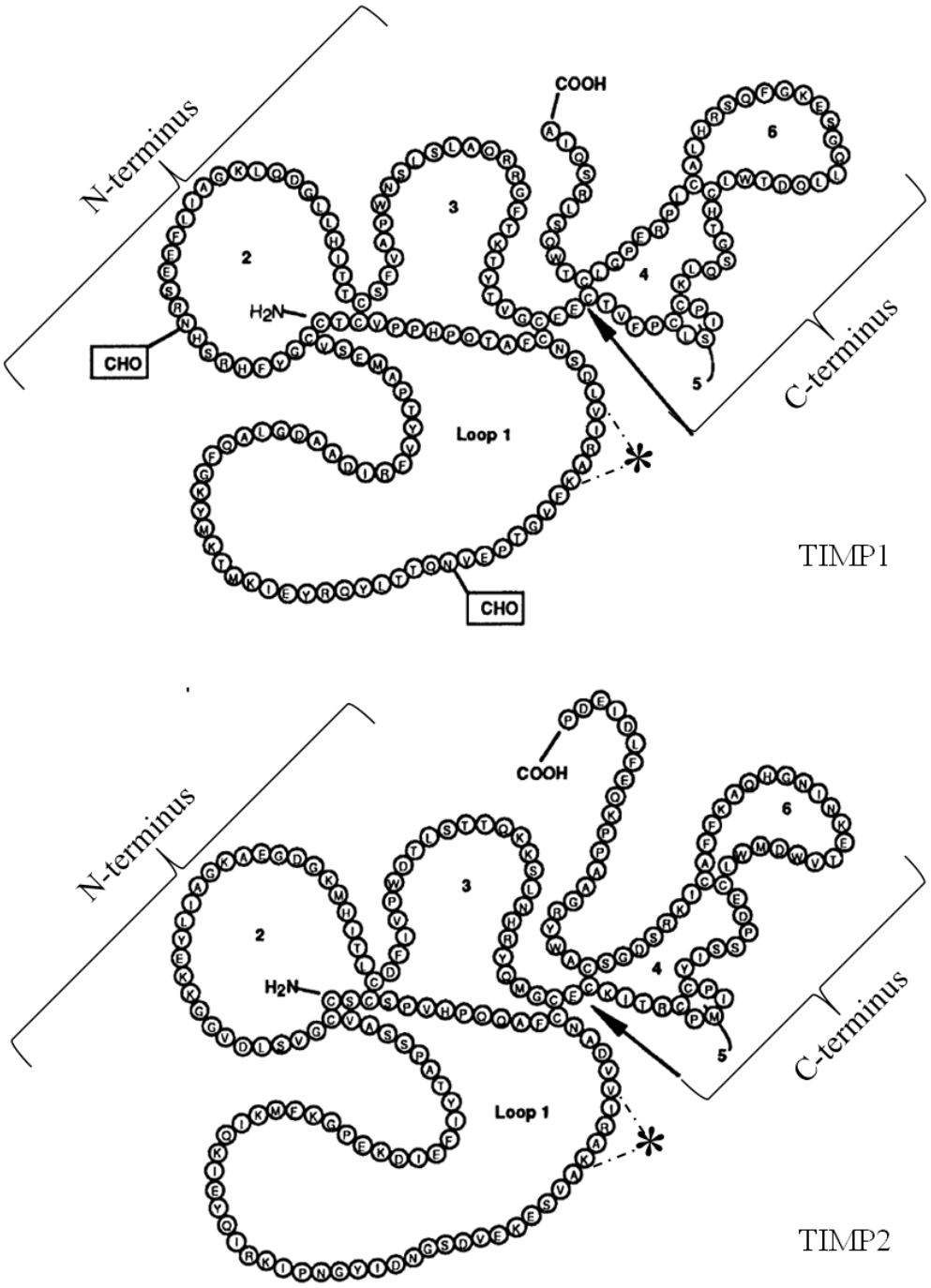

Figure 1.3 Molecular structure of TIMP1 and TIMP2.

TIMP1 (A) and TIMP2 (B) sequence. Arrow indicates N- and C-terminus junction. Adapted from Moore *et al*. [43].

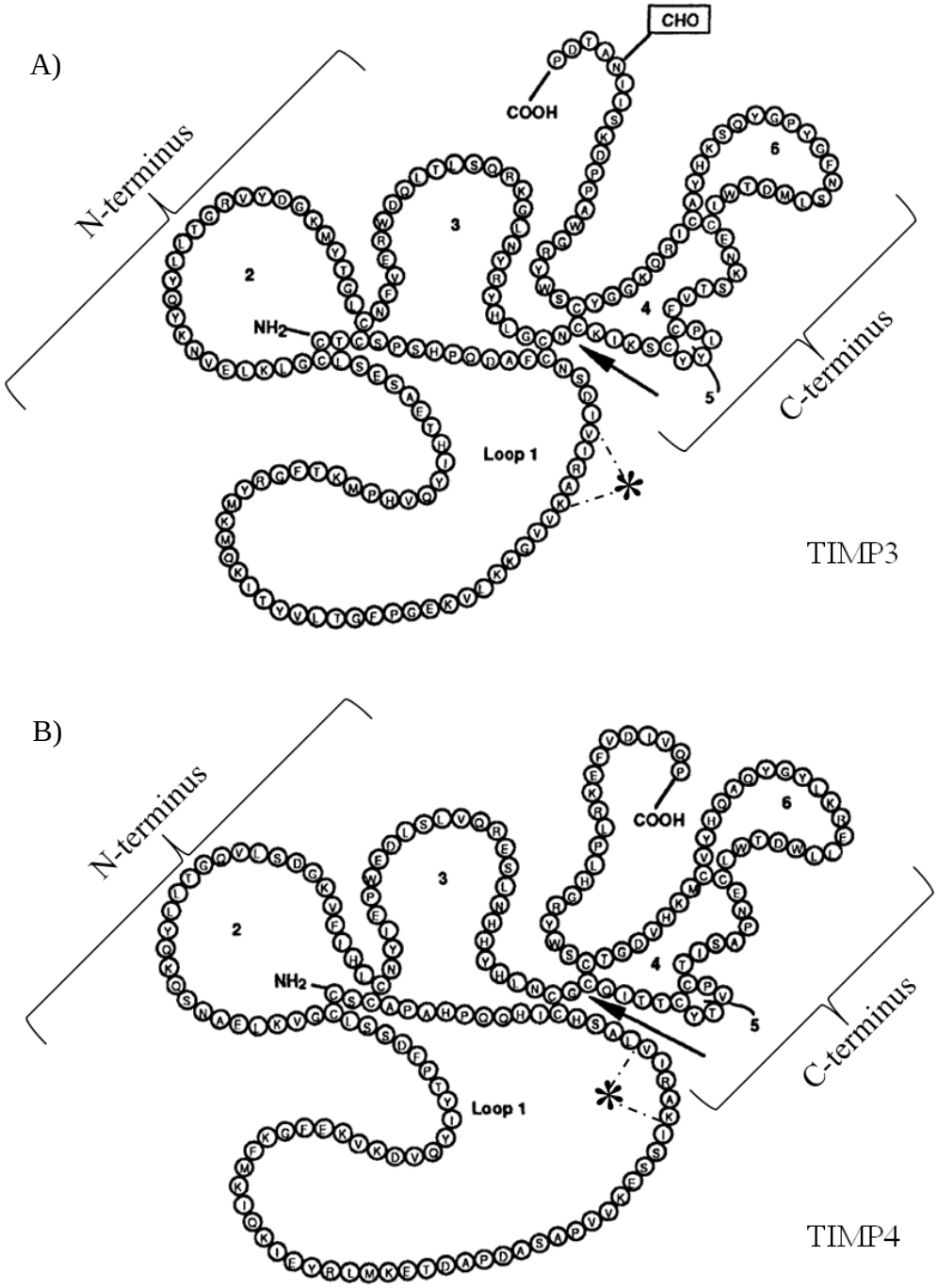

Figure 1.4 Computational structure of TIMP3 and TIMP4.

TIMP3 (A) and TIMP4 (B) sequence. Arrow indicates N- and C-terminus junction Adapted from Moore *et al*. [43].

1.6 Types of vascular remodeling

Structure and function of arteries undergo vascular remodeling as an adaptive response to stress including exercise, hypertension, stenosis, kidney diseases or aneurysm[44]. Impaired integrity of the vascular ECM due to alterations in the homeostasis of MMPs and TIMPs leads to physiological and pathological vascular remodeling. The changes associated with a decrease or an increase in the circumference can be classified as inward or outward vascular remodeling, respectively (Figure 1.5). The change in wall mass can be classified as hypotrophic (decreased in wall mass which leads to lower wall to lumen ratio than normal), eutrophic (constant wall mass), or hypertrophic (increased in wall mass which leads to higher wall to lumen ratio than normal). Inward eutrophic or hypertrophic remodeling is associated with hypertension, and outward remodeling is often associated with aneurysm.

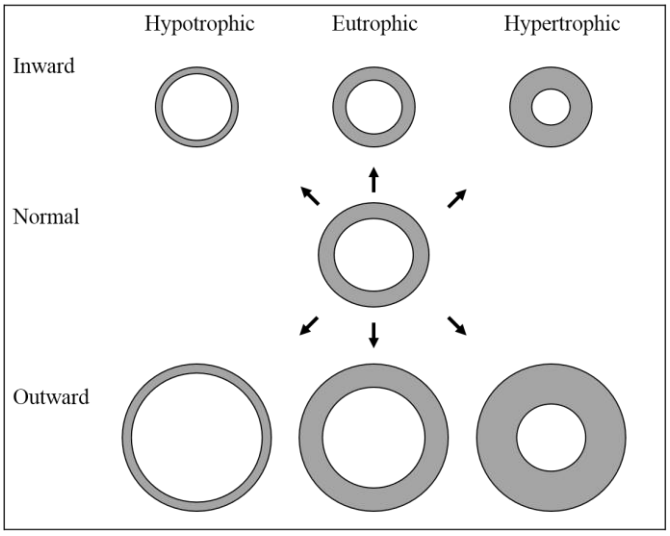

Figure 1.5 Types of vascular remodeling

Different types of pathological vascular remodeling showing changes in wall to lumen ratio. Adapted from Mulvany *et al*[44].

1.7 Aortic aneurysm

By convention, an enlargement of 50% or greater in aortic diameter compared to its baseline diameter is considered aneurysmal. Aortic aneurysm can affect any region of the aorta, including the aortic root, the ascending thoracic aorta, the aortic arch, the descending thoracic aorta, and the abdominal aorta. The most common form of aortic aneurysm is the infrarenal abdominal aortic aneurysm (AAAs), usually associated with atherosclerosis, while thoracic aortic aneurysm (TAAs) are also commonly encountered in clinical practice. Collectively, aortic aneurysm represents the 13th most common cause of death in the United States, and the prevalence of the disease is increasing[1]. Gender, race, age, smoking, hypertension, atherosclerosis, genetic disposition and family history are risk factors for aortic aneurysm. Generally, AAA is linked to modifiable risk factors including life style, age, gender and pre-existing health conditions, while TAA is more linked to genetic predisposition such as Marfan syndrome. As aortic aneurysm enlarges, the aorta can eventually rupture; since the aorta is the largest artery, delivering blood from the heart to the body, the mortality due to aortic rupture is extremely high. Thoracic aortas with aneurysm are also prone to acute aortic dissection, another potentially life-threatening event in which the intima and media are dissected off the adventitia, creating a false lumen and potentially pinching off branch vessels. Though AAAs and TAAs are common and potentially life threatening, to date there are no effective medical therapies to treat aneurysm or to prevent their growth. To monitor presence and progression of aortic aneurysm, imaging techniques such as ultrasonography, computed tomographic scanning, or

magnetic resonance scanning are recommended. Vascular surgeries such as open or endovascular repair are available for patients with sufficiently large aortic aneurysm.

1.8 Clinical studies on aortic aneurysm

1.8.1 Classification

Each type of aortic aneurysm shows distinctive characteristics of histopathology, disease mechanisms, and clinical presentations. Commonly used classification for aortic aneurysm is based on the anatomical location. According to the anatomic classification, aortic aneurysm can be divided into AAA and TAA depending on its location relative to the diaphragm.

1.8.2 Histopathology of aortic aneurysm

Aortic aneurysm is a degenerative disease which leads to progressive dilation of the aorta. Pathological vascular remodeling of aortic aneurysm, which mainly occurs in the medial layer of the aorta, includes disruption of elastin and collagen fibers, infiltration of inflammatory cells, and loss of SMCs[45-48]. In addition, deposition of collagen fiber correlates positively with aortic diameter in patients with AAA[49]. The association between wall thickness and aortic aneurysm is not clear. While wall thickness was decreased in aorta from patients with TAA, patients with AAA did not show thinning of the aortic wall[49, 50].

1.8.3 Abdominal aortic aneurysm

AAA is divided into suprarenal, pararenal or infrarenal; of these, the most common form is the infrarenal form. An aorta with a diameter greater than 3 cm is

considered aneurysmal in the abdominal segment, which is 1.5 times larger than the normal diameter for this segment. The average annual expansion rates are 1 to 4 mm for smaller aneurysmal aortas and 7 to 8mm for larger aneurysmal aortas[51]. Prevalence of AAA is 1.3% for men and 0% for women aged 45 to 54 years and 12.5% for men and 5.2% for women 75 to 94 years of age[52]. AAA are rare in Asian populations compared to white populations[53]. Aortic rupture is the main complication of AAA, though thromboembolic complications and dissection can occur.

Smoking shows a strong association to aortic aneurysm events[54]. Smoking is thought to promote the production of reactive oxygen species to activate MMPs, which have the capacity to disrupt aortic wall structure[55-57]. Prevalence and mortality increase with smoking due to increased rate of aneurysm expansion[58, 59]. Interestingly, after cessation of smoking, risk of prevalence of AAA decreased minimally[60].

Hypertension is another risk factor for aortic aneurysm. More than 80% of patients with aortic aneurysm have hypertension[61]. However, direct association between aortic aneurysm and hypertension is not clear. AAA patients with 4 to 5.5 cm in aortic diameter showed significant association between risk of rupture and mean blood pressure[62]. However, in another study, hypertension did not show any association to the expansion rate of AAA[59].

Atherosclerosis, or lipid deposition in the aortic wall, also correlates with aortic aneurysm. Progression of type 2 lesions to advanced lesions is faster and

greater in quantity in aortic tissues from patients with AAA compared to TAA[63]. In addition, prevalence of aortic aneurysm with atherosclerosis disease is higher in AAA than in TAA[61]. The lipoprotein level in serum, a risk factor for atherosclerosis, is generally elevated in patients with AAA[64].

Genetic predisposition has been shown to play a role in AAA where 15 to 30% of patients with AAA had first-degree relatives suffering aneurysm[65, 66]. While genetic syndromes such as Marfan syndrome, Loeys-Dietz syndrome, and Ehlers-Danlos syndrome mostly exhibit abnormalities in thoracic region of the aorta, degenerative changes can occur in the abdominal aorta as well.

Inflammation is also associated with AAA, forming a specific sub-type of AAA called an inflammatory AAA. Abnormal accumulation of macrophages and cytokines in the aneurysmal aortic tissue leads to inflammation. Patients with inflammatory aneurysm showed larger aortic diameter compared to patients with non-inflammatory aneurysm[67]. Inflammatory aortic aneurysm was present in approximately 5% of patients with AAA[68, 69]. Staphylococcus and Salmonella are the common cause of infectious, or mycotic, aneurysm but the prevalence of this type of disease is rare.

1.8.4 Thoracic aortic aneurysm

TAA is divided into ascending, arch, or descending TAA. As in other segments of the aorta, an increase of greater than 50% in thoracic aortic diameter is considered aneurysmal. Prevalence of TAA is 10.4 cases per 100000 population. Aortic diameter increases with age and the difference between genders decreases

with age[70]. The average rate of TAA expansion is about 0.1 to 0.42 cm/year[71]. Possible complications associated with TAA are aortic rupture and aortic dissection. Similar to AAA, smoking and hypertension show correlation with development and progression of TAA.

Bicuspid aortic valve (BAV), where the aortic valve has two leaflets as opposed to three leaflets in normal aortic valve, is the most common congenital abnormality of the aortic valve affecting about 1 in 100 of the population[72, 73]. Due to structural abnormalities of the ascending aorta, BAV is frequently associated with aortic aneurysm such that individuals with BAV are prone to developing aortic aneurysm and aortic dissection[74, 75]. The congenital BAV predisposes to majority of TAA development[76, 77]. Patients with BAV showed larger diameter of aortic root and ascending aorta[78]. In addition, TAA patients with BAV showed greater elastin degradation than patients with tricuspid aortic valve (TAV)[79]. Elevated MMP2 and MMP9 levels were observed in BAV patients with TAA[80, 81].

Genetic predisposition plays a role where 10% of patients with TAA have a first-degree relatives with TAA[82]. About 20% of TAA are due to genetic syndromes[1]. Genetic syndromes with abnormal connective tissue are associated with TAA. Marfan syndrome is a heritable disorder of the connective tissue due to mutations in the Fibrillin-1 which is an essential protein for elastin assembly in the medial layer of the aorta. Abnormalities in the connective tissue lead to abnormal manifestations in cardiovascular, ocular and skeletal systems. Patients with Marfan syndrome are highly predisposed to TAA and aortic dissection where most patients

present dilation of the aortic root/ascending aorta or Type A dissection[83]. Loeys-Dietz syndrome is an autosomal dominant disorder with mutations in either the Transforming growth factor receptor type I or II genes where most patients exhibit aortic root aneurysm[84]. Ehlers-Danlos syndrome is a rare autosomal dominant disorder with defect in type III collagen which is encoded by the COL3A1 gene. Characteristic symptoms of this disorder are hyperelasticity of skin, and rupture of arteries or other organs. The most fatal complication is arterial rupture which attributes to aortic ruptures and dissections. Since Fibrilin-1 and collagen are crucial for assembly and production of ECM structural proteins within the aortic wall, patients with these genetic syndromes are prone to aortic rupture and dissection.

Inflammatory diseases are also associated with TAA. Takayasu arteritis is an idiopathic vasculitis of elastic arteries where aorta can develop either aneurysm or stenosis. Prevalence of aortic aneurysm in the abdominal region is higher than in the thoracic region where 23% of patients with Takayasu arteritis exhibit AAA[85]. This disease affects all ethnic groups with moderately higher prevalence in Asian population[84]. Unlike other cardiovascular disease, women are affected about 10 times more than men[83, 86]. Giant cell arteritis is an elastic vessel vasculitis involving the aorta and its secondary and tertiary branches. The main difference between this disease and Takayasu arteritis is the distribution of patients' age. While patients above the age of 50 years are mostly affected by giant cell arteritis, patient with age below 40 years old are affected by Takayasu arteritis[87].

Infectious etiology are rare but it can affect the entire thoracic aorta and lead to TAA. Saccular aneurysm is the most common type of infectious aneurysm. The clinical outcome is poor with 30% to 50% mortality. Although thoracic aortic atherosclerosis is less common than abdominal aortic atherosclerosis, atherosclerotic TAA showed association with prevalence of ischemic stroke[88].

1.8.5 Clinical presentations and complications

Aortic aneurysm is usually asymptomatic until the size of aneurysm is noticeably large and it is close to rupturing. Depending on the anatomical location of aneurysm, symptoms vary. Following aortic expansion or rupture, symptoms might occur more frequently with severe extent.

A common symptom of AAA is pain especially at the back and abdomen. Fullness or pulsations in the abdomen may occur as well[89]. The most common complication of AAA is aortic rupture. Common symptoms of TAA include chest pain or upper back pain[90]. Dyspnea (shortness of breath) or dysphagia (difficulty in swallowing) can occur due to an increased pressure on the trachea. However, most symptoms are not specific to TAA. The most common complication of TAA is aortic dissection, though aortic rupture can still occur.

