OVEREXPRESSION OF ANG-(1-7) OR CARDIAC-SELECTIVE OVEREXPRESSION OF ANGIOTENSIN TYPE 2 RECEPTOR IMPROVES CARDIAC FUNCTION AND ATTENUATES LEFT VENTRICULAR REMODELING

By

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I dedicate this work to my parents, Xiangqian Qi and Yuanli Deng and my husband, Xuan Liu

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Despite the significant advances in pharmacological and interventional therapies for acute myocardial infarction, myocardial infarction is still one of the most debilitating cardiovascular diseases with tremendous economic impacts on society. It is well established that intricate regulation of the cardiac renin angiotensin system (RAS) is critical in normal heart functions, and an aberrant activity of the RAS is associated with deterioration of heart function and pathological cardiac remodeling post myocardial infarction.

Angiotensin-(1-7) (Ang-(1-7)) has been implicated to play a cardioprotective role in cardiovascular diseases. Ang-(1-7) decreases incidence and duration of ischemia-reperfusion arrhythmias and improvement of the postischemic contractile. Investigations into the mechanisms of action of ACE inhibitors and angiotensin II type 1 receptor (AT1R) antagonists in cardiovascular disease have revealed that both of these treatments cause increased circulating levels of Ang-(1-7). Infusion of Ang-(1-7) has been shown to improve endothelial aortic function and coronary artery perfusion and preserved cardiac function in rats with heart failure. Agonist for the Ang-(1-7) receptor

Mas (AVE0991) attenuates postischemic heart failure in rats. There are two distinct arms of the RAS, the ACE2-Ang-(1-7)-Mas axis and the ACE-AngII-AT1R axis. The ACE-AngII-AT1R axis has been implicated in pathological cardiac remodeling, vasoconstriction, salt and water reabsorption, and inflammatory and proliferative affects of components of the cardiovascular system. The ACE2-Ang-(1-7)-Mas axis on the other hand has been hypothesized to work in opposition to the ACE-AngII-AT1R axis.

In addition to the AT1R there is also a corresponding receptor that, when stimulated, acts in opposition to that of AT1R activation. The role of this angiotensin II type 2 receptor (AT2R) in cardiovascular disease remains elusive, despite recent advances in understanding the RAS. It is widely accepted that AT2R counteracts AT1R-mediated actions, which is beneficial for the organism. It has been speculated that AT2R contributes to the beneficial effects of AT1R blockade because AT1R blockade causes Angiotensin II to bind unopposed to the AT2R. However, role of the AT2R in myocardial infarction is controversial. Although most studies claim that AT2R improves postinfarct cardiac function, others report either no effect on the outcome or even deterioration.

The present studies were designed to characterize the cardioprotective actions of Ang-(1-7) and AT2R in a rat myocardial infarction model. Ang-(1-7) was overexpressed by the lenti-viral vector. AT2R was overexpressed by cardiac-selective recombinant adeno associated virus serotype 9 (rAAV9).

The results of studies indicate that the both Ang-(1-7) and AT2R play a cardioprotective role in myocardial infarction. Overexpression of either Ang-(1-7) or AT2R in the heart attenuates the development of cardiac hypertrophy and heart failure

in rat myocardial infarction model. The cardioprotective effects of Ang-(1-7) were mediated through restoring the balance between ACE-AngII-AT1R axis and ACE2-Ang-(1-7)-Mas axis, and upregulating protective factors (BKR2 and IL10). The cardioprotective effects of AT2R were also mediated through restoring the ACE/ ACE2 balance, reducing the TGF-β, Collagen I, and Collagen III expression, and upregulating Mas and BKR2 receptor levels. Our results indicate that both Ang-(1-7) and AT2R may represent a new class of targets for pharmacological intervention post myocardial infarction and during progression of heart failure.

CHAPTER 1
GENERAL INTRODUCTION

Myocardial Infarction

Myocardial infarction (MI), commonly known as a heart attack, a disorder that causes damage and potential death of heart tissue as a result of a lack of supply of oxygen and other nutrients causing by the sudden blockage of a coronary artery. The blockage is mostly due to occlusion of a coronary artery following the disruption of an atherosclerotic plaque. Risk factors include previous history of vascular disease (such as atherosclerosis), angina, a previous heart attack or stroke, and age (especially men over 40 and women over 50 years). According to a report from the American Heart Association Statistics Committee, an estimated 785 000 Americans will have a new heart attack and about 470 000 will have a recurrent attack in their lifetime (Lloyd-Jones et al., 2009) (Rosamond et al., 2007). Prompt recognition and treatment is critical for preventing further damage to the myocardium.

Heart Failure

Heart failure is a condition when the heart can not pump enough blood to meet the body's demand. The most common cause for the heart failure is ischemic heart diseases-myocardial infarction. More than 60% of the heart failure that occurs in the US may be attributable to ischemic heart diseases (He et al., 2001). Other causes include hereditary cardiomyopathies, valvular malfunctions and congenital aberrations. Heart failure is a growing healthcare problem all over the world. In the United States, heart failure currently afflicts > 5 millions patients, with another 550,000 new cases diagnosed annually (Thom et al., 2006). After myocardial infarction, a viable myocardium needs to

compensate the function of the dysfunctional myocardium. Local and systemic compensating mechanisms are triggered to cope with the sustained body's demand.

Cardiac Remodeling Post-Myocardial Infarction

Following an MI, characteristic morphological and patho-physiological changes occur, namely cardiac remodeling (Pfeffer and Braunwald, 1990) (Patten et al., 1998). This remodeling process includes cardiac fibrosis (Weber and Brilla, 1992), left ventricular (LV) dilation caused by an expansion of the infarct zone, and the development of an eccentric hypertrophy in the non-infarcted area (McKay et al., 1986). Cardiac remodeling post-myocardial infarction, which is characterized by cardiac hypertrophy and reactive fibrosis, results in wall stiffness and diminished cardiac performance. Myocardial infarct size, followed by adverse left ventricular (LV) remodeling (dilation and fibrosis) and cardiac dysfunction are major determinants in the pathogenesis of cardiac diseases. Therefore, to improve clinical outcomes among patients with MI, it is essential to develop therapies that effectively modulate the post-MI LV remodeling.

