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Matrix metalloproteinase-2 mediates angiotensin II-induced hypertension

by

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Abstract

Angiotensin II signals cardiovascular disease through metalloproteinases including MMP-2, MMP-7 and ADAM-17/TACE. We hypothesized that these metalloproteinases regulate each other at the transcriptional level. Further, MMP-2, being a major gelatinase in cardiac and vascular tissue, could mediate angiotensin II-induced cardiovascular disease.

We studied the development of hypertension (by tail cuff plethysmography), cardiac hypertrophy (by M-mode echocardiography and qRT-PCR analysis of hypertrophy marker genes) and fibrosis (by collagen staining and qRT-PCR analysis of fibrosis marker genes) in mice receiving angiotensin II.

Angiotensin II induced hypertension, cardiac hypertrophy and fibrosis which correlated with an upregulation of MMP-2. Downregulation of MMP-2 by pharmacological inhibition and RNA interference attenuated hypertension but not cardiac hypertrophy or fibrosis. Downregulation of MMP-7 or ADAM-17/TACE by RNA interference attenuated angiotensin II-induced MMP-2 upregulation as well as hypertension, cardiac hypertrophy and fibrosis.

We conclude that MMP-2 selectively mediates angiotensin II-induced hypertension under the transcriptional control of MMP-7 and ADAM-17/TACE.

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Abbreviations

ADAM	A disintegrin and metalloproteinase
a-sk-actin	alpha-skeletal-actin
Ang I	Angiotensin I
Ang II	Angiotensin II
ACE	Angiotensin converting enzyme
ACE-2	Angiotensin converting enzyme-2
b-MHC	beta-myosin heavy chain
BNP	Brain natriuretic peptide
Col I	Collagen type I
Col III	Collagen type III
DMSO	Dimethyl sulfoxide
Dox	Doxycycline
ECM	Extracellular matrix
EGF(R)	Epidermal growth factor (receptor)
Erk	Extracellular regulated kinase
Fn-1	Fibronectin-1
GqPCR	Gq protein coupled receptor
kDa	kiloDalton
LV	Left ventricle
Luc	Luciferase
MMP	Matrix metalloproteinase
mmHg	millimeter of mercury

MAPK	Mitogen activated protein kinase
ND	No difference
PE	Phenylephrine
PBS	Phosphate buffered saline
qRT-PCR	Quantitative real-time polymerase chain reaction
RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic Acid
siRNA	small interference RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHR	Spontaneously hypertensive rat
SNS	Sympathetic nervous system
TACE	Tumor Necrosis Factor-alpha converting enzyme
TIMP	Tissue inhibitor of metalloproteinase

Chapter 1

Introduction

1.1 Hypertension

1.1.1 Definition and classification

Hypertension is a systemic condition characterized by elevated arterial blood pressure. Although blood pressure fluctuates depending on environmental conditions and can be elevated at any given time, hypertension is a medical condition defined as a persistent elevation in blood pressure[1]. The severity of hypertension, according to the seventh report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, can be divided into four categories: normal, pre-hypertension, stage 1 hypertension and stage 2 hypertension (**Table 1.1**)[2]. In addition to being classified by severity, hypertension can also be classified by cause. Primary (essential) hypertension accounts for approximately 90-95% of all cases and refers to persistently elevated blood pressure where the medical cause is unknown[3]. Secondary hypertension accounts for the remaining 5-10% of hypertensive cases and is the result of identifiable medical conditions including metabolic syndrome, renal disease and pre-eclampsia (during pregnancy)[4-5].

1.1.2 Risk factors of hypertension

Although no direct cause can be attributed in most cases of hypertension, certain risk factors exist that increase the likelihood of an individual becoming hypertensive. Hypertensive risk factors consist of both uncontrollable variables (i.e. age, sex, other predisposing medical conditions and ethnic background) and controllable variables (i.e. lifestyle, diet and alcohol/tobacco use).

One of the most well-established risk factors of hypertension is obesity. It is thought that obese individuals develop hypertension as a result of insulin resistance, increased sympathetic neural activity, renin levels and sodium retention[6-7]. Increased adipocyte secretion of leptin in obese individuals may be a contributing factor to increased sympathetic neural activity and subsequent elevation of blood pressure[8-9]. Obese individuals also have increased renin, aldosterone and angiotensin converting enzyme levels, which contribute to increased blood pressure[10]. In adults, obese individuals are 3.5 fold more likely than lean individuals to become hypertensive[11-12]. Lifestyle factors, including exercise, diet, alcohol intake and tobacco use also influence the risk of an individual developing hypertension by mechanisms that are, in part, linked to the presence of obesity. Sedentary lifestyle, high sodium intake, and excessive use of alcohol and tobacco products are all controllable factors that are associated with obesity and predispose individuals to becoming hypertensive[13].

Genetic and heritable factors including sex, endocrine mutations and ethnic background also influence the likelihood of an individual becoming hypertensive. For example, the prevalence of hypertension is much higher in African Americans than in Caucasians[14]. Other heritable genetic traits, such as glucocorticoid-remediable aldosterism, mineralocorticoid receptor mutations and mutations in sodium transport proteins also influence the susceptibility of an individual to becoming hypertensive in an autosomal-dominant manner[1].

There is also substantial correlation between individuals with diabetes who are also hypertensive[15]. Insulin resistance is associated with increased sodium

retention, aldosterone secretion and sympathetic nervous system activity, all of which contribute to hypertension[16] (as described in chapter 3).

1.1.3 Health and economic burdens associated with hypertension

In 2007, over 30% of American adults were hypertensive and the prevalence of hypertension is currently increasing[17-18]. Due to the simultaneous increasing prevalence of several hypertensive risk factors, it is difficult to attribute which risk factors are predominantly responsible for the increasing incidence and severity of hypertension. As described in chapter 1.1.4, hypertension is a substantial risk factor for other cardiovascular diseases including cerebrovascular stroke, arterial disease, congestive heart failure and renal disease[2]. Together, cardiovascular disease is the world's leading cause of death[17], therefore minimizing the occurrence and severity of risk factors such as hypertension is an enormous priority. In addition to the tremendous health burden, hypertension also imposes substantial socioeconomic and political concerns. Between direct costs (pharmaceuticals, medical services, etc.) and indirect costs (utility loss, quality / duration of life adjustments, etc.), the cost of hypertension in the United States was estimated at $66 billion in 2007[17]. Hypertension therefore represents a significant portion of health care expenditures which is sufficient justification for encouraging research in the development of cost-effective and efficient therapeutic strategies.

1.1.4 Clinical complications associated with hypertension

Hypertension, often termed "the silent killer", has very few direct signs and symptoms. Rather, hypertension is a predominant underlying factor for other clinical conditions, especially renal disease and other cardiovascular diseases. The mechanical hemodynamic overload is thought to strongly contribute to the deterioration of renal function in hypertensive patients (hypertensive nephropathy)[19]. Increased stimulation of angiotensin type 1 and alpha-adrenergic receptors in the efferent renal arterioles contributes to increased glomerular pressure and subsequent plasma filtration fraction[20-21]. Ultimately, hypertensive nephropathy results in interstitial fibrosis, afferent arteriolar hyalinosis (accumulation of the pink proteinaceous material hyaline) and renal failure[22].

Increased signaling of the renin-angiotensin-aldosterone and sympathetic nervous systems in hypertensive patients increases cardiac and arterial remodelling (described in chapter 1.2). This hypertensive remodelling leads to the hardening of arteries (arteriosclerosis), increased susceptibility to ischemic emergencies (cerebrovascular stroke and myocardial infarction/heart attack) and heart failure[2,23]. For each increase of 20 mmHg (systolic) or 10 mmHg (diastolic) above 115/75 mmHg, the risk of other cardiovascular diseases increases by twofold[24].

1.1.5 Research models of hypertension

A number of *in vitro* and rodent models exist to study vascular tone and hypertension, respectively. Small mesenteric arteries isolated from wild type Sprague Dawley rats (inner diameter of approximately 200 μm) can be mounted

on a microperfusion arteriograph where the inner diameter and changes in vasoconstriction / vasodilation can be measured in real-time or at predefined intervals[25]. Typically, arteries are constricted with adrenergic agonists in the bath (such as phenylephrine, 10 μM on the adventitial side of the artery) and the potential properties of compounds to induce vasodilation are assessed by delivering the compound of interest either intraluminally or in the bath (as done in this study).

To study hypertension *in vivo*, two common rodent models are typically used: the mouse model of angiotensin II (Ang II)-induced hypertension (as done in this study) and the spontaneously hypertensive rat. Briefly, as described in chapter 2, the mouse model of Ang II induced hypertension uses a surgically implanted subcutaneous osmotic minipump to deliver recombinant human Ang II (1.4 mg/kg/d) for up to 28 days[26]. As described in this study, these mice develop hypertension and hypertensive cardiac remodelling within 14 days following Ang II infusion. A larger scale rodent model that encompasses multiple agonist systems is the spontaneously hypertensive rat, which is genetically predisposed to developing hypertension detectible within 5-6 weeks after birth[27]. In both models, test compounds can be delivered by a number of means including oral administration (as done in this study for pharmacological inhibitors), subcutaneous osmotic minipumps (as done in this study for small interference RNAs, siRNAs) or bolus injection (intraperitoneal, intramuscular or intravenous).

1.1.6 Diagnosis and measurement techniques in the clinic and laboratory

The pressure of blood against the arterial wall (blood pressure) can be determined either directly (by invasively measuring blood pressure within the vasculature) or indirectly (by monitoring the presence / absence of a pulse distal to an occlusion site). One direct approach to measuring blood pressure is radiotelemetry. A catheter is surgically placed within the femoral artery which directly measures the arterial blood pressure and transmits real-time blood pressure information in conscious, unrestrained animals[28].

Indirect blood pressure measurement is achieved by pressure occlusion plethysmography. Occlusion plethysmography in rodents works by monitoring reperfusion of blood to the tail with a pulse sensor after proximal occlusion[29]. Blood flow is occluded by an inflatable cuff connected to a pump and a pressure gauge. Following occlusion, the pulse signal disappears (blood flow is occluded) and the cuff pressure is slowly decreased while reperfusion of blood is monitored by a pulse sensor placed distally to the occlusion cuff. When the pressure of the occlusion cuff is equal to the systolic blood pressure, a signal (pulse) can be detected (i.e. the pulse signal re-appears in the rodent pulse sensor). New developments are being made to improve the detection of tail reperfusion using photoplethysmography (light sensor to detect blood flow / reperfusion) and volume-pressure recording (mechanical sensor to detect blood flow / reperfusion) to enhance the sensitivity of these systems.

The same principles apply in the clinical setting to measure blood pressure in humans. An occlusion cuff (sphygmomanometer) is placed on the bicep of one

arm and used to occlude blood flow to the forearm. During deflation, a stethoscope is used to listen for a pulse. The audible pulse heard using the stethoscope indicates the point at which the systolic blood pressure is equal to the cuff pressure. The audible pulse continues to be heard until the occlusion cuff pressure is equal to the diastolic blood pressure, at which point the sounds are dampened and the diastolic blood pressure is recorded.

1.1.7 Treatment strategies

Current guidelines used to treat hypertension utilize one or more anti-hypertensive agents that function to reduce blood pressure by: i) lifestyle modifications, ii) receptor antagonism of pro-hypertensive agonist receptors, iii) inhibition of pro-hypertensive agonist synthesis or iv) decreasing salt / water retention.

A recent review on obesity-induced hypertension proposes lifestyle modifications as a first measure of anti-hypertensive therapy[6]. Lifestyle changes including the implementation of an exercise regime, caloric restriction, decreased sodium intake and minimization of alcohol / tobacco use can substantially decrease the severity of hypertension[30].

Hypertension can also be targeted pharmaceutically. Since Ang II is such a potent hypertensive agent, a large degree of pharmaceutical development has been done to investigate therapies that: i) inhibit Ang II synthesis (i.e. ramipril, an angiotensin converting enzyme inhibitor[31]), ii) antagonize Ang II receptors (i.e. losartan, an AT1R antagonist[32]) and iii) accelerate Ang II degradation (i.e.

exogenous administration of recombinant angiotensin converting enzyme 2 (ACE-2)[33]). The metabolic pathway of Ang II synthesis and degradation is reviewed in chapter 1.3. Targeting the Ang II pathway is an effective means of lowering blood pressure and is typically used as the initial pharmacological agent in treating hypertension. In cases where hypertension remains (resistant hypertension), additional anti-hypertensive agents (described below) are used in combination until treatment goals are met[6].

Similar to receptor antagonism of Ang II receptors, adrenergic receptor antagonists are also good therapeutic candidates for anti-hypertensive therapy. Beta adrenergic antagonists (i.e. nebivolol[34-35]), alpha adrenergic antagonists (i.e. doxazosin[36]) and broad spectrum adrenergic antagonists (i.e. carvedilol[37-38]) can all be used as potent vasodilators to reduce blood pressure. One drawback of adrenergic antagonists is that they are often not cardioselective and thus confer systemic side effects including fatigue, depression and impaired glucose tolerance[35]. As such, adrenergic antagonists are not used as initial treatment lines of hypertension, but rather in combination with other therapies in resistant hypertension.

In addition to preventing systemic vasoconstriction, other approaches aim at reducing salt and water retention in the kidney. Thiazide diuretics (i.e. hydrochlorothiazide[39]) and calcium channel blockers (i.e. verapamil[40]) are typically used in combination with drugs targeting the angiotensin system to target hypertension using complimentary approaches[41-42]. Decreasing the retention of salt and water lowers blood volume (hypovolemia) and subsequently decreases

blood pressure. Thiazide diuretics function by inhibiting thiazide-sensitive Na^+/Cl^- symporters, decreasing the retention of these ions within the distal convoluted tubule of the kidney. As a side effect of excess sodium in the kidney collecting ducts, Na^+/K^+ antiporters exchange sodium for potassium, which can result in hypokalemia (low blood potassium)[43]. As such, potassium supplements or hyperkalemic agents can be used in conjunction with thiazide diuretics to prevent these effects[44].

In cases of severe hypertensive emergencies (sudden acute increase of blood pressure), rapid vasodilators such as nitroglycerin can be administered intravenously to provide a rapid decrease in blood pressure[45-46]. The pharmaceuticals described above are summarized in **table 1.2**.

Although increasing, less than 60% of diagnosed patients are meeting treatment goals (maintaining blood pressure <140/90 mmHg)[2]. It is evident that current treatment strategies, although effective on a case-by-case trial and error basis, are insufficiently meeting the strong demand for universal approaches that effectively lower blood pressure among the entire hypertensive population.

1.2 Hypertensive cardiac remodelling

One of the most common complications of chronic hypertension is the concurrent development of pathological cardiac remodelling due to the development of cellular hypertrophy and fibrosis[47-48]. Pathological cardiac remodelling leads to myocardial stiffness, decreased cardiac output and increased

risk of heart failure[49-51], all of which must be considered when devising anti-hypertensive strategies.

1.2.1 Cardiac hypertrophy

Cardiac hypertrophy results from the hypertrophic growth of individual cardiomyocytes within the heart. This hypertrophic growth is stimulated by growth factor signaling (described in chapter 1.4) and results in increased cell size, increased heart and left ventricle weight to body weight ratios, as well as increased expression of fetal genes (described in chapter 1.2.5).

1.2.2 Cardiac fibrosis

The excessive deposition of extracellular matrix (ECM) proteins in the interstitial space of the heart defines cardiac fibrosis. This accumulation of collagens and fibronectins is caused by: i) increased synthesis and secretion of fibrous proteins by fibroblast cells, ii) decreased turnover (degradation) of these proteins in the extracellular space or both in combination[48,52-53].

1.2.3 Risk factors of cardiac remodelling

It is clear that hemodynamic overload (hypertension) induces the development of hypertensive cardiac remodelling; however, the mechanisms by which this occurs are not fully understood. Whether hypertension induces cardiac hypertrophy directly by hemodynamic overload or indirectly by agonist signaling associated with hypertension in the heart is discussed in chapter 4.2.

Despite the detrimental effects of cardiac remodelling in hypertension, other instances of cardiac hypertrophy exist in nature which can be beneficial to the heart (i.e. the heart of a well-trained athlete[54]). A major factor leading to cardiac failure in subjects with hypertension is the excessive deposition of extracellular matrix proteins (cardiac fibrosis) that impairs cardiac contractility. This process of hypertension-associated cardiac fibrosis occurs in parallel to the hypertrophy process. However, despite developing within similar time frames and having common agonists, cardiac fibrosis and hypertrophy can be pharmacologically separated. Zeisberg et al.[55] have demonstrated that administration of recombinant human bone morphogenic protein-7 to mice with transverse aortic constriction (described in chapter 1.2.4) prevents cardiac fibrosis and improves cardiac function, despite the presence of cardiac hypertrophy.

1.2.4 Research models of hypertensive cardiac remodelling

In addition to the research models of chronic hypertension described in chapter 1.1.5, load-induced hypertrophy can be mechanically stimulated by transverse aortic constriction (TAC)[32]. In these models, partial surgical occlusion of the aorta creates a hemodynamic overload on the left ventricle of the heart, creating an exaggerated model of increased systemic vascular resistance. As a result, animals with TAC develop severe cardiac hypertrophy and fibrosis within 2 weeks of surgery without the presence of systemic hypertension[32]. Treatment

experiments can be conducted in conjunction with this model in the same manner as described in chapter 1.1.5.

1.2.5 Diagnosis and measurement techniques in the clinic and laboratory

In the clinic and in the laboratory, anatomical features of the heart can be visualized by echocardiography. This ultrasound technique provides 2-dimensional imaging of the left ventricle (posterior wall, ventricle chamber and interventricular septum). Images taken at systole and diastole can then be used to calculate the thickness of ventricular walls, ventricle volume, ventricle mass, fractional shortening of cardiomyocytes as well as overall ejection fraction of the heart[56-57]. This information provides researchers with real-time data describing anatomical changes *in vivo. Ex vivo*, researchers can also assess cardiac hypertrophy by overall weight of the heart. For both left ventricle (determined by echocardiography) and whole heart (determined by *ex vivo* pathology) weights, data is normalized to total body weight. Increases in left ventricle weight (or whole heart weight) to body weight ratio indicate the presence of cardiac hypertrophy.

Histological analysis of cardiac samples can also provide insight into the pathogenesis of hypertensive cardiac remodelling. Cellular cross sectional area (cellular hypertrophy) can be determined by staining sections with hematoxylin and eosin, although improved contrast of cellular borders can be visualized using fluorescein-conjugated wheat germ agglutinin, a carbohydrate-binding protein that recognizes N-acetylglucosaminyl sugars on the plasma membrane[58-59].

Collagen deposition (fibrosis) in heart samples can be visualized by staining heart sections with picrosirius red, a dye that binds extracellular collagen, therefore indicating the relative severity of cardiac fibrosis[60].

At the molecular level, cardiac hypertrophy and fibrosis can be assessed in the laboratory by measuring expression of hypertrophy and fibrosis marker genes by qRT-PCR. In left ventricle samples, increased expression of the fetal genes alpha-skeletal actin (a-sk-actin), brain natriuretic peptide (BNP) and beta-myosin heavy chain (b-MHC) are molecular markers of cardiac hypertrophy[26,61-62]. Similarly, increased expression of collagen type I and III (Col I and Col III, respectively) as well as fibronectin-1 (Fn-1) are molecular indications of cardiac fibrosis[62].

1.2.6 Treatment strategies

Currently, treatment of hypertensive cardiac remodelling is largely overlapping with treatment of hypertension (i.e. ACE inhibitors, diuretics etc.)[63-64]. Because of the increased risk of developing arrhythmias in hypertensive cardiac remodelling and cardiomyopathy, artificial pacemakers may be implanted to regulate heart rhythm[65]. In advanced cases of disease, cardiac transplantation may be required to avoid lethal heart failure.

1.3 Physiological control of blood pressure and cardiac remodelling

1.3.1 Factors affecting systemic blood pressure

The physiological control of blood pressure is essential to maintain adequate perfusion of blood to all body organs in varying environmental conditions. Each time the left ventricle contracts, a bolus of blood is pumped through the aorta to the systemic vasculature[1]. As such, the pressure exerted on the arterial wall (blood pressure) fluctuates depending on the real-time state of the heart in its output cycle. During systole, when the heart is ejecting blood, pressure is highest (systolic blood pressure) and when the heart ventricle is filling (no blood pumping), the blood pressure is lowest (diastolic blood pressure). Thus, blood pressure can vary depending on cardiac output. Additional factors, such as contractile strength and stroke volume also affect cardiac output and therefore blood pressure. Increased cardiac output results in increased blood pressure, provided vascular resistance is unchanged[66].

Blood pressure is also highly dependent on vascular resistance. Similar to fluid flow in pipes, alterations in the diameter and vascular tone of arterial blood vessels changes blood pressure. When smooth muscle cells surrounding the arterial vasculature contract (as described in chapter 1.4) vasoconstriction occurs, decreasing the arterial diameter of the vessel, increasing the vascular resistance and subsequently increasing the blood pressure[66]. Likewise, relaxation of these smooth muscle cells increases arterial diameter, thus decreasing the vascular resistance and subsequently decreasing the blood pressure. The regulated contraction and relaxation of arterial smooth muscle cells is controlled by a multitude of endocrine, biochemical and neurological stimuli. Those directly related to the content of this thesis are elaborated below (chapter 1.3.3 and 1.3.4).

Another factor that can influence blood pressure is blood volume. During blood loss (hypovolemia), an insufficient quantity of blood limits cardiac output and subsequently lowers blood pressure. Hormones that regulate salt and water retention in the kidney (i.e. aldosterone) function by increasing the re-uptake of Na^+, Cl^- and H_2O in the distal convoluted tubule of the kidney, increasing plasma volume and leading to a subsequent increase in blood pressure[67].

1.3.2 Factors affecting cardiac remodelling

Hypertrophic growth and interstitial fibrosis develop simultaneously in response to common stimuli; however, the biochemical signaling pathways in each process are distinct. For example, hypertensive agonists such as Ang II initiate the activation of growth pathways (leading to hypertrophy) as well as fibrous protein synthesis (leading to fibrosis)[68]. Hypertrophic growth depends on growth factor receptor activation and subsequent kinase cascades (i.e. stimulation of the epidermal growth factor receptor and activation of the mitogen activated protein kinase pathway, MAPK)[69]. Ultimately, overall protein synthesis is accelerated by the phosphorylation of the S6 protein subunit of the ribosome, a characteristic that is enhanced by MAPK[70]. Simultaneously, upon stimulation, fibrotic factors such as transforming growth factor-beta (TGF-β) are released by proteolytic cleavage of regulatory binding proteins[71]. Once released, TGF-β acts on cell surface receptors and increases the phosphorylation of SMAD transcription factors (i.e. SMAD-3) [72]. Phosphorylation of SMAD-3 confers its ability to act as a transcription factor to allow the increased expression of

extracellular matrix proteins[72]. In addition to SMAD-mediated transcription, TGF-β also mediates the activation of kinases such as extracellular regulated kinase (Erk) and p-38 MAPK[73].