Since the aorta is the largest artery that delivers blood to the entire body, aortic rupture may lead to a life-threatening event following massive hemorrhaging. Paralysis can occur when the aneurysm is large enough to compress surrounding spinal cord or after aortic rupture. Less common complications are embolization,

fistulae or thrombosis. In addition, presence of aortic aneurysm increases prevalence of other cardiovascular diseases.

1.8.6 Treatments

Treatment approaches for aortic aneurysm vary depending on the subtype of aneurysm. The ultimate goal of the treatment is to slow down the progression of the aortic expansion and aortic rupture. Available treatment options for aortic aneurysm are surgical intervention (open and endovascular aortic aneurysm repairs), risk factor reduction (smoking, hypertension, or hyperlipidemia), and therapeutic interventions. In this review, therapeutic interventions will be discussed in detail.

There are clinical trials on the different therapeutic interventions for AAA. One of the approaches is to control risk factors such as hypertension or hyperlipidemia. Propranolol is used as a non-selective β adrenergic blocker. Patients with AAA showed no significant differences in the annual growth rate and mortality rate between placebo and propranolol groups[91]. Angiotensin-converting enzyme (ACE) inhibitors are used to block the conversion of Angiotensin I to Angiotensin II. In a small study, aneurysm growth was fastened in patients taking ACE inhibitors[92]. However, in a large study, patients who took ACE inhibitors prior to admission showed significantly less prevalence of aortic rupture[93]. In this study, researchers found out that other anti-hypertensive agents including β blockers, angiotensin II receptor blocker (ARB), thiazide diuretics, calcium channel blocker, and α blocker did not show any significant prevention against aortic rupture.

However, in another study, while ACE inhibitors lower the abdominal aortic rupture, ARB did not show any protection against aortic rupture compared to placebo group[93]. Statin is used to lower cholesterol level in patients with AAA. However, after 5 years of follow-up, no association was found between statin and expansion rate of AAA[94]. Another approach is to inhibit MMPs which have been shown to be altered in patients with aortic aneurysm. Doxycycline is an antibiotic which can inhibit broad range of MMPs. Doxycycline was given in patients with AAA (3.0 to 5.5 cm in diameter) for 6 months but there were no significant changes in the aortic diameter and overall expansion rate between treatment and placebo groups[95]. However, in another study, taking doxycycline for 3 months significantly reduced expansion rate of AAA (3.0 cm in diameter) over the 18 months surveillance[96]. Roxithromycin is a semi-synthetic macrolide antibiotic and patients with small AAA showed reduced annual expansion rate following the treatment[97].

There are limited clinical trials on the therapeutic interventions for TAA. Patients with Marfan syndrome showed significantly lower rate of aortic dilation as well as better survival rate following propranolol treatment[98]. 24 weeks of perindopril, an ACE inhibitor, treatment reduced aortic stiffness and aortic root diameter in patients with Marfan syndrome[99]. Losartan, an ARB, showed slower rate of progression in thoracic aorta in patients with Marfan syndrome[100]. In another study, statin treatment did not show improvement in the long-term mortality in patients with TAA[101]. However, regardless of various clinical trials on medical therapy, there is insufficient evidence to prove effectiveness of therapeutic interventions on the expansion of aortic aneurysm.

1.9 Role of the ECM in aortic aneurysm

Development of aortic aneurysm is a complicated process which involves pathological changes in both cellular and extracellular levels. Common pathological changes in the aneurysmal aortic wall are destruction of elastin and collagen fibers, and loss of SMCs[90, 102, 103]. MMPs and TIMPs play a significant role in the development of aortic aneurysm such that alteration in MMPs and TIMPs levels disrupts collagen and/or elastin structures in aortic wall and leads aorta prone to aneurysm. In addition, polymorphism of the genes associated with MMPs and TIMPs have been constantly reported in patients with aortic aneurysm[104].

1.9.1 Alterations in expression of MMPs and TIMPs in aortic aneurysm

Expression patterns of MMPs and TIMPs vary depending on the anatomical location of aortic aneurysm (abdominal vs. thoracic region)[105]. Abdominal aortic wall tissue from patients with aneurysm showed increased MMP2 and MMP9 complementary DNA expression compared to normal aortic tissue[106]. In another study, mRNA level of MMP2 was significantly increased in the inferior mesenteric arteries, which is in close proximity to the abdominal aorta, from patients with AAA compared to control[107].

Expression patterns of MMPs and TIMPs were examined in patients with TAA with BAV or TAV. Compared to control, MMP2 levels were significantly elevated only in patients with BAV whereas TIMP2 level was significantly reduced in patients with TAV[79]. In this paper, the relationship between MMPs and TIMPs expression levels and aortic diameter was examined where the alteration of MMPs

and TIMPs were different between patients with BAV and TAV. While patients with BAV showed alterations in MMP2, MT1-MMP and TIMP1 levels, patients with TAV showed alterations in MMP3, MMP7, MMP13 and TIMP2 levels. Taken together, these data imply that expression of MMPs and TIMPs are not only dependent on the anatomical location but also specific pathology of the disease.

1.9.2 Role of MMP2 in aortic aneurysm

Among 26 MMPs that have been discovered, MMP2, known as a gelatinase, works as a collagenase and an elastase[108], has been consistently reported to be linked to TAA and AAA[109-112]. In addition, abdominal and thoracic aortas show different expression patterns of MMP2 gene in the normal and disease state[9]. mRNA levels and activity of MMP2 were lower in patients with atherosclerotic and non-atherosclerotic TAA than control[113]. Elevated MMP2 levels were observed in aortic biopsies collected from small AAA[110]. Smooth muscle cells taken from patients with AAA showed increased MMP2 production in culture medium[111]. Isolated aortic fibroblast from $CaCl_2$-exposed TAA showed elevated expression of MMP2[112]. In addition, $MMP2^{-/-}$ mice did not develop AAA following adventitial $CaCl_2$ exposure[114]. However, the role of MMP2 in TAA has not been yet explored. The abundance and expression of MMP2 vary depending on not only the types but also the size of aortic aneurysm as such the direct role of MMPs including MMP2 is still not clear[115-117].

1.9.3 Role of MMP2 in TGFβ signaling pathway

One of the upstream regulators of ECM synthesis is the Transforming Growth Factor-beta (TGFβ) signaling pathway. TGFβ is a soluble anti-inflammatory cytokine that is synthesized by many cell types in its latent form. Latent TGFβ and latency-associated peptide (LAP) form a non-covalent complex called a small latent complex (SLC). SLC forms a disulphide bond with a latent TGFβ binding protein (LTBP) to form a large latent complex (LLC). This complex is sequestered to the ECM components including Fibrilin-1. In normal physiological state, Fibrilin-1 forms a cross-linkage with the LLC to stabilize its microfibriliar structure and sequesters TGFβ. Latent TGFβ can be activated by heat[118], acid[119], reactive oxygen species[120], and proteases[121-124]. MMP2, MMP9 and MT1-MMP have been shown to cleave the bond between LAP and LTBP to release TGFβ[121-123]. The released (active) TGFβ binds to type II TGFβ receptor (TGFβ R2) which then recruits and phosphorylates type I TGFβ receptor (TGFβ R1). Subsequently, the activated TGFβ R1 phosphorylates and activates Smad2/3. Following phosphorylation, Smad2/3 interacts with Smad4 and then this complex translocates to the nucleus where regulation of matrix-associated protein expression which includes elastin, collagen and MMPs occurs (Figure 1. 6)[125-127].

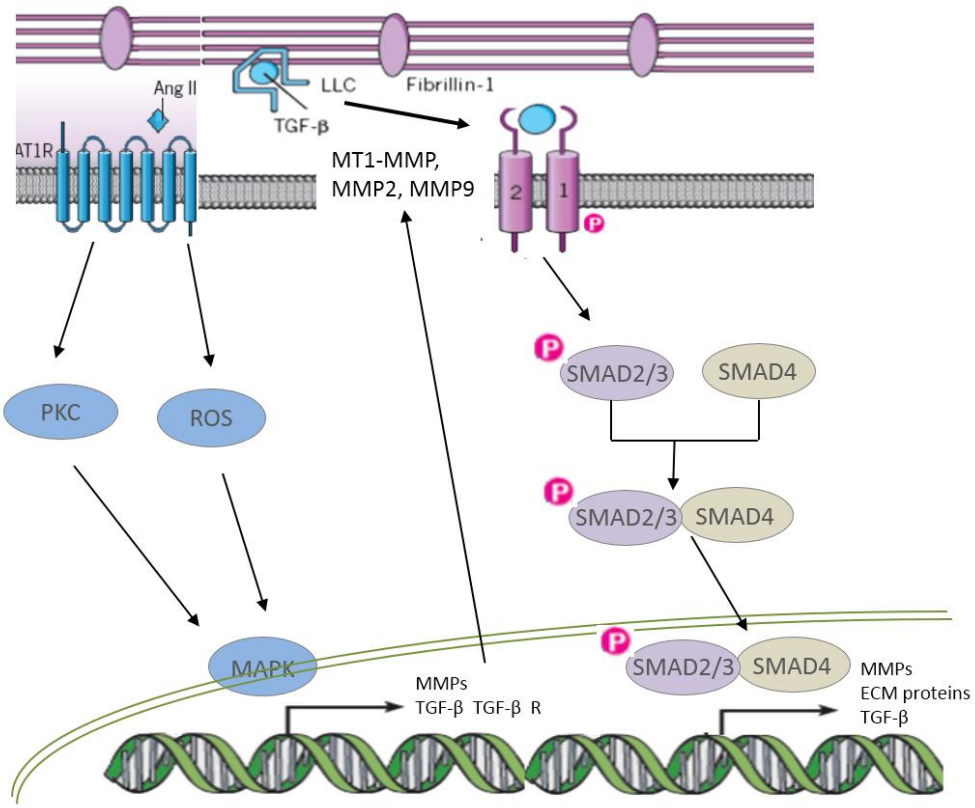

Figure 1.6 TGFβ-Smad signaling pathway.

The TGFβ binds to its receptor and activates Smad signaling pathway which regulates matrix-associated protein expression. Modified from Lindsay & Dietz[128].

TGFβ participates in various cellular responses such as proliferation[129], angiogenesis via regulating proliferation and migration of endothelial cells[130, 131], would repair, apoptosis[132, 133] and inflammation[134]. In addition, TGFβ plays a crucial role in vascular remodeling via regulating matrix deposition and degradation. TGFβ1$^{-/-}$ mice die about 20 days after birth due to inflammatory responses[135]. Mutations in TGFβ receptor genes have been observed in patients with AAA[136]. TGFβ expression is upregulated in abdominal aneurysmal aortic wall[137].

On the other hand, complementary DNA level of TGFβ is significantly decreased in patients with AAA compared to patients with TAA or normal aorta[105]. Patients with Marfan syndrome (mutation in Fibrilin-1) showed increased circulating TGFβ1 and TGFβ receptor 2 levels compared to control group[115, 138]. In TAA samples taken from Marfan syndrome patients, a decrease in MMP2 activity was found compared to normal aortic samples[109]. Patients with Loeys-Dietz syndrome (mutation in the gene coding for TGFβ receptor 1 or receptor 2) showed increased activity of TGFβ[139, 140]. Interestingly, patients with heterozygous mutations of TGFβ receptor 1 or 2 showed enhanced TGFβ signaling in the aortic wall[139]. Alterations in TGFβ signaling pathway is examined in patients with AAA. Since TGFβ signaling pathway has been shown to stimulate collagen and elastin production, these alterations in TGFβ signaling molecules might be the mechanism underlying reduction in elastin and collagen in aneurysmal aortic wall[126, 141]. Histological analyses showed decreased elastin content and fragmented elastin fiber in aortic tissue from patients with Marfan syndrome[142]. Moreover, alteration of collagen architecture has been observed in patients with AAA and TAA associated with Marfan syndrome[143].

In addition, VSMCs from rat thoracic aorta show enhanced Smad activation through AT1 receptor following Ang II treatment[144]. In another study, Ang II-induced activation of latent TGFβ1[145] and phosphorylation of Smad2 and Smad3[146, 147] has been reported in kidney and cardiac fibroblast[148]. In cardiac fibroblast, losartan (AT1 blocker) attenuates activation of TGFβ1[149].

1.10 The renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system (RASS) that regulates blood pressure, fluid and electrolyte homeostasis in body. Alterations in the RASS has been reported to promote physiological and pathological changes in the cardiovascular diseases[150]. Angiotensin, which is released from the liver, is cleaved by renin, which is a proteolytic enzyme secreted from the granulated cells of the juxtaglomerular cells, and forms the decapeptide Angiotensin I (Ang I) (Figure 1.7)[151-153]. Subsequently, angiotensin converting enzyme (ACE) converts Ang I to Ang II. Ang I and Ang II can be cleaved by ACE2 to generate Ang-(1-7). ACE2 converts Ang I to Ang-(1-9) which then subsequently degraded to Ang-(1-7) by ACE. ACE2 also coverts Ang II to Ang-(1-7)[154, 155]. Ang II predominantly binds to angiotensin II receptor, type-1 (AT1) and promotes vasoconstriction, cell growth and oxidative stress[156-159]. Ang II also binds to angiotensin II receptor, type-2 (AT2) and promotes vasodilation, nitric oxide (NO) release, and apoptosis[158-161]. Ang-(1-7) binds to Mas, G protein-coupled receptor to mediate vasodilation, anti-proliferation and anti-hypertrophy[162-164].

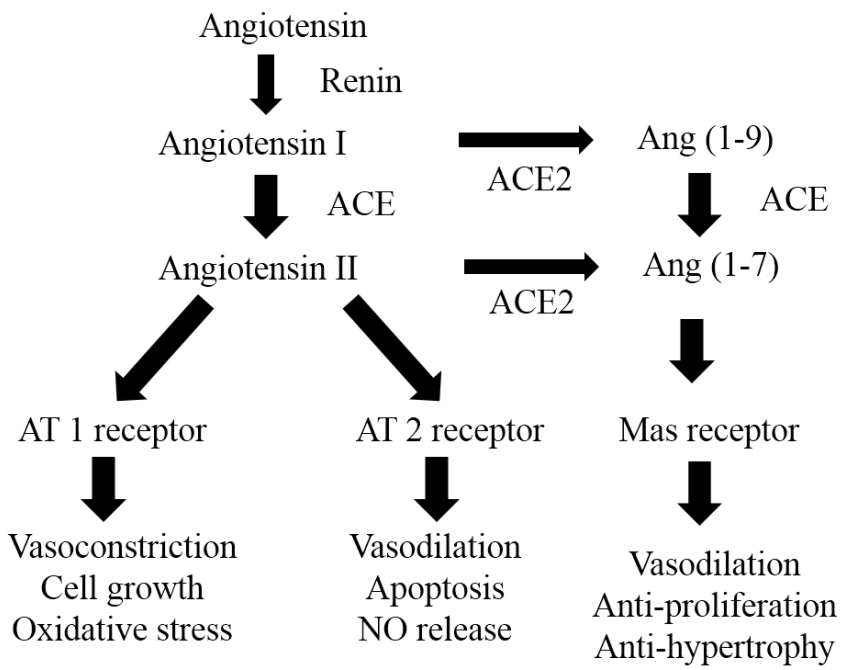

Figure 1.7 The renin-angiotensin-aldosterone system

ACE : angiotensin converting enzyme, AT 1 receptor : angiotensin II receptor type 1, AT 2 receptor : angiotensin II receptor type 2. Adapted from Lemarie & Schiffrin[165].

Ang II, primary agonist of the RAAS, affects function of many organs including heart, kidneys, and vasculature. Ang II induces nitric oxide (NO) synthesis[166], inflammation[167, 168], hypertrophy[169, 170], and ECM remodeling[171, 172] in VSMCs, endothelial cells, and cardiomyocytes. Ang II induced elevations in pro-inflammatory cytokines (TNF-α)[173] and oxidative stress in turn upregulate expression of MMP2[174, 175]. Co-localization of Ang II and MMP2 atherosclerotic lesions has been reported[176]. Upregulation of Ang II has been shown to promote aneurysm formation in the aorta[177-179].

1.11 Animal studies on aortic aneurysm

1.11.1 Experimental models to induced aortic aneurysm

Several experimental models have been used to study aortic aneurysm in rodents. Intraluminal elastase perfusion or adventitial calcium chloride ($CaCl_2$) exposure models have been used to induce aortic aneurysm[180-182]. Since disruption of elastin structure is commonly observed in the aorta from patients with aortic aneurysm, elastase is used to mimic the pathology of the disease. Elastase perfusion model is mostly used to study AAA. Abdominal aorta is clamped near the renal vein and the segment of isolated aorta is perfused with elastase through an aortotomy at the bifurcation. Elastase is perfused for 5 minutes from the lumen to the adventitia wall of the aorta[180, 183]. Since the elastase is extracted from porcine pancreas, it is hard to conclude that development of aortic aneurysm is purely due to elastin disruption by elastase or inflammatory response against porcine elastase. In addition, elastase perfusion model is invasive and it can only applied to the specific region of the aorta to induce internal injury. Thus, this is not the best experimental model to study underlying mechanism on the formation of aortic aneurysm.

$CaCl_2$ exposure model is widely used to study AAA and TAA. $CaCl_2$ is applied for 15 minutes on the outer surface of the abdominal aorta between renal arteries and the bifurcation of the iliac arteries for AAA or distal half of descending thoracic aorta for TAA[181, 182]. $CaCl_2$ leads to structural degradation of medial layer by activating MMPs which have binding site for calcium[184, 185]. In addition to dilation of the lumen of the aorta, aortic wall becomes more susceptible to aneurysm

development. Similar to elastase infusion model, this model is limited to examine systematic effect of $CaCl_2$ in the development of aortic aneurysm.

Another experimental model of aortic aneurysm is systemic infusion of Ang II via an osmotic pump allowing the release of Ang II into the entire body at a constant rate[186]. This allows examination of the systematic effect of Ang II in the development of aortic aneurysm in both abdominal and thoracic regions at the same time. Since Ang II is a physiological hormone, which is elevated in patients with cardiovascular disease[187,188], this experimental model is physiologically relevant. Therefore, Ang II infusion is the most promising study model to induce aortic aneurysm in animal model to examine underlying mechanisms of the development of aortic aneurysm.

1.11.2 Genetically engineered mice

In order to understand the underlying mechanisms of aortic aneurysm, genetically engineered mice have been used. Whole body knockout mice that show alterations in ECM components are widely used to study aortic aneurysm. TIMP1$^{-/-}$ mice exhibited increased TAA size compared to wild-type following $CaCl_2$ infusion[189]. In addition, TIMP3$^{-/-}$ mice developed AAA following Ang II infusion for 4 weeks and by deleting both TIMP3 and MMP2, mice with Ang II developed more severe AAA than TIMP3$^{-/-}$ mice[177]. MMP2$^{-/-}$ and MMP9$^{-/-}$ mice did not show dilation of the abdominal aorta following $CaCl_2$ infusion[114]. However, there is no reports on TAA formation in MMP2$^{-/-}$ mice. In a different study, MMP9$^{-/-}$ mice were protected against TAA following $CaCl_2$ infusion[181].

As prominent ECM components in aortic wall, mice with defects in collagen or elastin structures are used to study aortic aneurysm. Lysyl oxidase (Lox) maintains ECM integrity by initiating covalent cross-linking of collagen and elastin[190]. Most of $Lox^{-/-}$ mice died at the end of gestation or as neonates but live born pups showed large aortic aneurysm[191]. Deletion of Fibrilin-1, which is essential for elastin assembly, has been used as an animal model of aortic aneurysm associated with Marfan syndrome[192, 193].

Hyperlipidemic mice are also used to study atherosclerotic aortic aneurysm. Following high fat diet, $Apolipoprotein^{-/-}$ ($ApoE^{-/-}$) mice developed AAA with atherosclerotic lesions[194-196]. $ApoE^{-/-}$/;$MMP3^{-/-}$ mice showed less frequent TAA but larger atherosclerotic lesions in the abdominal aorta was observed compared to $ApoE^{-/-}$/;$MMP3^{+/+}$ mice following a high fat diet for 6 months[195]. In addition, $ApoE^{-/-}$/;$TIMP1^{-/-}$ mice showed less atherosclerotic lesions throughout the aorta but more frequent AAA and TAA compared to $ApoE^{-/-}$/;$TIMP1^{+/+}$ mice[194].