Ventricular remodeling is strongly correlated with improvement in other heart failure outcomes. The impact of myocardial infarction(MI) on ventricular geometry and function in an animal model of coronary artery ligation was described by Pfeffer et al. in 1985 (Pfeffer et al., 1985) , who demonstrated a strong relationship between the size of the myocardial injury and degree of left ventricular (LV) remodeling and mortality. Pfeffer et al. also showed a beneficial reduction in LV end-diastolic volume index in rats treated with the angiotensin-covnerting enzyme inhibitor (ACEi) -captopril. Others have confirmed the relationship between myocardial injury, activation of neurohormonal pathways, and the pathologic cardiac phenotype associated with heart failure

(Sadoshima et al., 1993) (McKay et al., 1986) (Katada et al., 2005) (Francis et al., 1990). Furthermore, it has been demonstrated that treatment strategies resulting in preventing the progression of pathologic remodeling typically also reduce heart failure mortality, whereas therapies with adverse impact on survival have minimal or no effect on ventricular remodeling (Cohn et al., 1991) (Greenberg et al., 1995) (Metra et al., 2003) (Wong et al., 2004) (Cintron et al., 1993).

Cardiac fibrosis is a hallmark for pathological cardiac remodeling in cardiovascular diseases. Reactive cardiac fibrosis interferes with the normal function and structure of the myocardium (Brilla et al., 1991) (Weber, 2000). Increased deposition of collagen in cardiac tissue results in an increase in cardiac tissue stiffness.This remodeling predisposes the patient to an increased risk of adverse cardiac events, including myocardial ischemia, infarction, arrhythmias and sudden cardiac death (Weber, 2000) . Thus, prevention and reversal of cardiac fibrosis are essential to preserve heart function and prevent pathological cardiac remodeling in cardiovascular disease.

It is well established that cardiac renin angiotensin system (RAS) is critical in regulating normal heart functions, and aberrant activity of the RAS is associated with pathological cardiac remodeling post myocardial infarction. Remodeling and functional impairment of myocardial contractility have been associated with high intramyocardial levels of angiotensin, norepinephrine, and aldosterone (Cohn et al., 2000). Thus, pharmacological inhibition of dysregulated RAS might play an important role in stabilization or reversal of pathological remodeling and improve clinical outcomes. The inhibition for the RAS provides not only a proof for the role of RAS in cardiovascular pathophysiology but also therapeutic treatments for heart failure patients. As noted

above, addition of an ACEi following MI attenuated the remodeling process and reduced mortality in animal model (Pfeffer et al., 1985). The effects of enalapril (an ACEi) were examined in the patients with clinical heart failure (Anonymous 1991). Enalapril-treated patients experienced significantly fewer deaths, primarily as a result of reduction in progressive heart failure, and fewer hospitalizations. An echocardiography substudy examining the impact of enalapril on LV function and geometry revealed that patients in the placebo arm had progressive LV dilation, whereas those treated with enalapril had sustained reductions in LV dimensions (Konstam et al., 1992) (Konstam et al., 1993). Captopril and ramipril (Anonymous 1993) demonstrated similar effects as enalapril, which proves that ACEi exhibits reversing ventricular remodeling and reducing mortality.

Blockade of the effects of the renin–angiotensin system utilizing angiotensin receptor blockers (ARBs) also appears to be linked to reverse ventricular remodeling and mortality benefit. Patients intolerant of ACEi treated with valsartan benefited from reduction in morbidity and mortality compared with those treated with placebo. Furthermore, patients in the valsartan group demonstrated improvement in remodeling exhibits a significantly smaller mean LV internal diastolic dimension index than patients randomized to placebo (Maggioni et al., 2002). Treatment with candesartan also showed similar reduction in morbidity and mortality in a study that assessed the benefits of candesartan in patients intolerant of ACEi (Granger et al., 2003) . Moreover, ACEi is reported to increase cardiac ACE2 mRNA level (Ferrario et al., 2005). ARB treatment has been shown to result in a significant upregulation of ACE2 mRNA level in the viable myocardium in the myocardial infarction model (Ishiyama et al., 2004b). ARB treatment has been reported to increase cardaic ACE2 mRNA level and cardiac ACE2 activity;

and a combination of ARB and ACEi exerted similar effects (Ferrario et al., 2005). ACEi and ARB thus may provide LV protective effects by not only inhibiting ACE-AngII-AT1R pathway but also increasing ACE2-Ang-(1-7)-Mas pathway. It has been also speculated that AT2R contributes to the beneficial effects of AT1R blockade because AT1R blockade causes Angiotensin II to bind unopposed to the AT2R.

A variety of cytokines are activated and are well-established mediators for cardiac remodeling during a number of cardiac pathophysiological conditions including MI, ischemia/reperfusion injury, and heart failure. The inflammatory response ultimately leads to healing and repair of the injured territory. Thus, the molecular signals induced post-MI may mediate suppression of tissue injury and regulate scar formation. The increased cytokine gene expression during acute phase of inflammation evokes a secondary, self-sustaining autocrine and paracrine growth factor and cytokine expression.

Proinflammatory cytokines (e.g., tumor necrosis factor [TNF]-α, interleukin [IL]-1, and IL-6), anti-inflammatory cytokine (e.g. IL-10), and cytokines having both pro- and anti-inflammatory effects (e.g. transforming growth factor-β [TGF-β]) play a critical role in mediating homeostasis within the heart in response to cardiac injury. TNF-α has been extensively investigated after cardiac injury. TNF-α RNA and protein is elevated in patients and in animal models with advanced heart failure (Testa et al., 1996) (Torre-Amione et al., 1996). TNF-α transgenic mice showed several symptoms of heart failure with cardiac specific overexpression of TNF-α (Kubota et al., 1997) (Bryant et al., 1998). Systemic infusion of recombinant TNF-α into rats depressed left ventricular function and caused left ventricular dilation at the dose yielding blood concentrations of TNFα seen in

patients with advanced heart failure (Bozkurt et al., 1998). Another pro-inflammatory cytokine is IL-1 involving in progression of myocardial infarction. Expression levels of IL-1β in blood and/or myocardial tissues are increased also in patients with coronary artery disease (Hasdai et al., 1996) , acute MI (Guillen et al., 1995) ,dilated cardiomyopathy (Han et al., 1991) (Francis et al., 1990), and in patients and animal models of congestive heart failure (Testa et al., 1996). IL-1 knockout mice exhibited less development of adverse left ventricular remodeling after MI and decrease in myofibroblast infiltration and collagen deposition (Bujak et al., 2008).