1.3.3 The renin-angiotensin-aldosterone system

Angiotensin II (Ang II), a potent vasoconstrictive agonist, is the central agonist molecule of the renin-angiotensin-aldosterone system (RAAS) and is produced by the multi-step proteolytic cleavage of the pro-peptide angiotensinogen[74]. Circulating angiotensinogen is a 452 amino acid peptide produced in the liver; however, angiotensinogen can also be produced locally in epithelial cells of the kidney[75]. Renin, a proteolytic enzyme produced in juxtaglomerular cells in the kidney, cleaves angiotensinogen yielding angiotensin I (Ang I), a ten amino acid peptide[75-76]. Ang II can then be produced by the subsequent cleavage of two residues from the C terminus of Ang I by circulating angiotensinases (i.e. angiotensin converting enzyme, ACE) secreted primarily by lung endothelial cells[77-78]. Ang II can then act as a vasoconstrictive agonist in the systemic arterial vasculature by activation of the angiotensin receptor subtypes 1 and 2 (AT1R and AT2R, respectively), as described in chapter 4.1. Ang II is metabolized by the enzyme ACE 2, which is produced by endothelial cells in the heart and kidneys and further cleaves Ang II to form angiotensin-(1-7), a far less vasoactive peptide[79-82]. In addition to being able to cleave Ang II, ACE-2 can also cleave a single amino acid from Ang I, forming angiotensin-(1-9), which is also relatively inactive[75]. Thus, ACE-2 activity suppresses the activity of Ang II by; i)

accelerating the metabolism of Ang II and ii) cleaving Ang I, the substrate of ACE required to make Ang II. In the kidney, Ang II also stimulates the synthesis of aldosterone, a mineralocorticoid hormone that acts to increase water and solute retention in the distal tubules of the kidney[83]. This action of aldosterone increases plasma volume and contributes to the hypertensive effects of Ang II. Intrinsic regulation of the RAAS is controlled by renin production in response to changes in: baroceptor stimulation (blood volume), salt retention and sympathetic nervous system activity[84-85]. For example when blood volume / pressure is low, renal baroreceptors mediate an increase in renin production, which activates the production of Ang II and increases blood pressure by: i) systemic vasoconstriction and ii) aldosterone-mediated increased salt / water retention in the kidney. Pathological overactivation of the RAAS is a common hallmark of hypertensive disorders and has led to multiple anti-hypertensive therapeutic approaches targeting mediators of the angiotensin metabolic pathway (described in 1.1.7). A visualisation of the above pathway is demonstrated in **figure 1.1** and the enzymes / peptides are summarized in **table 1.3**.

1.3.4 The sympathetic nervous system

The sympathetic nervous system is part of the autonomic (involuntary) response that reacts to environmental stress by initiating a "fight-or-flight" response. Neuronal or hormonal stress signals the release of acetylcholine (Ach) from preganglionic neural fibres which stimulate nicotinic Ach receptors[86]. After the signal propagates to the postganglionic neurons, catecholamines

(norepinephrine and epinephrine) are released to target tissues[87]. Additionally, the adrenal medulla acts as a sympathetic ganglion, where preganglionic release of Ach stimulates postganglionic release of catecholamines from chromaffin cells directly into the bloodstream, where they are able to act systemically[88]. Catecholamines activate adrenergic receptors which, in turn, induce vasoconstriction. Excessive adrenergic stimulation is associated with early stages of human essential hypertension, offering an additional avenue for therapeutic intervention[89-90].

1.4 Cellular signaling pathways in hypertension and hypertensive cardiac remodelling

The parallel development of cardiac hypertrophy and fibrosis with hypertension suggests overlapping mechanisms with common inducers and mediators. Among the common inducers of hypertension, cardiac hypertrophy and fibrosis are vasoconstrictive Gq protein-coupled receptor (GqPCR) agonists such as catecholamines, endothelins, and Ang II. These agonists are thought to modulate vascular tone by stimulating traditional phospholipase C pathways, although novel signaling mediators (including metalloproteinases) have been identified[91-93]. A basic overview of each pathway is described below.

1.4.1 Traditional G-protein coupled receptor pathway

In response to stimuli such as Ang II, Gq-protein coupled receptors (GqPCRs) catalyze a nucleotide replacement (GDP-GTP) in G proteins, which

induce a conformational change, allowing the G protein to dissociate from the GqPCR[94]. Following stimulation, G proteins such as Gαq can then associate with and activate phospholipase C (PLC)[95]. PLC activation induces the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3)[96]. Subsequent cellular signaling events are carried out by activation of DAG-dependent protein kinase C (PKC) isoforms and IP_3-dependent release of calcium from the sarcoplasmic reticulum[97]. This pathway is visualized in **figure 1.2**. In smooth muscle cells (such as those surrounding the arterial vasculature), Gq-mediated calcium release promotes the calmodulin-dependent activation of myosin light chain kinase, which phosphorylates myosin subunits causing constriction of smooth muscle cells[98-99].

1.4.2 Transactivation of growth factor receptors by metalloproteinases

Recently, alternative pathways of GqPCR agonists have been proposed whereby novel signaling mediators (such as metalloproteinases) mediate signaling events by the shedding of growth factors and cleavage of vasoactive peptides[91-93,100-104]. It is known that metalloproteinases can be activated in response to vasoconstrictive agonists both acutely (i.e. matrix metalloproteinase-7, MMP-7) [26] and in the long term (i.e. matrix metalloproteinase-2, MMP-2)[105-106]. Activation of metalloproteinases has been proposed to modulate cell signaling by transactivation of growth receptors (such as the epidermal growth factor receptor, EGFR)[101] through shedding of epidermal growth factor (EGF) by cleavage of Heparin-Binding EGF (HB-EGF)[100]. This transactivation pathway leads to

activation of the growth kinase cascades described in chapter 1.3.2 and is visualized in **figure 1.3**.

1.4.3 Pathological effects of excessive agonist signaling

Although the aforementioned pathways are important in the maintenance of vascular homeostasis, excessive stimulation can be pathogenic, as in the case of hypertension. As described in chapter 1.3.3 and 1.3.4, excessive stimulation of the RAAS and SNS is common in hypertensive patients and targeting these systems has the capacity, at least in some cases, to lower blood pressure. Due to functional redundancy and cross-talk between vasoconstrictive agonist systems (described in chapter 4.5.2), identifying common downstream mediators of these pathways offers a novel and valuable approach in treating hypertensive disorders.

1.5 Metalloproteinase families: Matrix metalloproteinases (MMPs) and disintegrin metalloproteinases (ADAMs)

1.5.1 Classification of metalloproteinases

Metalloproteinases are a class of proteases whose catalytic activity depends on the presence of metal ions such as zinc or cobalt at the active site. Examples of this class of enzymes include the matrix metalloproteinases (MMP) and the disintegrin metalloproteinases (ADAMs)[107-108]. Although metalloproteinases in general share structural and functional characteristics, they can be further sub classified according to their domain structure and substrate specificity[107]. Examples of these groups include the gelatin-binding MMPs, types

I / II transmembrane MMPs and the hemopexin domain-containing MMPs[109]. Between all members, matrix metalloproteinases are able to cleave essentially all components of the ECM[109]. All MMPs are synthesized as pro-enzymes and require the proteolytic cleavage of a propeptide for complete activation (described in chapter 1.5.2).

1.5.2 Activation and regulation of metalloproteinases

Metalloproteinases (such as MMP-2) are regulated by a variety of mechanisms including proteolytic activation, interaction with regulatory proteins, transcriptional regulation and post translational modifications (i.e. phosphorylation)[110-113]. As is the case with all matrix metalloproteinases, MMP-2 has an N terminal propeptide domain that acts as an autoinhibitory domain. Cleavage of this prodomain is required for the complete activation of MMP-2 and can be inititated by other MMPs such as MT1-MMP[109,114]. The regulation of metalloproteinases by propeptides can be explained by a cysteine-switch mechanism, where a critical cysteine residue on the propeptide acts as a ligand of the catalytic metal ion[110]. Cleavage of the propeptide (by other proteases or by autolysis), oxidative stress or other conformational perturbations can disrupt the interaction of the cysteine residue with the catalytic metal ion, exposing the active site[110]. This process has been proposed to occur in two steps. First, serine proteases cleave a portion of the propeptide from the prodomain resulting in a intermediate activation form of MMP-2, which is not completely active. Following this initial cleavage, the remaining propeptide can be cleaved by

autolysis, resulting in the complete activation of MMP-2[115]. Additional mechanisms of proteolytic activation of MMP-2, including proteolysis by metalloproteinases such as MT1-MMP have also been proposed[116-117]. The activation of MMP-2 has also been shown to occur without the proteolytic cleavage of the propeptide. Modification of the critical cysteine residue in the propeptide by perturbants such as hypochlorous acid, oxidants and detergents are also able to disrupt the interaction of the propeptide with the catalytic domain, allowing activation of the metalloproteinase without cleavage of the prepeptide[118]. A summary of MMP-2 activation by propeptide cleavage is shown in **figure 1.4**.

Another aspect of metalloproteinase regulation arises from their interactions with regulatory proteins such as the tissue inhibitors of metalloproteinases (TIMPs), which reversibly inhibit the proteolytic activities of metalloproteinases in a 1:1 stoichiometric ratio[109]. All currently known MMPs have been shown to be inhibited by at least one of the four currently identified TIMP isoforms (TIMP 1, 2, 3 and 4)[119]. Specifically, TIMP-2 has been shown to inhibit MMP-2 by binding to both the pro and active forms of MMP-2, although additional roles of TIMP-2 have been identified by formation of a trimolecular activation complex involving MMP-2, TIMP-2 and MT1-MMP[111,117,120]. In this complex, TIMP-2 interacts with MT1-MMP and recruits pro-MMP-2 for proteolytic activation by MT1-MMP. When TIMP-2 levels are low, the active site of some MT1-MMP enzymes is exposed and able to cleave a portion of the propeptide of MMP-2, leading to its activation[121]. Conversely, when TIMP-2 levels are elevated, the active site of most MT1-MMP enzymes is inhibited and

therefore cannot initiate the activation of MMP-2[121]. Thus, TIMP-2 has bifunctional roles in the activation and inhibition of MMP-2.

In addition to being regulated post-translationally, MMPs can also be regulated by transcriptional activation[112]. This transcriptional upregulation depends on the activation of transcription factors such as activating transcription factor-2 (ATF-2), activating protein-1, JunB-Fra1and JunB-FosB [122-123]. These transcription factors can be activated by phosphorylation mediated by MAPK and JNK pathways which are triggered by growth factors (i.e. EGF) and some cytokines (i.e. transforming growth factor beta, TGF-β)[106,122].

1.5.3 Molecular function of metalloproteinases

Metalloproteinases have been widely accepted as mediators of ECM remodelling[124]. However, over the past 20 years, the molecular functions of metalloproteinases have expanded from acting strictly as ECM degrading enzymes to becoming accepted as mediators of cell signaling pathways. Metalloproteinases have been demonstrated to act as molecular "sheddases" where they can cleave membrane bound growth factors and cytokines, releasing and allowing them to transactivate their respective receptors[125-128]. In this context, previous work from our lab has demonstrated that metalloproteinases (i.e. MMP-7) can transactivate the EGFR through shedding HB-EGF in agonist-induced hypertension[92-93].

1.5.4 Pathophysiological roles of metalloproteinases

Excess activities of metalloproteinases have been associated with a number of diseases including cancer, cardiovascular disease and inflammatory diseases[129-132]. Because of the strong upregulation of metalloproteinases in these pathological conditions, metalloproteinases have been investigated as potential therapeutic targets. Despite reasonable success in the laboratory, clinical applications of metalloproteinases in the cancer field have had very little success[133-134]. Currently, the only approved metalloproteinase inhibitor used is periostat (doxycycline hyclate), which is used to treat periodontal disease[134]. Although initially thought to improve periodontal disease by acting as an antibiotic, it has now been established that periostat functions by broad-spectrum inhibition of metalloproteinases[135-136]. Since the failures of metalloproteinase-based therapeutic in the cancer field can be, at least in part, attributable to their lack of specificity, further research is merited to determine the clinical value of selective metalloproteinase inhibition.

1.5.5 Emerging roles of MMP-2 in the cardiovascular system

We have previously suggested that MMPs and ADAMs are transcriptionally networked to mediate pro-hypertensive, pro-hypertrophic and pro-fibrotic signalling in response to GqPCR agonists[26,137-138], however, the exact roles of individual metalloproteinases in the development of cardiovascular disease remain unclear. Of these metalloproteinases, MMP-2 has been shown to regulate vascular function by cleavage of vasoactive peptides (such as big

endothelin, calcitonin gene related peptide and adrenomedullin[102-104]) and cell signalling receptors (such as vascular endothelial growth factor and insulin receptors[139-141]). Since MMP-2 is a known protease of vasoactive peptides and is highly expressed in the heart and vasculature, we therefore focused our studies of metalloproteinases in Ang II-induced hypertension on MMP-2.

1.6 Protein kinase B (Akt) as a regulator of vascular tone

1.6.1 Molecular function of Akt

Protein kinase B (Akt) is a ubiquitously expressed serine/threonine protein kinase that plays important roles in cell survival, growth and metabolism[142-144].

Cell survival can be enhanced by suppressing the pro-apoptotic activities of proteins such as Bcl-2-associated death promoter (Bad). Upon stimulation with growth/survival factors, Akt prevents apoptosis and enhances cell survival by phosphorylating BAD at serine 75 and 99 which prevents the apoptotic function of BAD by regulating its interaction with other proteins such as Bcl-xL and 14-3-3 proteins[145-146]. Conversely, cell growth can be stimulated by growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) which also activate Akt[147-148]. Cellular metabolism of carbohydrates, fatty acids and triacylglycerols is regulated by phosphorylation of metabolic enzymes such as ATP citrate lyase and glycogen synthase kinase-3 (GSK-3), which are substrates of Akt[144,149]. In response to insulin (and subsequent Akt activation), phosphorylation of these enzymes increases the synthesis of glycogen, fatty acids and triacylglycerols.

1.6.2 Activation and regulation of Akt

Akt can be post-transcriptionally activated by reversible phosphorylation initiated by signaling of growth factors and GqPCRs[150-151]. Akt activation depends on the recruitment of Akt to the plasma membrane through interactions of an internal Pleckstrin homology (PH) domain and phosphatidylinositol (3, 4, 5)-triphosphate (PIP_3)[152]. PIP_3 is formed by the phosphorylation of PIP_2 by phosphatidylinositol-3 kinase (PI3K) and is is necessary but insufficient for Akt activation[153]. Activation of Akt can thus be inhibited using the well characterized PI3K inhibitors wortmannin and LY294002[154-155]. Following recruitment to the plasma membrane, Akt is activated by phosphorylation at two major regulatory residues, threonine 308 and serine 473[156-157]. Threonine 308 is phosphorylated by phosphoinositide dependent kinase 1 (PDK-1)[158-159] at the cell surface, in a phosphoinositide dependent manner. Serine 473 is phosphorylated by the mammalian target of rapamycin complex 2 (mTORC2)[160-161]. Further, complete activation of Akt requires phosphorylation at serine 473[162] thus, phosphorylation at serine 473 is an appropriate marker of Akt activation that can be detected by immunoblotting using commercially available phospho-specific antibodies.

1.6.3 Role of Akt in the maintenance of vascular tone

Recently, Akt has also been identified as a mediator of vascular tone. In response to pro-hypertensive agonists such as phenylephrine and Ang II, Akt is phosphorylated at serine 473[151,163]. Although the involvement of Akt activation in the maintenance of arterial tone has been demonstrated in physiological studies,

the molecular mechanism by which this occurs is unclear. It has been proposed that Akt regulates vascular tone by controlling the bioavailability of ATP required for vasoconstriction[163], although the involvement of other mediators (including MMP-2) in this pathway are not fully understood.

1.7 Rationale, hypotheses and research objectives

1.7.1 Rationale and hypotheses

Excessive GqPCR agonist signaling in the heart and vasculature leads to the development of hypertension and hypertensive cardiac remodelling. Since MMP-2 is upregulated by Ang II and is one of the major metalloproteinases expressed in cardiac and vascular tissue, we developed the following hypotheses:

Hypothesis #1: Ang II induces hypertension, cardiac hypertrophy and fibrosis by engaging metalloproteinases such as MMP-2.

Hypothesis #2: Metalloproteinases that are upregulated by Ang II are likely to be key mediators of Ang II-induced hypertension and hypertensive cardiac remodelling.

1.7.2 Research objectives

Objective #1: To determine if metalloproteinases such as MMP-2 are upregulated in Ang II-induced hypertension.

Objective #2: To determine if targeting these metalloproteinases has the potential to attenuate or prevent Ang II-induced hypertension or hypertensive cardiac remodelling.

Objective #3: To better understand the mechanistic role of metalloproteinase signaling in Ang II-induced hypertension.

Table 1.1 Classification of hypertensive disorders by severity

Category	**Systolic Blood Pressure** (mmHg)	**Diastolic Blood Pressure** (mmHg)
Normal	<120	and <80
Pre-hypertension	120-139	or 80-89
Stage 1 hypertension	140-159	or 90-99
Stage 2 hypertension	≥160	or ≥100

Modified from: The seventh report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, 2003[2].

Table 1.2 Description of example anti-hypertensive pharmaceuticals

Name	Function	References
Losartan	Ang II receptor (AT1R) antagonist	32
Ramipril	Angiotensin converting enzyme (ACE) inhibitor	31
ACE 2	Accelerates metabolism of Ang II	33
Doxazosin	Alpha adrenergic receptor antagonist	36
Nebivolol	Beta adrenergic receptor antagonist	34
Verapamil	Calcium channel blocker	40
Hydrochloro-thiazide	Thiazide diuretic	39
Nitroglycerin	Nitric oxide (NO) donor, potent acute vasodilator	45

Table 1.3 Description of hormones in the renin-angiotensin-aldosterone system

Name	Function	Source tissue	References
Renin	Protease that cleaves angiotensinogen to form Ang I	Kidney	75
ACE	Protease that converts Ang I to Ang II	Lung, kidney	78
ACE-2	Protease that converts Ang II to Ang-(1-7)	Heart, kidney	82
Angiotensinogen	Zymogen of Ang I, substrate of renin	Liver, kidney	75
Ang I	Pro-form of Ang II lacking vasoconstrictive properties, substrate of ACE	Metabolite of angiotensinogen	76
Ang II	Potent vasoconstrictor, stimulates synthesis of aldosterone, substrate of ACE-2	Metabolite of Ang I	75
Ang-(1-7)	Metabolite of Ang II lacking vasoconstrictive properties	Metabolite of Ang II	79
Aldosterone	Renal salt / water retention	Adrenal cortex	83

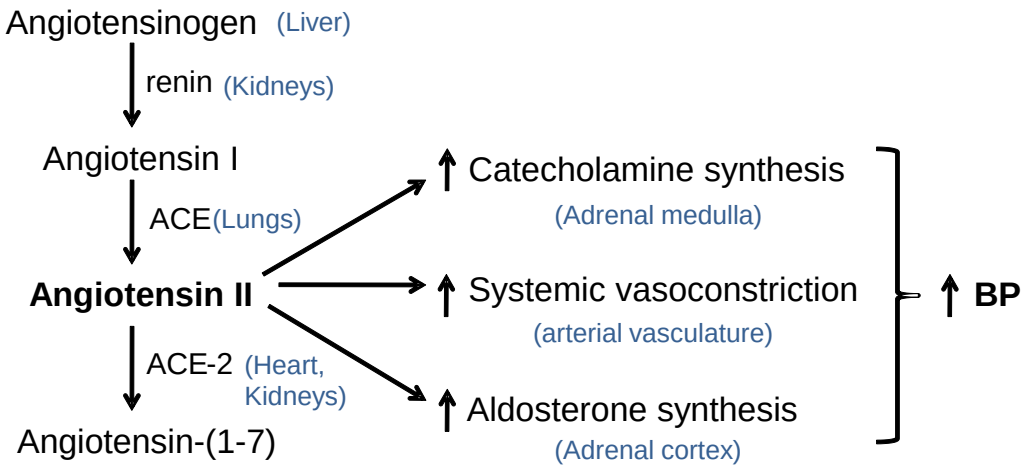

Figure 1.1 The renin-angiotensin-aldosterone system. Angiotensinogen is metabolised by renin to form angiotensin I, which is subsequently converted into angiotensin II by ACE. Angiotensin II exhibits bioactive effects such as increasing sympathetic nervous activity, acting as a vasoconstrictor and increasing aldosterone / vasopressin synthesis. Angiotensin II is subsequently metabolised by ACE-2 into angtiotensin-(1-7), a far less active biomolecule. Source tissues are indicated in parentheses. ACE: Angiotensin converting enzyme. ACE-2: Angiotensin converting enzyme 2. BP: blood pressure.

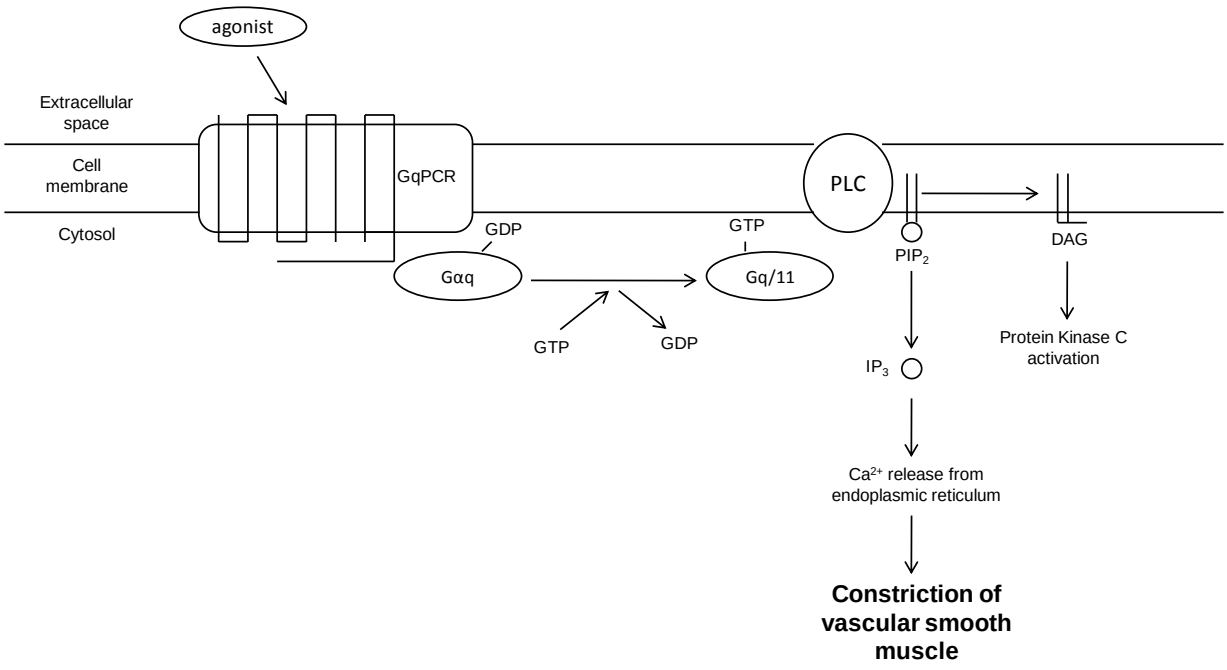

Figure 1.2 GqPCR agonists stimulate the phospholipase C $\rightarrow$ Ca^{2+} pathway. GqPCR agonists stimulate the substitution of guanosine nucleotides on Gαq proteins. Activated Gαq then dissociates from the receptor and activates PLC. PLC cleaves PIP_2 to form the products IP_3 and DAG. DAG then induces protein kinase C activation and IP_3 causes the release of Ca^{2+} from the endoplasmic reticulum, causing constriction of smooth muscle cells. GqPCR: Gq protein coupled receptor. GDP/GTP: Guanosine diphosphate / triphosphate. PLC: Phospholipase C. PIP_2: Phosphatidylinositol 4, 5-bisphosphate. IP_3: Inositol 1, 4, 5-triphosphate. DAG: diacylglycerol. Modified from Dowal et al., 2006 and Sternweis et al., 1993[94,Sternweis, 1993 #7306].