1.12 Hypothesis and objective

1.12.1 Hypothesis

Since MMP2 can degrade a number of ECM proteins, such as collagen and elastin fibers, $MMP2^{-/-}$ mice will be protected against excess proteolysis and aortic aneurysm following Ang II infusion, due to the suppressed ECM-degrading function of MMP2.

1.12.2 Objective

The main objective of this study was to examine the role of MMP2 in vascular remodeling and development of aortic aneurysm. This would provide crucial information on the underlying mechanism and therapeutic interventions for aortic aneurysm.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal Care

WT and MMP2-deficient ($MMP2^{-/-}$)[197] mice in C57BL/6 background (The Jackson Laboratory; Bar Harbor, MN, USA) were housed in our animal facility at University of Alberta. WT and $MMP2^{-/-}$ mice were bred to generate WT, heterozygous, and homozygous knockout age-matched littermates. All animal experiments were performed in accordance with Canadian Council on Animal Care Guidelines and regulations of Animal Policy and Welfare committee at University of Alberta.

2.2 Genotyping

2.2.1 Isolation of DNA from toe

Small piece of toe was cut from mice at 2 weeks of age. Toe was placed in eppendorf tube with 175 µL of toe digestion buffer (Table 2.1) and 0.12mg of Proteinase K and incubate overnight at 56 ℃ for digestion. Samples were chilled on ice for 5 minutes. 67.5 µL of saturated 5 M NaCl was added and samples were mixed by inversion. Samples were centrifuged at 14,500 $\times g$ for 15 minutes at 4 ℃. Supernatant was transferred to eppendorf tube. 150 µL of isopropanol was added. Tubes were gently inverted to precipitate DNA. Samples were centrifuged at 14,000 $\times g$ for 15 minutes at 4 ℃. The supernatant was removed and discarded. 0.25 mL of cold 70% ethanol was added to wash pellet and samples were pipetted until the pellet was completely dislodged. Samples were centrifuged at 14,000 $\times g$ for 15 minutes at 4 ℃. The supernatant was removed and discarded. 20 µL of TE buffer was added to re-suspend pellet. 2 µL of DNA was diluted into 200 µL of dH_2O for quantification.

Table 2.1 Toe digestion buffer

Chemical name	M.W. (g/mol)	$Conc._{stock}$	$Conc._{final}$
Tris-HCl	121.14	N/A	50 mM
Sodium chloride (NaCl)	58.44	N/A	100 mM
SDS	288.38	N/A	1%
EDTA	372.24	N/A	100mM
Proteinase K	N/A	5mg/mL	0.4mg/mL

2.2.2 MMP2 Multiplex Polymerase chain reaction (PCR)

Primer sequences are shown in Table 2.2[197, 198]. Reaction mix was prepared and ran for PCR reaction (Table 2.3 and 2.4). Upon completion of PCR reaction, 6× DNA loading dye (Thermo Scientific; Willmington, DE, USA.) was added to samples. Samples were loaded into 1.5% agarose in TAE buffer containing SYBR® Safe DNA Gel Stain (Invitrogen; Burlington, ON, Canada.) and ran for 30 minutes at 80V. Gel was scanned using ImageQuant LAS 4000 (GE ImageQuant LAS 4000; GE).

Table 2.2 Primer sequences for MMP2 genotyping

Primer sequences 5’ to 3’	
MMP2 WT	CAG GGA GCC AGA ACA GAA GTG GGA
MMP2 Anchor	CCA GGT CGT GAG AAG GCA CAG A
MMP2 NeoA	TGG CGG CGA ATG GGC TGA

Table 2.3 Reaction mix for MMP2 genotyping

ddH_2O	17.5 µL
10x PCR Buffer	2.5 µL
$MgCl_2$ (50mM)	1 µL
dNTP (10µM)	0.5 µL
MMP2 WT (10pmol/µL)	0.75 µL
MMP2 Anc (10pmol/µL)	0.75 µL
MMP2 NeoA (10pmol/µL)	0.75 µL
Taq (5U/µL)	0.25 µL
DNA	1 µL

Table 2.4 Polymerase chain reaction (PCR) program for MMP2 genotyping

	PCR Program	
1	94 ℃	2 min
2	94 ℃	1 min
3	67 ℃	1 min
4	72 ℃	1 min
5	Repeat step 2-4 x40	
6	72 ℃	10 min
7	4 ℃	10 min

2.3 Experimental animal disease model

Eight-week old male WT and MMP2$^{-/-}$ mice were anesthetized with 1% isoflurane using 100% oxygen. A 1 cm incision was made on dorsal region and Alzet micro-osmotic pumps (Model 1002, Durect Co.) were implanted subcutaneously to deliver 1.5 mg/kg/day of Angiotensin II (Ang II, Sigma-Aldrich; Oakville, ON, Canada) [199-201], 30mg/kg/day of Phenylephrine (PE, Sigma-Aldrich; Oakville, ON, Canada) or saline (control). The incision was closed using a 6-0 silk suture. The pumps were replaced at day 14 to allow for continuous infusion for 28 days.

2.4 Tissue Collection

2.4.1 Mortality and autopsy

WT and MMP2$^{-/-}$ mice were monitored regularly and autopsy was performed on each mouse found dead throughout the course of the experiment. Mortality due to aortic rupture was confirmed by the presence of blood clot in the chest cavity close to the heart. The aorta was isolated and confirmed directly under a stereomicroscope.

2.4.2 Tissue collection from WT and MMP2$^{-/-}$ mice

At 2 weeks or 4 weeks after Ang II infusion, mice were anesthetized with 0.2 mL of Ketamine-Xylazine combination (2 mL of 100 mg/mL Ketamine stock, 1mL of 20 mg/mL Xylazine stock, and 7mL of Normal saline 0.9% (Hospira Inc. Montreal. QC. Canada). Prior to any tissue collection, status of anesthesia was checked by pinching toes to confirm that the animal was in surgical plane. The heart, the entire aorta from the right beneath of the atria to the suprarenal arteries, mesenteric arteries, and carotid arteries were harvested. Samples were flash-frozen in liquid nitrogen and stored at -80 ℃ for further molecular analyses. Tibial length was measured as a measure of growth. Body weight, heart weight and kidney weight were measured and normalized to tibial length to examine hypertrophy in response to Ang II treatments.

2.5 *In vivo* aortic structure and function assessment

2.5.1 Tail Cuff Blood Pressure Measurement

Blood pressure was recorded using the non-invasive tail-cuff system (iiTC Life Sciences; Woodland Hills, CA). Mice were acclimatized be being placed in the restrainer tube 3 days prior to the actual blood pressure measurement (10 minutes/day). Baseline blood pressure was measured over one week before implantation of osmotic pumps (three measurements). Temperature inside chamber was maintained at 30 ℃. During the first 2 weeks of Ang II infusion, systolic blood pressure, diastolic blood pressure and heart rate were measured. In order to minimize any variations due to circadian rhythm, measurement was done at the same time of the day. Each measurement was done triplicates.

2.5.2 Ultrasound Imaging of aorta

In vivo systolic and diastolic aortic diameters of thoracic, proximal and abdominal regions were measured non-invasively by ultrasound recording (Vevo770 high-resolution in vivo micro-imaging system; VisualSonics, Toronto, Canada) as previously described[202, 203]. Mice were anaesthetized 1% isoflurane in 100% oxygen. Hair was removed using hair removal cream. Ultrasound gel was applied on the chest and abdomen of the mouse. The aortic diameters were measured by M-mode at three different regions: between Brachiocephalic artery and left common carotid artery for thoracic diameter, between aortic arch and the diaphragm for proximal diameter, and suprarenal region for abdominal diameter. The maximum aortic lumen diameter (aortic systolic diameter corresponding to cardiac systole) and the minimum aortic lumen diameter (aortic diastolic diameter

corresponding to cardiac diastole) were measured. Aortic expansion index (%) was calculated using the following equation:

$$\text{Aortic expansion index (\%)} = \frac{\text{systolic aortic diameter} - \text{diastolic aortic diameter}}{\text{systolic aortic diameter}} \times 100$$

2.6 Histological analysis

For histological analysis, 1mL formalin was directly injected from the left atrium and aorta was slowly perfuse-fixed with formalin (Buffered, 10%, phosphate buffer) for 30 minutes at 80mmHg. Aorta was carefully harvested and cleaned for any adipose tissue and fixed in formalin for 48 hours. Perfuse-fixed aortas were paraffin-embedded for Gomori Trichrome (Alberta Diabetes Institute Histology Core; University of Alberta, Edmonton, AB, Canada) and Verhoeff-Van Gieson (VVG) staining (University of Alberta Department of Laboratory Medicine and Pathology; Edmonton, AB, Canada).

2.6.1 Gomori Trichrome (GT) stain

Paraffin-embedded aortas were cross-sectioned (5μm in thickness) for Gomori trichrome staining (Alberta Diabetes Institute Histology Core; University of Alberta, Edmonton, AB, Canada). Slides were deparaffinised and hydrated with distilled water. Slides were placed in 60 °C Bouin's solution (Sigma-Aldrich; Oakville, ON, Canada) for 30 minutes and cool it at room temperature for another 30 minutes. Slides were washed with water for 5 minutes and stained with trichrome stain (1.2 g of chromotrope 2R, 0.6 g of light green SF, 1.6 g of dodecatungstophospho -ric acid, and 20 mL of distil water) for 20 minutes. Slides

were placed in 0.5% glacial acetic acid for 2 minutes and dehydrated through 100% ethanol.

2.6.2 Verhoeff-Van Gieson (VVG) stain

Slides were deparaffinised and hydrated with water. Slides were placed in Verhoeff's solution (20 mL of 5% alcoholic hematoxylin, 8mL of 10% ferric chloride and 8mL of Weigert's iodine solution (2% potassium iodide and 1% iodine in distill water)) for 1 hour. Slides were rinsed with water and incubated with 2% ferric chloride for 2 minutes. Slides were washed with water and treated with 5% sodium thiosulphate for 1 minute. Slides were washed in water for 5 minutes and counterstained in Van Geison's solution (15 mL of 1% aqueous acid fuschin, 50 mL saturated aqueous picric acid and 50mL of distill water) for 3 minutes. Slides were dehydrated in 95% and 100% alcohol and cleared in xylene for 3 minutes each.

2.7 RNA expression analysis

2.7.1 RNA extraction

Prior to any molecular work, blood in the vessels were flushed away. Any adipose tissue or nerves were removed as well. For aorta, side branches including brachiocephalic artery, left and right common carotid arteries were removed. Samples were homogenized by using tissue crusher. 500 µl of ice-cold Trizol (Invitrogen; Burlington, ON, Canada) was added to each tube. Tubes were incubated in ice for 5 minutes and centrifuged at 12,000 $\times g$ for 10 minutes at 4 ℃. Supernatant was transferred to new tube and 500 µl of ice-cold Trizol was added to

each tube and the mixture was pipetted up and down. Tubes were incubated in ice for 5 minutes and centrifuged at 12,000 ×*g* for 10 minutes at 4 ℃. Supernatant was transferred to the tube with which contains supernatant from previous step and 200 µl of chloroform was added. Tubes vigorously were shook for 15 seconds and incubated in ice for 5 minutes. Tubes were centrifuged at 12,000 ×*g* for 15 minutes at 4 °C and the upper colorless phase was transferred to new tube. 500µl of isopropanol was added to each tube and tubes were incubated at -20 ℃ for 7 days. After incubation, tubes were centrifuged at 12,000 ×*g* for 10minutes at 4 ℃. The supernatant was removed and discarded. 1mL of 75% ethanol was added to each tube and the mixture was pipetted until the pellet was completely dislodged. Tubes were centrifuged at 7,500 ×*g* for 5 minutes at 4 ℃ and the supernatant was removed and discarded. The pellet was dried for 5 minutes to remove any ethanol remnant. 12 µl of RNA –free water was added to dissolve RNA. 1 µl of RNA was loaded to NanoDrop 1000 Spectrophotometer (Thermo Scientific; Willmington, DE, USA) to measure the concentration. Samples were stored at -80 ℃.

2.7.2 Taqman RT-PCR

In order to generate complementary DNA (cDNA), RNA was reverse transcribed. PCR water was added to 1 µg of RNA to make final volume of 9 µl. 2 µl of random hexamers was added. Samples were incubated at 70 ℃ for 10 minutes and centrifuged briefly at 10,000 ×*g* at 4 °C. 9 µl of mixture (4 µl of 5× buffer, 2 µl of DTT from 0.1M stock, 1 µl dNTP from 25 mM stock, 1 µl SuperScript II Reverse Transcriptase(Invitrogen; Burlington, ON, Canada), 1 µl RNase inhibitor from 40 U/µl stock(Invitrogen; Burlington, ON, Canada). Tubes were incubated at

40 ℃ for 1 hour. 396 µl of PCR water was added to 4 µl of RT products. 10µl of 6 serial dilutions of standard (0.625, 1.25, 2.5, 5, 10 and 20µg) were added to each well of 384-well white bottom plates. 4.17µl of Mastermix (Applied Biosystems; Carlsbad, CA, USA), 0.35µl of forward primer, 0.35µL of reverse primer, 0.35µl of probe, 2.78µl of H2O, and 5µl of sample cDNA were added to each well. All samples were run in triplicates. Optical adhesive film was used to cover the plate. Real-time PCR reaction was performed for 2 hours using the LightCycler® 480 Real-Time PCR System. Readings were normalized by control HPRT (hypoxanthine-guanine phosphoribosyltransferase-1). The primers and probes of genes used in our study are listed in Table 2.5.

Table 2.5 Taqman primers and probe sequences.

Gene	Primer/Probe	Sequence
HPRT	Forward:	5'-AGC TTG CTG GTG AAA AGG AC-3'
	Reverse:	5'-CAA CTT GCG CTC ATC TTA GG-3'
	Probe:	5'-FAM-CAA CAA AGT CTG GCC TGT ATC CAA C-TAM RA-3'
TIMP1	Forward:	5'-CAT GGA AAG CCT CTG TGG ATA TG-3'
	Reverse:	5'-AAG CTG CAG GCA CTG ATG TG-3'
	Probe:	5'-FAM-CTC ATC ACG GGC CGC CTA AGG AAC-TAM RA-3'

TIMP2	Forward: Reverse: Probe:	5'-CCA GAA GAA GAG CCT GAA CCA-3' 5'-GTC CAT CCA GAG GCA CTC ATC-3' 5'-FAM-ACT CGCT GTC CCA TGA TCC CTT GC-TAM RA-3'
TIMP3	Forward: Reverse: Probe:	5'-GGC CTC AAT TAC CGC TAC CA-3' 5'-CTG ATA GCC AGG GTA CCC AAA A-3' 5'-FAM-TGC TAC TAC TTG CCT TGT TTT GTG ACC TCC A-TAM RA-3'
TIMP4	Forward: Reverse: Probe:	5'-TGC AGA GGG AGA GCC TGA A-3' 5'-GGT ACA TGG CAC TGC ATA GCA-3' 5'-FAM-CCA CCA GAA CTG TGG CTG CCA AAT C-TAMRA-3'
MMP2	Forward: Reverse: Probe:	5’-AAC TAC GAT GAT GAC CGG AAG TG-3’ 5’-TGG CAT GGC CGA ACT CA-3’ 5’-FAM-TCT GTC CTG ACC AAG GAT ATA GCC TAT TCC TCG-TAM RA-3’
MMP9	Forward:	5’-CGA ACT TCG ACA CTG ACA AGA AGT -3’

	Reverse: Probe:	5’- GCA CGC TGG AAT GAT CTA AGC-3’ 5’-FAM-TCT GTC CAG ACC AAG GGT ACA GCC TGT TC-TAM RA-3’
IL-1β	Forward: Reverse: Probe:	5'-AAC CTG CTG GTG TGT GAC GTT C-3’ 5'-CAG CAC GAG GCT TTT TTG TTG T-3’ 5'FAM-TAG ACA GCT GCA CTA CAG GCT CCG AGA TG-TAMRA-3’
IL-6	Forward: Reverse: Probe:	5'-ACA ACC ACG GCC TTC CCT ACT T-3’ 5'-CAC GAT TTC CCA GAG AAC ATG TG-3’ 5'FAM TTC ACA GAG GAT ACC ACT CCC AAC AGA CCT-TAMRA-3’
pro-collagen I-α1	Forward: Reverse: Probe:	5’-CTTCACCTACAGCACCCTTGTG-3’ 5’-TGACTGTCTTGCCCCAAGTTC-3’ 5’-FAM-CTGCACGAGTCACACC-TAMRA-3’
MCP-1	Forward: Reverse: Probe:	5’- GTT GGC TCA GCC AGA TGC A-3’ 5’-AG CCT ACT CAT TGG GAT CAT CTT G-3’ 5’-FAM-TTAACGCCCCACTCACCTGCTGCTACT-TAMRA-3’
Elastin		Applied Biosystems Assay ID Mm00514670_m1

		(premixed primers/probe)
TGF-β		Applied Biosystems Assay ID Mm01178819_m1 (premixed primers/probe)

TGF-β: transforming growth factor-beta; TNF-α: tumor necrosis factor-alpha; MCP-1: monocyte chemoattractant protein-1; IL-1β: interleukin-1 beta; IL-6: interleukin-6;

2.8 Protein analysis

2.8.1 Protein extraction

Samples were homogenized by using tissue crusher. Liquid nitrogen was constantly applied to prevent samples from thawing. Eppendorf containing homogenized sample was centrifuged for 5 seconds. 80 µl of RIPA without EDTA extraction buffer (Table2.6) containing protease inhibitor (Cocktail Set III; Calbiochem; San Diego, CA, USA), phosphatase inhibitors (Cocktail Set II; Sigma-Aldrich, Oakville, ON, Canada, and Cocktails Set IV; Calbiochem; San Diego, CA, USA) was added to each tube. For elastase activity assay, Cytobuster Protein Extraction Buffer (Novagen; Madison, WI, USA) was used to extract protein. Tubes were vortexed for 1 minute and kept in the ice for 5 minutes. This step was repeated three times. Samples were centrifuged at 14,000 $\times g$ for 15 minutes at 4 ℃. Supernatant was transferred to a new tube and samples were dilute in 1 to 5 ratios for quantification.

Table 2.6 RIPA Protein extraction buffer pH 7.4 in ddH_2O

Chemical name	M.W. (g/mol)	$Conc._{stock}$	$Conc._{final}$
Tris-HCl	121.14	N/A	50 mM
Sodium chloride (NaCl)	58.44	N/A	150 mM
Triton X-100 (detergent)	624.00	N/A	1%
SDS (detergent)	288.38	N/A	0.1%
NP40 (detergent)	N/A	N/A	1%
Sodium deoxycholate (detergent)	414.55	N/A	1%

2.8.2. Protein quantification

5 µl of protein standard (series dilution of BSA in ddH2O starting from 2.0 µg/mL) and samples to each well of 96-well clear bottom plates. Protein concentration was measured by using the Bio-Rad DC protein assay kit (Bio-Rad; Missisauga, ON, Canada). 25 µl of Solution A' (mixture of 1000 µl of Solution A to 20 µl of Solution B) and 200 µl of Folin Reagent were added to each well. All samples were run in triplicates. The plate was wrapped with foil and incubated at the room temperature for 10 minutes. Absorbance was measured at 750 nm using SoftMax Pro. Based on the absorbance (optical density), concentration of protein was calculated. Data was plotted in scatter plots with concentration on X-axis and optical density on Y-axis. Actual concentration was calculated based on the slope

of the regression/trend line. Samples were stored at -80℃ for western blot or *in vitro* gelatinase analyses.

2.8.3 Western blotting

Samples were prepared by combining 25 µg of protein, protein loading buffer (Table.2.7) and PBS (Table.2.8) and boiled for 5 minutes to denature the protein. Samples were loaded into 5% sodium dodecyl sulphate-based polyacrylamide (SDS) stacking gel and separated through SDS loading gel (8-15% gradient gel) via electrophoresis at 120V (Bio Rad; Mississauga, ON, Canada) in the presence of running buffer solution (Table 2.9).