Necrosis and apoptosis are both occurring post myocardial infarction and involving in inflammatory response. In contrast to necrosis that triggers an inflammatory response, apoptotic cells lead to the production of anti-inflammatory cytokines such as IL-10. IL-10, a potent anti-inflammatory cytokine, is a strong deactivator for monocytes and suppressor of various pro-inflammatory mediators (Frangogiannis et al., 2000) (Yao et al., 2008) . IL-10 suppresses the inflammatory responses through inhibiting the production pro-inflammatory cytokines such as IL-1, TNF-α, IL-6 and IL-8 (Yang et al., 2000),. IL-10 also modulates expression of metalloproteinases and their inhibitors, so that IL-10 may have a significant role in extracelluar matrix formation (Lacraz et al., 1995). IL-10 level in the plasma was prominently elevated in patients with myocardial ischemia/reperfusion (Shibata et al., 1997) (Seghaye et al., 1996). The potential role of IL-10 in experimental myocardial infarction has recently been investigation. IL-10 deficient mice exhibited an enhanced inflammatory response, as demonstrated by increased neutrophil recruitment, elevated plasma level of TNF-α , and high mortality following myocardial infarction (Yang et al., 2000). IL-10 mRNA and protein upregulation

was demonstrated in the reperfused infarcted myocardium using a canine model of myocardial infarction (Frangogiannis et al., 2000).Thus, IL-10 may have a protective role after myocardial ischemia/reperfusion through the suppression of the acute inflammatory process. TGF-β is a locally generated cytokine and is likely to affect multiple pathways in the healing infarct, serving to suppress inflammatory signals, but also to induce fibrous tissue deposition in the infarct and to stabilize the extracellular matrix. Thus, TGF-β signaling may be crucial for resolution of inflammation preventing injury (Lefer et al., 1990) (Lefer et al., 1993), but may also promote fibrous tissue deposition in the remodeling myocardium increasing dysfunction. Understanding the respective signaling pathways involved in each of its distinct actions will be critical in order to design therapeutic interventions that target specific TGF-β mediated effects. TGF-β expression is increased in the ischemic as well as hypertrophied hearts (Deten et al., 2001) (Kuwahara et al., 2002). TGF-β has an important function for healing after MI as it stimulates fibroblast proliferation and extracellular matrix production (Rosenkranz, 2004). Anti-TGF-β treatment in the first days after coronary artery ligation increases mortality and worsens left ventricular remodeling in mice with MI due to alteration in the extracellular matrix (Frantz et al., 2008).

The Renin Angiotensin System

Discovery of the renin angiotensin system (RAS) started when a link between renal disease and left ventricular hypertrophy was observed in 1836 by Richard Bright. This link was also reported by two other research groups in 1868 and 1872 respectively (Basso and Terragno, 2001). Tigerstedt and Bergman in 1898 found a pressor compound in rabbit renal extracts that they named renin (Phillips and Schmidt-Ott, 1999). They also reported the role of renin in association between renal disease and

cardiac hypertrophy. After the discovery of renin, two independent research groups simultaneously discovered and described another pressor substance that would later become known as angiotensin (Basso and Terragno, 2001). Many components of the RAS and their functions have been discovered over past years. However, new components and functions of the renin-angiotensin system are still being uncovered. Detailed examination of the RAS is given later in the text.

The RAS system presents both systemically in the circulating system, locally in the tissues exerting autocrine and paracrine functions, and intracellularly exerting intracrine functions. The systemic RAS is seen as a regulator of systemic volume and electrolyte balance, and of blood pressure homeostasis. Local tissue RAS have effects involving proliferation, growth, protein synthesis and organ functions, e.g. in kidney, heart, brain, reproductive organs and pancreas (Paul et al., 2006) (Leung, 2007). The role of intracellular RAS is presently unclear, though the existence of complete and functional intracellular RAS has been reported in several tissues (De Mello, 2004) (Re and Cook, 2006). Intracellular RAS is reported to mediate changes in Ca^{2+} fluxes and activation of genes (Haller et al., 1996). Intracellular upregulation of cardiac Ang II has been attributed to cardiac hypertrophy *in vivo* in mice (Baker et al., 2004).

In the systemic RAS, angiotensinogen, which is largely produced in the liver, is converted to the decapeptide angiotensin I (AngI) by the proteolytic enzyme renin that is produced mainly in the kidney. Angiotensin I is then cleaved by a second proteolytic enzyme, mainly produced in the lungs, angiotensin converting enzyme (ACE), to generate the physiologically-active hormone angiotensin II (AngII). AngII elicits most of biological actions of the RAS by binding to either the angiotensin II type 1 receptor

(AT1R) or the angiotensin II type 2 receptor (AT2R). In general, AngII binding to the Angiotensin type I receptors (AT1Rs) is associated with the development of cardiovascular pathophysiologies, while binding to the Angiotensin type II receptor (AT2Rs) is thought to counteract the AT1R and elicit cardioprotective effects. AngII has approximately the same binding affinity to these receptors (Carey and Siragy, 2003). AT1Rs, the best-elucidated receptor, account for the majority of hemodynamic effects such as vasoconstriction, aldosterone secretion, sodium retention and non-haemodynamic effects, like cardiac and vascular cell proliferation (Carey et al., 2000). Angiotensin converting enzyme 2 (ACE2) generates Ang 1–7 from Ang II. Ang 1–7 may also be generated from Ang I or Ang II by other peptidases. Ang (1–7) was found to have actions opposing those of Ang II, namely vasodilation and antitrophic effects and amplification of vasodilation caused by bradykinin. A more detailed examination of Ang-(1-7) and AT2R is discussed later in the text.

Figure 1-1 summarizes the presently recognized components of the RAS.