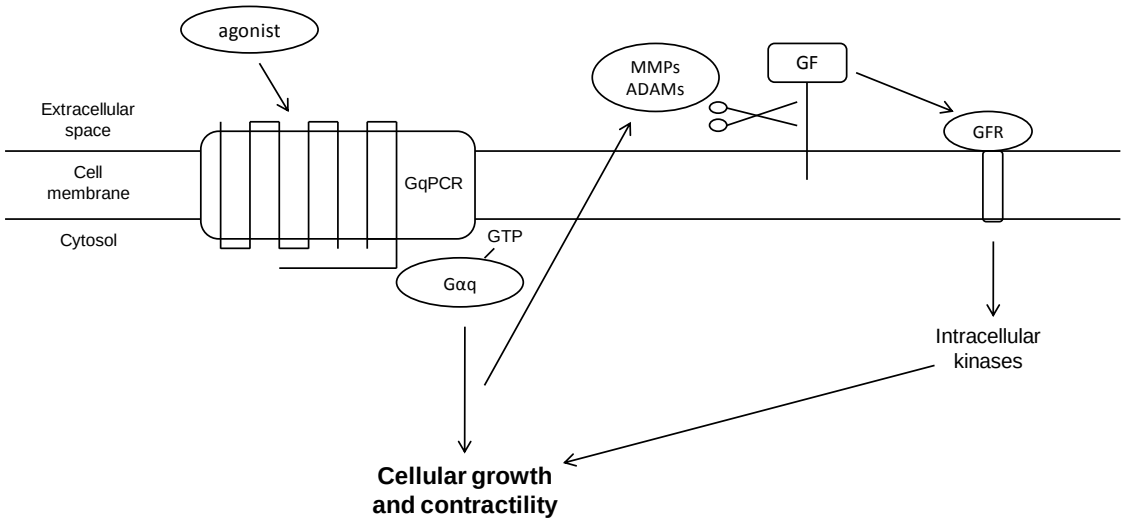

Figure 1.3 Transactivation of growth factor receptors by metalloproteinases. GqPCR agonists stimulate the activation of metalloproteinases (MMPs and ADAMs) which can shed extracellular growth factors. Upon metalloproteinase shedding, growth factors transactivate their respective receptors and initiate cell growth and contractility mediated by intracellular kinase signaling. GqPCR: Gq protein coupled receptor. GTP: Guanosine triphosphate. GF: growth factor. GFR: growth factor receptor. Modified from Fernandez-Patron, 2007[92].

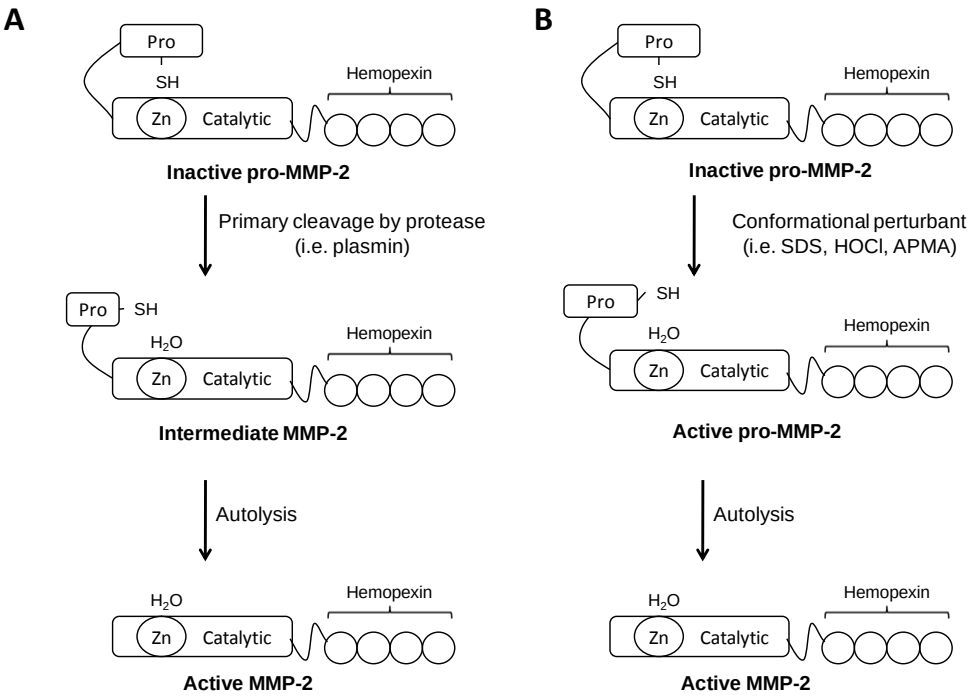

Figure 1.4 Schematic of proteolytic activation of MMP-2. (**A**) Pro-MMP-2 (inactive) is partially cleaved by proteases (such as plasmin). This cleavage disrupts the interaction of a cysteine residue with the catalytic zinc atom and allows an autolysis step to remove the remaining section pro-domain and causing complete activation of MMP-2. (**B**) Pro-MMP-2 (inactive) undergoes a conformational change when exposed to perturbants such as sodium dodecyl sulfate (SDS), hypochlorous acid (HOCl) or aminophenylmercuric acid (APMA), disturbing the interaction of the critical cysteine in the pro-domain with the catalytic zinc ion. Similar to the partial proteolysis mechanism, the now-exposed active site of MMP-2 can cleave the pro-domain by autolysis, resulting in complete activation of MMP-2. Modified from Chakraborti et al., 2003[115].

Chapter 2

Materials and Methods

2.1 Materials

MMP-2 inhibitors I / III and Ang II were obtained from Calbiochem (Gibbstown, NJ, USA). Phenylephrine was obtained from Sigma (Oakville, ON, Canada). All siRNAs were synthesized by Sigma-Aldrich (Paris, France) and dissolved in sterile PBS prior to use. The first two nucleotides of each strand were 2'-O methylated and the final two nucleotides were deoxy nucleotides to increase stability of the siRNAs. Sequences of siRNAs were adapted from [26,164-165] and are shown in Table 2.1. All primers and probes used for qRT-PCR analysis were obtained as kits from Applied Biosystems (Carlsbad, CA, USA). A7R5 rat aortic smooth muscle cells were purchased from ATCC (Manassas, VA)**.** NIH-3T3 cells were donated by the Michalak lab at the University of Alberta. Antibodies against Akt (SC-8312) and phospho-Akt (SC-7985-R) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MMP-2 antibodies (MAB3308) were purchased from Millipore (Billerica, MA, USA).

2.2 Animals

Animal protocols were conducted in accordance with institutional guidelines issued by the Canada Council on Animal Care. Male C57BL/6 mice and Sprague Dawley rats were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and Biosciences (University of Alberta, AB, Canada), respectively. All animals were anesthetised by inhalation (2-4% Isoflurane) before and during all surgical procedures. Ang II (1.4 mg/kg/d) and siRNA (0.4 mg/kg/d) Corporation, Cupertino, CA, USA) were administered by subcutaneous osmotic

minipumps, as described in our earlier papers[26,137]. Briefly, Ang II and siRNAs were dissolved in sterile PBS prior to use and were loaded the day before use to allow for overnight priming. Osmotic minipumps were then surgically implanted on the posterior midsection of mice and siRNA / Ang II was delivered at a rate of 0.25 μl/hr for the times indicated. MMP-2 inhibitor I (40 mg/kg/d) and doxycycline (20 or 50 mg/kg/d, as indicated) were administered by incorporation into pellets of standard rodent chow and given once daily. Mice were euthanized at pre-determined endpoints by sodium pentobarbital (Euthanyl, Bimeda-MTC, Cambridge, ON, Canada) overdose (100 mg/kg). Intact mice, sham operated mice and mice implanted with vehicle-delivering only pumps were used as a control. No differences in blood pressure, heart weight to body weight, left ventricle weight to body weight or expression of hypertrophy or fibrosis marker genes was observed between these control groups (data not shown).

2.3 Microperfusion arteriograph

Mesenteric arteries of adult Sprague Dawley Rats (6-month old, male) were dissected and mounted on a Danish MyoTechnology arteriograph system (Aarhus, Denmark). As we have described previously[93], arteries were perfused at 2 μL/min with standard HEPES-PSS (142 mM sodium chloride, 4.7 mM potassium chloride, 1.17 mM magnesium sulfate, 1.56 mM calcium chloride, 1.18 mM potassium phosphate, 10 mmol/L HEPES, 5.5 mM glucose, pH 7.4) at 37°C. Phenylephrine (10 μmol/L) was added to the bath (adventitia side) to constrict arteries for 5 min. Following constriction, MMP-2 inhibitor III (100 μmol/L) or

DMSO (1%) was added to the bath for an additional 5 min. Changes in arterial diameter were recorded using Vediview acquisition software (Danish MyoTechnology, Aarhus, Denmark).

2.4 Blood pressure measurement

Systolic blood pressure was measured indirectly using a computerized tail cuff plethysmography system (Kent Scientific Corporation, Torrington, CT, USA) as described previously[26,93,137]. Briefly, conscious mice were maintained at 32-35ºC using a heating pad and restrained during all blood pressure measurements. Averages of 10 inflation/deflation cycles were conducted to obtain mean systolic blood pressure. Every effort was made to ensure consistency between time of day, duration of measurement and number of inflation/deflation cycles for each mouse.

2.5 Echocardiography

Anatomical and hemodynamic properties of hearts were monitored *in vivo* using M-mode echocardiography as described previously[26,137]. Prior to and during analysis, animals were anaesthetized by inhalation (2-4% isoflurane) and cardiac parameters were measured using a Vevo 770 high-resolution imaging system (Visualsonics, Toronto, ON, Canada). Corrected left ventricle (LV) mass (in mg) was calculated using the equation below:

$$\text{Corrected LV mass} = 0.84 \times [(\text{LVID} + \text{LVPW} + \text{IVS})^3 - (\text{LVID})^3]$$

ID- diastolic internal diameter (in mm). PW- diastolic posterior wall thickness (in mm). IVS- diastolic interventricular septum thickness (in mm).

2.6 Histological analysis

Mouse hearts were embedded in Tissue-Tek (Sakura, Torrance, CA, USA), frozen on dry ice and stored at -70ºC. Heart sections (10 μm) were cut on a Leica microtome, fixed with ice-cold acetone, brought back to water and collagen was stained using picrosirius red (1 g/L Direct Red 80 in saturated picric acid solution) for 1 hr. Slides were then rinsed in acidified water, dehydrated, mounted on cover slips and photographed using a DCM500 microscope camera and ScopePhoto software (Madell Technology, Beijing, China).

2.7 Tissue sample preparation

Hearts, aortas, livers and kidneys from mice were collected, rinsed in phosphate buffered saline, weighed and snap-frozen. Samples were then homogenized in 5-10 volumes of homogenization buffer (50 mM Tris, 50 mM NaCl, 1.25 mM PMSF, 62.5 mM Glycerol-2-phosphate, 12.5 mM sodium pyrophosphate, 125 μM NaF, 6.25 μg/ml leupeptine, 312.5 μM sodium orthovanadate, 12.5% glycerol, 1% SDS, 0.1% Triton X-100 at pH 7.4). Homogenates were spun at 10 000 rpm for 5 min at 4ºC to isolate any debris. Supernatants were combined with standard 5x zymography loading buffer (non-reducing) or 5x SDS-PAGE loading buffer (reducing) accordingly.

2.8 Gelatin zymography

MMP-2 enzymatic activity was determined by gelatin zymography. Samples were loaded on 7.5% SDS PAGE gels co-polymerized with gelatin and run under standard electrophoresis conditions. Following electrophoresis, gels were washed thrice in 2.5% Triton X-100. Gels were then incubated overnight at 37ºC in zymography development buffer (25 mM Tris, 5 mM $CaCl_2$, 142 mM NaCl, 0.5 mM NaN_3, pH 7.6) and stained with coomassie blue. Enzymatic activity was visualized as clear bands against a blue background in the gel. Normalized enzyme activity was calculated by dividing the densitometric band intensity of the MMP-2 activity band on the zymography gel by the SDS-PAGE loading control band intensity.

2.9 Immunoblotting

Immunoblotting of Akt, phospho-Akt (Ser 473) and MMP-2 was performed by electrotransfer of unstained SDS PAGE gels onto nitrocellulose membranes using a semi-dry transfer apparatus at a constant current of 2 mA/cm^2 for 45 minutes. Following electrotransfer, membranes were blocked in 5% skim milk powder for 1 hour at room temperature and incubated with primary antibodies (described in chapter 2.1). Detection was achieved using secondary antibodies conjugated to horseradish peroxidase and standard development protocols.

2.10 Gene expression analysis by qRT-PCR

Expression of hypertrophy marker genes, fibrosis marker genes, metalloproteinases and TIMP-2 were measured by quantitative real-time polymerase chain reaction (qRT-PCR) using an ABI 7900 sequence detection system (Applied Biosystems, Carlsbad, CA, USA). RNA from frozen left ventricle samples was extracted with Trizol reagent (Invitrogen, Burlington, ON, Canada) and reverse transcription was preformed with 1 µg RNA using a random hexamer as described previously[166]. Briefly, synthesized cDNA was combined with gene specific primers and fluorometric probes (Applied Biosystems, Carlsbad, CA, USA) in 384 well plates. Fluorescence was measured during PCR thermocycling and relative amounts of cDNA were quantitated by comparing fluorescence intensity to a standard curve. For all experiments, 18S rRNA was used as an internal standard to normalize mRNA values.

2.11 Cell Culture

A7R5 rat aortic smooth muscle cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified eagle medium supplemented with 10% FBS at 37ºC and 5% CO_2. Cells were passaged using 0.05% trypsin-EDTA solution and seeded in 6 well plates. Cells were transfected with siRNA using DharmaFECT 2 transfection reagent as per manufacturer's protocol (Thermo Scientific, Rockford, IL, USA). Briefly, serum-starved cells were treated with a pre-mixed solution 100 nmol/L siRNA and 0.4% DharmaFECT 2 transfection reagent in serum-free

media for 24 hours and MMP-2 activity was measured by gelatin zymography in conditioned media and cell lysates.

2.12 ELISA

Inflammatory cytokine levels (Interleukin-12, IL-12 and interferon gamma, IFN-g) were measured using commercially available enzyme linked immunosorbent assay (ELISA) kits (eBiosciences, San Diego, CA, USA). Briefly, protein concentration of plasma samples and tissue homogenates were determined by Bradford protein assay and diluted to 2 mg/ml. 100 µl of samples and standards were incubated in 96 well plates pre-coated with capture antibody. Detection was achieved by using biotin-conjugated detection antibodies and avidin-conjugated horseradish peroxidase enzyme.

2.13 Statistical analysis

Results were analyzed using one-way ANOVA or *t*-test (Jandel SigmaStat 3.5 statistical software) as appropriate. Time course experiments utilized one-way repeated measures ANOVA whereas treatment experiments used student's t-test. All data are reported as means +/- sem. $p<0.05$ was considered significant for all experiments. The number of mice/trials and statistical symbols are indicated within the corresponding figure legends.

Table 2.1 Nucleotide sequences of siRNAs used in experiments

Gene	siRNA oligonucleotide sequences	
MMP-2	Sense:	5’-mCmAUACAGGAUCAUUGGUUAdTdT
	Antisense:	5’-mUmAACCAAUGAUCCUGUAUGdTdT
MMP-7	Sense:	5’-mCmCUACAGAAUUGUAUCCUAdTdT
	Antisense:	5’-mUmAGGAUACAAUUCUGUAGGdTdG
TACE	Sense:	5’-mGmAGAAGCUUGAUUCUUUGCdTdT
	Antisense:	5’-mGmCAAAGAAUCAAGCUUCUCdAdA
Luciferase	Sense:	5’-mUmAAGGCUAUGAAGAGAUACdTdT
	Antisense:	5’-mGmUAUCUCUUCAUAGCCUUAdTdT

“m” denotes O-methylation of following nucleotide. “d” indicates deoxynucleotide.

Chapter 3

Results

3.1 Characterization of a rodent model of Ang II-induced hypertension and hypertensive cardiac remodelling

To characterize the progression of Ang II-induced cardiovascular disease, we used a mouse model of Ang II-induced hypertension and monitored the development of hypertension, hypertensive cardiac remodelling and gene expression of metalloproteinases over a 16 day time course. We surgically implanted subcutaneous osmotic minipumps containing 1.4 mg/kg/d Ang II in male C57BL/6 mice and mice were euthanized at 0, 4, 8, 12 and 16 days after surgery (**Figure 3.1**). Ang II infusion resulted in a time dependent elevation in blood pressure (**Figure 3.2**) as well as development of cardiac hypertrophy and fibrosis over a 16 day timecourse. Cardiac hypertrophy was confirmed by increased heart weight (by *ex vivo* pathology, **Figure 3.3 A**) and left ventricle weight (by M-mode echocardiography, **Figure 3.3 B**) to body weight ratios and increased expression of the hypertrophy marker genes a-sk-actin and BNP by qRT-PCR (**Figure 3.4**). Similarly, development of cardiac fibrosis was demonstrated by increased collagen deposition (picrosirius red staining, **Figure 3.5**) and expression of the fibrosis marker genes Col I, Col III and Fn-1 by qRT-PCR (**Figure 3.6**).

3.2 Expression profile of MMP-2 in Ang II-induced hypertension

It is known that GqPCR agonists such as Ang II can increase the expression of metalloproteinases. However, whether or not these upregulated metalloproteinases play a role in mediating Ang II-induced hypertension remains

unclear. Previous evidence has identified an upregulation of MMP-2 by Ang II both *in vitro*[105-106,167] and *in vivo*[168], although the functional relevance of this upregulation by Ang II is not fully understood.

Analysis of gelatinase expression (by qRT-PCR, **Figure 3.7**) and enzymatic activity (by gelatin zymography, **Figure 3.8**) of heart and aorta homogenates revealed that MMP-2 was upregulated during Ang II infusion. In contrast to MMP-2, Ang II downregulated another major gelatinase, MMP-9. We therefore hypothesized that upregulation of MMP-2 may be a contributing factor in Ang II-induced cardiovascular disease.

Since MMP-2 activity *in vivo* is dependent on the relative abundance of TIMP-2[120,169-170], we assessed whether TIMP-2 expression was substantially changed in our model of Ang II-induced hypertension. In left ventricle samples, no difference of TIMP-2 mRNA levels was observed between control mice and mice treated with Ang II, as measured by qRT-PCR (**Figure 3.9**).

3.3 Functional studies to examine the involvement of MMP-2 in hypertension

Since MMP-2 is a major gelatinase in vascular tissue and is upregulated by Ang II, we hypothesized that targeting MMP-2 using either pharmacological inhibition or RNA interference could prevent the physiological effects of Ang II. To examine the involvement of MMP-2 in hypertension, we studied both isolated arteries and mice with agonist-induced hypertension.

3.3.1 Pharmacological studies to establish the role of MMP-2 in regulation of vascular tone of isolated arteries

Previous evidence indicates that MMP inhibition is a viable approach to decrease blood pressure by modulating vascular tone[26,93,171]. Our group has shown that broad-spectrum MMP inhibitors such as doxycycline, phenanthroline and GM 6001 induce a dose-dependent relaxation of microperfused small mesenteric arteries constricted with phenylephrine[172]. The cyclic peptide inhibitor CTT, which selectively inhibits gelatinases (i.e. MMP-2 and MMP-9), also produces similar results[102-103]. Here, we confirm these results using a biphenylsulfonamido-hydroxamate inhibitor selective for MMP-2 (MMP-2 inhibitor III) in isolated small rat mesenteric arteries mounted on a microperfusion arteriograph. There are numerous metalloproteinase inhibitors with various degrees of selectivity ranging from broad spectrum to individual metalloproteinase specificity, however, the biphenylsulfonamido compound used in our studies (MMP-2 inhibitor III) is over 4000 and 17 fold more selective for MMP-2 than MMP-1 or MMP-9, respectively[173].

In our studies of isolated small mesenteric arteries, treatment with a zinc binding site targeting MMP-2 inhibitor III (100 μmol/L), but not vehicle (DMSO, 1%), caused relaxation of arteries pre-constricted with 10 μmol/L phenylephrine (**Figure 3.10**). Taken together with our earlier studies, these results strongly suggest that vasoconstrictive agonists engage MMP-2 to maintain arterial tone.

3.3.2 *In vivo* studies of MMP-2 in Ang II-induced hypertension using pharmacological inhibitors

To determine whether targeting MMP-2 attenuates Ang II-induced hypertension and/or hypertensive cardiac remodelling *in vivo*, mice receiving Ang II were treated with either a pharmacological inhibitor or small interference RNA (siRNA) against MMP-2.

We targeted MMP-2 pharmacologically using both a broad spectrum metalloproteinase inhibitor (Doxycycline, Dox, 20 or 50 mg/kg/d, as indicated) and a lipid analogue MMP-2 inhibitor (MMP-2 inhibitor I, MMP-2i, 40 mg/kg/d). Inhibitors were given once per day by oral administration beginning the day before Ang II infusion began (**Figure 3.11**). Pharmacological inhibition of metalloproteinases by Dox attenuated Ang II-induced hypertension in a dose-dependent manner over a 12 day time course (**Figure 3.12 A**). Similarly, selective inhibition of MMP-2 by MMP-2i attenuated Ang II-induced hypertension by 75% (**Figure 3.12 B**).

Despite the protective effects of MMP-2 inhibition on blood pressure, no protection from either cardiac hypertrophy (by M-mode echocardiography or qRT-PCR analysis of hypertrophy marker genes) **Figures 3.13, 3.14**) or fibrosis (by picrosirius red staining and qRT-PCR analysis of fibrosis marker genes, **Figure 3.15, 3.16**) was observed. These results indicate that MMP-2 mediates Ang II-induced hypertension, but not cardiac hypertrophy or fibrosis.