Table 2.7 Sample loading buffer pH 6.8 in ddH_2O - Western blot

Chemical name	M.W. (g/mol)	$Conc._{stock}$	$Conc._{final}$
Tris-HCl	121.14	130 mM	65 mM
SDS	288.38	4.6%	2.3%
Bromophenol Blue	669.96	0.2%	0.1%
Glycerol	92.09	20%	10%
Dithiothreitol (DTT)	154.25	2%	1%

Table 2.8 Phosphate-buffered Saline (PBS) pH 7.4 in ddH_2O

Chemical name	M.W. (g/mol)	$Conc._{stock}$	$Conc._{final}$
Sodium chloride (NaCl)	58.44	1370 mM	137mM
Potassium chloride (KCl)	74.55	27 mM	2.7 mM
Sodium phosphate dibasic (Na_2HPO_4)	141.96	100 mM	10 mM
Potassium phosphate monobasic (KH_2PO_4)	136.09	18 mM	1.8 mM

Table 2.9 Running buffer pH 8.3 in ddH_2O

Chemical name	M.W. (g/mol)	$Conc._{stock}$	$Conc._{final}$
Tris-HCl	121.14	250 mM	25 mM
Glycine	75.07	1920 mM	192 mM
Sodium dodecyl sulfate (SDS)	288.38	10%	1%

Samples was transferred polyvinylidene fluoride (PVDF) membrane at 200 mAs for 90 minutes in the presence of transfer buffer solution (Table 2.10). The gel was stained with commasie blue staining solution (Table 2.11) for 2 hours at room temperature and destained with water. This gel was used for loading control. The membrane was blocked with 5% skim milk in TBS (Table 2.12) with 0.1% Tween (TBST) for 2 hours at room temperature.

Table 2.10 Transfer buffer pH 8.3 in ddH_2O

Chemical name	M.W. (g/mol)	$Conc._{stock}$	$Conc._{final}$
Tris- HCl	121.14	200 mM	20 mM
Glycine	75.07	1500 mM	150 mM
Methanol	32.04	N/A	20%

Table 2.11 Coomassie blue staining solution in ddH_2O

Chemical name	M.W. (g/mol)	$Conc._{stock}$	$Conc._{final}$
Coomassie Brilliant Blue	825.97	N/A	2%
Methanol	32.04	N/A	25%
Acetic acid	60.05	N/A	10%

Table 2.12 Tris-buffered Saline (TBS) pH 8.0 in ddH_2O

Chemical name	M.W. (g/mol)	$Conc._{stock}$	$Conc._{final}$
Sodium chloride (NaCl)	58.44	1250 mM	125 mM
Tris-HCl	121.14	250 mM	25 mM

Primary antibody, diluted in 5% skim milk in TBST, was applied overnight at 4 ℃. Next day, the membrane was washed with TBST three times for 10 minutes to remove leftover primary antibody. Appropriate species-based horse radish peroxidase (HRP) linked-secondary antibody, diluted 5% skim milk in TBST, was applied for 2 hours at room temperature. The membrane was washed with TBST three times for 10 minutes. Enhanced Chemiluminescence prime (GE Amersham; Baie d'Urfe, QC, Canada) was applied to the membrane for 5 minutes and the

membrane was developed and imaged using a luminescent image analyzer housing a chemiluminescence-sensitive camera. (GE ImageQuant LAS 4000; GE). The PVDF membrane was then stripped with a mild stripping buffer (Table 2.13) for 30 min at 55 ℃ for subsequent immunoblotting of the membrane.

Table 2.13 Western blot Membrane Stripping buffer pH 6.8 in ddH_2O

Chemical name	M.W. (g/mol)	Conc.$_{stock}$	Conc.$_{final}$
Tris-HCl	121.14	1000 mM (pH 6.8)	62.5 mM
SDS	288.38	20%	2%
β-mercaptoethanol	78.13	14300mM	100mM

2.8.4 *In vitro* Gelatin zymography

Samples were prepared by combining 20 μg of protein, protein loading buffer (Table.2.14) and PBS (Table.2.8). Samples were quickly centrifuged and loaded to 8% gelatin-based polyacrylamide gel. HT-1080, a fibrosarcoma cell line which produce high levels of MMPs, in particular MMP2 and MMP9, was used as a positive control. The gel was ran at 60 mA for 75 minutes. After electrophoresis, the gel was rinsed with 2.5% Triton X-100 for 1 hour to remove remaining SDS. After another washing with ice-cold substrate buffer (Table 2.15) for 40 minutes, the gel was incubated in cold substrate buffer (Table 2.16) at 37 ℃ for 48 hours. The substrate buffer was changed after first 24-hour incubation. The gel was stained with staining solution (Table 2.11) overnight at room temperature. The gel was destained for 1 hour using destaining solution (Table 2.17) and imaged using a luminescent image

analyzer housing a chemiluminescence-sensitive camera. (GE ImageQuant LAS 4000; GE).

Table 2.14 Sample loading buffer pH 6.8 in ddH_2O - Zymography)[38]

Chemical name	M.W. (g/mol)	Conc.$_{stock}$	Conc.$_{final}$
Tris-HCl	121.14	125 mM	62.5 mM
Glycerol	92.09	20%	10%
SDS	288.38	4%	2%
Bromophenol Blue	669.96	0.02%	0.01%

Table 2.15 Substrate buffer in ddH_2O - Zymography

Chemical name	M.W. (g/mol)	Conc.$_{stock}$	Conc.$_{final}$
Tris-HCl	121.14	2000 mM	50 mM
Calcium chloride ($CaCl_2$)•$2H_2O$	147.02	2000 mM	5 mM
Sodium chloride (NaCl)	58.44	N/A	150 mM
Sodium azide (NaN_3)	65.01	5%	0.05%

Table 2.16 Polyacrylamide Gel Staining solution in ddH_2O

Chemical name	M.W. (g/mol)	Conc.$_{stock}$	Conc.$_{final}$
Coomassie blue	854.00	N/A	2%
Methanol	32.04	N/A	25%
Acetic acid	60.05	N/A	10%

Table 2.17 Polyacrylamide Gel Destaining solution in ddH_2O

Chemical name	M.W. (g/mol)	Conc.$_{stock}$	Conc.$_{final}$
Methanol	32.04	N/A	30%
Acetic acid	60.05	N/A	1%

2.8.5 Quantification of bands on Western blots and gelatin zymography

Quantification of the protein bands on either the western blot or zymography was measured using densitometry analysis software (ImageQuant TL 7.0; GE). Intensity of target bands relative to the background were measured. This value was normalized to the density of its corresponding loading control.

2.9 Elastase *in vitro* activity assay

Total elastase activity was measured using fluorescent-based EnzChek® Elastase Assay Kit (Invitrogen; Burlingtion, ON, Canada). Soluble elastin from the kit is labeled with dye which quench fluorescence from conjugate and non-fluroescent substrate is digested by elastase which gives off fluorescence. Proteins were extracted using Cytobuster Protein Extraction Buffer (Novagen; Madison, WI, USA). 50 µl of 1× reaction buffer, 50 µl of 100 µg/mL of elastin working solution, and 100 µl of the diluted elastase were added to each well. 35 µg of protein was added to each well. Fluorescence was measured every 10 minutes for 4 hours with excitation at 485 nm and emission at 520 nm using SoftMax Pro. Data was plotted in scatter plots with time on X-axis and amount of fluorescence on Y axis. Actual activity was calculated based on the slope of the regression/trend line.

2.10 Isolation of vascular smooth muscle cells (VSMCs) for cell culture

Mice were injected with 50 µL Heparin (10 units/ml; LEO Inc. Thornhill. ON. Canada) to prevent any blood clot formation inside of the aorta. After 10 minutes, mice were anesthetized with 0.2 mL of Ketamine-Xylazine combination (2 mL of 100 mg/mL Ketamine stock, 1mL of 20mg/mL Xylazine stock, and 7 mL of Normal saline 0.9%). Incision site was sterilized with 70% ethanol. Thoracic aorta (from the beneath of the atria to the diaphragm) and abdominal aorta (from the diaphragm to renal arteries) were carefully harvested in small Petri dishes containing 2 mL of PBS with gentamicin. Any adipose tissue or nerves were removed carefully. Aortic wall was exposed by cutting open in longitudinal way. Adventitial layer was peeled off with a forceps and endothelial layer was scraped off. The aorta was cut into small pieces (1mm × 1mm) and aortic explants were placed carefully into a Petri dish. The dish was incubated in a humidified atmosphere at 37 ℃ in 5% CO_2 and 95% O_2 for 15 minutes. 3 mL of Dulbecco's modified Eagle medium (DMEM; Invitrogen; Burlingtion, ON, Canada.) supplemented with 20% fetal bovine serum (FBS; Sigma-Aldrich; Oakville, ON, Canada), Gentamicin sulphate (Santa Cruz; Santa Cruz, CA, USA) and Fungizone (Invitrogen; Burlingtion, ON, Canada) were added to the dish. Dish was incubated for first 7 days and the medium was replaced every two days (DMEM with 10% FBS, Gentamicin sulphate and Fungizone). When cells reached greater than 90% confluency, cells were split 1:1 using 0.12% trypsin (Worthington Biochemical Corporation; Lakewood, NJ.) and 0.005% Ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich; Oakville, ON, Canada) in PBS.

2.11 Statistical analysis

An unpaired *t* test was used to compare the effects of regional heterogeneity in figure 3..1 Two-way analysis of variance (ANOVA) was performed to compare data sets with two factors (genotype and treatment). Two-way repeated-measures ANOVA analysis was performed for blood pressure data in Figures 3.5 and 3.21. Statistical analyses were performed using SPSS software (Chicago, Illinois, Version 10.1). Normality test (Sahpir-Wilk) was performed to confirm normal distribution of all data. Averaged values are represented as mean ± SEM. Statistical significance is recognized at $p<0.05$.

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CHAPTER 3

RESULTS

3.1 Regional heterogeneity of the expression levels of ECM structural proteins, MMPs and TIMPs between thoracic and abdominal aortas from WT mice

To examine regional heterogeneity of expression levels of ECM structural proteins, MMPs and TIMPs in WT mice, aortas were separated into thoracic and abdominal regions. mRNA expression levels of Collagen I, elastin, MMP2, MMP9, MMP13, MT1-MMP, TIMP1, TIMP2, TIMP3, and TIMP4 in thoracic and abdominal aortas from WT mice were measured (Figure 3.1). Compared to thoracic aorta, abdominal aorta shows significantly decreased expression levels of Collagen I, elastin. MMP2, TIMP1, TIMP2, and TIMP3. No significant differences is observed in expression levels of MMP7, MMP9, MT1-MMP and TIMP4.

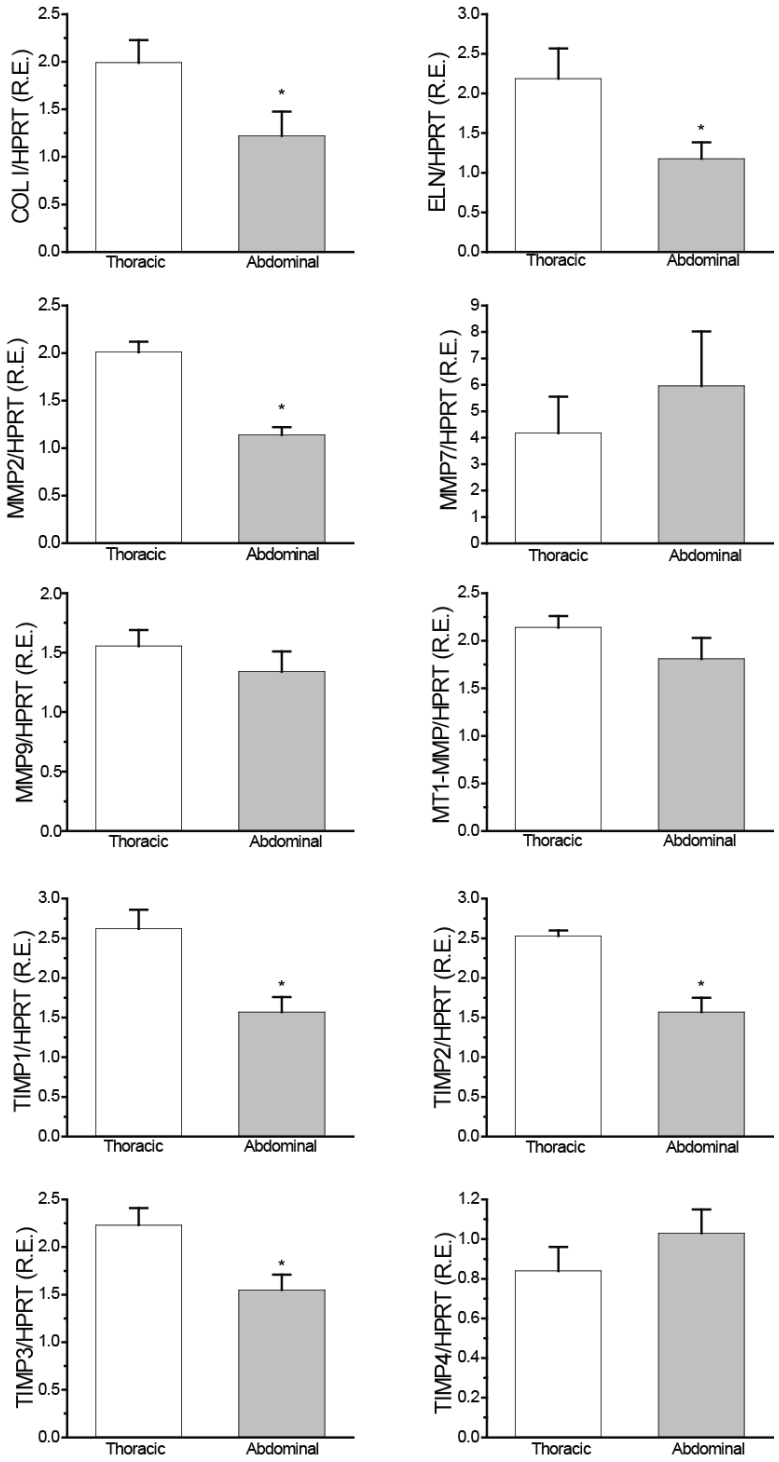

Figure 3.1 mRNA expression levels of ECM structural proteins, MMPs and TIMPs in WT aortas.

Averaged mRNA expression levels of Collagen I, elastin, MMP2, MMP9, MMP13, MT1-MMP, TIMP1, TIMP2, TIMP3, and TIMP4 from WT aortas (N=5/group/genotype). *P<0.05 for the main effect.

3.2 MMP2-deficient mice develop thoracic aortic aneurysm (TAA) following 4 weeks of systemic Ang II infusion

The entire aortas from WT and MMP2$^{-/-}$ mice were dissected for images (Figure 3.2). We did not find differences in baseline phenotype between WT and MMP2$^{-/-}$ aortas. Following 4 weeks of systemic Ang II infusion (1.5mg/kg/day), WT aortas underwent constructive remodeling with uniform dilation throughout the aorta. None of the WT mice developed thoracic or abdominal aortic aneurysm. We found that MMP2$^{-/-}$ aortas underwent destructive remodeling and as such, 70% of these mice developed thoracic aortic aneurysm (TAA). Abdominal aortic aneurysm was not detected in MMP2$^{-/-}$ mice.

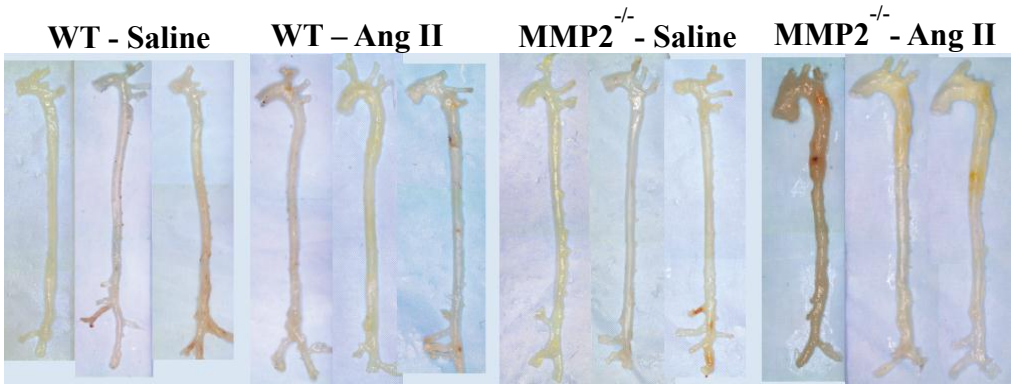

Figure 3.2 MMP2$^{-/-}$ - Ang II mice developed thoracic aortic aneurysm.

Representative images of the entire aorta from saline and Ang II-infused WT and MMP2$^{-/-}$ mice.

3.3 MMP2-deficient mice show impaired recoil property in thoracic aorta following Ang II infusion

Aortic diameters were measured using ultrasound recording in B-mode (A) and M-mode (B) (Figure 3.3). Baseline aortic diameters were similar between WT and $MMP2^{-/-}$ mice. $MMP2^{-/-}$ - Ang II aortas exhibited greater dilation than WT – Ang II mice. Compared to other groups, $MMP2^{-/-}$ - Ang II aortas showed significantly reduced aortic wall motion which is indicated by the flat line in M mode (Figure 3.3B). Following Ang II infusion, both genotypes showed significantly increased systolic and diastolic aortic diameters compared to the parallel saline groups (Figure 3.4A and B). Diastolic aortic diameter was significantly different between the two genotypes following Ang II infusion (Figure 3.4Bi). Aortic expansion index, which is calculated as the percent difference between systolic and diastolic diameters, was markedly suppressed in $MMP2^{-/-}$ - Ang II aortas compared to WT - Ang II aortas (Figure 3.4Ci). Aortic expansion index of proximal aorta was similarly reduced in both genotypes following Ang II infusion (Figure 3.4Cii). These data indicate impaired recoil property of $MMP2^{-/-}$ - Ang II thoracic aortas.

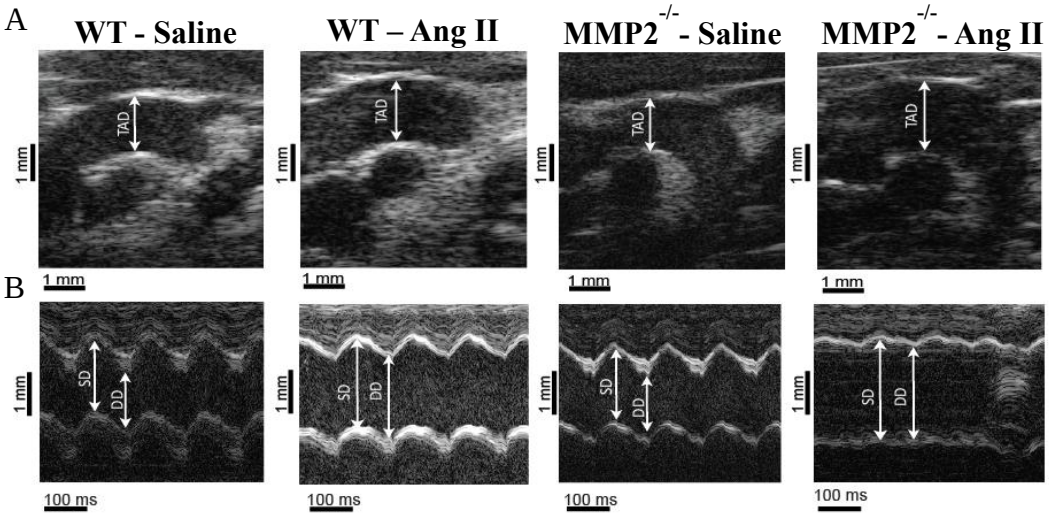

Figure 3.3 Representative ultrasound images of the thoracic aortas from WT and $MMP2^{-/-}$ mice.