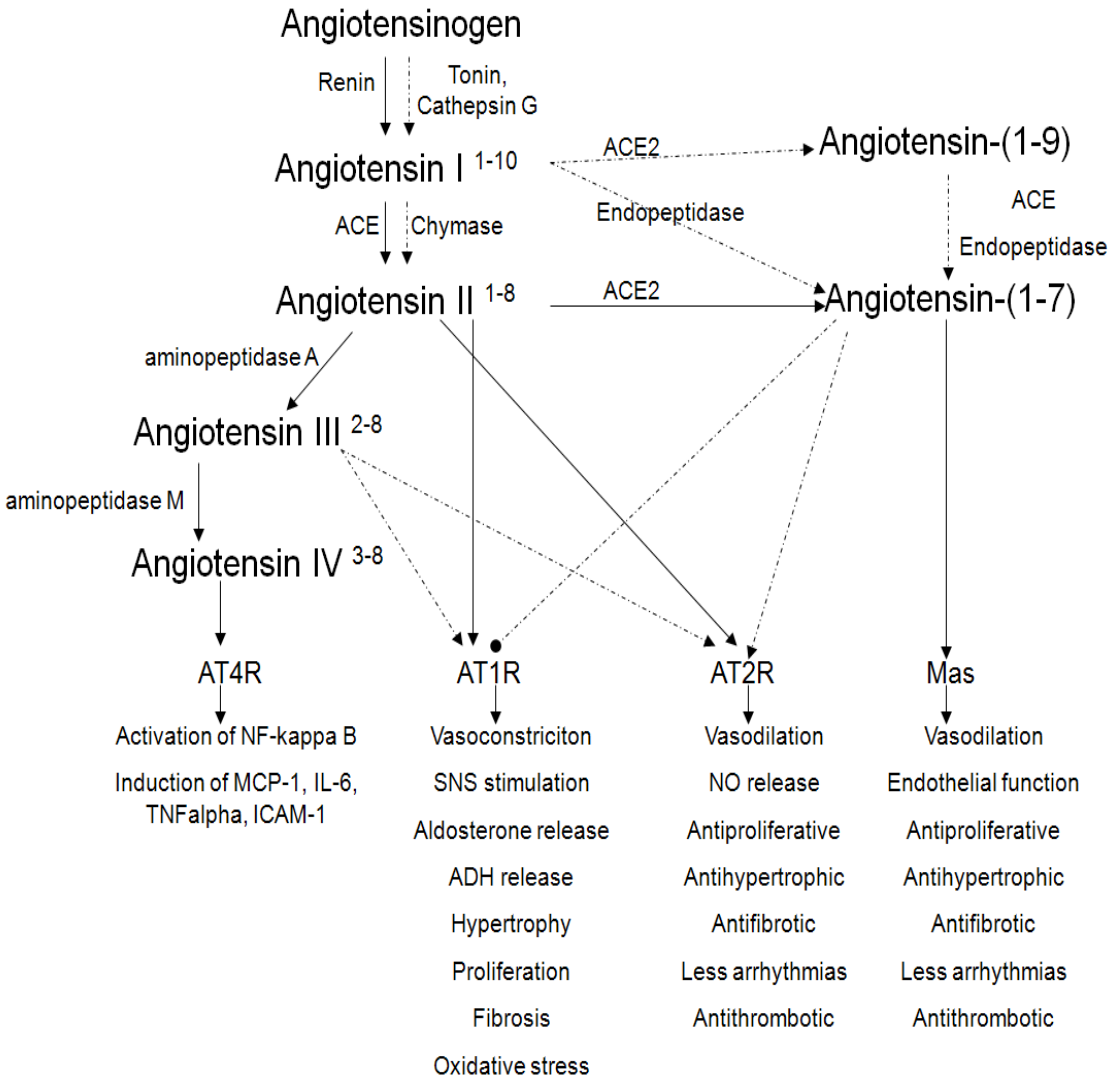

Figure 1-1 The Renin Angiotensin System and its physiological effects mediated by corresponding peptides and receptors. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Mas, Ang-(1-7) receptor; AT2R, angiotensin type 2 receptor; AT1R, angiotensin type 1 receptor; AT4R, Ang IV receptor; ADH, MCP-1, antidiuretichormone; monocyte chemotactic protein-1; ICAM-1, Inter-Cellular Adhesion Molecule 1; SNS, sympathetic nervous system; NO, nitric oxide.

Taking into account all the new components discovered in the RAS, the ACE-AngII-AT1R axis mainly regulates vasoconstriction, salt and water reabsorption, oxidative stress, fibrosis, proliferation, and hypertrophic and proliferative effects (Ferrario et al., 1997) (Ferreira et al., 2007); while AT2R binding counteracts actions

mediated by AT1R. The ACE2-Ang-(1-7)-Mas axis also counter-regulates the ACE-AngII-AT1R axis (Ferrario et al., 1997) (Ferreira et al., 2007). A proper balance between regulating and counter-regulating factors of the RAS appears quite important in maintaining normal physiological functions of many organs.

Ang-(1-7)

Ang-(1–7) was first discovered more than 30 years ago (Semple et al., 1976a) (Semple et al., 1976b) (Semple and Morton, 1976). Ang-(1-7) was thought for a long time to be devoid of biological functions despite early reports on its biological effects (le Tran and Forster, 1997). The importance of Ang-(1–7) was emphasized by the relatively recent discovery of a 'new' ACE2 and since then physiological functions of Ang-(1–7) has been widely investigated. Ang (1–7) was found to have actions counteract those of Ang II, namely vasodilation, antitrophic and antifibrotic effects, and amplification of vasodilation caused by bradykinin (Schiavone et al., 1988) (Santos et al., 2000) (Schmaier, 2003). Thus, Ang-(1-7) is an excellent target for experimental and pharmacological research for cardiovascular diseases.

Formation of Ang (1–7)

Ang-(1-7) can be formed from several routes as indicated in Figure 1-1. Ang II is hydrolyzed by ACE2 to form Ang-(1-7) (Tipnis et al., 2000) (Donoghue et al., 2000) (Vickers et al., 2002a). This route is not very favorable because the affinity of Ang II for its receptors is much higher than that for ACE2 (Reudelhuber, 2006). The route of formation from AngII to Ang-(1-7) by the enzymatic action of ACE2 is responsible for most of the angiotensin-(1–7) formed from angiotensin II (Trask et al., 2007). Other

enzymes, including prolylendopeptidase and prolylcarboxypeptidase can also mediate this route to form Ang-(1-7) from AngII (Tan et al., 1993).