Similar to the effects of Dox on blood pressure, broad-spectrum metalloproteinase inhibition decreased MMP-2 activity in aortic homogenates in a dose-dependent manner (**Figure 3.17**).

3.3.3 *In vivo* studies of MMP-2 in Ang II-induced hypertension using siRNAs

Supplemental studies were conducted to validate these pharmacological results. We used an RNA interference approach with a siRNA (**Table 2.1**) shown to knock down MMP-2 in cultures of aortic smooth muscle cells (**Appendix I: siRNA design**).

To determine if the effects of Ang II could be prevented by targeting MMP-2, we designed an experiment where MMP-2 siRNA treatment (0.4 mg/kg/d) started 5 days prior to Ang II infusion (pre-treatment protocol, **Figure 3.18 A**). Further, to assess if the effects of Ang II could be reversed in already-hypertensive mice, we designed a rescue experiment where MMP-2 siRNA treatment started 5 days after Ang II infusion began (rescue protocol, **Figure 3.18 B**). In both protocols, MMP-2 siRNA attenuated the Ang II-induced upregulation of cardiac and aortic MMP-2 enzymatic activity (**Figure 3.19 upper panels**). We also examined MMP-2 at two distal sites (ie. the kidney and liver). In these tissues, MMP-2 was not upregulated by Ang II and we were unable to detect any attenuation of MMP-2 activity using siRNA (**Figure 3.19 lower panels**).

Interestingly, in both pre-treatment and rescue protocols, administration of MMP-2 siRNA decreased the severity of Ang II-induced hypertension (**Figure 3.20**). MMP-2 siRNA treatment did not attenuate the development of cardiac

hypertrophy or fibrosis by either protocol (pre-treatment, **Figures 3.21 A, 3.22 A, 3.23 A, 3.24 A** or rescue, **Figures 3.21 B, 3.22 B, 3.23 B, 3.24 B**). Therefore, MMP-2 siRNA had similar anti-hypertensive (but not anti-hypertrophic or anti-fibrotic) effects whether given before or after hypertension was initiated (pre-treatment or rescue protocols, respectively).

Confirming the target-specific effects of MMP-2 siRNA, siRNA against the non-mammalian gene luciferase (Luc)[164] did not prevent Ang II-induced hypertension or MMP-2 upregulation (**Figure AII.I**). No changes in blood pressure or cardiac remodelling parameters were observed in mice receiving Ang II under basal conditions (**Figure AII.II**).

3.4 MMP-7 and TACE as upstream mediators of MMP-2

We next investigated how and why Ang II induces MMP-2 upregulation with a focus on metalloproteinases upstream of MMP-2. Previously, we reported that TACE is a key mediator in cardiac hypertrophy and fibrosis and that knockdown of TACE partially attenuates Ang-II induced MMP-2 upregulation[137]. We have also shown that MMP-7 is involved in early stages of agonist-induced hypertension, but whether this role of MMP-7 is dependent on MMP-2 remains unclear[26,137]. We therefore revisited the effects of siRNAs targeting MMP-7 and TACE either individually or together on expression of MMP-2 using previously validated siRNAs and treatment protocols[26,137].

In mice receiving MMP-7 siRNA, TACE siRNA or both siRNAs together, the upregulation of MMP-2 by Ang II was prevented at both the mRNA (**Figure**

3.25 A) and enzymatic activity (**Figure 3.25 B**) levels. Similar to the results obtained by targeting MMP-2 directly, simultaneous knockdown of MMP-7 and TACE attenuated Ang II-induced hypertension (**Figure 3.26**). In contrast to siRNA against MMP-2, siRNAs against MMP-7 and TACE attenuated the development of cardiac hypertrophy (by echocardiography and qRT-PCR analysis of hypertrophy marker genes, **Figures 3.27**) and fibrosis (by picrosirius red staining of heart sections and qRT-PCR analysis of fibrosis marker genes, **Figure 3.28**). These findings indicate that Ang II upregulates MMP-2 through MMP-7 and TACE.

3.5 Akt as a signaling mediator of Ang II induced hypertension downstream of MMP-2

Although we provide strong evidence that MMP-2 mediates agonist-induced hypertension, the molecular mechanisms by which this process occurs remain unclear.

Recent research has demonstrated that GqPCR agonists such as phenylephrine and Ang II induce protein kinase B (Akt) activation[151] which can be attenuated by broad spectrum metalloproteinase inhibition using GM6001 [163]. Further, preventing Akt activation using phosphatidylinositol 3-kinase (PI3K) inhibitors such as wortmannin and LY 294002 induces vasorelaxation in arteries constricted with phenylephrine[163]. Taken together, these results indicate that Akt signals downstream of metalloproteinases in agonist-induced hypertension.

Since targeting either MMP-2 or Akt has similar effects on blood pressure and vasorelaxation of isolated pre-constricted arteries, we hypothesized that

MMP-2 and Akt are mediators of a common pathway. Specifically, we hypothesized that, downstream of agonists such as Ang II, MMP-2 signals vasoconstriction through Akt. To test our hypothesis, we examined Akt activation (by immunoblotting for phospho-Akt at serine 473) in aortic homogenates from control mice, mice receiving Ang II and mice receiving Ang II and either MMP-2 inhibitor I or MMP-2 siRNA.

We found that mice with Ang II-induced hypertension had both increased MMP-2 activity and increased phosphorylation of Akt at serine 473. These results were in agreement with previous research[151,163,174]. We also found that targeting MMP-2 using pharmacological inhibition (**Figure 3.29 A**) or RNA interference (**Figure 3.29 B**) attenuated Akt activation induced by Ang II.

We conclude that MMP-2 contributes to Akt activation downstream of Ang II, however, determining the exact involvement of this pathway in Ang II-induced hypertension warrants further research.

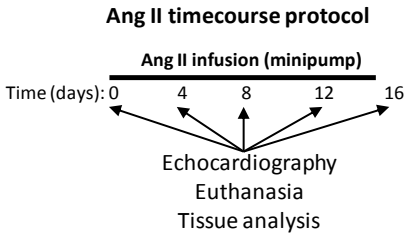

Figure 3.1 Experimental protocol: Time course studies of Ang II-induced hypertension. Subcutaneous osmotic minipumps containing Ang II (1.4 mg/kg/d) were surgically implanted in male C57BL/6 mice. Blood pressure and echocardiography data were collected throughout the experiment and mice were euthanized at 0, 4, 8, 12 and 16 days after surgery.

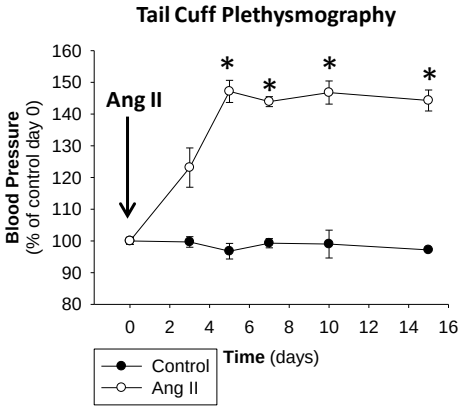

Figure 3.2 Development of Ang II-induced hypertension over a 16 day time course. Time course of systolic blood pressure in control mice and mice treated with Ang II (1.4 mg/kg/d) over 16 days, as measured by tail cuff plethysmography. n=4 mice for each time point. * indicates $p<0.05$ vs. day 0. Blood pressure data was obtained by collaboration with Xiang Wang (described in Appendix III).

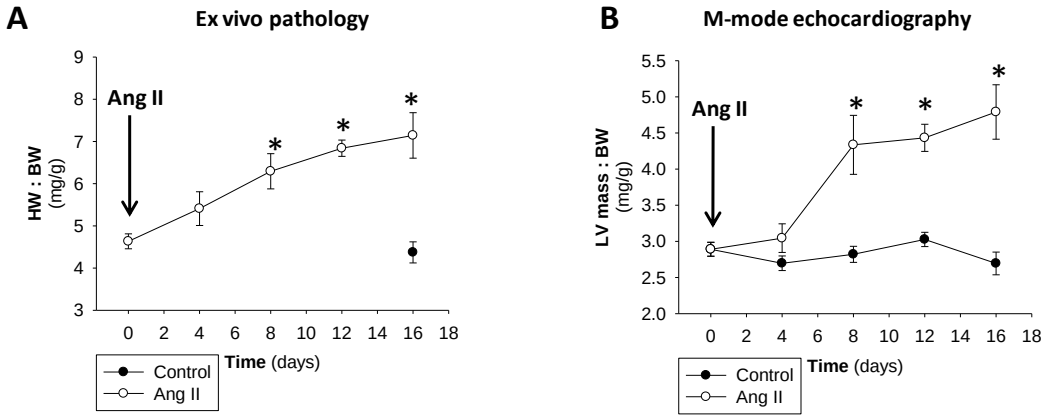

Figure 3.3 Development of cardiac hypertrophy induced by Ang II: organ-scale effects. Time course of heart weight (**A**) and left ventricle weight (**B**) to body weight ratios of control mice and mice receiving Ang II (1.4 mg/kg/d) as determined by *ex vivo* pathology and M-mode echocardiography, respectively. n=4 mice for each time point. * indicates $p<0.05$ vs. day 0. Echocardiography data was obtained by collaboration with Dr. Tatsujiro Oka (described in Appendix III).

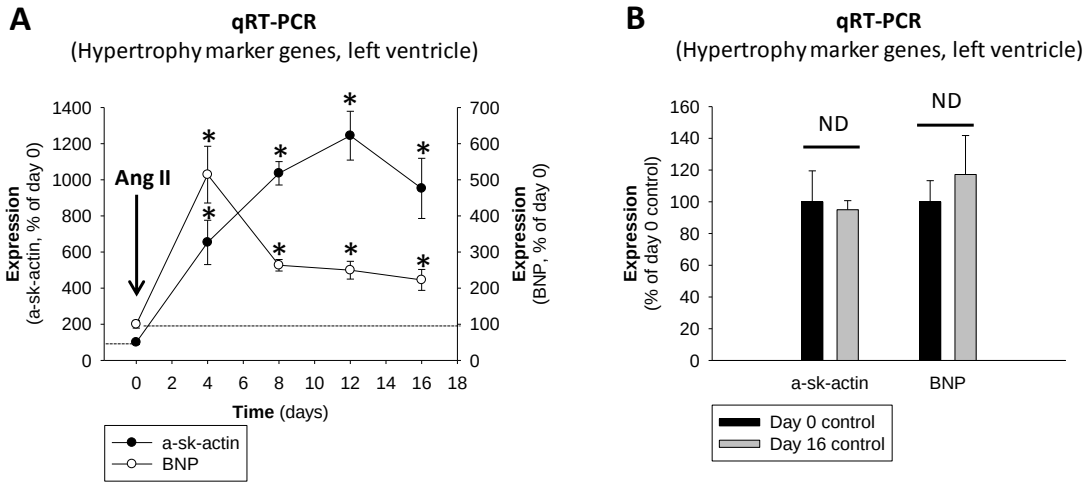

Figure 3.4 Development of cardiac hypertrophy induced by Ang II: molecular effects. (**A**) Expression profile of the hypertrophy marker genes alpha-skeletal-actin (a-sk-actin) and brain natriuretic peptide (BNP) in left ventricle samples of mice receiving Ang II (1.4 mg/kg/d) over a 16 day timecourse as measured by qRT-PCR. (**B**) Expression of the above genes in control mice at the beginning (day 0) and the end (day 16) of the experiment. n=4 mice for each time point. * indicates $p<0.05$ vs. day 0. qRT-PCR data was obtained by collaboration with Dr. Zamaneh Kassiri (described in Appendix III).

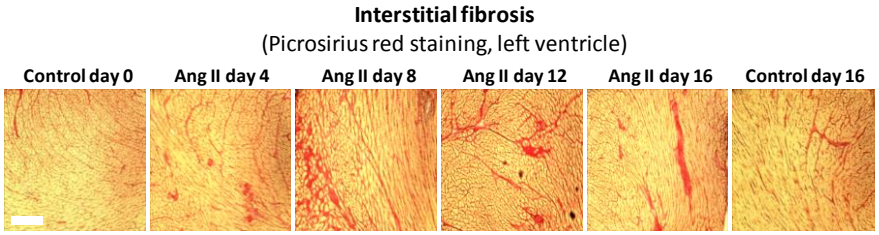

Figure 3.5 Development of cardiac fibrosis induced by Ang II: organ-scale effects. Light micrographs of heart sections (10 µm) stained with picrosirius red to indicate collagen deposition in control mice and mice receiving Ang II (1.4 mg/kg/d) over a 16 day timecourse. Images are representative of n=4 mice for each time point. Scale bar indicates 250 µm. Picrosirius red data was obtained by collaboration with Stephan Cooper (described in Appendix III).

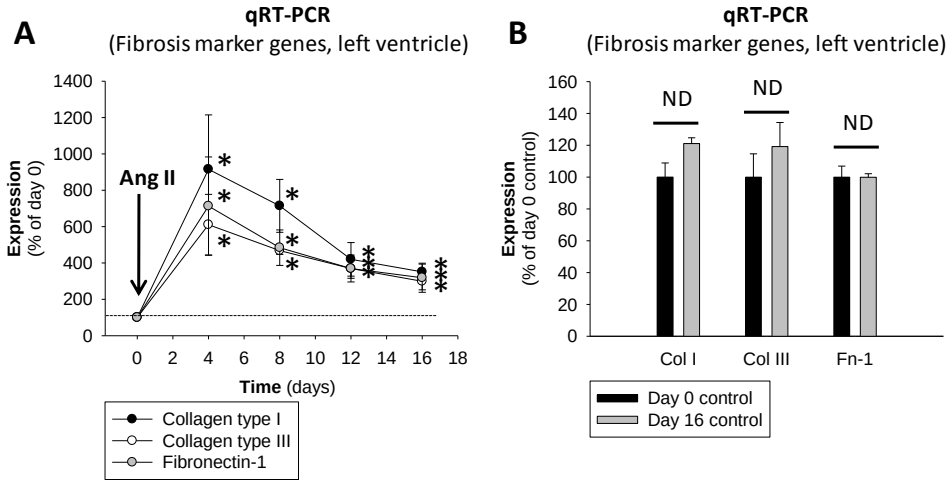

Figure 3.6 Development of cardiac fibrosis induced by Ang II: molecular effects. (**A**) Expression profile of the fibrosis marker genes collagen type I (Col I), collagen type III (Col III) and fibronectin-1 (Fn-1) in left ventricle samples of mice receiving Ang II (1.4 mg/kg/d) as measured by qRT-PCR. (**B**) Expression of the above genes in control mice at the beginning (day 0) and the end (day 16) of the experiment. n=4 mice for each time point. * indicates $p<0.05$ vs. day 0. qRT-PCR data was obtained by collaboration with Dr. Zamaneh Kassiri (described in Appendix III).

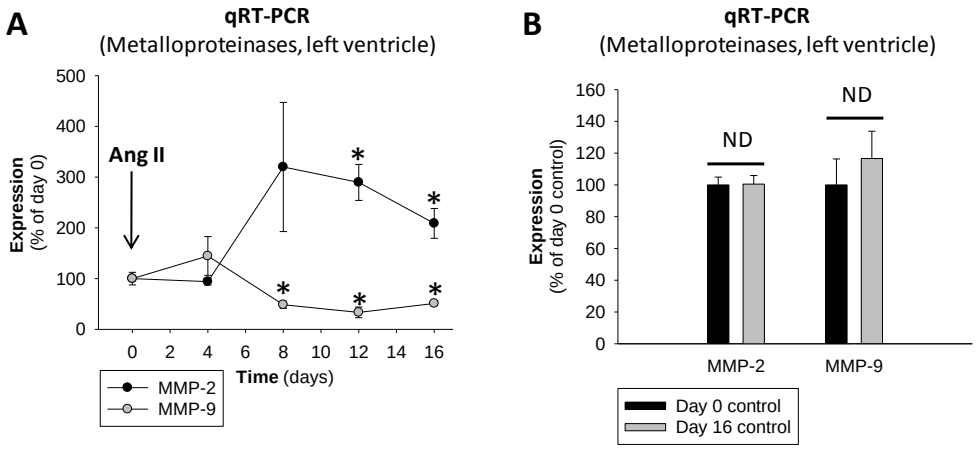

Figure 3.7 Molecular profile of gelatinase expression in response to Ang II. (**A**) Expression profile of the gelatinases MMP-2 and MMP-9 in left ventricle samples of mice receiving Ang II (1.4 mg/kg/d) as measured by qRT-PCR. (**B**) Expression of the above genes in control mice at the beginning (day 0) and the end (day 16) of the experiment. n=4 mice for each time point. * indicates $p<0.05$ vs. day 0. qRT-PCR data was obtained by collaboration with Dr. Zamaneh Kassiri (described in Appendix III).

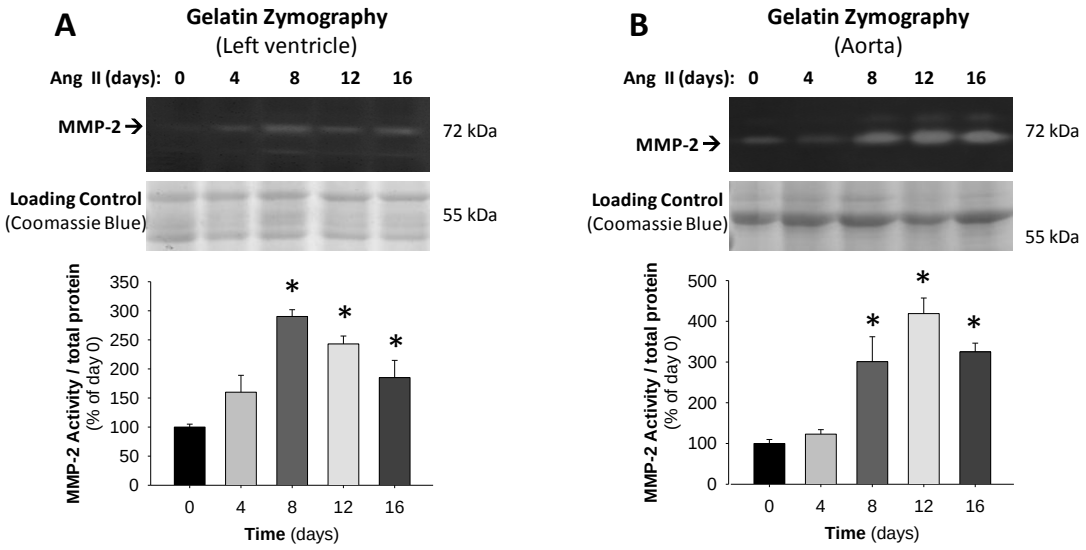

Figure 3.8 MMP-2 activity is upregulated in response to Ang II. Gelatin zymography analysis of left ventricular (**A**) and aortic (**B**) homogenates from control mice and mice receiving Ang II (1.4 mg/kg/d) over a 16 day time course. Quantitation of MMP-2 activity is shown in the form of bar graphs and is normalized to total protein. Images are representative of n=4 mice for each time point. * indicates $p<0.05$ vs. day 0.

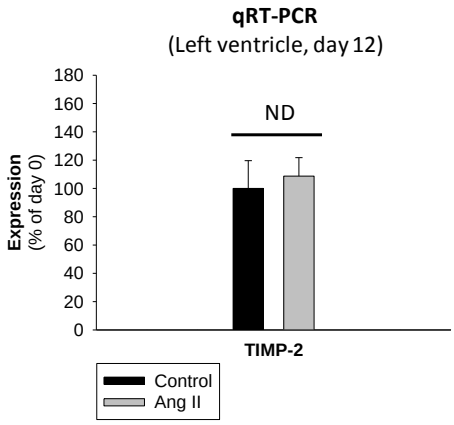

Figure 3.9 TIMP-2 expression is unchanged in Ang II-induced hypertension. qRT-PCR analysis of TIMP-2 mRNA levels in left ventricle samples of control mice and mice treated with Ang II (1.4 mg/kg/d) for 12 days. n=4 mice for each group. qRT-PCR data was obtained by collaboration with Dr. Zamaneh Kassiri (described in Appendix III).

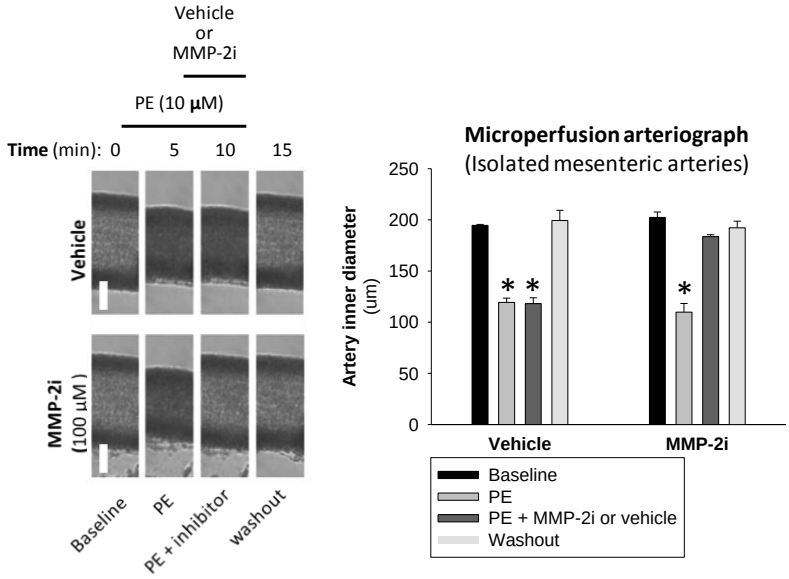

Figure 3.10 MMP-2 is involved in the maintenance of agonist-induced arterial constriction. Small rat mesenteric arteries were isolated and mounted on a microperfusion arteriograph system. Left panel- Light micrograph images of arteries at baseline (0 min), constricted with phenylephrine (PE, 10 µmol/L, 5 min), treated with either MMP-2 inhibitor III (100 µmol/L) or vehicle (1% DMSO, 10 min) and washed in triplicate (washout, 15 min). Right Panel- Quantitative analysis of artery inner diameter from acquired images. Images are representative of triplicate trials. Scale bar indicates 100 µm. * indicates $p<0.05$ vs baseline. Data was obtained in part through collaboration with Steve Ok as described in Appendix III.

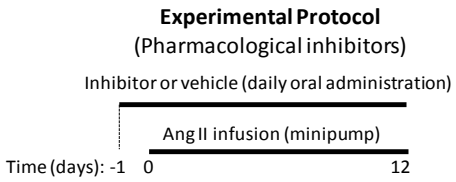

Figure 3.11 Experimental protocol: Treatment regimen of pharmacological inhibitors in Ang II-induced hypertension. Male C57BL/6 mice were orally administered doxycycline (20 or 50 mg/kg/d), MMP-2 inhibitor I (40 mg/kg/d) or vehicle once per day, for 12 days. After one day of treatment, mice were surgically implanted with subcutaneous osmotic minipumps containing 1.4 mg/kg/d Ang II for 12 days. Blood pressure data were collected throughout the experiment and animals were euthanized after 12 days of Ang II infusion.