Representative ultrasound images of the thoracic aorta taken at the B-mode (A) and M-mode (B) in saline and Ang II-infused WT and $MMP2^{-/-}$ mice. TAD indicates thoracic aortic diameter. SD indicates systolic diameter and DD indicates diastolic diameter.

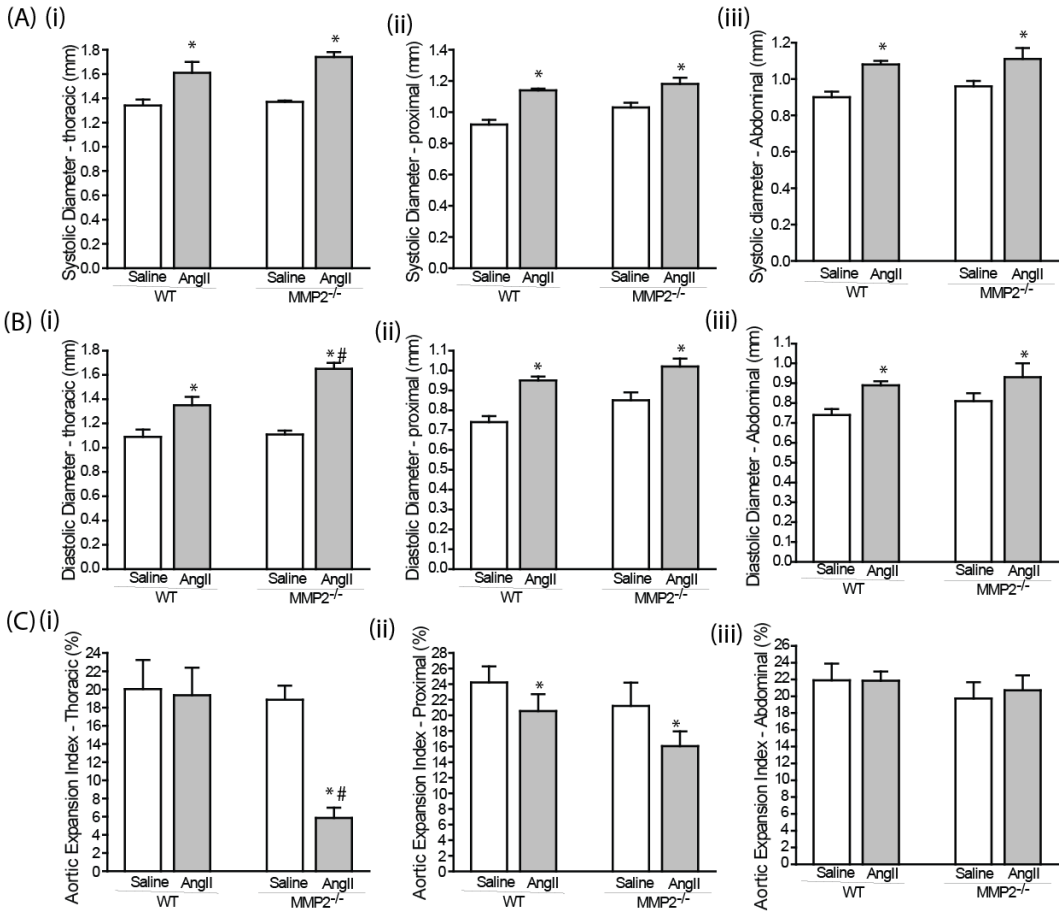

Figure 3.4 Averaged aortic diameter and expansion index of saline and Ang II-infused WT and MMP2$^{-/-}$ mice.

Averaged systolic (A), diastolic aortic diameters (B), and aortic expansion index (C) in thoracic (i), proximal (ii), and abdominal (iii) aortas of saline and Ang II-infused WT and MMP2$^{-/-}$ mice (N=7/group/genotype). *P<0.05 for the main effect, #p<0.05 for the interactions.

3.4 Blood pressure is similarly increased in WT and MMP2-deficient mice following Ang II infusion

During the first 2 weeks of Ang II infusion, blood pressure was measured, and WT and MMP2$^{-/-}$ mice showed a similar hypertensive response to Ang II infusion (WT: 164.5 ±1.12 mmHg and MMP2$^{-/-}$: 172.9 ±0.24 mmHg; Figure 3.5). We did not observe a significant difference in baseline blood pressure between genotypes.

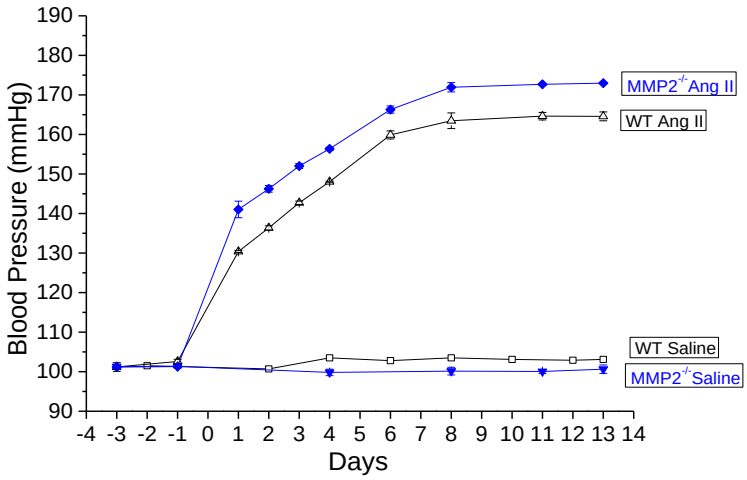

Figure 3.5 Blood pressure of WT and MMP2$^{-/-}$ mice following Ang II infusion

Blood pressure was measured using the non-invasive tail-cuff system from saline and Ang II infused WT and MMP2$^{-/-}$ mice (N=6/group/genotype). Repeated-measures analysis was performed for statistical analysis.

3.5 Thoracic aortas from MMP2-deficient mice exhibit adverse aortic remodeling following 4 weeks of Ang II infusion

We assessed aortic remodeling by analyzing the predominant arterial structural proteins, elastin and collagen fibers. Gomori trichrome (GT) staining, which indicates collagen structure in green, showed disorganization of collagen structure in the thoracic aorta of MMP2$^{-/-}$ - Ang II compared to WT - Ang II mice (Figure 3.6). Similarly, Verhoeff-Van Gieson (VVG) staining, where elastin fibers appear as black lines, showed more disruptions of elastin fibers in the thoracic aorta of MMP2$^{-/-}$ - Ang II compared to WT - Ang II group. We do not find differences in WT and MMP2$^{-/-}$ saline mice. Abdominal aortas from saline and Ang II infused WT and MMP2$^{-/-}$ mice show intact collagen and elastin fibers (Figure 3.7).

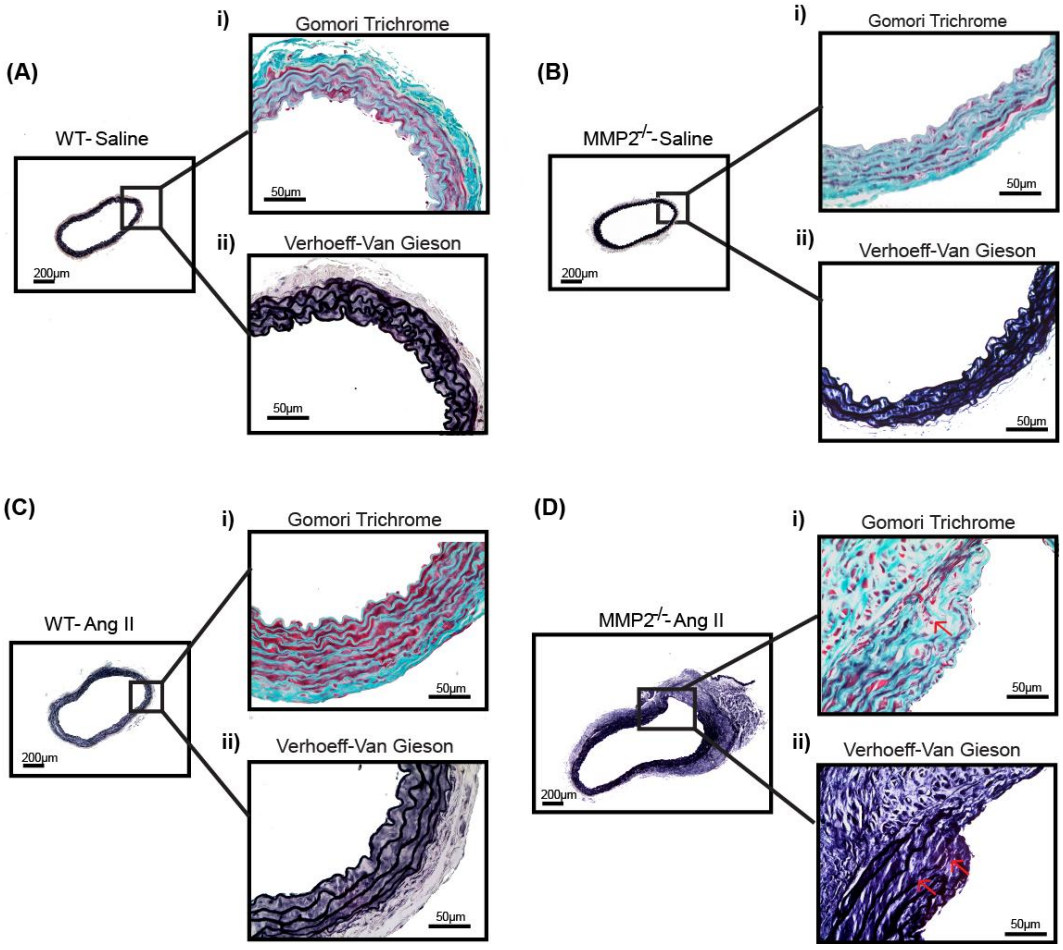

Figure 3.6 Gomori trichrome (GT) and Verhoeff-Van Gieson (VVG) staining of thoracic aortas from saline and Ang II infused WT and MMP2$^{-/-}$ mice.

Representative images of thoracic aortas from saline and Ang II infused WT (A and C) and MMP2$^{-/-}$ (B and D) mice stained with GT (i) and VVG staining (ii). Red arrows show disruption of corresponding structural proteins.

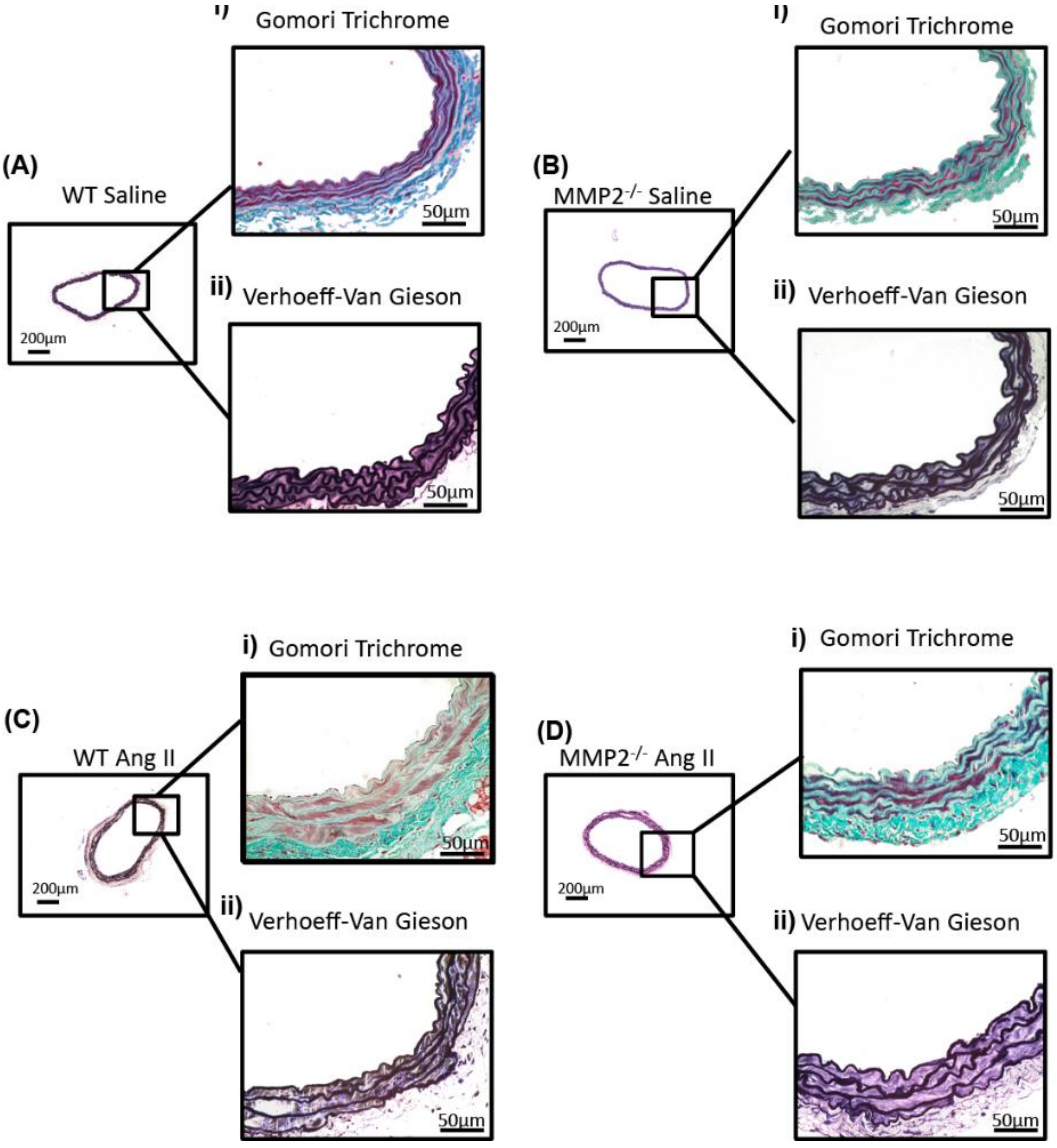

Figure 3.7 Gomori trichrome (GT) and Verhoeff-Van Gieson (VVG) staining of abdominal aortas from saline and Ang II infused WT and MMP2$^{-/-}$ mice.

Representative images of abdominal aortas from saline and Ang II infused WT (A and C) and MMP2$^{-/-}$ (B and D) mice stained with GT (i) and VVG staining (ii).

3.6 Aneurysmal thoracic aortas from MMP2-deficient mice show reduced mRNA and protein levels of elastin

In WT mice, compared to the saline group, following 4 weeks of Ang II infusion protein level of α-elastin in thoracic and abdominal aortas is significantly elevated (Figure 3.7 i). Compared to the saline group, in $MMP2^{-/-}$ mice, protein levels of α-elastin in abdominal but not thoracic aortas showed significant increase following 4 weeks of Ang II infusion (Figure 3.7 ii). Ang II infusion resulted in a significant increase in the mRNA expression of elastin in WT mice, but not in $MMP2^{-/-}$ mice (Figure 3. iii).

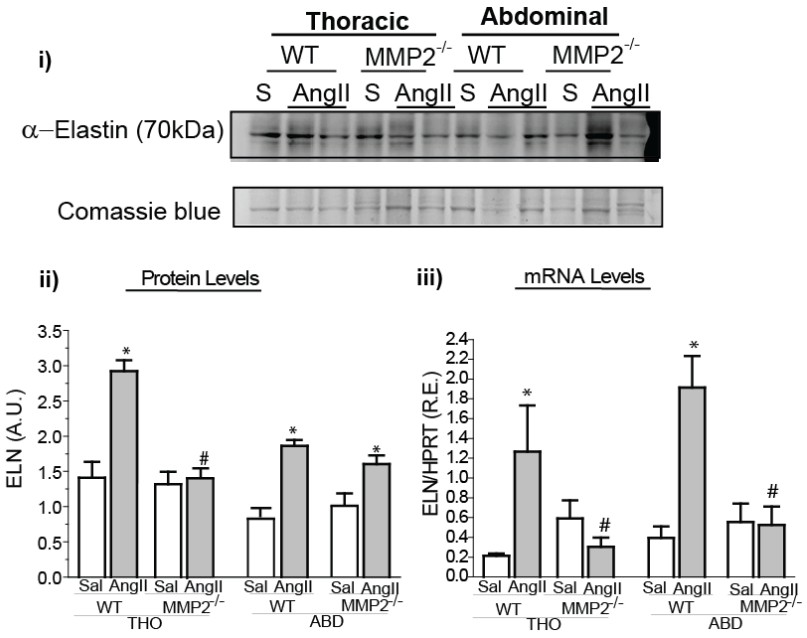

Figure 3.8 Protein and mRNA expression levels of α-elastin.

Representative Western blot image for α-elastin (i). Average protein (ii) and mRNA (iii) levels of α-elastin from saline and Ang II-infused WT and $MMP2^{-/-}$ mice (N=5/group/genotype). Coomassie blue-stained gel was used as the loading control. *P<0.05 for the main effect, #p<0.05 for the interactions.

3.7 WT and MMP2-deficient mice do not show significant differences in protein levels of Collagen I following 4 weeks of Ang II infusion

Saline and Ang II infused aortas from WT and MMP2$^{-/-}$ mice show similar changes in the protein levels of collagen type I (Figure 3.8 i and ii). Following Ang II infusion, Collagen I protein levels is significantly increased in WT and MMP2$^{-/-}$ mice compared to the parallel saline groups. mRNA expression of Collagen I is significantly increased in both WT and MMP2$^{-/-}$ mice following Ang II infusion and significant difference between the genotypes is observed in abdominal aorta (Figure 3.8 iii).

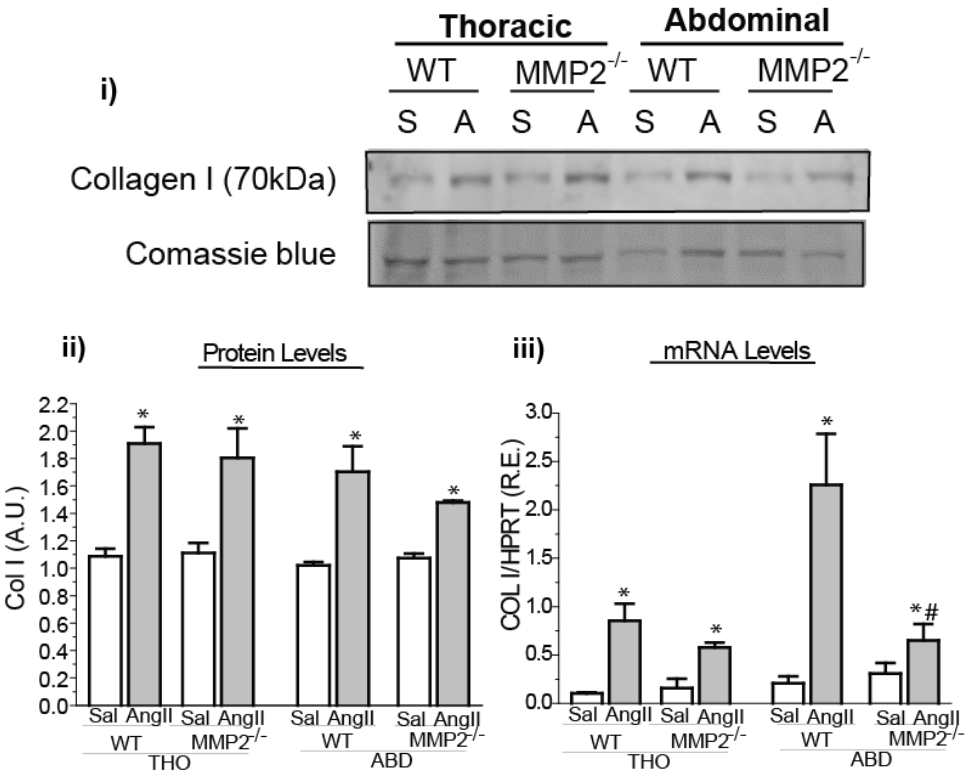

Figure 3.9 Protein and mRNA expression levels of Collagen I.