Another route to obtain Ang-(1-7) is from hydroysis AngI and requires both ACE and ACE2. Ang I is first hydrolyzed by ACE2 to form Ang-(1-9), which might function as an endogenous ACE inhibitior in the heart (Donoghue et al., 2000). Then Ang-(1-9) is converted to Ang-(1-7) by ACE or endopeptidase (Rice et al., 2004) . This route of generating Ang-(1-7) was shown to be activated in failing human heart and in macrophages from chronic heart failure patients (Zisman et al., 2003b).

In addition, a direct conversion of Ang I to Ang-(1–7) was shown to be mediated by several enzymes such as: prolyl-endopeptidase in vascular endothelial cells, neutral endopeptidase (neprilysin) in the circulation or kidney, and thimet oligopeptidase in vascular smooth muscle cell (Santos et al., 1992) (Yamamoto et al., 1992).

Receptors for Ang-(1-7)

It was first shown that Ang-(1-7) effects are mediated through AT1R and AT2R (Jaiswal et al., 1992) (Jaiswal et al., 1993) (Muthalif et al., 1998) (Rowe et al., 1995) (Tallant et al., 1997). Ang-(1-7) binds to the AT1R (Gironacci et al., 1999) and leads to the downregulation of a subtype of AT1R (Clark et al., 2001). In addition, Ang-(1-7) seems to be involved in stimulation of the AT2R as Ang-(1-7) mediates prostaglandin release from vascular smooth muscle cells and endothelial cells (Jaiswal et al., 1992) (Muthalif et al., 1998). This pathway is probably involved in the non-competitive antagonism of AngII-induced vasoconstriction (Roks et al., 2004) and inhibition of proliferation of vascular smooth muscle cells (Brogelli et al., 2002).

When a specific antagonist of Ang-(1-7) [d-Ala^7-angiotensin-(1–7) (A779)] was identified, other receptors for Ang-(1-7), other than the AT1R and AT2R, were

specultated to exist (Silva et al., 2007). The existence of a receptor for Ang-(1-7) that is different from AT1R and AT2R was first demonstrated by using A779 (Santos et al., 1994). A779 blockage induces the following effects: upregulation of AT1R (Neves et al., 2000), inhibition of angiogenesis (Cardini et al., 1988), interaction with insulin signal transduction (Giani et al., 2007), and the antithrombotic effect of Ang-(1-7) (Fraga-Silva et al., 2008) (Kucharewicz et al., 2002). The Mas receptor was recently identified as a receptor mediating angiotensin-(1–7) effects and its activation is sensitive to A779 (Santos et al., 2003). It was also confirmed that AVE 0991 is an agonist of the Mas receptor as it can mimic some of the effects of angiotensin-(1–7) (Wiemer et al., 2002) (Pinheiro et al., 2004). The *in vivo* role of the Mas receptor has been studied in Mas receptor knockout mice. It has been shown that this receptor is crucial for heart function, as Mas receptor knockout mice exhibit a severely impaired heart function, with lower systolic tension and increased dimensions of the left ventricle (Santos et al., 2006), and a slower post-ischemic cardiac recovery (Santos et al., 2006) (Castro et al., 2006). This underscores the importance of Mas receptors in cardiovascular physiology.

Role of Ang-(1-7) in cardiovascular system

Counteraction of Ang-(1–7) to AngII initiates from the moment of Ang-(1–7) generation. As described above, Ang-(1–7) was generated through routes that either bypass the formation of Ang II or utilize AngII as a main substrate to generate Ang-(1–7). Both routes will result in reduction of AngII in the circulation system and/or tissue level. This might partially explains the beneficial effects of Ang-(1–7) in cardiovascular system.

Established effects of angiotensin-(1–7) are regulation of blood pressure, cardiac functions, and smooth muscle and myocardial cell growth. Several studies

demonstrated the antihypertensive action of Ang-(1-7) (Dobruch et al., 2003) (Schiavone et al., 1988). This vasorelaxative effects was endothelium-dependent (Santos et al., 2003). Cardioprotective effects mediated by Ang-(1-7) are demonstrated by several studies. Ang-(1-7) reduces the incidence and the duration of reperfusion arrhythmias (Ferreira et al., 2001) (De Mello, 2004). Ang-(1-7) has been shown to preserve cardiac function, coronary perfusion, and aortic endothelial function in a rat model for heart failure (Loot et al., 2002). Ang-(1-7) also effectively prevented the cardiac myocardial and perivascular fibrosis in the DOCA–salt hypertensive rat model (Grobe et al., 2006).

Angiotensin Type 2 Receptor

Despite recent advances in understanding AT2R-mediated actions, the functions of AT2Rs in cardiovascular diseases still remains elusive. Generally, AT2Rs have been described as having opposite effects to AT1Rs. For example, AT2Rs inhibit cell growth and has vasodilating action (Carey et al., 2000) (Carey and Siragy, 2003). A better understanding of AT2R-mediated effects could elucidate both AT2R functions in the RAS and their therapeutic implications for cardiac diseases.

Distribution of AT2Rs

Although the identification date of AT2Rs can be retrieved back to 1989, their functions are less studied because they are only ubiquitously expressed at high level in the fetus (Grady et al., 1991). After birth, their expression declines to low levels in the healthy adult. Moreover, their expression pattern in normal adults has tissue-specific properties. The expression is limited in certain cell types and certain tissues such as vascular endothelial cells, the adrenal, selected renal, selected cardiac structures, ovaries and certain brain areas (de Gasparo et al., 2000). However, AT2Rs are

upregulated under pathophysiological conditions such as myocardial infarction and mechanical injury (Gallinat et al., 1998) (Nio et al., 1995). Even though the expression level decreases to low level, AT2Rs can still be detected and measured in selected tissues in the healthy adults, such as in the adrenal, ovary, kidney, heart, and brain (de Gasparo et al., 2000).