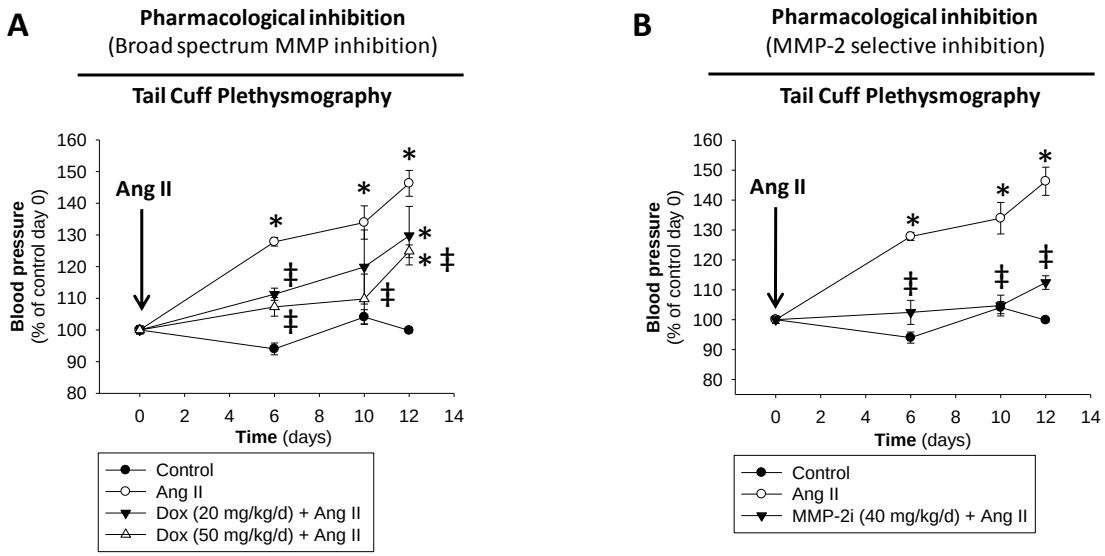

Figure 3.12 Pharmacological inhibition of MMP-2 attenuates Ang II-induced hypertension. Systolic blood pressure of mice treated with doxycycline (Dox, **A**) or MMP-2 inhibitor I (MMP-2i, **B**) during Ang II infusion, as measured by tail cuff plethysmography. n=4 mice for each group. * indicates $p<0.05$ vs. control. ‡ Indicates $p<0.05$ vs Ang II.

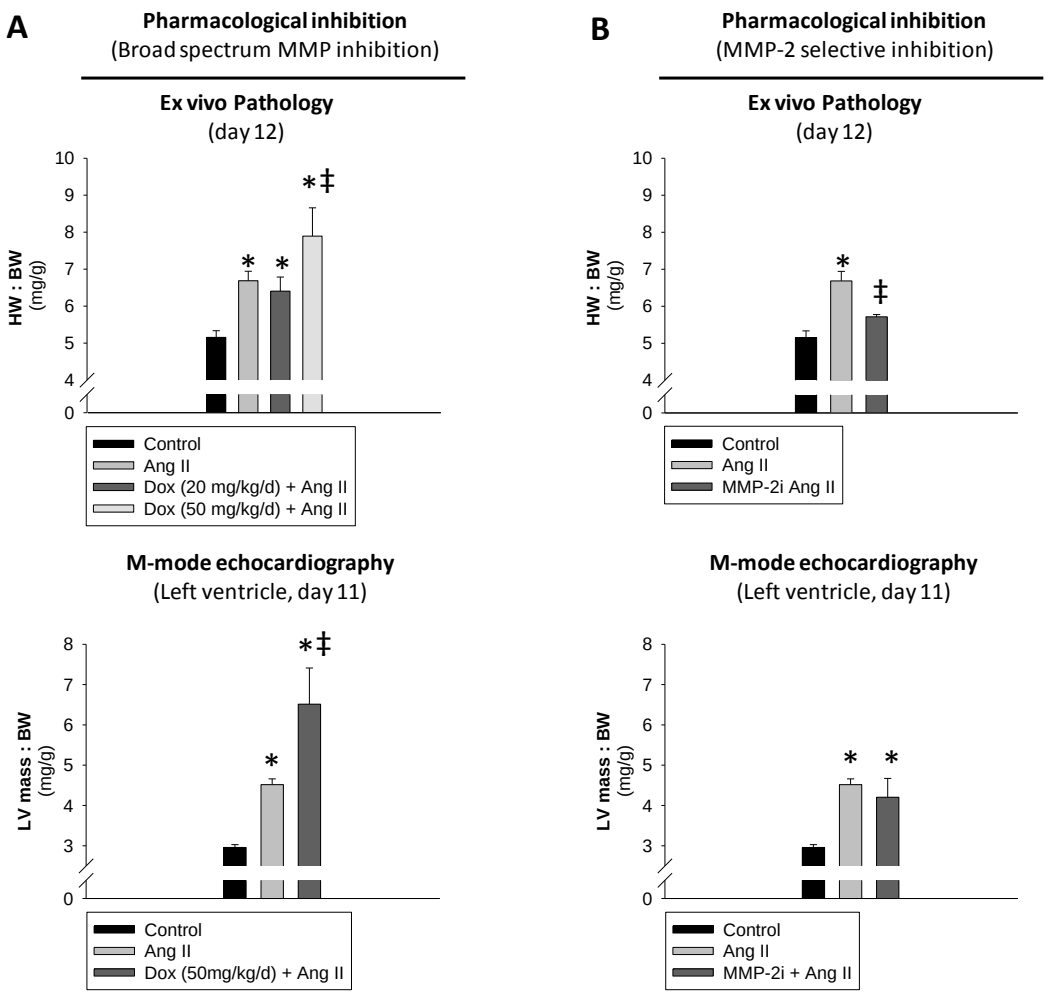

Figure 3.13 Pharmacological inhibition of MMP-2 does not prevent Ang II-induced hypertrophic growth of the heart. *Ex vivo* pathology and M-mode echocardiography analysis of heart weight (upper panels) and left ventricle weight (lower panels) to body weight ratios of mice treated with doxycycline (Dox, **A**) or MMP-2 inhibitor I (MMP-2i, **B**) during Ang II infusion. n=4 mice for each group. * indicates $p<0.05$ vs. control. Echocardiography data was obtained by collaboration with Donna Beker as described in Appendix III.

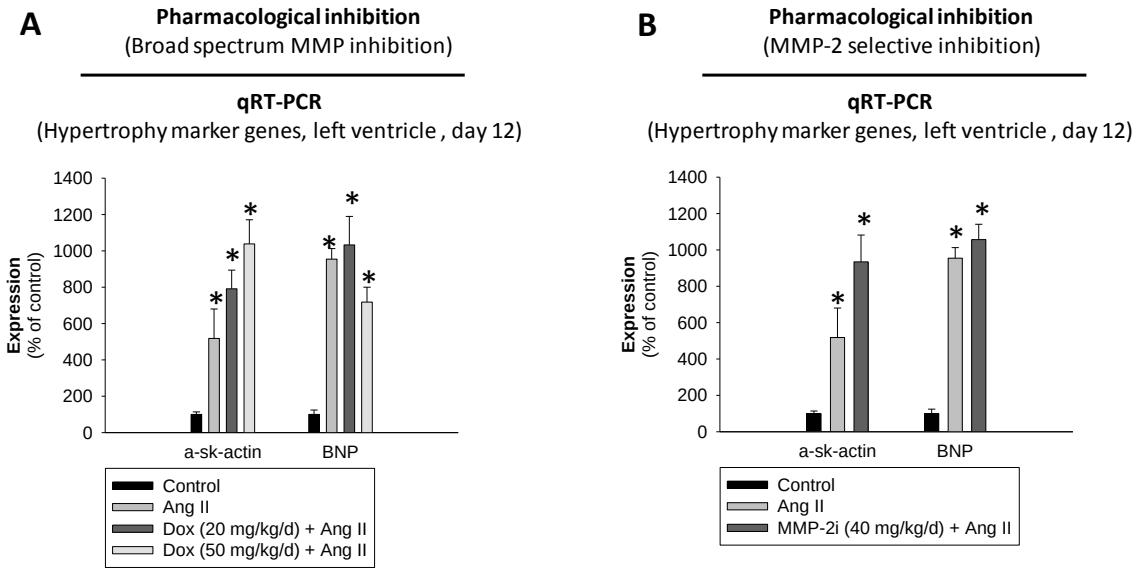

Figure 3.14 Pharmacological inhibition of MMP-2 does not prevent Ang II-induced expression of hypertrophy marker genes in the heart . qRT-PCR analysis of the hypertrophy marker genes a-sk-actin and BNP in left ventricle samples from mice treated with doxycycline (Dox, **A**) or MMP-2 inhibitor I (MMP-2i, **B**) during Ang II infusion. n=4 mice for each group. * indicates $p<0.05$ vs. control. qRT-PCR data was obtained by collaboration with Joanne Zhao as described in Appendix III.

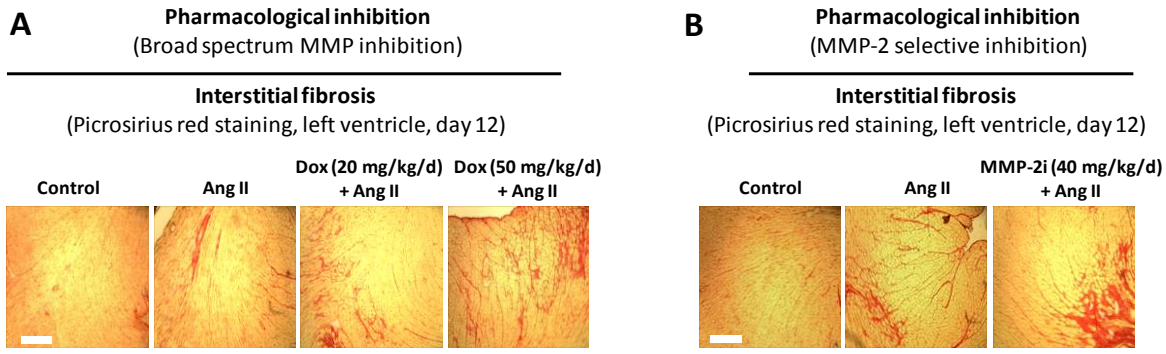

Figure 3.15 Pharmacological inhibition of MMP-2 does not prevent Ang II-induced collagen deposition in the heart. Light micrographs of heart sections (10 µm) stained with picrosirius red to indicate collagen deposition in mice treated with doxycycline (Dox, **A**) or MMP-2 inhibitor I (MMP-2i, **B**) during Ang II infusion. Scale bar indicates 250 µm. Images are representative of n=4 mice for each group. Picrosirius red data was obtained by collaboration with Stephan Cooper as described in Appendix III.

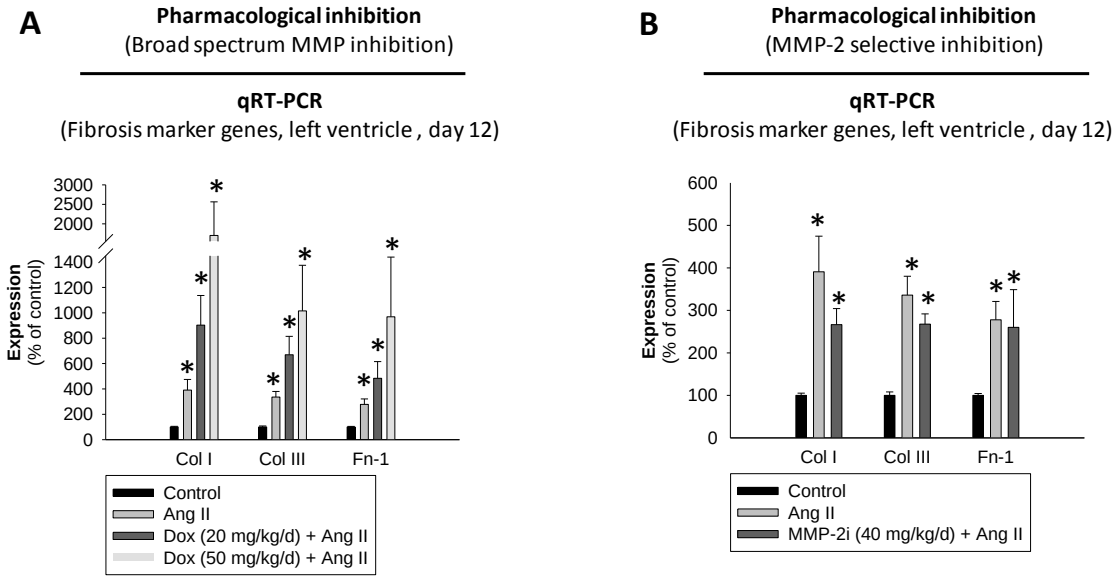

Figure 3.16 Pharmacological inhibition of MMP-2 does not prevent Ang II-induced expression of fibrosis marker genes in the heart. qRT-PCR analysis of the fibrosis marker genes Col I, Col III and Fn-1in left ventricle samples from mice treated with doxycycline (Dox, **A**) or MMP-2 inhibitor I (MMP-2i, **B**) during Ang II infusion. n=4 mice for each group. * indicates $p<0.05$ vs. control. qRT-PCR data was obtained by collaboration with Joanne Zhao as described in Appendix III.

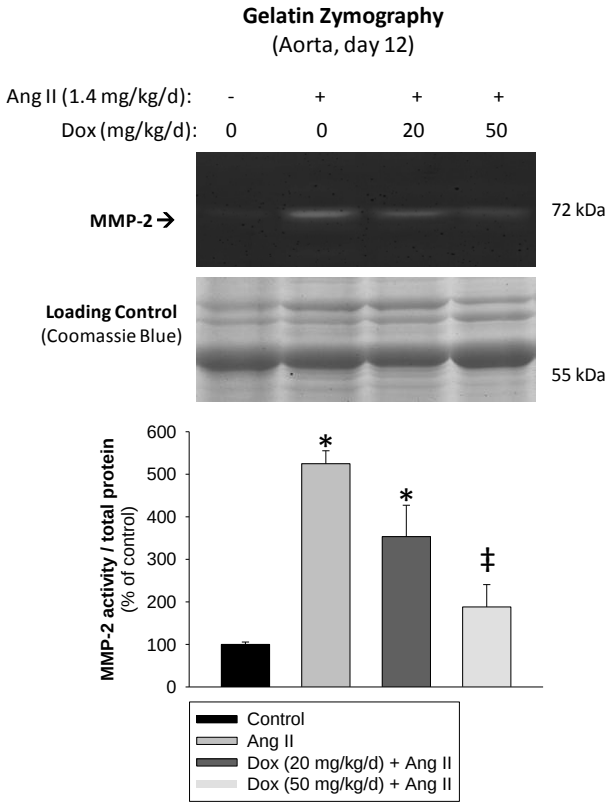

Figure 3.17 Doxycycline administration decreases aortic MMP-2 activity *in vivo*. Gelatin zymography analysis of aortic homogenates from mice treated with doxycycline (Dox) during Ang II infusion. Quantitation of MMP-2 activity is shown in the form of bar graphs and is normalized to total protein. Images are representative of n=4 mice for each group. * indicates $p<0.05$ vs. control. ‡ Indicates $p<0.05$ vs Ang II.

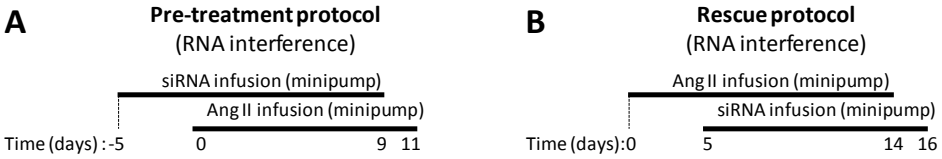

Figure 3.18 Experimental protocol: Treatment regimen of siRNAs in Ang II-induced hypertension. Male C57BL/6 mice were surgically implanted with subcutaneous osmotic minipumps containing 1.4 mg/kg/d Ang II. Mice were implanted with second minipump containing siRNAs (0.4 mg/kg/d) against MMP-2, MMP-7, TACE or luciferase beginning 5 days prior to (pre-treatment protocol, **A**) or 5 days after (rescue protocol, **B**) Ang II infusion. Blood pressure data were collected throughout the experiment and animals were euthanized 16 days after surgery.

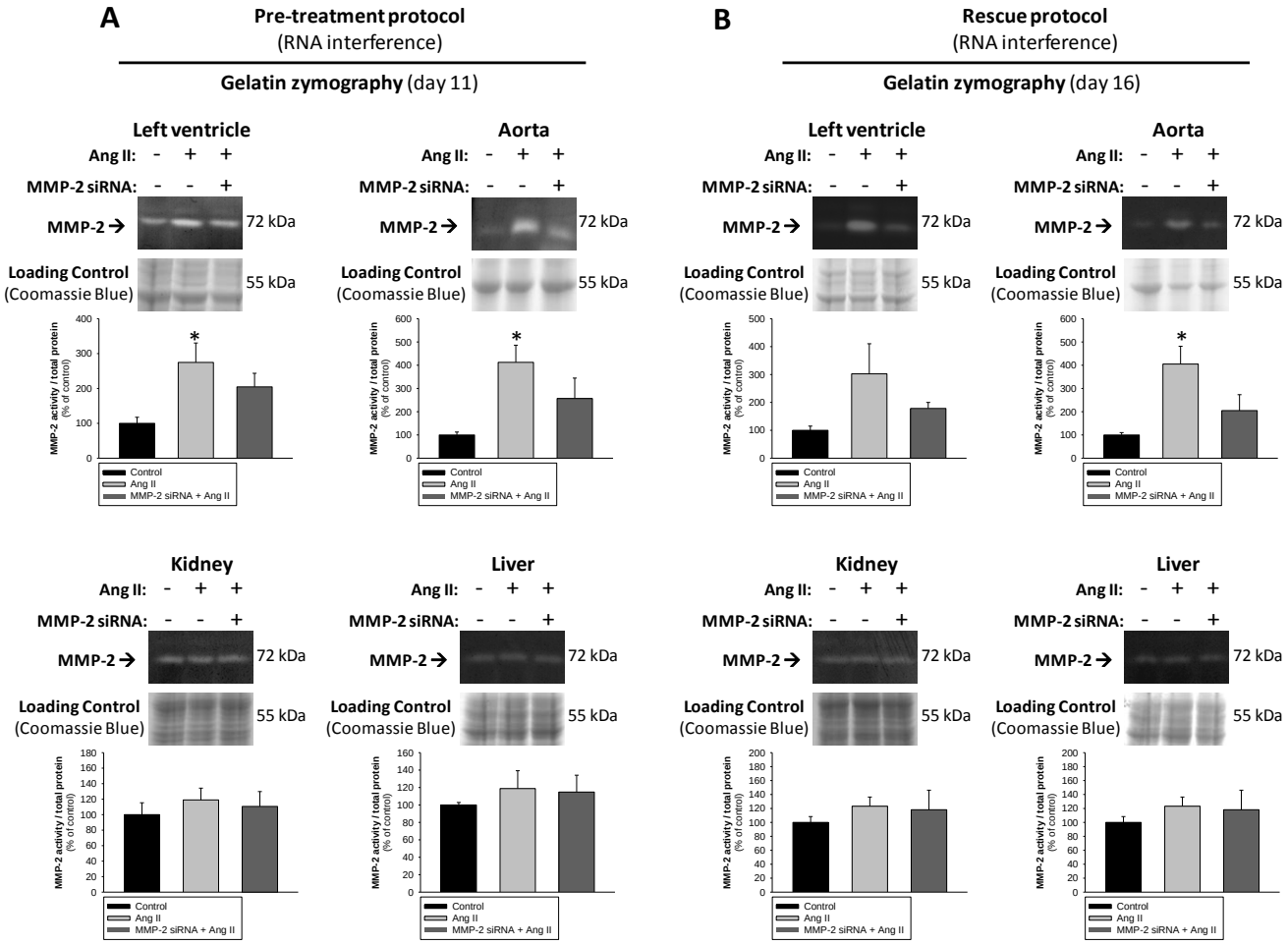

Figure 3.19 Systemic effects of MMP-2 siRNA on MMP-2 enzymatic activity in Ang II-induced hypertension. Gelatin zymography analysis of left ventricular, aortic, renal and hepatic homogenates of control mice, mice receiving Ang II (1.4 mg/kg/d) and mice receiving Ang II and MMP-2 siRNA (0.4 mg/kg/d) in both pre-treatment (**A**) and rescue (**B**) protocols on days 11 and 16, respectively. Images are representative of n=3-4 mice for each group. Quantitation of MMP-2

activity is shown in the form of bar graphs and is normalized to total protein. * indicates p<0.05 vs. control.

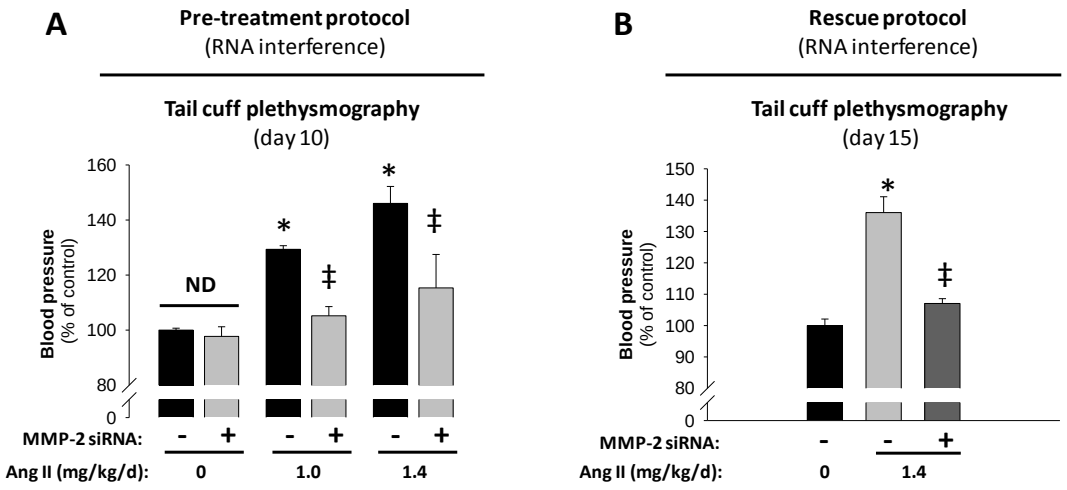

Figure 3.20 MMP-2 siRNA attenuates Ang II-induced hypertension. Systolic blood pressure of control mice, mice receiving Ang II (1.0 or 1.4 mg/kg/d) and mice receiving Ang II and MMP-2 siRNA (0.4 mg/kg/d) in both pre-treatment (**A**) and rescue (**B**) protocols, as measured by tail cuff plethysmography. n=3-4 mice for each group. * indicates p<0.05 vs. control. ‡ Indicates p<0.05 vs Ang II. Blood pressure data were collected in part through collaboration with Xiang Wang as described in Appendix III.