Representative western blot image for Collagen I (i). Averaged protein (i) and mRNA (ii) levels of Collagen I from saline and Ang II-infused WT and MMP2$^{-/-}$ mice (N=5/group/genotype). Coomassie blue-stained gel was used as the loading control. *P<0.05 for the main effect, #p<0.05 for the interactions.

3.8 Expression profile of MMPs in thoracic and abdominal aortas from WT and MMP2-deficient mice

Expression of MMPs (MMP2, MMP9, MMP13 and MT1-MMP), which have been consistently reported in aneurysmal aortas, are examined to study the role of these MMPs in TAA formation in $MMP2^{-/-}$ Ang II mice. mRNA expression levels of MMP2, MMP9, MMP13, and MT1-MMP were measured in saline and Ang II infused thoracic and abdominal aortas from WT and $MMP2^{-/-}$ mice (Figure 3.10). MMP2 levels were significantly elevated in both thoracic and abdominal aortas from WT - Ang II mice compared to the saline group (Figure 3.10 i). MMP9 levels were significantly elevated in WT mice following Ang II infusion but this elevation is not observed in $MMP2^{-/-}$ mice (Figure 3.10 ii). MMP13 levels were significantly elevated following Ang II infusion in all groups (Figure 3.10 iii). Abdominal aorta from WT mice showed significantly higher MMP13 levels compared to other parallel groups. MT1-MMP levels were significantly elevated in WT and $MMP2^{-/-}$ mice following Ang II infusion (Figure 3.10 iv). Taken together, mRNA expression patterns of MMP2, MMP9, MMP13, and MT1-MMP in thoracic aorta from $MMP2^{-/-}$ -Ang II mice does not explain TAA formation in $MMP2^{-/-}$ mice.

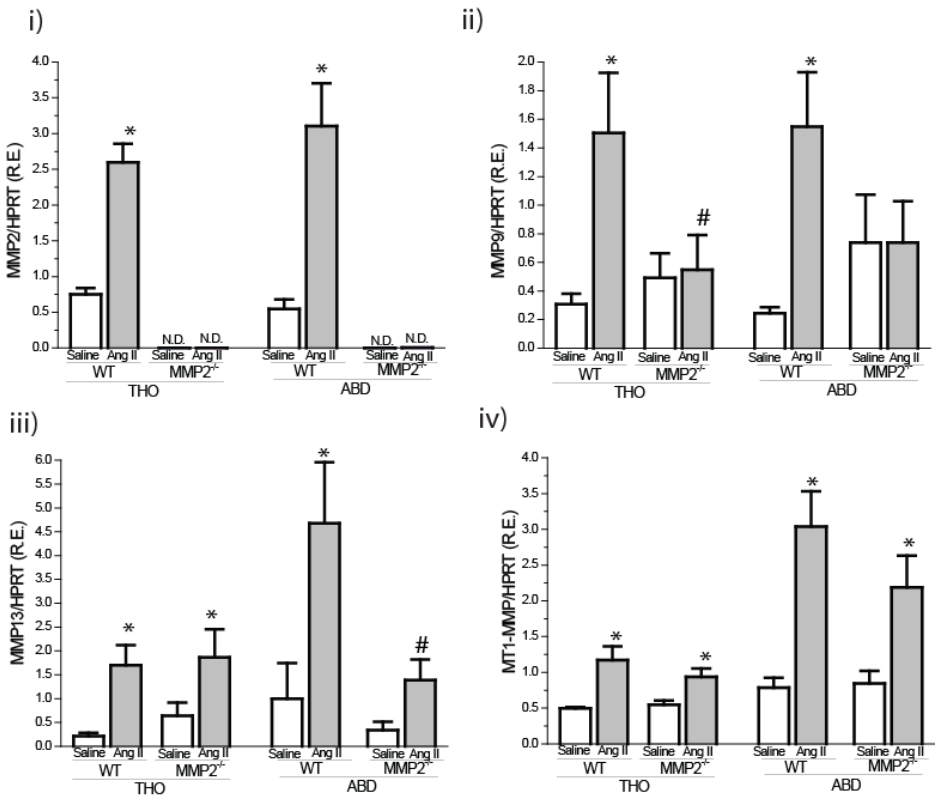

Figure 3.10 mRNA expression levels of MMP2, MMP9, MMP13 and MT1-MMP

Averaged mRNA expression levels of MMP2 (i), MMP9 (ii), MMP13 (iii), and MT1-MMP (iv) from saline and Ang II-infused WT and $MMP2^{-/-}$ aortas (N=5/group/genotype). *P<0.05 for the main effect, #p<0.05 for the interactions.

3.9 mRNA expression levels of TIMPs in thoracic and abdominal aortas from WT and MMP2-deficient mice

To examine the balance between MMPs and TIMPs, mRNA expression levels of endogenous MMP inhibitors, TIMPs were measured in saline and Ang II infused thoracic and abdominal aortas from WT and $MMP2^{-/-}$ mice (Figure 3.11). mRNA expression of TIMP1 was significantly elevated in thoracic and abdominal aortas from WT-Ang II mice but in $MMP2^{-/-}$ mice, this increase was only observed in abdominal aortas (Figure 3.11 i). mRNA expression of TIMP2 was significantly

elevated in WT aortas but the Ang II-induced increase was not observed in MMP2$^{-/-}$ aortas (Figure 3.11 ii). TIMP3 mRNA expression levels were only increased in Ang II infused WT abdominal aorta (Figure 3.11 iii). Ang II infused MMP2$^{-/-}$ aortas showed significantly reduced TIMP3 levels compare to the parallel WT groups (Figure 3.11 iii). mRNA expression of TIMP4 was significantly elevated following Ang II infusion in WT aortas but this increase was not observed in MMP2$^{-/-}$ mice (Figure 3.11 iv).

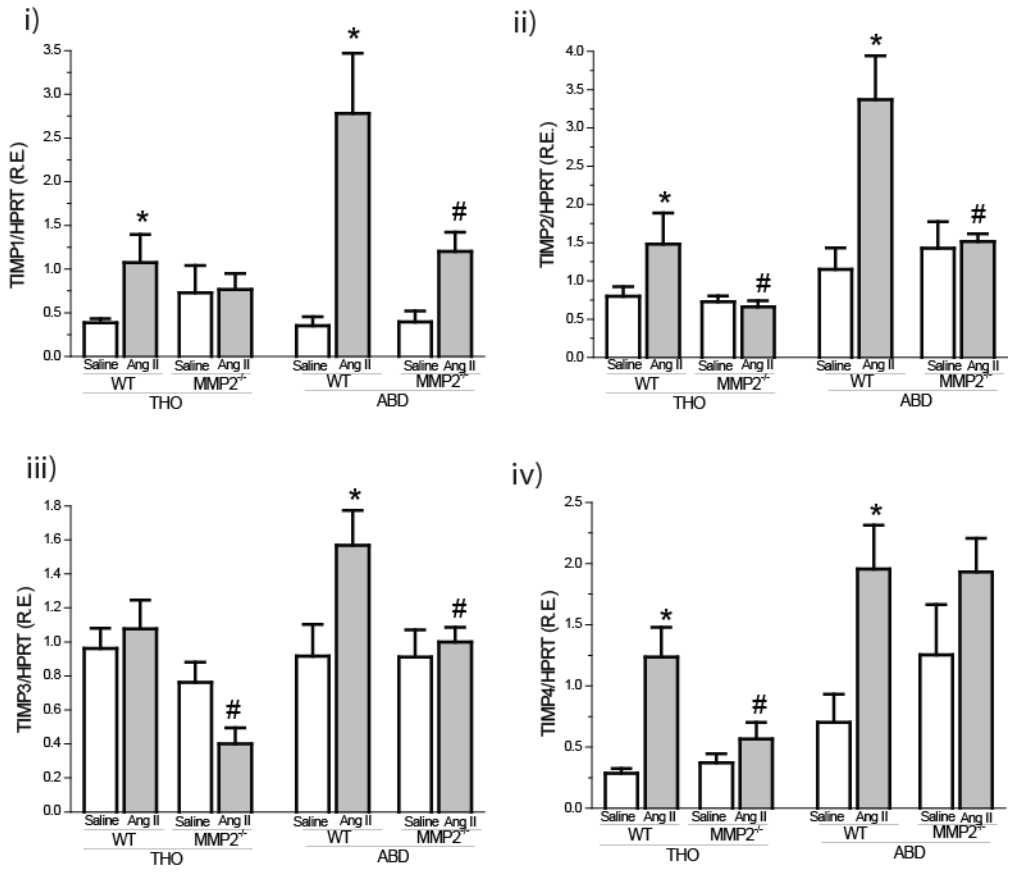

Figure 3.11 mRNA expression levels of TIMP1, TIMP2, TIMP3, and TIMP4 from saline and Ang II infused WT and MMP2$^{-/-}$ aortas.

Averaged mRNA expression levels of TIMP1 (i), TIMP2 (ii), TIMP3 (iii), and TIMP4 (iv) from saline and Ang II-infused WT and MMP2$^{-/-}$ mice (N=5/group/genotype). *P<0.05 for the main effect, #p<0.05 for the interactions.

3.10 Thoracic aortas from MMP2-deficient mice do not show enhanced ECM degradation

In order to determine the mechanism underlying the reduction of elastin in thoracic aortic wall of MMP2$^{-/-}$ mice, the contributions of proteases in thoracic aortas were investigated. *In vitro* gelatin zymography showed no significant differences in MMP9 levels between WT and MMP2$^{-/-}$ mice (Figure 3.12). Following Ang II infusion, WT mice showed increased levels of pro-MMP2 and cleaved/active MMP2. There are no significant difference in MMP9 activation between groups. Total elastase activity was elevated in Ang II infusion groups compared to the parallel saline groups and the extent of elevation was significantly reduced in MMP2$^{-/-}$ -Ang II mice compared to WT-Ang II mice (Figure 3.13).

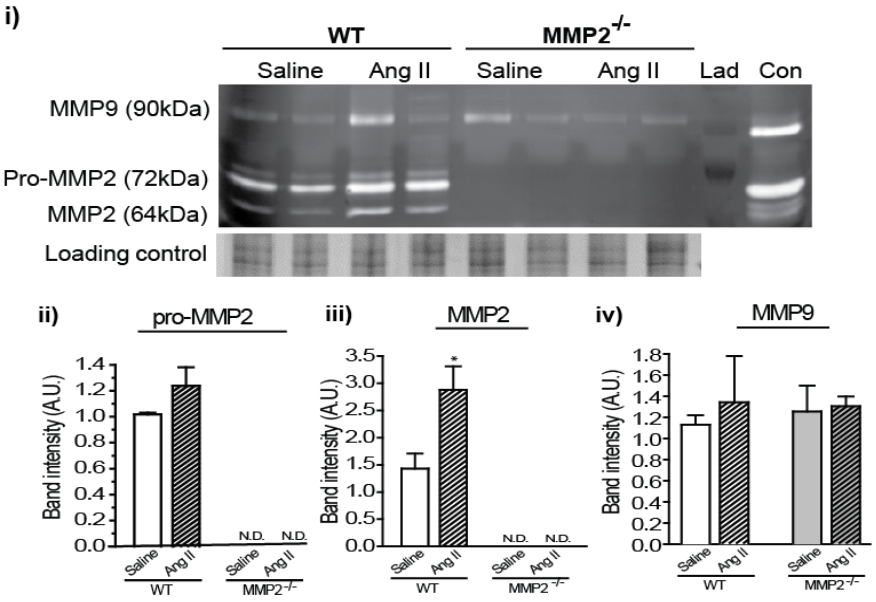

Figure 3.12 No significant differences in activation of MMP9

Representative images of *in vitro* gelatin zymography (i). Averaged activation levels of pro-MMP2 (ii), MMP2 (iii), and MMP9 (iv) (N=6/group/genotype). Coomassie blue-stained gel was used as the loading control *P<0.05 for the main effect.

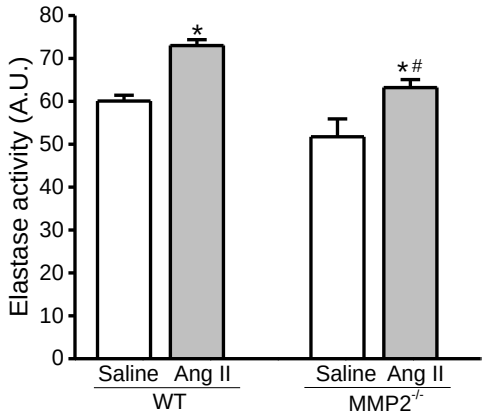

Figure 3.13. No significant differences in total elastase activity

Total elastase activity of the thoracic aortas from saline and Ang II infused WT and MMP2$^{-/-}$ mice (N=5/group/genotype). *P<0.05 for the main effect, #p<0.05 for the interactions.

3.11 Thoracic aortas from MMP2-deficient mice show reduced active TGFβ levels

In order to determine the underlying molecular mechanism of the development of TAA in MMP2$^{-/-}$ - Ang II mice, we examined TGFβ signaling pathway in thoracic aortas from WT and MMP2$^{-/-}$ mice. Following Ang II infusion, protein level of active TGFβ were significantly elevated in WT and MMP2$^{-/-}$ mice (Figure 3.14). However, MMP2$^{-/-}$ - Ang II aortas showed a significantly lower levels of active TGFβ protein compared to the parallel WT – Ang II aortas.

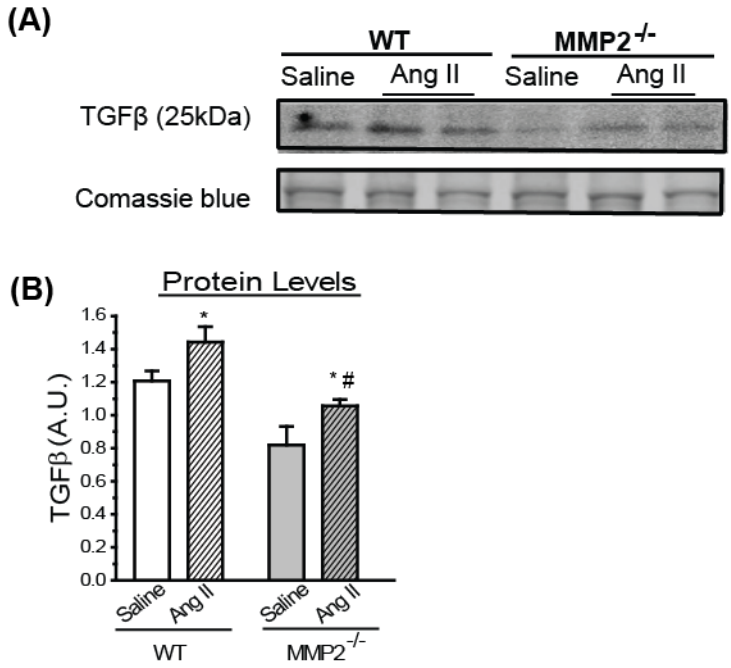

Figure 3.14 Protein levels of active TGFβ

Representative image of western blot of active TGFβ (A). Averaged protein levels (B) of TGFβ (N=6/group/genotype). *p<0.05 for the main effect, #p<0.05 for the interactions.

3.12 Thoracic aortas from MMP2-deficient mice show impaired Smad signaling pathway

In order to examine downstream of TGFβ signaling pathway, protein levels of phosphorylated-Smad2 (P-Smad2), phosphorylated-Smad3 (P-Smad3), total Smad2 and total Smad3 were measured in thoracic aortas from WT and MMP2$^{-/-}$ mice (Figure 3.15). WT, but not MMP2$^{-/-}$ mice showed significantly elevated protein levels of P-Smad2 and P-Smad3 following 4 weeks of Ang II infusion (Figure 3.15 ii and iii). Protein levels of total Smad2 and Smad3 were also

significantly elevated in WT - Ang II compared to WT saline whereas this increase was not observed in MMP2$^{-/-}$ mice (Figure 3.15 iv and v).

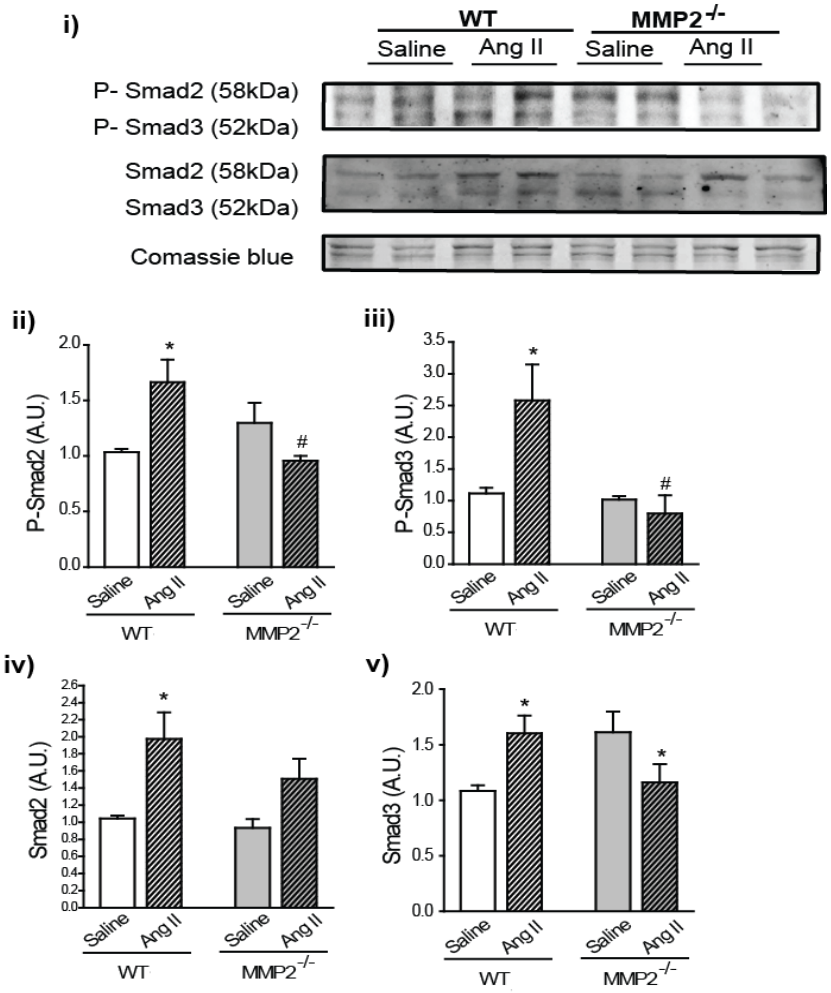

Figure 3.15 Protein levels of P-Smad2, P-Smad3, Smad2, and Smad3.

Representative image of western blot of P-Smad2, P-Smad3, Smad2, and Smad3 (i). Averaged protein levels of P-Smad2 (ii), P-Smad3 (iii), Smad2 (iv), and Smad3 (v) (N=6/group/genotype). *P<0.05 for the main effect, #p<0.05 for the interactions.

3.13 Inflammatory cells do not contribute to the development of thoracic aortic aneurysm in MMP2-deficient mice

We examined the mRNA expression levels of inflammatory markers in thoracic aortas from WT and MMP2$^{-/-}$ mice (Figure 3.16). Following Ang II

infusion, WT and MMP2$^{-/-}$ aortas showed elevated expression levels of MCP1, and IL-1β. MMP2$^{-/-}$-Ang II, but not WT-Ang II mice showed significant elevation in expression levels of TNF and IL-6. Compared to WT-Ang II, MMP2$^{-/-}$-Ang II aortas showed lower levels of inflammatory markers but this difference does not reach statistical significance. Immunofluorescence staining for neutrophils was performed to examine localization of inflammatory cells in thoracic aortas from WT and MMP2$^{-/-}$ mice and positively stained neutrophils are comparable between the four groups. (Figure 3.17).