Cardiovascular system

AT2Rs are found in many different kind of vessels such as aorta (Chang and Lotti, 1991), mesenteric (Matrougui et al., 1999) (Touyz et al., 1999), and uterine arteries (McMullen et al., 1999). They are abundantly expressed in both vascular endothelium and the muscular layers of blood vessels (Utsunomiya et al., 2005). In the heart, AT2Rs are present in ventricular and atrial myocardium, and coronary arteries (Wang et al., 1998). For the myocytes and fibroblast in the adult-rat heart, AT2Rs have less expression level than AT1R, as AT1Rs account for 35% of the total AngII binding sites and less than 5% of the binding sites for AT2R (Villarreal et al., 1993). AT2Rs are upregulated about 153% in the heart of the cardiomyopathic hamster (Ohkubo et al., 1997). In the human, AT2Rs are increased about 3 fold in dilated-cardiomyopathy patients (Tsutsumi et al., 1998). In addition, AT2Rs are the dominant receptors in the human end-stage failing heart. They account for 69% of the total AngII binding sites, while AT1Rs only comprise about 33 % of the binding sites (Regitz-Zagrosek et al., 1995). Although both of these receptors are downregulated at this heart failure stage, the extent of downregulation for AT1Rs is more severe than AT2Rs. The selective and reversible expression of AT2Rs in cardiac-vascular system indicates that AT2Rs are important regulators in physiological conditions as well as in pathological conditions.

Kidney

Since the renin (a major component in the RAS) is produced in the kidney, the expression of AT2Rs in this organ will be discussed here too. AT2Rs are distributed through tubular and vascular segments of the renal cortex and medulla (Ozono et al., 1997). They may participate in regulating glomerular blood flow as they are mainly found in interlobular arteries and afferent arterioles (Carey et al., 2000) . Moreover, AT2Rs, found in the juxtaglomerular cells in the afferent arterioles, play a role in regulating the RAS activity. AT2Rs in juxtaglomerular cells inhibit renin biosynthesis and secretion (Ichihara et al., 2003) (Ishiyama et al., 2004a) (Siragy et al., 2005). This inhibition effects may be mediated by cGMP, which is a known inhibitor for renin secretion and key second messenger in one of the three AT2R-mediated signaling pathways.

In vivo studies have complicated conditions caused by cellular, circulating, and paracrine factors to hinder the actual AT2R effects. The in vitro studies could better demonstrate the AT2R effects as they exclude these influence factors. Primary cells and cell lines, expressing AT2Rs, are used to investigate signaling mechanism and functional significance of the AT2Rs. The primary cells include neuronal cells from neonatal hypothalamus, neonatal rat cardiomyocytes and adult rat cardiac microvascular endothelial cells, and rat fetal fibroblasts (Steckelings et al., 2005) . Researchers also use cell lines to study AT2Rs, including PC12W (Steckelings et al., 2005), (Speth and Kim, 1990), R3T3 (Dudley et al., 1991), NG108-15 (Buisson et al., 1992), and Neuro-2a (Hoffmann and Cool, 2003). The PC12W cell line expresses only

AT2Rs and is widely used to study AT2Rs signaling pathways, because it unveils the AT2Rs-mediated actions by abolishing AT1Rs-mediated actions.

AT2Rs Signaling and functions

AT2Rs act as a modulator of complex biological programs involved in embryonic development, cell differentiation, apoptosis, regulation of renal function and blood pressure. They are also play a role in pathophysiological processes such as cardiovascular remodeling after myocardial infarction, hypertension, heart failure and stroke. Though intracellular-signaling pathways of AT2Rs have been extensively studied, confusions about their actions still exist. The confusions are due to many unconventional signaling cascades involved in AT2R signaling pathways and some AT2R–signaling mechanisms cannot be clearly attributed to AT2Rs functions. Yet, it is generally recognized that AT2R-signaling pathways are completely different from AT1R-coupled signaling pathways. To date, at least three pathways and other non-traditional signaling transudation mechanisms are involved in AT2Rs signaling.

Activation of Phosphatase

Growth factors promote growth and proliferation of cells by tyrosine-kinase receptors and several kinase-driven phosphorylation steps in signaling cascades. Among these signaling cascades, extracellular regulated kinase (ERK)1/2 plays an important role in these phosphorylation cascades. AT2R-mediated actions could activate phosphatases and then dephosphorylate ERK. This may explain why AT2Rs stimulation has anti-growth and anti-proliferation effects. AT2Rs play a pivotal role in directly inhibiting the protein kinase pathways activated by AT1R or growth factors (Horiuchi et al., 1999). Activation of various protein phosphatases by stimulating AT2R works to prevent undesired growth of normal tissues.

Up to now, there are three phosphatases have been identified: mitogen-activated protein kinase phosphastase (MKP-1), SH2 domain-containing phosphatase 1 (SHP-1), and protein phosphotase 2A (PP2A). MKP-1 is vanadate-sensitive, dual-specificity tyrosine/threonine phosphatase (Tsuzuki et al., 1996) (Yamada et al., 1996). SHP-1 is vanadate-sensitive tyrosine phosphatase (Bedecs et al., 1997) and PP2A okadaic acid-sensitive serine/threonine phosphatase (Huang et al., 1996).

Activation of Kinin /NO/cGMP

It has been confirmed that AT2Rs have vasodilatory actions (Brede et al., 2003). The AT2R-mediated vasodilation is related to bradykinin (BK), nitric oxide (NO) and cGMP. The indirect evidence is that the AT2R antagonist (PD123319) induced vasoconstriction is duplicated by the BK-B2 receptor antagonist and NO synthase inhibitor (Brede et al., 2003). Whereas, the direct evidence demonstrates that stimulation of AT2Rs in vascular smooth muscle cells increases BK production by activating kininogenase in transgenic mice (Kurisu et al., 2003). Researchers also found that the AT2Rs possess constitutive activity, because overexpressing AT2Rs in vascular smooth muscle cells activates the vascular kinin system and causes vasodilation in transgenic mice (Tsutsumi et al., 1999). AT2R stimulation may also lead to changes in Na+/H+ exchange activity, causing acidification of the intracellular environment, then stimulating kininogenase. The consequent increase in bradykinin (BK) synthesis and nitric oxide (NO) accumulation leads to enhance cyclic guanosine monophosphate (cGMP) production (Tsutsumi et al., 1999). The kinin/NO/cGMP signaling pathway is also suggested in attenuating perivascular fibrosis (Brede et al., 2003).