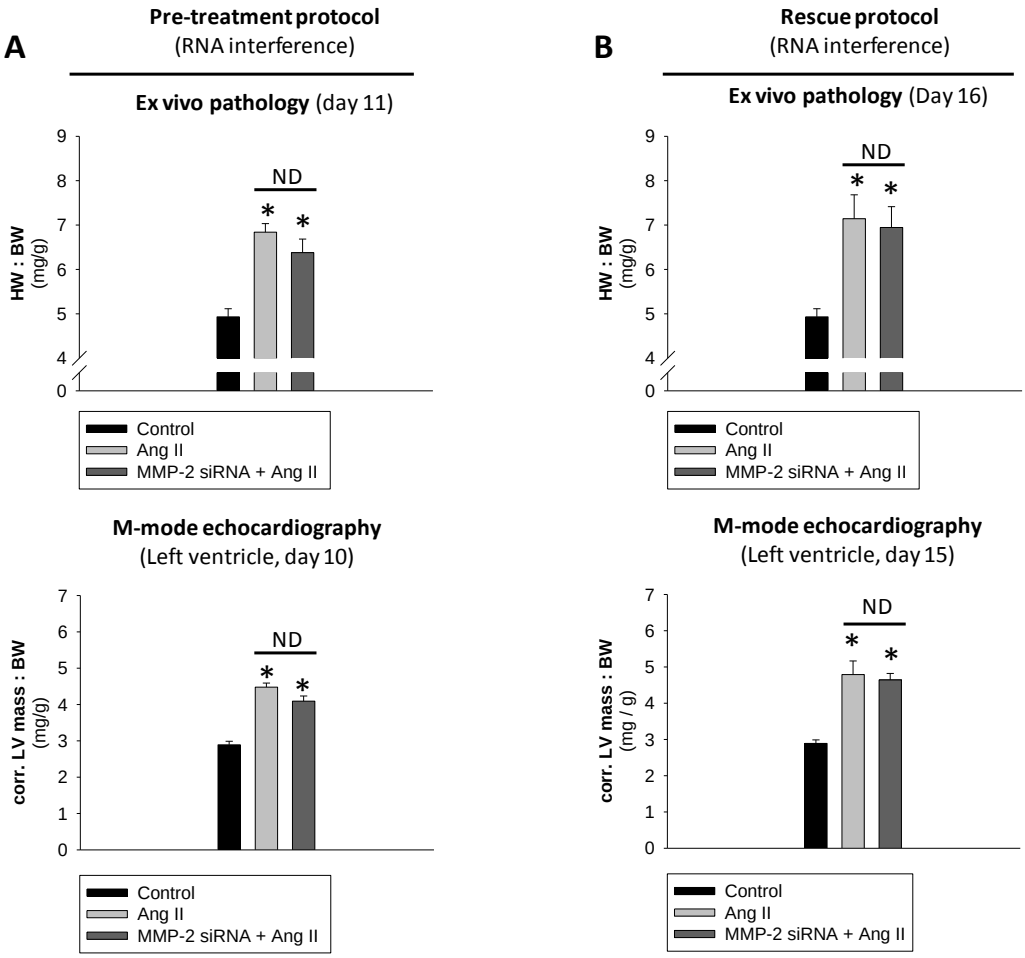

Figure 3.21 MMP-2 siRNA does not prevent Ang II-induced hypertrophic growth of the heart. *Ex vivo* pathology and M-mode echocardiography analysis of heart weight (upper panels) and left ventricle weight (lower panels) to body weight ratios in control mice, mice receiving Ang II (1.4 mg/kg/d) and mice receiving Ang II and MMP-2 siRNA (0.4 mg/kg/d) in both pre-treatment (**A**) and rescue (**B**) protocols. n=3-4 mice for each group. * indicates $p<0.05$ vs. control.

Echocardiography data was obtained by collaboration with Dr. Tatsujiro Oka as described in Appendix III.

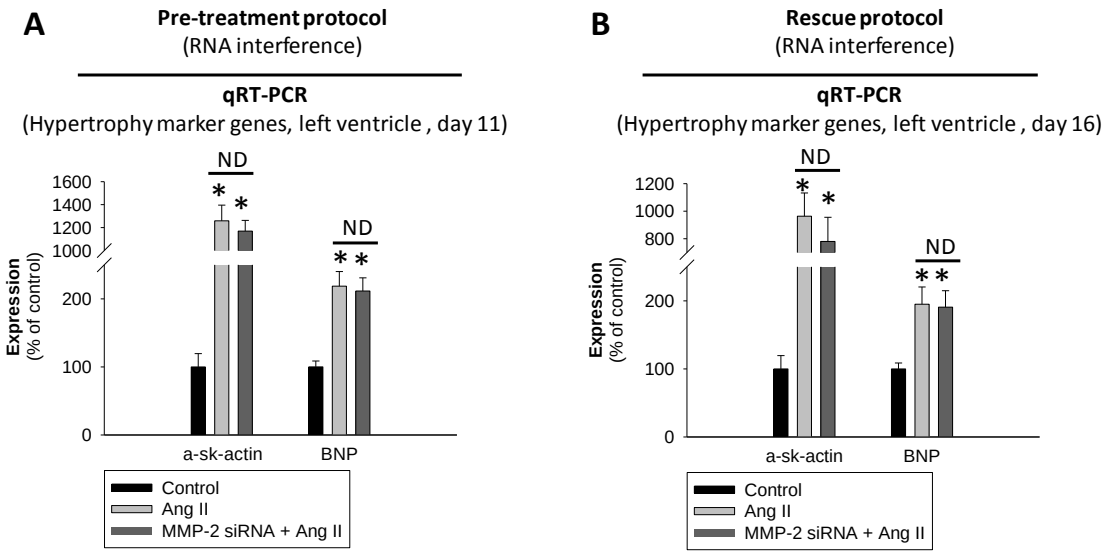

Figure 3.22 MMP-2 siRNA does not prevent Ang II-induced expression of hypertrophy marker genes in the heart. qRT-PCR analysis of the hypertrophy marker genes a-sk-actin and BNP in left ventricle samples from control mice, mice receiving Ang II (1.4 mg/kg/d) and mice receiving Ang II and MMP-2 siRNA (0.4 mg/kg/d) in both pre-treatment (**A**) and rescue (**B**) protocols. n=3-4 mice for each group. * indicates $p<0.05$ vs. control. qRT-PCR data was obtained by collaboration with Dr. Zamaneh Kassiri as described in Appendix III.

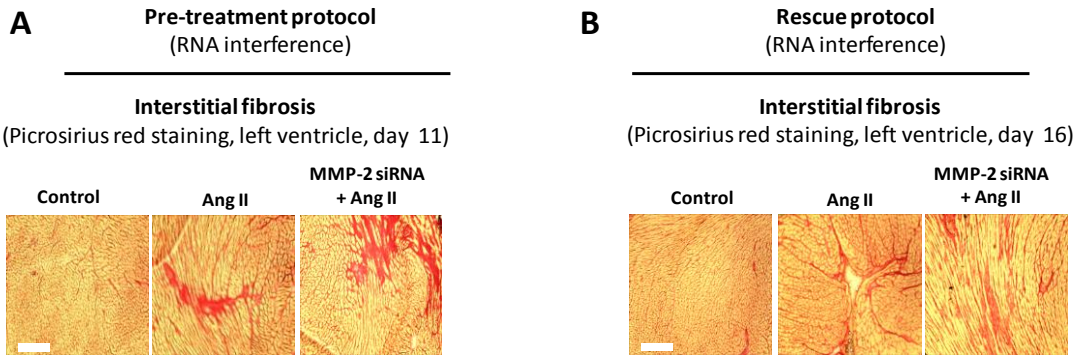

Figure 3.23 MMP-2 siRNA does not prevent Ang II-induced collagen deposition in the heart. Light micrographs of heart sections (10 µm) stained with picrosirius red to indicate collagen deposition in control mice, mice receiving Ang II (1.4 mg/kg/d) and mice receiving Ang II and MMP-2 siRNA (0.4 mg/kg/d) in both pre-treatment (**A**) and rescue (**B**) protocols. Scale bar indicates 250 µm. Images are representative of n=3-4 mice for each group. Picrosirius red data was obtained by collaboration with Stephan Cooper as described in Appendix III.

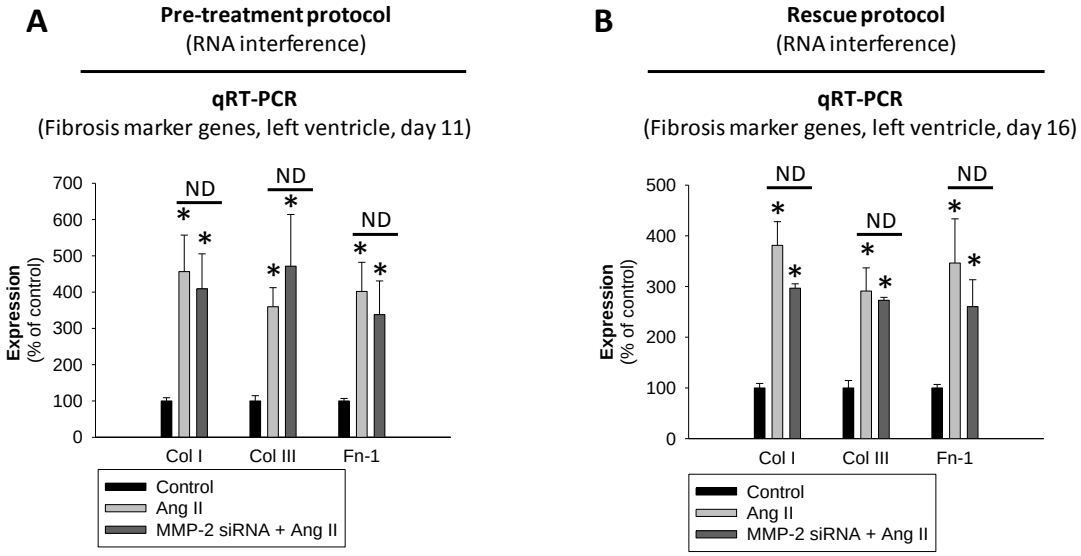

Figure 3.24 MMP-2 siRNA does not prevent Ang II-induced expression of fibrosis marker genes in the heart. qRT-PCR analysis of the fibrosis marker genes Col I, Col III and Fn-1in left ventricle samples from control mice, mice receiving Ang II (1.4 mg/kg/d) and mice receiving Ang II and MMP-2 siRNA (0.4 mg/kg/d) in both pre-treatment (**A**) and rescue (**B**) protocols. n=3-4 mice for each group. * indicates $p<0.05$ vs. control. qRT-PCR data was obtained by collaboration with Dr. Zamaneh Kassiri as described in Appendix III.

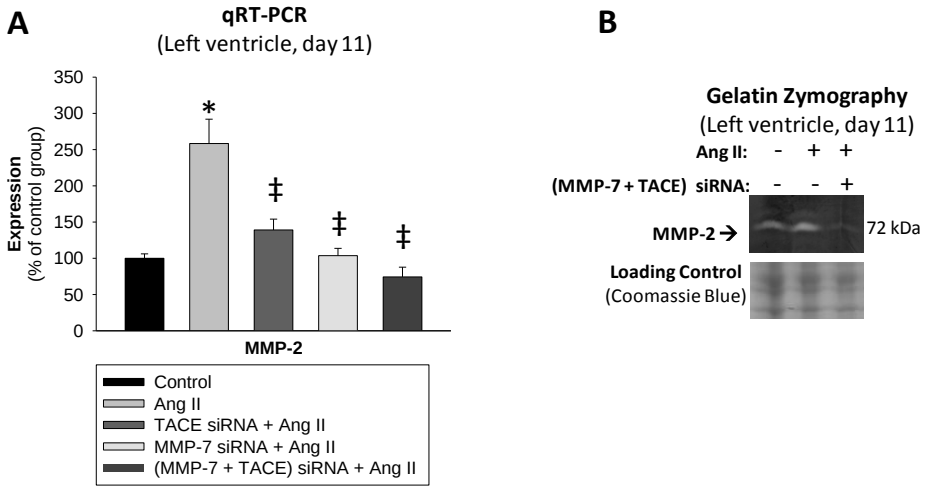

Figure 3.25 MMP-7 and TACE mediate Ang II-induced MMP-2 upregulation in the heart. qRT-PCR (**A**) and gelatin zymography (**B**) analysis of MMP-2 expression and activity in left ventricle samples from control mice, mice receiving Ang II (1.4 mg/kg/d) and mice receiving Ang II and siRNAs (0.4 mg/kg/d) against MMP-7, TACE or both siRNAs together. n=4 mice for each group. * indicates $p<0.05$ vs. control. ‡ Indicates $p<0.05$ vs Ang II. qRT-PCR data was obtained by collaboration with Dr. Zamaneh Kassiri as described in Appendix III.

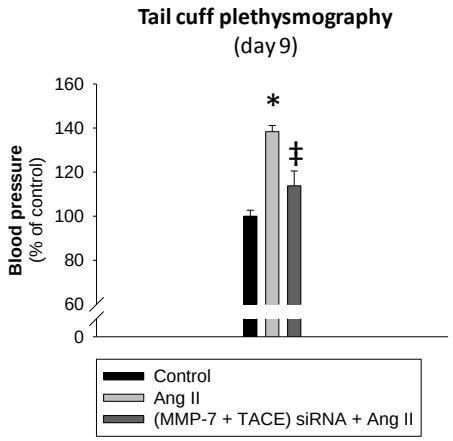

Figure 3.26 siRNAs against MMP-7 and TACE attenuate Ang II-induced hypertension. Systolic blood pressure of control mice, mice receiving Ang II (1.0 or 1.4 mg/kg/d) and mice receiving Ang II and siRNAs (0.4 mg/kg/d) against MMP-7 and TACE, as measured by tail cuff plethysmography. n=4 mice for each group. * indicates $p<0.05$ vs. control. ‡ Indicates $p<0.05$ vs Ang II. Blood pressure data was collected in collaboration with Xiang Wang and Fung Lan Chow, respectively (as described in Appendix III).

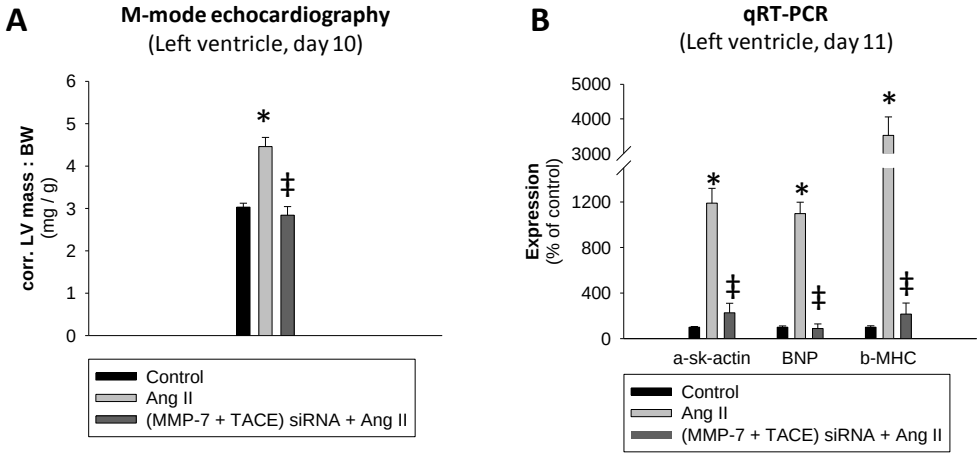

Figure 3.27 siRNAs against MMP-7 and TACE attenuate Ang II-induced cardiac hypertrophy. M-mode echocardiography analysis of left ventricle weight to body weight ratio (**A**) and qRT-PCR analysis of hypertrophy marker gene expression (**B**) in left ventricle samples of control mice, mice receiving Ang II (1.4 mg/kg/d) and mice receiving Ang II and siRNAs (0.4 mg/kg/d) against MMP-7 and TACE. n=4 mice for each group. * indicates $p<0.05$ vs. control. ‡ Indicates $p<0.05$ vs Ang II. Echocardiocardiography and qRT-PCR were obtained by collaboration with Dr. Tatsujiro Oka and Dr. Zamaneh Kassiri, respectively (as described in Appendix III).

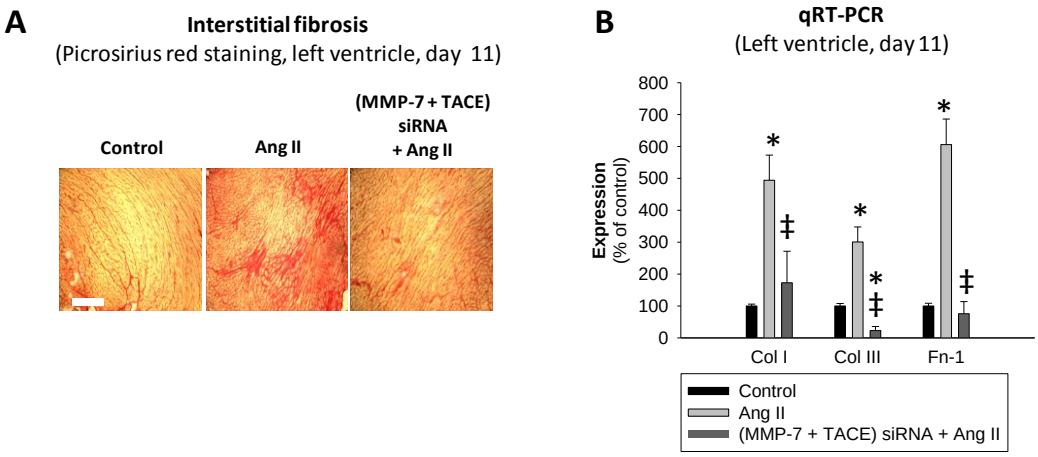

Figure 3.28 siRNAs against MMP-7 and TACE attenuate Ang II-induced cardiac fibrosis. Light micrographs of heart sections (10 µm) stained with picrosirius red (**A**) and qRT-PCR analysis of fibrosis marker gene expression (**B**) in left ventricle samples of control mice, mice receiving Ang II (1.4 mg/kg/d) and mice receiving Ang II and siRNAs (0.4 mg/kg/d) against MMP-7 and TACE. Scale bar indicates 250 µm. Images are representative of n=4 mice for each group. * indicates $p<0.05$ vs. control. ‡ Indicates $p<0.05$ vs Ang II. qRT-PCR and picrosirius red data were obtained by collaboration with Dr. Zamaneh Kassiri and Stephan Cooper, respectively (as described in Appendix III).

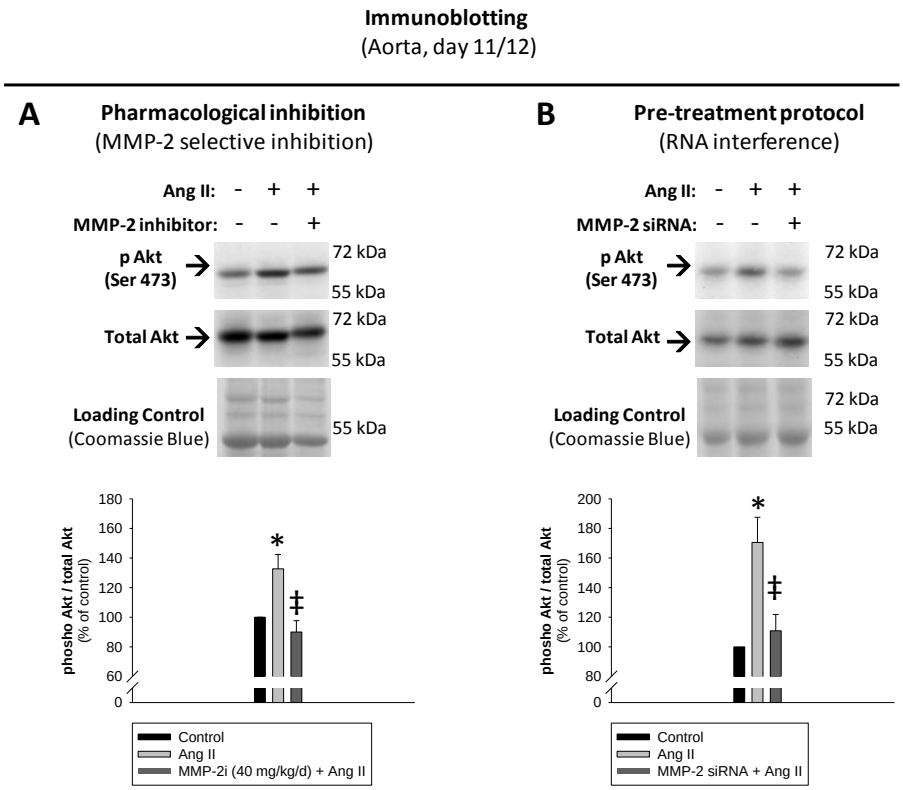

Figure 3.29 MMP-2 contributes to Akt activation in Ang II-induced hypertension. Western blot analysis of phospho-Akt and total Akt levels (upper panels), SDS-PAGE analysis of total protein (middle panels) and densitometric quantitation of phospho-Akt / total Akt ratios in aortic homogenates of mice with MMP-2 inhibitor I (MMP-2i, 40 mg/kg/d, **A**) or MMP-2 siRNA (0.4 mg/kg/d, **B**) during Ang II infusion. n=3-4 mice for each group. * indicates $p<0.05$ vs. control. ‡ Indicates $p<0.05$ vs Ang II.

Chapter 4

Discussion

4.1 Summary of results

This research provides new evidence that agonist-induced cardiovascular disease is signalled by multiple metalloproteinases. We demonstrate that MMP-2 inhibition (by pharmacological means) and gene knockdown (by RNA interference) attenuate Ang II-induced hypertension. These manipulations did not prevent any of the following pro-hypertrophic and pro-fibrotic responses: i) increases in left ventricle mass, ii) increases in overall heart weight, iii) increases in collagen deposition or, iv) increases in expression of hypertrophy and fibrosis marker genes. Importantly, we observed a novel transcriptional regulation of MMP-2 by two other metalloproteinases, MMP-7 and TACE. Paradoxically, while direct blockade of MMP-2 only attenuated Ang II-induced hypertension, indirect blockade of MMP-2 (by knocking down MMP-7 and TACE) attenuated Ang II-induced hypertension as well as the development of cardiac hypertrophy and fibrosis. We also observed that targeting MMP-2 in Ang II-induced hypertension attenuated Ang II-induced Akt activation. These findings suggest a signaling bifurcation where Ang II-induced hypertension diverges from development of cardiac hypertrophy and fibrosis. Therefore, we propose a model where Ang II signals through MMP-7 and TACE to induce: i) hypertension mediated by MMP-2 and, ii) cardiac hypertrophy and fibrosis independent of MMP-2 (**Figure 4.1**).