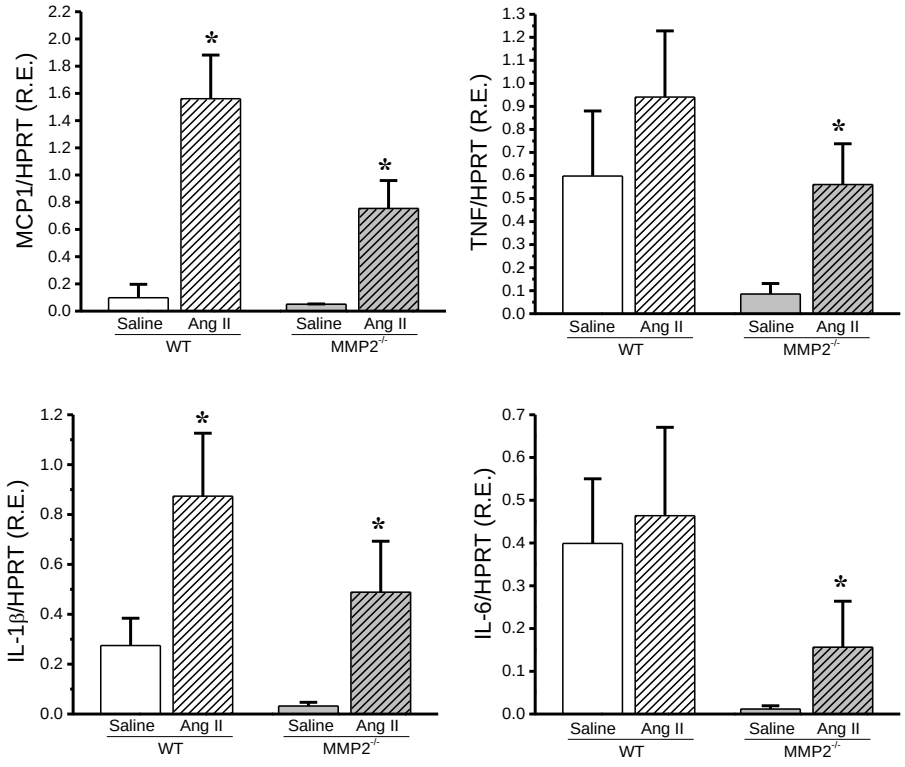

Figure 3.16 mRNA expression levels of inflammatory markers from WT and MMP2$^{-/-}$ thoracic aorta

Averaged mRNA expression levels of MCP1, TNF, IL-1β, and IL-6 from WT and MMP2$^{-/-}$ thoracic aorta in response to Ang II (N=5/group/genotype). *P<0.05 for the main effect.

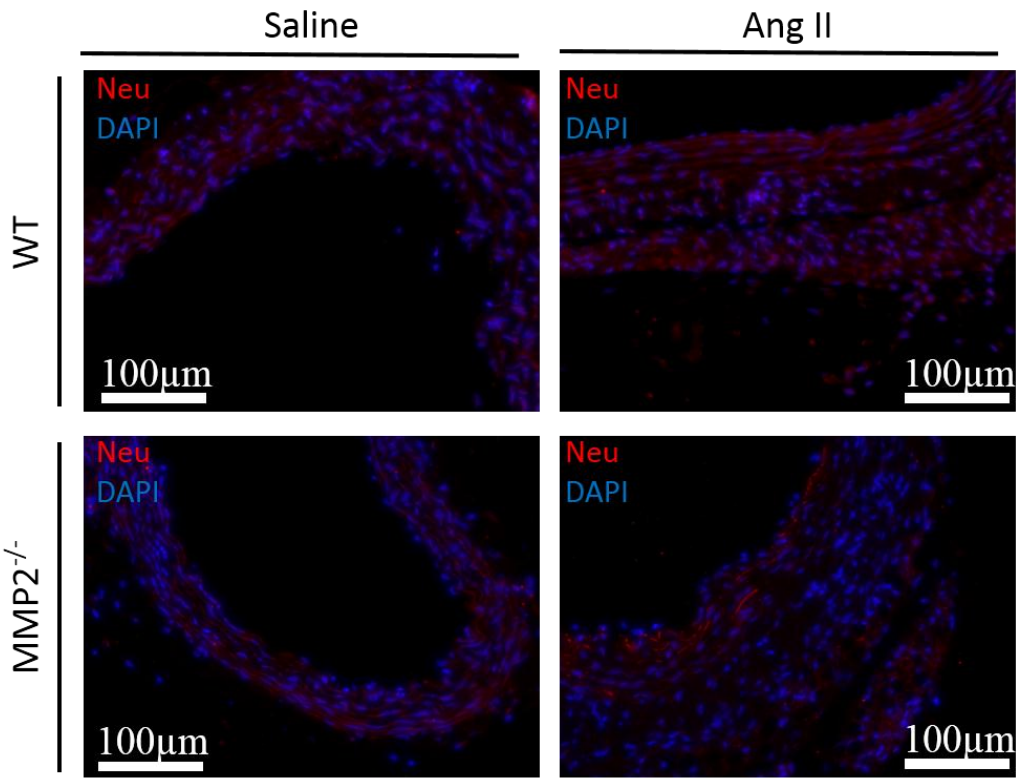

Figure 3.17 Immunofluorescence staining for neutrophils

Immunofluorescence staining for neutrophils (red) superimposed with DAPI nucleus staining (blue) in thoracic aortas from WT and MMP2$^{-/-}$ mice.

3.14 Effect of Ang II or TGFβ on vascular smooth muscle cells from WT and MMP2$^{-/-}$ thoracic aorta

To identify the role of vascular smooth muscle cells (VSMCs) in susceptibility of MMP2$^{-/-}$ mice to thoracic aortic aneurysm, cultured VSMCs from WT and MMP2$^{-/-}$ thoracic aortas were treated with Ang II (10μM) or TGFβ (10ng/mL). mRNA expression levels of ECM proteins, elastin and collagen, were measured in response to Ang II or TGFβ (Figure 3.19). Ang II treatment did not increase mRNA expression of collagen type I in WT VSMCs, but resulted in a significant increase in MMP2$^{-/-}$ VSMCs. Elastin levels were markedly elevated in both WT and MMP2$^{-/-}$ VSMCs following Ang II treatment (Figure 3.18 A). In

respond to TGFβ, both WT and MMP2$^{-/-}$ VSMCs showed significantly increased Col1 and elastin levels, indicating that the downstream pathway of TGFβ is intact in the MMP2$^{-/-}$ VSMCs (Figure 3.18 B).

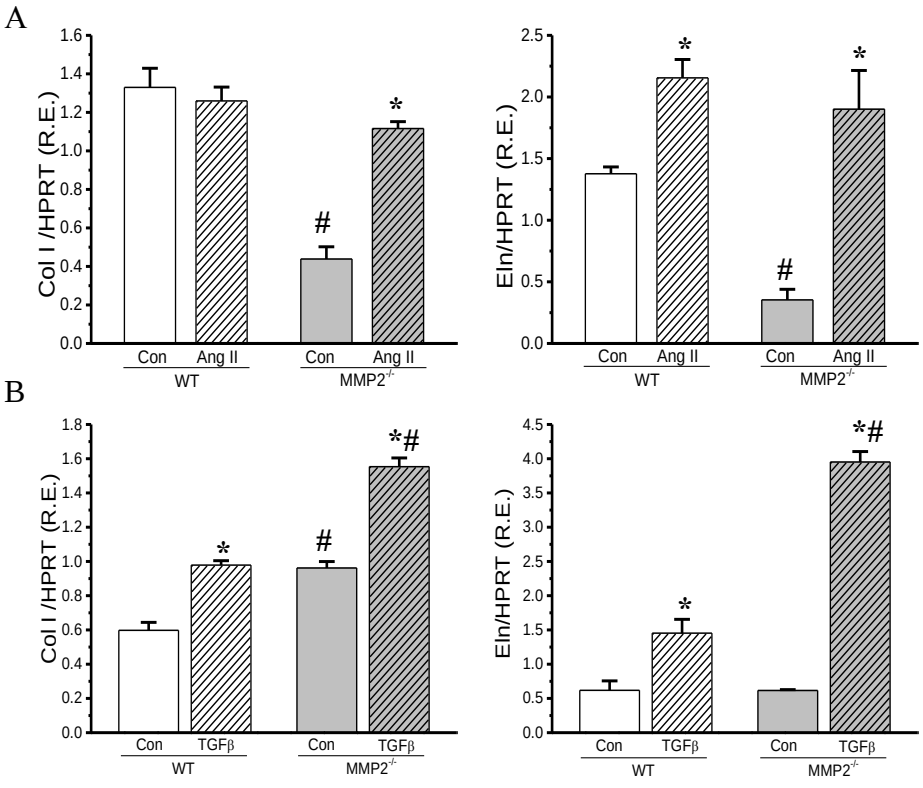

Figure 3.18 mRNA expression of collagen and elastin in VSMCs from WT and MMP2$^{-/-}$ thoracic aorta in response to Ang II or TGFβ

Averaged mRNA expression levels of elastin and collagen I in response to Ang II (A) or TGFβ (B) (N=3/group/genotype). *P<0.05 for the main effect, #p<0.05 for the interactions.

3.15 WT and MMP2-deficient mice are protected against aortic aneurysm following 6 weeks of nicotine infusion

To investigate the role of smoking in aortic aneurysm, nicotine (5mg/kg/day) was systemically infused to WT and MMP2$^{-/-}$ mice. Following 6 weeks of nicotine infusion, WT and MMP2$^{-/-}$ mice did not develop aortic aneurysm (Figure 3.19). Aortic diameter and expansion index were comparable between groups (Figure 3.20).

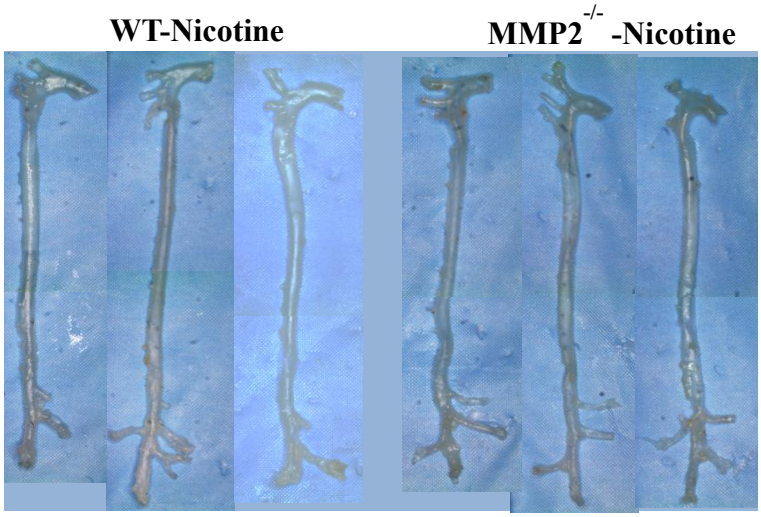

Figure 3.19 WT and MMP2$^{-/-}$ mice did not develop aortic aneurysm following nicotine infusion

Representative images of the entire aorta from WT and MMP2$^{-/-}$ mice following 6 weeks of nicotine infusion.

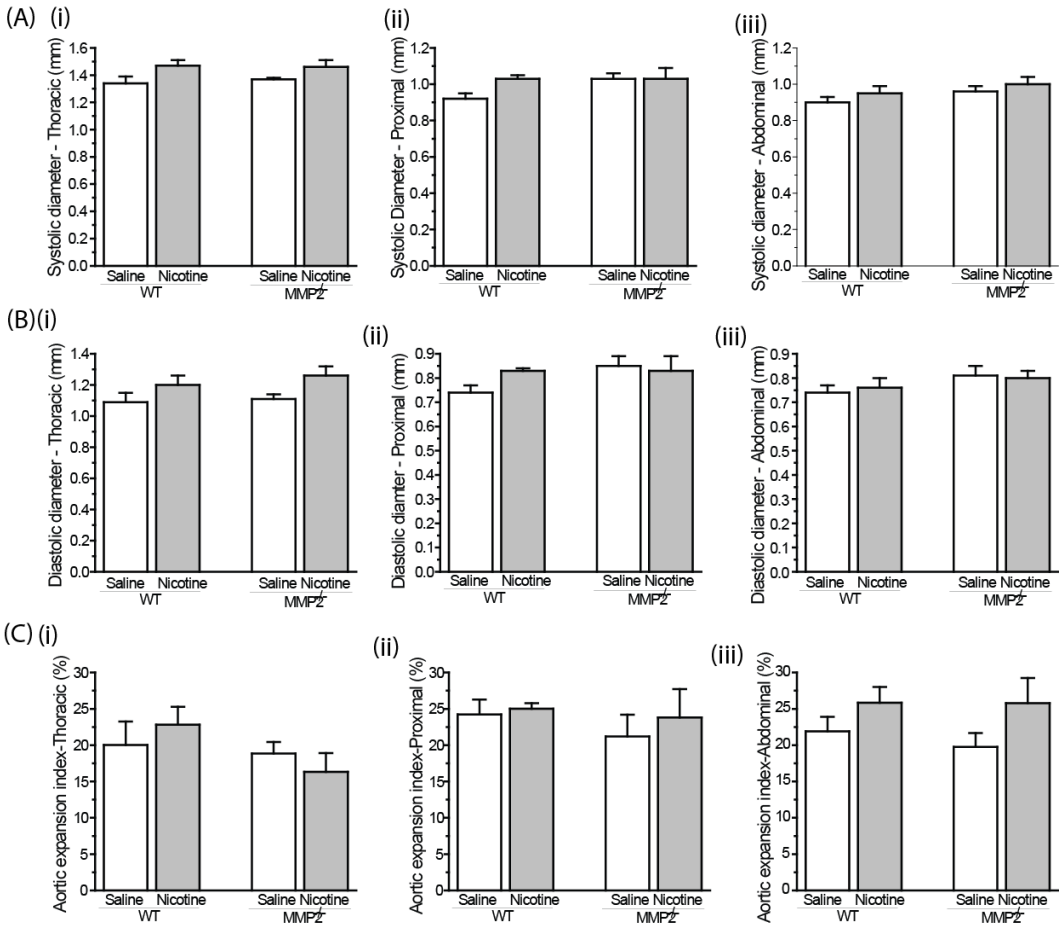

Figure 3.20 Averaged aortic diameters and expansion index of saline or nicotine-infused WT and MMP2$^{-/-}$ mice

Averaged systolic (A) and diastolic aortic diameter in thoracic (i), proximal (ii), and abdominal (iii) aortas of saline and nicotine-infused WT and MMP2$^{-/-}$ mice (N=7/group/genotype). Aortic expansion index (C) is measured.

3.16 WT and MMP2-deficient mice develop hypertension but not aortic aneurysm following 4 weeks of phenylephrine infusion

To investigate the role of hypertension in the development of aortic aneurysm, phenylephrine (PE; 30mg/kg/day), a sympathomimetic agent which elevates blood pressure, is systemically infused. During the first 2 weeks, blood pressure is measured and WT and MMP2$^{-/-}$ mice show similar hypertensive

response to PE (WT: 167.4±1.18 mmHg and MMP2$^{-/-}$: 166.0±1.75 mmHg; Figure 3.21). Following 4 weeks of PE infusion, both WT and MMP2$^{-/-}$ mice are protected against aortic aneurysm (Figure 3.22).

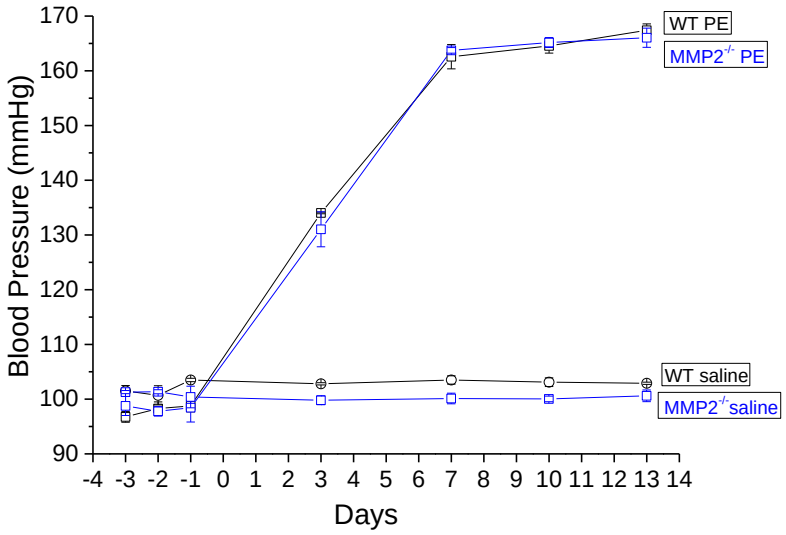

Figure 3.21 WT and MMP2$^{-/-}$ mice showed hypertensive response following PE infusion

Black lines represent WT mice and blue lines represent MMP2$^{-/-}$ mice (N=5/group/genotype). Blood pressure were recorded using the non-invasive tail-cuff system.

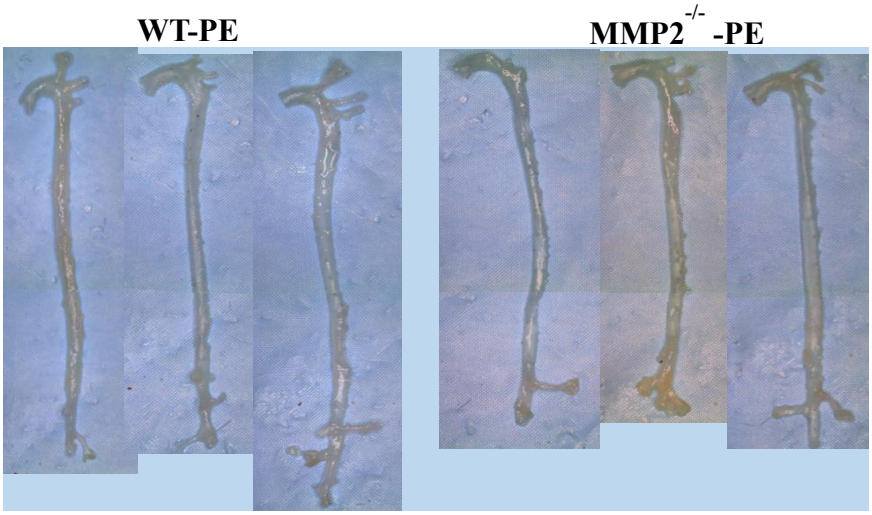

Figure 3.22 WT and MMP2$^{-/-}$ mice do not develop aortic aneurysm following PE infusion

Representative images of the entire aorta from WT and MMP2$^{-/-}$ mice following 4 weeks of PE infusion.

CHAPTER 4

DISCUSSION

Development of aortic aneurysm is a complicated process which involves pathological changes in both cellular and extracellular levels. Integrity of the ECM is important for keeping the aortic wall intact. Homeostasis of MMPs and TIMPs levels regulates the constant turnover of the ECM under physiological condition. When this balance between MMPs and TIMPs is disrupted, excessive deposition or degradation of ECM structural proteins lead to stiffening or progressive weakening of aortic wall respectively. Mechanical strength and integrity of the aortic wall is impaired such that the aorta will undergo destructive remodeling and become prone to developing aneurysm. My interest was to study the role of MMP2 in vascular ECM remodeling and development of aortic aneurysm.

4.1 Summary of important findings

With MMP2 deficiency, we observed thoracic aortic aneurysm in 70% of $MMP2^{-/-}$ mice but no abdominal aortic aneurysm following four weeks of Ang II infusion. The Ang II infusion model is widely used and is a physiological relevant method to induce vascular remodeling in murine animal models. We found markedly suppressed recoil properties in the thoracic aorta of $MMP2^{-/-}$- Ang II mice. Histological analyses showed disorganization and disruption of elastin and collagen fibers in the thoracic aorta of $MMP2^{-/-}$- Ang II compared to WT- Ang II mice. In addition, mRNA and protein levels of elastin, but not collagen type I, were significantly reduced in the thoracic aorta of $MMP2^{-/-}$-Ang II compared to WT-Ang II mice. This reduction in elastin levels was not due to excess degradation since *in vitro* gelatin zymography and total elastase activity were not elevated in the $MMP2^{-/-}$-Ang II mice. Our study demonstrates that loss of MMP2 impaired TGFβ-Smad

signaling pathway that mediates synthesis of elastin and collagen in response to stimuli such as Ang II, resulting in thoracic aortic aneurysm. Compared to WT-Ang II, MMP2$^{-/-}$-Ang II showed reduced active TGFβ, and phospho-Smad2/3 levels. Therefore, the adverse remodeling in the thoracic aorta of MMP2-deficient mice was associated with decreased synthesis of ECM structural proteins without concomitant upregulation of proteolytic activities. This was the first study to demonstrate the protective role of MMP2 in Ang II-induced aortic aneurysm.