Phospolipase A2

In phospolipase A2 pathway, AT2R-mediated activation is coupled to kinase instead of phosphatase-activation. AT2R stimulation activates membrane-associated phospholipase A2 (PLA2) in renal proximal tubule epithelial cells (Dulin et al., 1998) (Alexander et al., 2004). Activated PLA2 induces the release of arachidonic acid (AA). Then PLA2-dependent release of AA regulates, with/without its cytochrome P450-dependent metabolites, phosphorylation of MAPK and additional kinases activation further downstream the RAS-signaling pathway (Dulin et al., 1998).

Ceramides and caspase

Ceramides are intracellular lipid second messengers, which have been implicated as an important mediator of programmed cell death. AT2R stimulation leads to the generation of ceramides (Gallinat et al., 1999). As a pro-apoptotic signal, ceramides can induce apoptosis in many cell types. This pathway is further confirmed in that blockading sphingolipid synthesis abolishes AT2R mediated programmed cell death (Dimmeler et al., 1997). Since AT2R-stimulation mediated apoptosis is associated with the activation of caspase-3, ceramides may induce apoptosis via activating caspase 3, a central downstream effector of the caspase cascade (Lehtonen et al., 1999).

Promyelocytic Zinc finger protein

AT2Rs are not only involved in apoptosis and vasodilation, but they also play a pivotal role in development and regeneration. The development and regeneration effects mediated by AT2R are associated with a zinc-finger homoeodomain enhancer binding protein gene (*Zfhep*), which is induced by AT2R-mediated activation in cells of neuronal and vascular origin (Stoll et al., 2002). *Zfhep* is required for differentiation of

the central nervous system and regulates cell fate (Lai et al., 1993). It is highly expressed in the heart and encodes for a transcription factor regulating cellular differentiation and protein synthesis.

Actions of AT2Rs in cardiac-vascular diseases

Since AT2Rs were found, studies have been done to elucidate their roles in the cardiovascular diseases. Conclusive evidence during the late 1990s through 2003 indicates that AT2Rs mediate vasodilation. During that time, AT2Rs were simply thought to antagonize AT1R-mediated actions. With ongoing research on AT2Rs, more effects of AT2Rs are uncovered, such as AT2Rs induction of apoptosis and neuron differentiation. Generally, AT2Rs are considered to have cardiac-protective effects. AT2Rs mediated effects in hypertension and in cardiac remodeling after myocardial infarction are discussed below.

Role in hypertension

The AT2R-mediated vasodilation has been confirmed. From the first evidence in 1992, this effect has been documented by AT2R antagonist blockade of the vasodilation action of AT1R antagonist (Buisson et al., 1992). This effect is also supported by experiments, demonstrating that AT2R-knockout mice have a slight but significant increase in blood pressure and increased constrictor response (Hein et al., 1995) (Ichiki et al., 1995). Pharmacological studies show that the blood pressure decrease observed in the SHR (spontaneously hypertensive rats) by administration of AT1R antagonist is enhanced in combination with the AT2R agonist CGP42112 and abolished by AT2R antagonist PD 123319 (Barber et al., 1999). “Knockdown”of AT2R by using antisense gene transfer technology significantly elevated systolic blood pressure and the pressor response to AngII in normotensive Sprague-Dawley (SD) rats (Wang et al., 2004).

Peripheral overexpression of AT2R also has been reported to potentiate the antihypertensive action of losartan (Li et al., 2007).

AT2R-mediated actions are critical when considering its potential physiological and therapeutic roles in the antihypertensive effects of AT1R antagonists. AT1R blockade markedly increases circulating and tissue levels of AngII around 20–30-fold (Ford et al., 2000). The accumulated AngII can then work as an AT2R agonist leading to a vasodilator response (Ford et al., 2000).

In addition, the expression level of the AT2Rs is relatively low in the adult, but the expression of AT2Rs is upregulated under pathological conditions. Therefore, we may speculate that AT2R-mediated anti-hypertensive effects are more apparent in pathological states (Tsutsumi et al., 1999) (Bautista et al., 2001) (Schuijt et al., 2001). This hypothesis is further confirmed in a study showing that AT2R-mediated vasodilatation only occurred in SHR but not in WKY rats (Barber et al., 1999). The results of this study is consistent with investigation that AT2R-mediated increases in vascular cGMP production occurred in SHRSP (Gohlke et al., 1998), but not in WKY rats (Moores et al., 2003). Moreover, female SHR rats have lower blood pressure (BP) than the male SHR rats and their AT2Rs are expressed at higher level than the AT1Rs (Silva-Antonialli et al., 2004).

In summary, AT2Rs work with AT1Rs in regulating blood vessel tension. Their selective expression in the arteries and upregulated expression under pathological conditions may indicate their therapeutic application in treating hypertension.

Role in cardiac remodeling

Pathological conditions, such as hypertension, cardiomyopathy and myocardial infarction, induce cardiac remodeling, which results in left ventricular hypertrophy (LVH) and fibrosis. These cardiac structural changes compensate hemodynamic performance but ultimately lead to an increased incidence of heart failure. As mentioned above, the expression level of AT2Rs changes after MI. Thus, it is necessary to understand the roles of AT2Rs in this process. Although AT2R-mediated actions are generally thought as counteracting AT1R-mediated effects in hypertension, the patho-physiological relevance of AT2R for cardiac remodeling has not yet been firmly understood.

Most studies are consistent with the hypothesis that AT2R mediates cardiac protective effects post-MI. Overexpression of AT2Rs in the mouse is associated with improved LV function at baseline and heart function during post-MI remodeling (Yang et al., 2002). AT2R knock out mice develop more cardiac remodeling, LV dysfunction and mortality than WT mice (Oishi et al., 2003). Furthermore, studies show that the AT2Rs may interact with AT1R in post-MI mice and AT2R-overexpression in attenuating post-MI remodeling is equally as effective as AT1R blockade treatment (Voros et al., 2006).