4.2 The crucial role of agonist signaling in hypertensive cardiac remodelling

Pathological cardiac remodelling is a clinically significant complication in hypertensive disorders[175]. Our research indicates that agonist-induced cardiovascular disease depends on metalloproteinases, which regulate the expression of genes involved in pathological cardiac remodelling (e.g. brain natriuretic peptide, alpha-skeletal actin, beta-myosin heavy chain, fibronectin-1 and collagen types I and III) as well as the expression of MMP-2. Moreover, our data show that targeting MMP-2 effectively uncouples Ang II-induced hypertension from the development of cardiac hypertrophy and fibrosis. Although the causal relationship between hypertension and cardiac remodelling is not fully understood, our findings support the notion that sustained agonist signalling leads to pathological cardiac remodelling that can occur in the absence of overt hypertension. In models of hypertension, the development of hypertensive cardiac remodelling is thought to be caused by: i) excessive systemic agonists (as done in this study) and, ii) hemodynamic pressure overload on the heart (as done in transverse aortic constriction, TAC)[176-180]. Strong evidence indicates that endogenous agonist production is required for TAC-induced remodelling. Indeed, TAC-induced cardiac hypertrophy is attenuated by blockade of Ang II production (ie., ACE inhibitors, ramipril[31]) or receptor antagonism (ie., AT1R antagonist, losartan[32]).

In addition, hypertrophic growth can be induced in cultured cardiomyocytes stimulated by GqPCR agonists in the absence of pressure overload[181-182]. Further supporting our claim, research in humans has

demonstrated a substantial increase in the incidence of cardiovascular disease in individuals with only mildly elevated blood pressure (i.e. 130-140 mmHg)[24,183]. Therefore, these data suggest that endogenous agonist signaling is necessary and sufficient for the development of hypertensive cardiac remodelling.

4.3 Mechanism of MMP-2 upregulation by Ang II: The emerging roles of MMP-7 and TACE

The concept that MMP-7 and TACE are upstream regulators of MMP-2 in Ang II-induced cardiovascular disease is supported by several unrelated lines of research. It is known that MMP-7 regulates MMP-2 activation *in vitro* (by direct proteolysis) and in myeloma cells to facilitate bone destruction and tumour spreading[184-185]. MMP-7 and TACE may also facilitate MMP-2 activation by releasing TNF-α to transcriptionally upregulate MT1-MMP[186-187]. Further, MMP-7 and TACE can shed growth factor receptor ligands such as HB-EGF[93,188] which have been involved in the transcriptional upregulation of MMP-2[189].

4.4 Clinical perspective

Pathological cardiac remodelling occurs concurrently with the development of hypertension. Because of the increased risk of mortality associated with pathological cardiac remodelling, the mediators of hypertensive cardiac remodelling are important targets to consider when treating hypertension. Our findings indicate that metalloproteinases are signaling mediators of GqPCR

agonists and are therefore candidate therapeutic targets in hypertensive cardiac remodelling.

Although targeting MMP-2 shows promise to lower high blood pressure, our inability to prevent the development of cardiac hypertrophy and fibrosis by blocking MMP-2 (both pharmacologically and through RNA interference) indicates the need for comprehensive and complimentary therapeutic strategies. One such strategy is suggested by novel findings made in this research. Targeting MMP-2 together with metalloproteinases upstream of MMP-2 (such as MMP-7 and TACE) should lower blood pressure with the added therapeutic benefits of preventing the development of cardiac hypertrophy and fibrosis. Further research is warranted to determine the biological roles and therapeutic potential of specific metalloproteinases in hypertensive cardiac disease.

The etiology of hypertension and hypertensive cardiac disease in the general population is generally complex or unknown. Modern therapeutic approaches focus on preventing signalling by presumed causative agonists by receptor antagonism, inhibition of agonist synthesis or acceleration of agonist degradation[31-32,190]. The agonist-specific therapeutic approaches are effective only if the agonist system targeted is primarily responsible for hypertensive signaling in each individual patient and tend to fail otherwise. Therefore, it is important to identify common downstream mediators of multiple pro-hypertensive agonist systems to provide potential targets when the etiology of hypertension is complex or unknown. Our data indicate that metalloproteinases (such as MMP-2, 7 and

TACE) could be candidate common downstream mediators of pro-hypertensive agonists and merit future research to determine their potential therapeutic values.

4.5 Limitations

The studies presented herein are subject to certain conceptual and technical limitations that limit the impact of these findings.

4.5.1 Technical limitations

Gelatin zymography:

The findings of this thesis rely heavily on being able to accurately measure MMP-2 activity by gelatin zymography. Gelatin zymography is a limited enzymatic activity assay to detect gelatinolytic activity with certain advantages and limitations with respect to other protease activity assays such as *in situ* zymography and fluorometric assays.

Advantages: One advantage of gelatin zymography is that protein samples are first subjected to gel electrophoresis, which allows gelatinases to be resolved by molecular weight. Since the substrates used to detect proteolysis by MMP-2 *in vitro* are also susceptible to cleavage by other gelatinases (such as MMP-9), separation of gelatinases by molecular weight (as done in gelatin zymography) allows the identification of individual gelatinases, rather than total gelatinolytic activity. Further, the pro and active forms of MMP-2 can be resolved, providing additional information regarding the activation state of MMP-2 which cannot be detected by fluorometric assays. In addition, gelatin zymography of tissue

provides a clear MMP-2 band compared to immunoblotting methods. When attempting immunoblotting for MMP-2 in both aortic and cardiac tissue homogenates, several non-specific bands were observed and therefore, this data was not used to quantitate MMP-2 levels in tissues (data not shown).

Disadvantages: The major limitation of gelatin zymography is that the regulation of gelatinases by TIMPs cannot be accounted for. TIMP regulatory proteins interact with gelatinases and alter their enzymatic activity by allostery and by favouring subcellular localization. During electrophoresis, interactions between TIMPs and gelatinases are broken and the proteins separate, allowing gelatinase activity to be measured without any effect of TIMPs. Although MMP-2 activity can be especially sensitive depending on the relative abundance of TIMP-2, it is unlikely that the observations reported in these studies are caused by changes in TIMP-2 levels, as TIMP-2 expression was unchanged by Ang II (**Figure 3.9**).

Another limiting factor of accurately measuring gelatinase activity measurement by gelatin zymography depends on the activity of the pro and active forms of MMP-2. In gelatin zymography, renaturation of gelatinases after electrophoresis can induce autolysis, thereby yielding activity measurements of pro-MMP-2 greater than would be observed *in vivo*[191-192]. This is likely due to pro-MMP-2 becoming cleaved to form active MMP-2, however, since this is after electrophoresis, this activity band appears at 72 kDa (corresponding to pro-MMP-2), rather than at 64 kDa (corresponding to active MMP-2). Nevertheless, MMP-2 levels can be estimated by gelatin zymography as increased MMP-2 levels yield

increased activity, which can be visualized by gelatin zymography[192]. It is ambiguous however, if gelatin zymography should be considered a protein assay to measure MMP-2 levels, an enzymatic assay to measure MMP-2 activity or a combination of the two (i.e. a limited activity assay).

Tail cuff plethysmography:

As described in chapter 1, tail cuff plethysmography is a non-invasive, indirect method to measure blood pressure by monitoring reperfusion of blood into the tail after occlusion. Compared to invasive (direct) methods to measure blood pressure, tail cuff plethysmography has the following advantages and disadvantages.

Advantages: One major advantage of using the tail cuff plethysmography method to measure blood pressure in large scale *in vivo* experiments is cost efficiency. Compared to invasive methods which require blood pressure monitors for each mouse, one tail cuff plethysmography machine can be used to measure the blood pressure of all the mice in an experiment. Using the same equipment for all measurements minimizes deviations in blood pressure due to variability between individual sensors and their positions implanted in each mouse. Additionally, equipment and training for tail cuff plethysmography are also much more accessible and less expensive.

Further, there is no need for surgical procedures to implant blood pressure measuring devices when using indirect methods, thereby minimizing surgical complications such as anaesthetic effects on blood pressure, effects of the implanted device on blood pressure and increased risk of infection[193].

Disadvantages: Measuring the blood pressure of each mouse on the same machine (as done in tail cuff plethysmography) can also be a disadvantage because only one mouse can be monitored at a time, allowing temporal variables such as time of day, environmental changes (temperature, noise and odours) and duration of measurement to vary between mice. Blood pressure can change acutely in response to these factors, which can increase the experimental error and decrease clarity in differences between experimental groups[194-195].

Compared to direct blood pressure measurements by invasive techniques, tail cuff plethysmography requires each mouse to be restrained for the duration of the measurement (10-15 minutes). This restraint can cause differing levels of stress to each mouse which can, in turn, affect blood pressure. Commonly, mice that are most stressed by the measurement (i.e. mice that are subjected to a longer period of restraint, mice that are heated and mice who are subjected to more inflation/deflation cycles) exhibit increased sympathetic nervous activity, leading to increased blood pressure[196].

Further, tail cuff plethysmography limits the timeframe of data acquisition to the time where blood pressure is actively being measured by the researcher. Invasive blood pressure measurements can provide data measurements in real time and for all mice simultaneously, thus eliminating changes in blood pressure due to time of day or acute environmental stress.

As described in chapter 2, every effort was made to minimize the impact of these factors by shuffling the order of mice when measuring blood pressure, acclimatizing the mice to the blood pressure device before the experiment and by

keeping the duration of measurement for each mouse as consistent as reasonably possible.

A potential complication of using tail cuff plethysmography to measure blood pressure is that the variable factors discussed above can mask smaller differences between experimental groups, thereby lowering the sensitivity of the system to experimental differences. Our data strongly suggest that targeting MMP-2 attenuates Ang II-induced hypertension, without affecting the development of cardiac hypertrophy or fibrosis. Although we were able to nearly fully attenuate Ang II-induced hypertension by targeting MMP-2, we cannot exclude the possibility that the residual increase in blood pressure of these mice contributed to the development of cardiac hypertrophy and fibrosis. However, mice challenged with Ang II and mice challenged with Ang II and MMP-2 inhibitor/siRNA developed cardiac hypertrophy and fibrosis to the same extent, suggesting that agonist signaling, and not residual hypertension, was ultimately responsible for cardiac remodelling in these mice.

Recent advances in non-invasive (indirect) blood pressure methods have been made to eliminate the need to attach two pieces of equipment to the tail of each animal (the occlusion cuff and the pulse sensor). Attaching these two pieces of equipment allows for additional sources of variance due to changes in the relative position between them. If the pulse sensor is not placed at a consistent distance relative to the occlusion cuff, detection of blood reperfusion can be different between mice, and cause erroneous blood pressure measurements. Movement of conscious mice within the restraining device during measurement

can enhance this effect. Newer systems use a built-in photometric sensor that is part of the occlusion cuff to detect blood flow instead of a separate pulse sensor. This new technology minimizes potential deviations caused by differential positioning of the pulse sensor on the tail and relative to the occlusion cuff.

RNA interference:

Gene function studies utilize two independent but complimentary approaches to determine the role of an individual gene in biological processes. First, enhanced function studies establish the function to a gene or its gene product by exogenously administering the gene (or gene product) of interest to a research system. Enhanced function studies can be achieved by delivery of purified recombinant protein or RNA (gene products), or vector delivery of the gene itself, for example using a viral vector. To enhance these studies, a complimentary approach is used to attribute a loss or decrease in function when a gene is down regulated. This approach can be achieved by genetically mutating a gene (i.e. gene knockout model), silencing or downregulating the expression of a gene (i.e. RNA interference) or by blocking enzymatic activity using small molecule inhibitors (i.e. pharmacological inhibition of gene products). The use of siRNA in cell culture applications has been widely used to enhance gene function assays by offering a new approach to loss of function studies. The practicality of using RNA interference approaches *in vivo* has limitations, although as we and other groups have shown[180,197], *in vivo* delivery of siRNA does have the capacity to substantially downregulate target genes.

Advantages: In contrast to pharmacological inhibition where only genes with known inhibitors can be targeted, exogenous administration of siRNA can target any gene whose mature mRNA sequence is known, however, this conceptual advantage does not come without drawbacks. Despite sequence specific design (one siRNA corresponds to only one gene), concerns regarding off-target effects of siRNA have hindered the widespread use of this technique.

Disadvantages: One potential complication of exogenous administration of siRNAs is the potential for activation of the innate immune system. Certain groups have proposed that structural homology between exogenous siRNA and viral RNA has conferred the ability of siRNAs to activate the innate immune system by recognizing siRNA as a foreign pathogen[164]. Kleinman et al.[164] have demonstrated that innate immune activation by siRNAs depended on toll-like receptor 3 and the inflammatory cytokines TNF-α and Interleukin-12. Interestingly, this activation depended on the length of the siRNAs, rather than their sequence, indicating the potential for off-target immune activation by siRNA.

It is also unclear how large polyanionic molecules such as siRNAs are able to cross biomembranes and enter cells *in vivo*. In cell culture, lipophilic transfection agents are used to increase transfection efficiency by forming micelle-like complexes. Previous research has demonstrated the effective use of siRNAs in adult mice by delivering siRNAs intravenously in cationic liposomes[198]. In our *in vivo* experiments, siRNAs were delivered without transfection agent or lipid material. In animals, it is possible that factors such as

arterial pressure and contractile movement in the cardiovascular system increases the flux of siRNAs across membranes into cells. It is also possible that some form of transport system functions to move small nucleotides across biomembranes. Future research is required to characterize how naked siRNAs are able to enter cells *in vivo* and to determine if this is a viable approach in rodent scale loss of function studies.

In this study, we have shown that MMP-2 is upregulated in response to Ang II in the heart and aorta but not in the liver or kidney. Interestingly, we were only able to detect a decrease in MMP-2 expression under stimulated conditions and only in the heart and aorta (**Figure 3.19**). One possible explanation for this is low protein turnover. SiRNAs function to attenuate synthesis of *de novo* proteins by mRNA degradation. If basal transcriptional levels are low because of low protein turnover, very little mRNA is present and therefore siRNAs have less potential to lower total protein level. We have also observed that MMP-2 siRNA did not lower basal MMP-2 levels in the heart and aorta (data not shown), which supports the possibility that low turnover of MMP-2 limits the ability of MMP-2 siRNA to knockdown total MMP-2 levels under basal conditions. When stimulated with Ang II, MMP-2 transcription increases and knockdown of MMP-2 becomes more prominent. This is likely because the increase in total MMP-2 is derived from an increase in MMP-2 mRNA, which can be attenuated by MMP-2 siRNA. We therefore believe that MMP-2 siRNA is able to attenuate the induction of newly synthesized MMP-2 that is stimulated by Ang II.

4.5.2 Conceptual Limitations

Contributions of organs distal to the heart and vasculature in hypertension and hypertensive cardiac disease:

A fascinating question in regards to development of hypertensive cardiac disease is the role played by organs distal to the heart such as the kidneys[199]. Interactions between the renin-angiotensin system and the sympathetic nervous system have been demonstrated by the increased synthesis of catecholamines and neurotransmitters in response to Ang II[200-201]. Conversely, sympathetic nervous activity can increase renin secretion and subsequent angiotensin maturation[202-203]. Recent research has also demonstrated direct interactions between the receptors of these two systems[202,204].

Based on our studies, we believe that elevated levels of pro-hypertensive agonists are ultimately causal of the development of hypertensive cardiac disease. In addition to acting directly on the heart and vasculature, agonists such as Ang II can stimulate secondary messaging systems (eg. the sympathetic nervous system) by acting at sites distal to the heart. These secondary messaging systems can contribute to the development of cardiac remodelling (eg. by acting on the heart) and hypertension (eg. by acting on the vasculature where they could stimulate MMP-2 dependent vasoconstriction) [204].

In contrast to our findings in the heart and vasculature, we found that MMP-2 was not elevated in the kidneys of mice with Ang II-induced hypertension. These data are in agreement with previous research showing that MMP-2 is not elevated in the renal medulla of the spontaneous hypertensive rat,

however, MMP-7 is[205]. Interestingly, there is evidence that TACE plays a role in chronic renal disease induced by Ang II[206]. Therefore, renal MMP-7 and TACE may be effectors of Ang II but might not act by upregulating MMP-2 in the kidney.

Metalloproteinases are strongly upregulated in many diseases including cancer, chronic renal disease and a pregnancy-induced hypertension condition termed pre-eclampsia[206-208]. Despite the strong upregulation of numerous metalloproteinases in cancer, the application of metalloproteinase inhibitors has had very little clinical success in the cancer field. This is likely a result of targeting metalloproteinases non-specifically and/or at the wrong stage of disease progression. Additionally, systemic administration of high doses of certain metalloproteinase inhibitors had secondary effects such as the development of musculoskeletal syndrome[209-210].

4.6 Future directions:

Studying models that more accurately represent essential hypertension, the most common forms of the condition in humans, should be investigated. Therefore, an important future direction is to explore the effects of targeting MMP-2 in the spontaneous hypertensive rat (SHR), a more clinically relevant model of disease. As described in chapter 1.1.5, disease development in this model depends on multiple agonist systems and endothelial dysfunction that act synergistically to induce hypertension and hypertensive cardiac remodelling. Using approaches similar to those done in this study (i.e. using pharmacological

inhibition and RNA interference), targeting MMP-2 in the SHR could determine if the anti-hypertensive effects of targeting MMP-2 are Ang II-specific or applicable to hypertensive agonists in general, indicating the potential for future therapeutic applications.

Interestingly, despite being strongly upregulated in chronic hypertension, MMP-2 is not rapidly activated in agonist-induced acute hypertension[26]. Although targeting MMP-2 induces immediate vasorelaxation in small mesenteric arteries constricted with phenylephrine (figure 3.10), the contribution of MMP-2 transcriptional upregulation in chronic hypertension is not fully understood. By targeting MMP-2 in models of acute hypertension, the role of MMP-2 at baseline levels could be determined. This would provide insight to determine whether the transcriptional upregulation of MMP-2 is required for MMP-2 to mediate hypertension.

Another avenue to further explore the role of MMP-2 in agonist-induced hypertension could be to conduct gain-of-function studies, where exogenous administration of MMP-2 (either recombinant or by viral vector) could enhance or exacerbate the hypertensive effects of agonists such as Ang II. Since downregulating MMP-2 (loss-of-function), attenuated Ang II-induced hypertension, we would expect that overexpression of MMP-2 may cause a further increase in blood pressure in response to Ang II or cause a hypertensive response at a lower dose of Ang II.

Although we provide new insight into the regulation of MMP-2 gene transcription by MMP-7 and TACE, the pathways by which MMP-2 regulates

vascular tone are complex and arise from a multitude of MMP-2 proteolysis substrates. As we and others have previously suggested, MMP-2 can cleave vasoactive peptides (such as big endothelin, calcitonin gene related peptide and adrenomedullin)[102-104] as well as extracellular receptors (such as vascular endothelial growth factor receptor-2, and insulin receptor)[139,141]. Future research should establish the specific contribution of these MMP-2 signaling mechanisms in agonist-induced hypertension.

We have provided evidence that MMP-2 contributes to Akt activation in Ang II-induced hypertension, although we have not proven that this regulation is causal to hypertension and not a consequence of lowering blood pressure. Since PI3K inhibitors have been shown to relax arteries, we hypothesize that this pathway is likely causal to hypertension. By treating mice with Ang II-induced hypertension with similar Akt activation inhibitors (i.e. wortmannin, LY294002), we could conclusively determine if Akt activation is part of the signaling pathway described in figure 4.1.

4.7 Conclusions

In short, MMP-2 mediates Ang II-induced hypertension under the transcriptional control of MMP-7 and TACE. We have shown that MMP-2 is transcriptionally upregulated in Ang II-induced hypertension and that this upregulation is dependent on MMP-7 and TACE. Targeting MMP-2 using pharmacological inhibition or RNA interference attenuates Ang II-induced hypertension, without preventing the development of cardiac hypertrophy or

fibrosis. Therefore, we believe that Ang II signals through MMP-7 and TACE to induce: i) hypertension mediated by MMP-2 and, ii) cardiac hypertrophy and fibrosis independent of MMP-2.

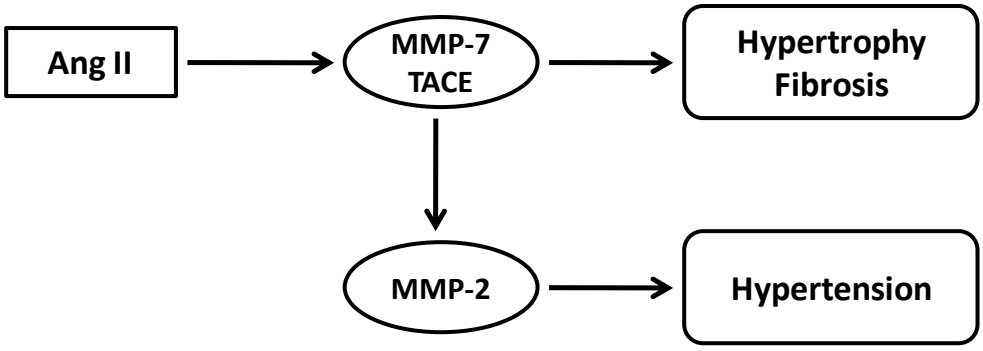

Figure 4.1 Model of metalloproteinase signaling in Ang II-induced cardiovascular disease. Ang II induces hypertension, cardiac hypertrophy and fibrosis which can be prevented by blockade of MMP-7 and TACE. A bifurcation in signaling exists downstream of MMP-7 and TACE where Ang II-induced hypertrophy and fibrosis occur independently of MMP-2 gene induction and the development of hypertension.