4.2 Regional heterogeneity leads to differential response to Ang II infusion between thoracic and abdominal aortas

Thoracic and abdominal regions of the aorta have developmental, structural and molecular heterogeneities which lead to differential histopathology between these two regions[6, 204]. In this study, we found that mRNA levels of MMP2 and elastin were significantly higher in WT thoracic aorta compared to abdominal aorta. This molecular heterogeneity leads to differential response to Ang II such that MMP2 deficiency promotes only TAA but not AAA. MMP2$^{-/-}$ mice has been reported to be protected against AAA, but thoracic aorta from MMP2$^{-/-}$ mice was not examined[114].

The inflammatory cells have a differential role in the thoracic and abdominal aortas. The inflammatory cells play an important role in development and progression of AAA where approximately 5% of patients with AAA were exhibited inflammatory aortic aneurysm[68, 69]. However, inflammatory aneurysm is rare in the thoracic aorta[205, 206]. Consistently, we observed comparable expression

levels of inflammatory markers including TNF-α, MCP-1, IL-1β and IL-6 between WT-Ang II and MMP2$^{-/-}$- Ang II thoracic aortas. Since inflammatory cells have been reported as the main source of MMP9[207, 208], it is consistent with no upregulation of MMP9 in the thoracic aorta of MMP2$^{-/-}$-Ang II mice was observed. These data collectively demonstrate a regional heterogeneity within the aorta such that therapeutic approaches need to be carefully applied to specific region of interest to prevent adverse side effects.

4.3 Imbalance between degradation and synthesis leads to thoracic aortic aneurysm in MMP2$^{-/-}$-Ang II mice

Aortic aneurysm is the outcome of destructive remodeling of the vascular wall ECM. The imbalance between MMPs and their endogenous inhibitors TIMPs is one of factors that mediates adverse ECM remodeling. When this balance is disrupted, the aorta undergoes pathological remodeling and becomes prone to develop aortic aneurysm.

In this study we used genetically modified MMP2-deficient mice as an animal model. Among 26 MMPs discovered to date[16, 17], MMP2 has been consistently reported to be linked to patients with TAA and AAA[109-112]. Since we hypothesized that MMP2-deficient mice would be protected against aortic aneurysm due to suppressed ECM degrading function of MMP2, TAA formation in MMP2-deficient mice was completely opposite to what we expected. We observed that elastin levels were markedly suppressed in MMP2$^{-/-}$-Ang II thoracic aortas which is consistent with aneurysmal aortic specimens from patients[50, 209].

Elastin plays an important role in vasculature development by regulating VSMC proliferation[210, 211] as such mice with a null mutation in the elastin gene (*Eln*$^{-/-}$) die shortly after birth due to occlusion of the blood vessel by over-proliferation of VSMC[212]. Elastin is critical in regulation of blood pressure by providing elasticity which allows the arterial wall to change the arterial diameter. Studies using elastin-haploinsufficient mice (Eln$^{+/-}$) showed that the arterial pressure and the number of elastin lamellae are increased in order to compensate for vessel stiffness[213, 214]. TGFβ has been reported to upregulate elastin synthesis by stabilizing elastin mRNA transcription[126, 127, 215, 216]. Surprisingly, aneurysmal VSMCs isolated from $CaCl_2$-exposed aorta showed significantly elevated elastin synthesis in the presence of TGFβ and hyaluronan oligomers which containing optimized ECM-derived bimolecular factors[217]. Overall, the compositional remodeling, reduced elastin and increased collagen, leads to stiffening of MMP2$^{-/-}$ - Ang II aortas and fails to withstand stress from repeated stretch and recoil during the cardiac cycle.

This is the first study to demonstrate the critical role of MMP2 in vascular ECM remodeling. MMP2, MMP9 and MT1-MMP have been shown to cleave and release latent TGFβ from the ECM[121-123]. Compared to WT-Ang II aortas, MMP2$^{-/-}$ - Ang II aortas showed significantly reduced mRNA levels of MMP9 and no significant differences in mRNA levels of MT1-MMP. Overall, these data suggest that MMP2 cleaves and activates latent TGFβ as such in the absence of MMP2, TGFβ signaling pathway is attenuated. Our finding on the protective role of MMP2 is opposite to predominant notion about the ECM degrading property of MMP2.

Thus, MMP2 levels need to be maintained at the optimal range because absence or overexpression of MMP2 would impair the integrity of the vascular ECM. Therefore, the broad spectrum MMP inhibitors or MMP2 specific inhibitors may not be a suitable therapeutic approach for TAA since reduced MMP2 levels could lead to adverse vascular remodeling which exacerbates vascular disease such as aortic aneurysm.

4.4 MMP2 mediates TGFβ-Smad signaling pathway by activating latent TGFβ

TGFβ, which regulates various cellular responses, can be activated by proteases such as MMP2, MMP9 and MT1-MMP[121-123, 218, 219]. TGFβ is synthesized in its latent form and is sequestered by Fibrilin-1 to the ECM. Aneurysmal patients with Marfan syndrome, a genetic disorder due to a mutation in Fibrilin-1 (FBN-1), have shown upregulation of TGFβ signaling.

FBN-1 is the major fibrillin isoform in elastin fibers which regulates deposition and the organization of elastin fibers[220, 221]. FBN-1 forms cross-links with the elastin precursor called tropoelastin and provides a structural scaffold of microfibrils for proper spatial and temporal arrangement of elastin fibers in the ECM. $Fbn1^{C1039G/+}$ mice show reduction in elastin levels[222, 223].

FBN-1 plays an important role in TGFβ signaling by regulating activation of TGFβ under physiological conditions. Elevation of TGFβ signaling has been reported in both human and animal with FBN malfunction[115, 138, 193, 224]. In addition, doxycycline, a broad-spectrum MMP inhibitor, has been reported as an effective treatment for $Fbn1^{C1039G/+}$ mice, well-validated animal model for Marfan

syndrome[193, 225]. The proposed mechanism is that doxycycline attenuates aneurysm expansion by blocking MMPs especially MMP2 and MMP9.

Studies have shown up-regulation of TGFβ signaling pathway with significantly reduced elastin levels in both humans and animal models with FBN-1 mutation. The underlying mechanism for this observation is that in the absence of FBN-1, increased elastin synthesis conjoined with significantly elevated availability of activated TGFβ is observed. However, tropoelastin is not capable of depositing into microfibrils to form the elastin fibers. Therefore, elastin synthesis is elevated but the amount and stability of elastin is markedly reduced, therefore making the aorta susceptible to aortic aneurysm in Marfan syndrome.

In $MMP2^{-/-}$ mice, however, the reduced elastin synthesis (rather than impaired elastin assembly as in Marfan syndrome) is associated with significantly reduced TGFβ activation and the downstream Smad signaling pathway activity, leading significantly reduced elastin levels.

Taken together, these data indicate that although histological phenotypes are similar between Marfan syndrome and $MMP2^{-/-}$ -Ang II mice, the underlying mechanisms are completely different. In Marfan syndrome, the defect is in elastin assembly, as opposed to elastin synthesis in $MMP2^{-/-}$ -Ang II mice. This suggests that idiopathic and genetic driven aortic aneurysm requires different therapeutic approaches and diagnostic options.

4.5 Elastin but not collagen synthesis is impaired in MMP2$^{-/-}$-Ang II aortas

We found that MMP2 plays a major role in cleaving and activating latent TGFβ. Since the TGFβ signaling pathway has been reported to regulate collagen[141, 226] and elastin[127, 227] synthesis in different cell types, it was unexpected to observe impaired elastin synthesis but intact collagen synthesis in MMP2$^{-/-}$-Ang II aortas. Smad signaling pathway is a canonical downstream TGFβ signaling pathway. It has been reported that Smad 2/3 and Smad 1/5/8 are activated by TGFβ but serve different roles[228]. Smad 1/5/8 is downstream of TGFβ R2 and Smad 2/3 is downstream of TGFβ R1[229, 230]. Bone morphogenetic proteins (BMP1) deficient mice showed abnormal collagen fiber formation[231]. Alterations in collagen expression levels were observed with TGFβ R2 mutation[139, 232, 233]. Studies on $CaCl_2$-exposed TAA mice model showed switching of Smad 2/3 to Smad 1/5/8 pathway[234]. Taken together, these data imply that in the absence of MMP2, impaired cleavage and activation of TGFβ mainly affects Smad 2/3 pathway that regulates elastin synthesis whereas Smad 1/5/8 pathway that regulates collagen synthesis is intact.

4.6 Disparity between *in vivo* and *in vitro* data in response to Ang II

To identify the role of VSMCs in susceptibility of MMP2$^{-/-}$ mice to TAA, we examined the response of VSMCs to Ang II or TGFβ and found lack of correlation between experimental data from aortic tissue and VSMCs in response to Ang II. In WT, Ang II-induced increase in Col I expression was only observed in aortic tissues but not in VSMCs. Moreover, we observed markedly suppressed elastin levels in MMP2$^{-/-}$-Ang II thoracic aortic tissues but this reduction was not

observed in MMP2$^{-/-}$ VSMCs. While isolating VSMCs for cell culture, adventitial and endothelial layers were scraped off. By culturing VSMCs alone, the influence of endothelial cells on VSMCs was excluded. Thus, the disparity between *in vivo* and *in vitro* data in response to Ang II might be due to the absence of endothelial cells which mediate VSMCs function[235-237].

4.7 Aneurysm formation is specific to Ang II infusion as MMP2-deficient mice do not develop aneurysm in response to nicotine or phenylephrine infusion

Smoking and hypertension are considered as risk factors of aortic aneurysm. Smoking is thought to promote the production of reactive oxygen species (ROS). ROS is tightly regulated to maintain cellular homeostasis under physiological condition. ROS regulates expression and activity of MMPs by disrupting the bond between the pro and catalytic domains to expose the catalytic site of MMP for proteolytic activity[238, 239]. Increased proteolytic activities alter aortic wall ECM, and as such the aorta becomes prone to expansion and rupture[55-60]. While smoking shows a strong association to aortic aneurysm events[54], the direct role of smoking in aortic aneurysm is still not clear.

In order to examine the role of smoking in aortic aneurysm, we infused nicotine via osmotic pumps. We found that nicotine alone was not sufficient to induced aortic aneurysm that we observed in Ang II infused MMP2$^{-/-}$ mice. We confirmed intact structural and functional integrities of WT and MMP2$^{-/-}$ aortas following nicotine infusion. In MMP2$^{-/-}$- Ang II mice, TAA formation is due to reduced elastin synthesis. Moreover, WT and MMP2$^{-/-}$ mice showed comparable

proteolytic activities at baseline and post-Ang II infusion. In contrast to Ang II which affects both ECM synthesis and degradation, smoking mainly alters proteolysis by activating MMPs[57, 238, 239]. Therefore, these data indicate that the extent of smoking-induced elastin degradation is comparable between WT and MMP2$^{-/-}$ mice, and as such, increased in proteolytic activities alone is insufficient to lead to aneurysm formation in MMP2$^{-/-}$ mice.

Hypertension is defined as blood pressure persistently higher than physiological range (140/90 mmHg). Hypertension is the most common cardiovascular disease which affects 1 in 3 adults in the United States and untreated hypertension leads to other cardiovascular diseases[240]. More than 80% of patients with aortic aneurysm have hypertension[61]. In response to hypertension, aortic wall undergoes remodeling which significantly changes structure, mechanical properties, and function of the aorta in order to maintain wall shear stress[241-243]. Thus, aortic wall thickness and stiffness are increased compared to baseline[244-246]. However, the direct relationship between hypertension and aneurysm is still unclear. Phenylephrine (PE) is a synthetically derived α1-adrenergic receptor agonist that mimics effects of norepinephrine. PE binds to post-synaptically located α-receptors on VSMCs and induces vasoconstriction[247, 248].

In order to examine the role of hypertension in aortic aneurysm, we infused PE. We found that BP was similarly elevated in MMP2$^{-/-}$ mice following Ang II or PE infusion, but none of PE mice developed TAA or AAA. We confirmed intact structural integrities of WT and MMP2$^{-/-}$ aortas following PE infusion. These data

indicate that the underlying mechanism of TAA formation in MMP2$^{-/-}$ mice following Ang II infusion is independent of hypertension.

Taken together, protection against aortic aneurysm following nicotine or PE infusion confirms that TAA development is specific response to Ang II infusion. From these data, we confirmed that TAA formation in MMP2$^{-/-}$ mice is due to reduced elastin synthesis but not due to increases in proteolysis nor hypertension.

4.8 Conclusion

The findings in this thesis show that the balance between degradation and synthesis of ECM components is crucial in maintaining the integrity of the ECM. MMP2 plays an important role in vascular remodeling by mediating TGFβ activation such that reduced MMP2 levels lead to formation of aortic aneurysm. Interestingly, aortic aneurysm is observed in thoracic but not abdominal aortas due to developmental, structural and molecular heterogeneities between these two regions of the aorta. The protective role of MMP2 in thoracic aortic aneurysm provides a new perspective of examining role of MMP2 in vascular remodeling.

4.9 Study limitations

4.9.1 Whole-body knockout mice as an experimental disease model

The usage of whole body knockout mice is widely used approach to examine direct role of specific gene. However, since many genes have multiple roles in producing different phenotypic characteristics, we took precautions to minimize possible implications of gene deletion in other organs including the heart and the kidneys in the development of aortic aneurysm. We used a large number of

MMP2-deficient mice where we found comparable baseline vascular function, and structural and molecular changes between WT and MMP2-deficient mice. In addition, with MMP2-deficient mice, we observed similar pathological conditions as human TAA patients present. We were certain that aneurysm formation is primary due to Ang II infusion as we did not observe baseline defects in cardiovascular structure or function. However, since the RAAS exists in several organs including the heart and the kidneys, organ specific or conditional knockout would be a better approach to limit the effect of systemically infused Ang II on other organs.

4.9.2 Studies on vascular smooth muscle cell culture

Elastin which is responsible for the mechanical properties of the aorta, is predominantly synthesized by VSMCs[11]. VSMCs are important in vascular function during pathological condition. VSMCs regulate contraction and blood vessel tone-diameter[249]. VSMCs exhibit phenotypic plasticity during pathological conditions to promote repair and remodeling of aortic wall via activation of cell migration and proliferation[250, 251]. Contractile function of VSMCs have been shown to prevent TAA and aortic dissections[252]. It has been reported that VSMCs apoptosis is one of the pathological vascular remodeling that result in TAA and AAA[10, 47, 253-255]. In pathological conditions, due to VSMCs apoptosis in the tunica media in aortic wall, the aorta is not capable of undergoing constructive vascular remodeling which then leads to thinning of the aortic wall.

In this study, we examined the response of VSMCs to Ang II or TGFβ. We expected impaired elastin synthesis in VSMCs from MMP2$^{-/-}$ mice, but we found that elastin expression levels were comparable between WT and MMP2$^{-/-}$ mice following Ang II treatment. VSMCs behave differently in culture than *in vivo* in the aortic wall since the aortic wall consists of VSMCs, endothelial cells, fibroblasts, and other ECM proteins. As a result, by excluding the interaction between the VSMCs and other cell types (e.g. endothelial cells and fibroblasts) within the aortic wall, our data from VSMCs are limited to examining what is happening in these cells alone.

4.10 Future directions

4.10.1 Rescue experiments

In this study, I mainly focused on identifying the role of MMP2 in aortic aneurysm so I did not perform any rescue experiments. Since MMP2, which is a main MMP, has already deleted and the underlying mechanism in the observed TAA involves reduced elastin synthesis with reduced proteolytic activities, using a broad spectrum MMP inhibitor is not a good rescue approach. In addition, in MMP2$^{-/-}$ mice, we observed reduced elastin levels due to impaired TGFβ signaling pathway. Thus, RNA interference (RNAi) technique using microRNA (miRNA) that regulates multiple mRNAs to reduce the protein levels of their target genes may not be useful in MMP2$^{-/-}$ mice[256, 257].

Since i*n vitro* experiment showed that intact downstream TGFβ signaling pathway in VSMCs from MMP2$^{-/-}$ thoracic aortas, a potential rescue approach in

preventing aneurysm progression could be replenishment of TGFβ using gene therapy such as adeno-associated viral vectors. Adenovirus is a non-enveloped virus containing a double-stranded DNA genome. The adenoviral vectors containing the gene of interest can be injected directly to the aneurysmal aortic tissue to introduce the specific gene of interest to synthesize functional proteins[258]. However, injected adenoviral vectors might initiates immune responses by activating inflammatory cytokines[259-261]. In addition, molecular and cellular changes vary depending on the location, etiology, and size of the aneurysm. Thus, more research is necessary to ensure safety and effectiveness of replenishing TGFβ to prevent progression of thoracic aortic aneurysm. Moreover, proper screening approach is needed to identify patients with thoracic aortic aneurysm due to significant reduction of MMP2 levels.

4.10.2 Influence of endothelial cells on vascular smooth muscle cells

Modification of the experimental protocol is needed to examine the disparity that we found between *in vivo* and *in vitro* data. The endothelial cells in the aortic wall can release paracrine factors to mediate VSMCs function[235-237]. In the future, it would be helpful to understand the interaction between endothelial cells and VSMCs by co-culturing VSMCs with endothelial cells, or to treat VSMCs with Endothelin-1 which has also been shown to activate VSMCs[262, 263]. In addition, it would be useful to see how these co-cultured cells respond to both Ang II and TGFβ. If cells show impaired elastin synthesis, addition of recombinant MMP2 can be used as a rescue option. This will provide role of VSMCs in the formation of aortic aneurysm.

4.10.3 TAA development in females

We used WT and MMP2$^{-/-}$ male mice to investigate the role of MMP2 in aortic aneurysm. Our preliminary data showed that MMP2$^{-/-}$ female mice were protected against AAA and TAA. These data suggest that MMP2 appears to have a regional and gender specific impact in Ang II-induced aortic remodeling, such that lack of MMP2 leads to thoracic aortic aneurysm in male but not in female mice. It has been reported that estrogen plays a protective role in cardiovascular diseases. Following estrogen treatment, reduction in cholesterol levels and aortic legion size were observed[264-266]. By regulating expression of MMPs, estrogen plays a role in vascular remodeling[266]. Several literatures showed the role of estrogen in renin-angiotensin system where estrogen reduced expression of Angiotensin II receptor type 1 and Angiotensin I-converting enzyme (ACE)[267-269]. It would be informative and useful to examine gender-specific heterogeneity in the expression pattern of the ECM components and other related factors in aortic wall. By comparing male and female mice, it will provide crucial information on the underlying mechanisms as well as possible therapeutic interventions for aortic aneurysm.

4.10.4 Temporal molecular and cellular events during progression of TAA in MMP2$^{-/-}$ mice post-Ang II infusion

In this study, we examined vascular remodeling in WT and MMP2$^{-/-}$ mice following 4 weeks of Ang II infusion. It would be useful to examine molecular and cellular changes at different time points (2 weeks and 8 weeks post-Ang II infusion). Temporal changes in the abundance of MMPs and TIMPs has been examined in from $CaCl_2$-exposed TAA in mice[270]. However, temporal changes in

infiltration of inflammatory cells, oxidative stress, and ECM remodeling have not been yet explored. Thus, it would be useful to profile temporal changes in MMPs, TIMPs, inflammatory cells, oxidative stress, proteolytic activities (elastase and collagenase activities), and synthesis of ECM components (TGFβ signaling pathway) in $MMP2^{-/-}$ mice at different time points. This will provide a better understanding of temporal relationship between molecular and cellular changes that occur throughout the progression of aortic aneurysm.

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