Chapter 5

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Appendix I

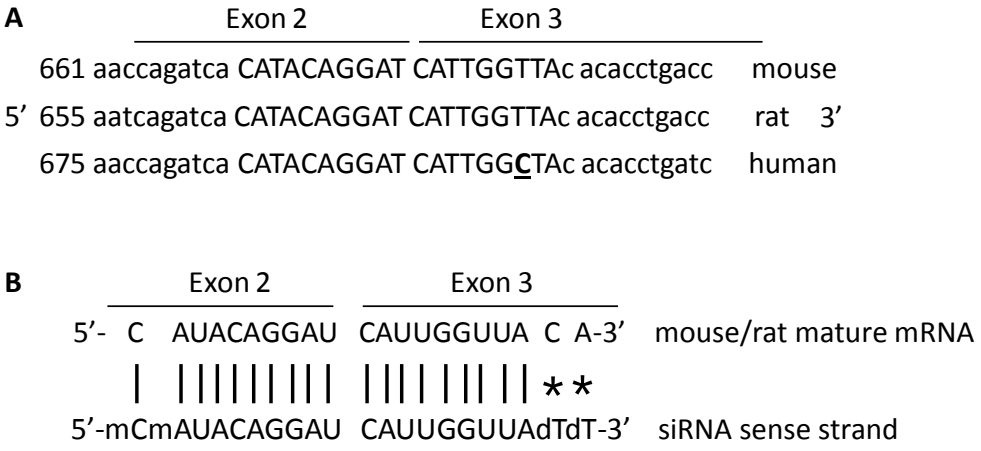
Design of MMP-2 siRNA

Based on previous research, we designed a siRNA against MMP-2 to fit the following criteria outlined by our lab and in previously published guidelines[1-2]:

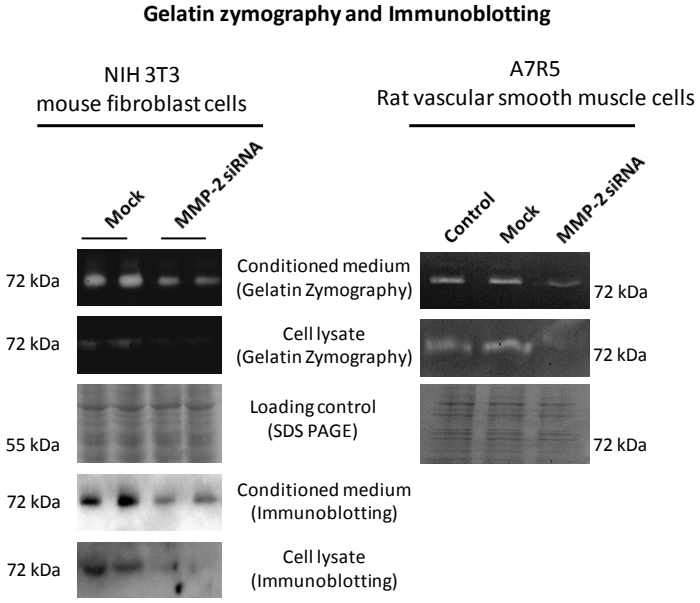
- The sequence should match both the mouse and rat mRNA.
- The sequence should have a low GC content (<50%).
- The sequence should have no other BLAST hits
- The sequence should have no predicted secondary structure.
- The sequence should have no section of 4 repeating bases.

We compared sequences of previously used siRNAs against MMP-2[3] to our top hits and elected to have our candidate siRNA synthesized on the basis of the criteria described above. Sequences alignments for mouse, rat and human MMP-2 along with our siRNA sequence are shown in **appendix figure I.I**.

Due to the expensive nature of siRNA experiments at the whole animal level, we first conducted *in vitro* experiments to confirm the effectiveness of our siRNA. We transfected two different cell lines (NIH 3T3 mouse fibroblast cells and A7R5 rat aortic smooth muscle cells) with MMP-2 siRNA and were able to significantly reduce the amount of MMP-2 activity (by gelatin zymography) and protein level (by immunoblotting) in both conditioned media and cell lysates (**Appendix figure I.II**). Following confirmation of RNA efficacy in cell culture, we then used the newly designed MMP-2 siRNA in our mouse model of Ang II-induced hypertension (Chapter 3).



Appendix figure I.I A-Sequence alignment of mouse, rat and human cDNA for the MMP-2 gene. Introns have been removed to align only the sequence relevant to the designed siRNA (in capital letters). Bold and underline indicates a sequence mismatch. **B**- Sequence alignment of mouse/rat MMP-2 mRNA and the sense strand of the designed MMP-2 siRNA. Lines indicate sequence matches and asterisks indicate mismatches.



Appendix figure I.II Validation of MMP-2 siRNA in cultured A7R5 cells. Serum starved A7r5 cell were transfected with MMP-2 siRNA (100 nmol/L) using DharmaFECT-2 transfection reagent. Conditioned medium and cell lysate were collected 24 hours after transfection. Representative images of conditioned media (upper panel) and cell lysates (middle panel) subjected to gelatin zymography. Equal loading was confirmed by SDS-PAGE (lower panel). Images are representative of triplicate experiments.

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Appendix II

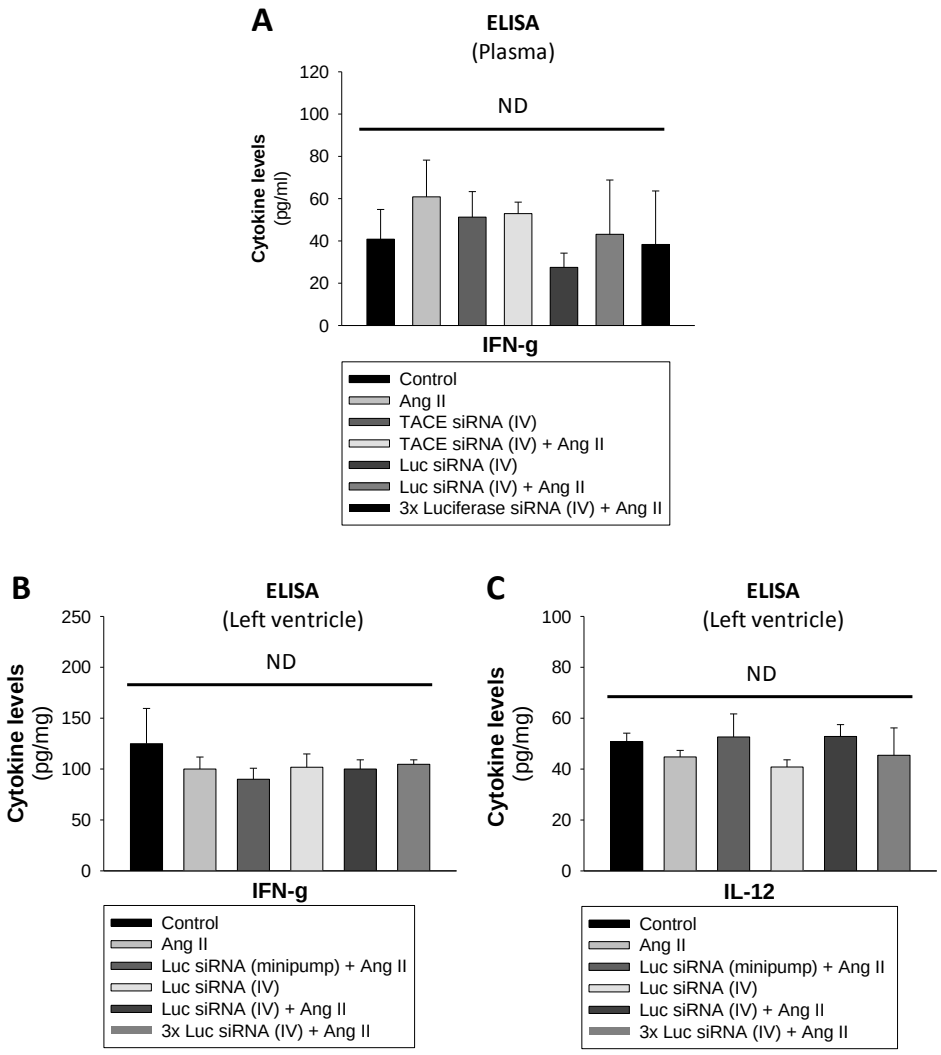
Control experiments involving siRNA

To exclude the possibility of our physiological results arising from an inflammatory response triggered by exogenous siRNA administration, we measured levels of the inflammatory cytokines TNF-α and Interleukin-12 (IL-12) in plasma samples and left ventricle homogenates by ELISA. In all instances (**Appendix figure II.I A-C**), we found no evidence of cytokine induction by siRNAs against TACE or luciferase when delivered by osmotic minipump (as done in chapter 3) or by bolus intravenous injection.

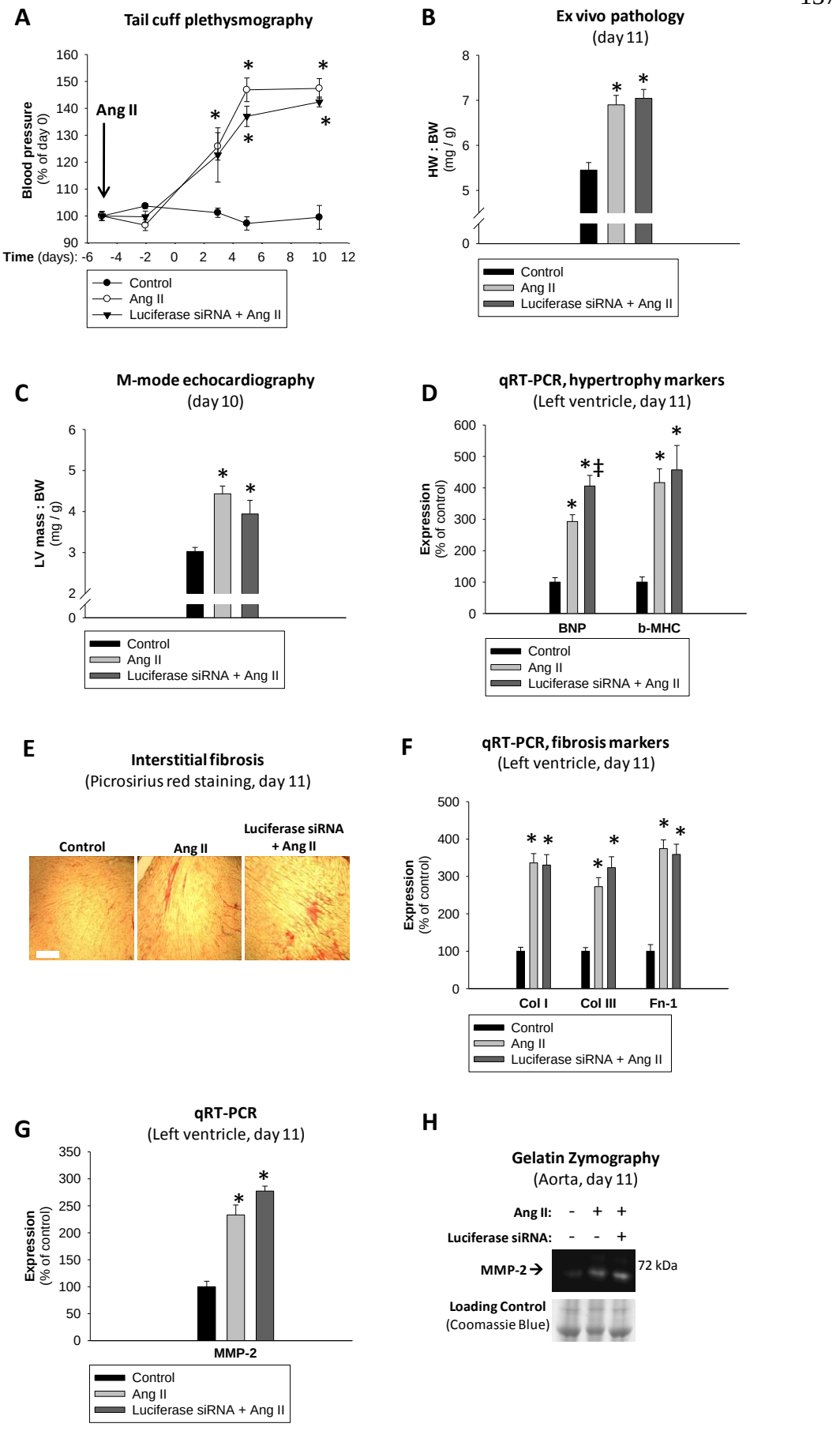
To confirm the target specific effects of MMP-2 siRNA, we used a control siRNA against the non-mammalian gene luciferase. Luciferase siRNA treatment (0.4 mg/kg/d) did not attenuate Ang II-induced hypertension (as measured by tail cuff plethysmography, **Appendix figure II.II A**), cardiac hypertrophy (as measured by *ex vivo* pathology, M-mode echocardiography and qRT-PCR analysis of hypertrophy marker genes, **Appendix figure II.II B-D**) or fibrosis (as measured by collagen staining of heart sections and qRT-PCR analysis of fibrosis marker genes, **Appendix figure II.II E, F**). Luciferase siRNA also had no effect on the induction of MMP-2 by Ang II (by qRT-PCR and gelatin zymography, **Appendix figure II.II G, H**). These results indicate that the physiological effects of MMP-2 siRNA presented in chapter 3 are sequence-dependent.

We also conducted a separate experiment to determine the physiological effects of MMP-2 siRNA in the absence of Ang II. MMP-2 siRNA alone had no effect on basal blood pressure (**Appendix figure II.III A**), heart weight

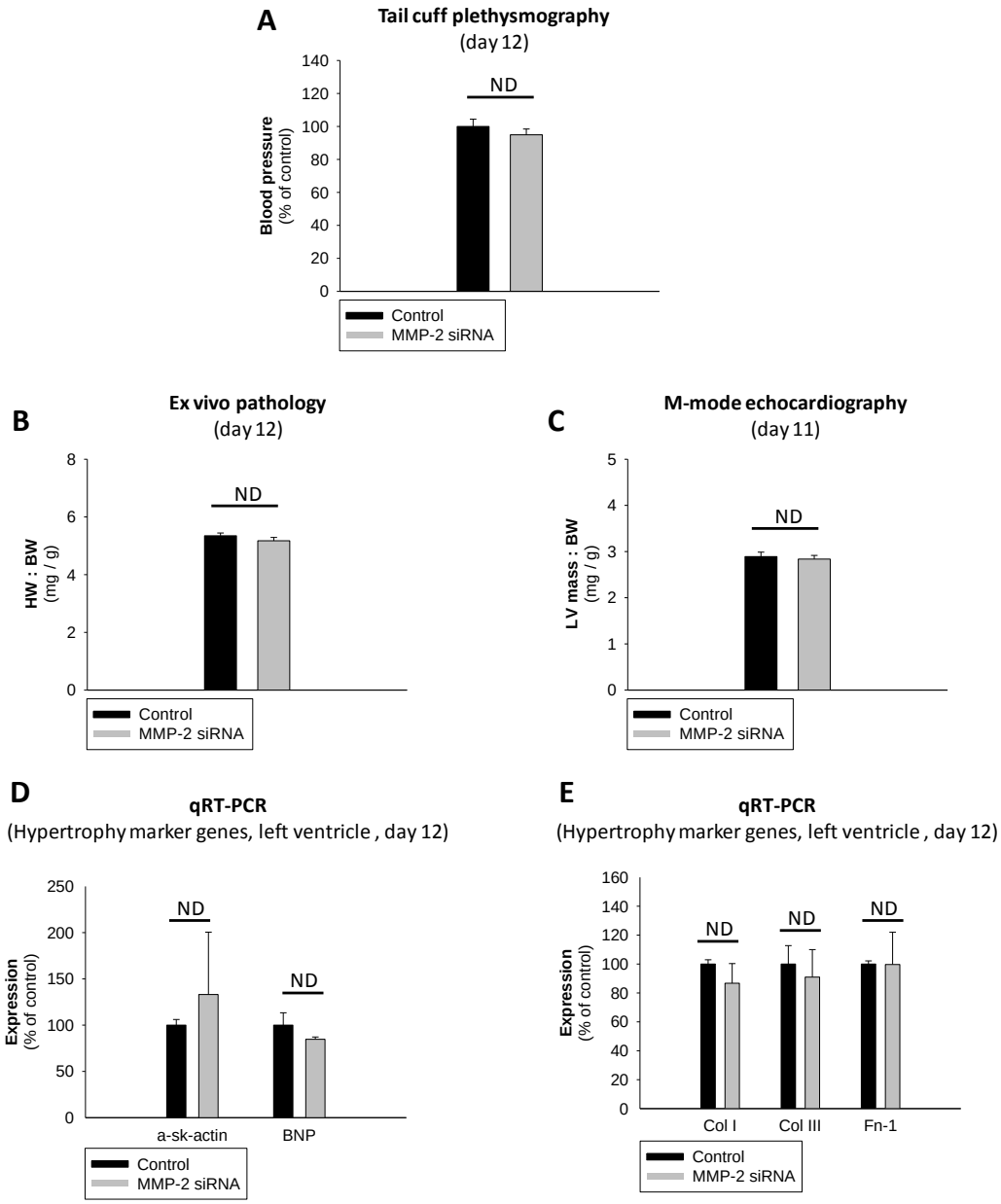
(**Appendix figure II.III B**) or left ventricle weight (**Appendix figure II.III C**) to body weight ratios or the expression of hypertrophy (**Appendix figure II.III D**) or fibrosis (**Appendix figure II.III E**) marker genes. Taken together with the siRNA data shown in chapter 3, these data indicate that MMP-2 siRNA has the capacity to attenuate agonist-induced hypertension, without any gross effects on basal blood pressure.



Appendix figure II.I Inflammatory cytokines are not increased in Ang II-induced hypertension or by siRNA treatment. ELISA analysis of IFN-g (**A, B**) and IL-12 (**C**) in mice treated with Ang II (1.4 mg/kg/d) by subcutaneous osmotic minipump and/or siRNA by either subcutaneous osmotic minipump (minipump, 0.4 mg/kg/d) or jugular vein injection (IV, 15 μg bolus) in plasma (**A**) or left ventricle (**B, C**) samples.


A
Tail cuff plethysmography
Blood pressure (% of day 0)
Ang II
Time (days): -6 -4 -2 0 2 4 6 8 10 12
Control
Ang II
Luciferase siRNA + Ang II
B
Ex vivo pathology (day 11)
HW : BW (mg / g)
Control
Ang II
Luciferase siRNA + Ang II
C
M-mode echocardiography (day 10)
LV mass : BW (mg / g)
Control
Ang II
Luciferase siRNA + Ang II
D
qRT-PCR, hypertrophy markers (Left ventricle, day 11)
Expression (% of control)
BNP
b-MHC
Control
Ang II
Luciferase siRNA + Ang II
E
Interstitial fibrosis (Picrosirius red staining, day 11)
Control
Ang II
Luciferase siRNA + Ang II
F
qRT-PCR, fibrosis markers (Left ventricle, day 11)
Expression (% of control)
Col I
Col III
Fn-1
Control
Ang II
Luciferase siRNA + Ang II
G
qRT-PCR (Left ventricle, day 11)
Expression (% of control)
MMP-2
Control
Ang II
Luciferase siRNA + Ang II
H
Gelatin Zymography (Aorta, day 11)
Ang II: - + +
Luciferase siRNA: - - +
MMP-2
72 kDa
Loading Control (Coomassie Blue)

Appendix figure II.II Luciferase siRNA does not attenuate Ang II-induced hypertension, cardiac hypertrophy or fibrosis. **A)** Time course of systolic blood pressure in control mice, mice treated with Ang II or mice treated with Ang II and luciferase siRNA. Cardiac hypertrophy as assessed by echocardiographic analysis of left ventricle weight to body weight ratio (**B**), gross pathology of heart weight to body weight ratio (**C**) and qRT-PCR analysis of hypertrophy marker genes (**D**). Fibrosis was assessed by histological analysis of heart sections (10 μm) stained with picrosirius red to determine collagen deposition (**E**) and qRT-PCR analysis of fibrosis marker genes (**F**). qRT-PCR (**G**) and gelatin zymography (**H**) analysis of gelatinase expression and activity in cardiac homogenates. Scale bars indicate 250 μm (Picrosirius red micrographs). n=4 mice for each group. * indicates $p<0.05$ vs. control group. Echocardiocardiography, qRT-PCR and picrosirius red data were obtained by collaboration with Dr. Tatsujiro Oka, Dr. Zamaneh Kassiri and Stephan Cooper, respectively (described in Appendix III).


A
Tail cuff plethysmography
(day 12)
Blood pressure
(% of control)
ND
Control
MMP-2 siRNA
B
Ex vivo pathology
(day 12)
HW : BW
(mg / g)
ND
Control
MMP-2 siRNA
C
M-mode echocardiography
(day 11)
LV mass : BW
(mg / g)
ND
Control
MMP-2 siRNA
D
qRT-PCR
(Hypertrophy marker genes, left ventricle , day 12)
Expression
(% of control)
ND
ND
a-sk-actin
BNP
Control
MMP-2 siRNA
E
qRT-PCR
(Hypertrophy marker genes, left ventricle , day 12)
Expression
(% of control)
ND
ND
ND
Col I
Col III
Fn-1
Control
MMP-2 siRNA

Appendix figure II.III MMP-2 siRNA does not affect basal blood pressure or cardiac remodelling. Male C57BL/6 mice were treated with MMP-2 siRNA (0.4 mg/kg/d) for 15 days and basal blood pressure was determined by tail cuff plethysmography (**A**). Cardiac hypertrophy as assessed by gross pathology of heart weight to body weight ratio (**B**), echocardiographic analysis of left ventricle weight to body weight ratio (**C**) and qRT-PCR analysis of hypertrophy marker genes (**D**). Fibrosis was assessed by qRT-PCR analysis of fibrosis marker genes (**E**). Echocardiocardiography and qRT-PCR data were obtained by collaboration with Dr. Tatsujiro Oka and Dr. Zamaneh Kassiri, respectively (described in Appendix III).

Appendix III

Contributions of collaborators

The results presented within this thesis are the product of a collaborative effort between multiple graduate students, technicians, core facilities and principal investigators. The contributions to this thesis that arise from the work of the collaborators are described below:

The design of all animal experiments was a collaborative effort of all present and former members of the Fernandez-Patron Lab (Xiang Wang, Stephan Cooper, Fung Lan Chow and Carlos Fernandez-Patron). Specifically, experiments involving MMP-7 and TACE siRNA treatment were done in collaboration with Fung Lan Chow (surgery), Xiang Wang (blood pressure analysis), Stephan Cooper (histological analysis). Ang II timecourse, control siRNA experiments and MMP-2 siRNA experiments were done in collaboration with Xiang Wang (blood pressure analysis) and Stephan Cooper (histological analysis). Experiments involving pharmacological inhibitors were done in collaboration with Stephan Cooper (histological analysis).

Studies of isolated mesenteric arteries were done in collaboration with Steve Ok (mounting of arteries and photographing).

Gene expression analysis (by qRT-PCR) was performed by Zamaneh Kassiri (and her lab) for the Ang II time course / siRNA experiments and Joanne Zhao (qRT-PCR core) for pharmacological inhibitor experiments.

Echocardiography analysis was performed by Dr. Tatsujiro Oka for the Ang II time course / siRNA experiments and Donna Beker (echocardiography core) for pharmacological inhibitor experiments.

Appendix IV

Sample echocardiography data

Appendix table IV.I Sample echocardiography data (raw data)

Parameter	Control	Ang II	MMP-2 siRNA + Ang II
IVS, d (mm)	0.64 +/- 0.02	0.89 +/- 0.03 *	0.87 +/- 0.02 *
LVID, d (mm)	4.63 +/- 0.13	4.67 +/- 0.09	4.50 +/- 0.03
LVPW, d (mm)	0.61 +/- 0.02	0.82 +/- 0.04 *	0.85 +/- 0.02 *
EF (%)	57.9 +/- 2.8	54.1 +/- 2.8	54.1 +/- 5.0
HR (bpm)	432 +/- 17	474 +/- 35	475 +/- 47
BW (g)	30.4 +/- 1.2	29.6 +/- 0.7	30.6 +/- 0.7

IVS indicates interventricular septum; LVID, left ventricle inner diameter; LVPW, left ventricle posterior wall; d, diastole; EF, ejection fraction; HR, heart rate; BW, body weight. Data are represented as means +/- sem. n=3-4 mice per group. * indicates $p<0.05$ vs. control group. Echocardiography data obtained by collaboration with Dr. Tatsujiro Oka as described in Appendix III.