Role in cardiac hypertrophy

LVH is an adaptive response for maintaining cardiac output and tissue perfusion when the heart is under high workload. Under high persistent workload, the LVH will finally be associated with interstitial fibrosis and apoptosis and increased heart rate. These changes indicate the heart is in a de-compensating state. At this time, LVH is recognized as a risk factor for cardiovascular mortality (Levy et al., 1990). Otherwise exercise induced hypertrophy is a compensative response to maintain cardiac output.

This point is important to help us to understand the controversial AT2R-mediated effects on hypertrophy.

Since the AT2Rs have anti-growth properties as stated above, AT2Rs are proposed to be anti-hypertrophic in the heart. However, it is controversial how AT2R-mediated effects modulate the hypertrophic condition. In vivo studies, using AT2-null mice have yielded mixed results. Overexpression of AT2Rs have been shown to inhibit LVH, prevent the increase in LV wall thickness, and reduce the heart: body weight ratio (Metcalfe et al., 2004b) (Sugino et al., 2001). However, other studies have demonstrated that AT2Rs have no effects on hypertrophy (Kurisu et al., 2003) (Sugino et al., 2001). Moreover, AT2Rs may have constitutive activities and might be required for hypertrophy. In some in vivo studies, utilizing the AT2R-knockout mice, Senbonmatsu et al (Senbonmatsu et al., 2000) reported that AT2Rs are required for the development of pressure-overload-induced cardiac hypertrophy. Over-expression of AT2Rs in the ventricular myocytes fail to demonstrate an antihypertrophic action (Yan et al., 2003). AT2R blockade by its specific antagonist PD123319 does not prevent the cardiac hypertrophy but decreases right ventricular- and pulmonary-to-body mass ratios in the proartrial natriuretic peptide (ANP) gene-disrupted mouse treated with high dietary salt (Angelis et al., 2006).

The signaling pathways regulating the AT2R-mediated effects on hypertrophy are also controversial. Two signaling pathways have been proposed to explain the AT2R-mediated action in LVH. The most commonly known and studied pathway is kinin/NO pathway, which exerts anti-hypertrophic effects of AT2Rs. As AT2R-stimulation results in increased production of NO, AT2R knock out mice have decreased eNOS and

blockade of NOS abolishes the antihypertrophic effects on cardiomyocytes (Brede et al., 2003). Zfhep signaling pathway may explain the AT2R-mediated hypertrophic effects. Nuclear Zfhep binds to and activates phosphatidylinositol-3 kinase p85α subunit (p85αPI3K) gene. Activation of p85αPI3K gene leads to subsequent activation of protein synthesis (Senbonmatsu et al., 2003). It is not clearly established which signaling pathway is dominant or if these two pathways regulate AT2R-mediated anti-hypertrophic or hypertrophic effects.

The discrepancies about AT2R-mediated effects on hypertrophy can be explained by both contradictory signaling pathways and the inconsistency among the experimental approaches. A lot of researchers use AT2R knockout mice or AT2R transgenic mice to study the effects mediated by AT2Rs. There may be compensatory mechanisms that take place in AT2R knock out or transgenic mice.

Role in fibrosis

During cardiac remodeling, there is an increase in fibrosis. AT2R actions in fibrosis are not as controversial as in cardiac hypertrophy. AT2Rs serve as cardiac-protective receptors, as they reduce fibrosis during cardiac remodeling process. A majority of studies show that AT2Rs have anti-fibrotic actions (Widdop et al., 1992). AT2R stimulation reduces extracellular matrix formation (de Gasparo et al., 2000). Selectively overexpressing AT2R in cardiac myocytes also demonstrates that AT2R-activation reduces perivascular fibrosis and interstitial fibrosis (Kurisu et al., 2003) (Metcalfe et al., 2004b). As mentioned in the AT2R-mediated signaling pathway, pharmacological experiments indicate that AT2R-mediated anti-fibrotic action might be regulated via kinin/NO signaling pathway.

Conclusions

The AT2Rs are now clearly established as vasodilator receptor via the kinin/NO/cGMP signaling pathway. There is increasing certainty to believe that AT2Rs are cardioprotective under pathological conditions. Functional studies prove that AT2Rs provide cardiac-protective effects through inhibiting detrimental cardiac remodeling after MI and reducing mortality. However, AT2R-mediated actions in cardiac hypertrophy have been controversial for a long time. There may be some underlying unknown signaling pathways existing, which are mediated by AT2Rs and other receptors that also may participate in the cardiac remodeling process. Although the cardiac remodeling mechanisms mediated by AT2Rs are not completely understood, it is clear that AT2R mediated actions can have obvious therapeutic implications. AT1R antagonists are more effective in hypertension patients, since the AT1R antagonists cause unbound AngII bind to the upregulated AT2Rs, which may enhance or amplify the favorable effects of AT1R antagonist. More experiments need to be done in order to elucidate AT2R functions and find out whether the activation of these receptors can be an effective treatment for human cardiac-vascular diseases.

CHAPTER 2
OVEREXPRESSION OF ANG-(1-7) IMPROVES CARDIAC FUNCTION AND ATTENUATES LEFT VENTRICULAR REMODELING

Abstract

OBJECTIVES: We assessed the hypothesis that the overexpression of the Angiotensin-(1-7) [Ang-(1-7)] may protect the myocardium following ischemia injury in the rat myocardial infarction model.

BACKGROUND: Myocardial ischemia causes significant myocardial damage, including myocyte death, fibrosis, and local wall thinning, leading to impaired ventricular function and cardiac failure. The renin-angiotensin system (RAS) plays a critical role in modulating heart functions following a myocardial infarction. Ang-(1-7), a component of the RAS has been shown to provide cardiac protective effects in various models of hypertension. Furthermore, it has been suggested that Ang-(1-7) may play a part in the beneficial effects attributed to angiotensin-converting enzyme inhibitors (ACEi) and Angiotensin type 1 receptor blocker (ARB) in cardiovascular disease.

METHODS: Over-expression of Ang-(1-7) mediated by lenti-viral vector was used to investigate the role of Ang-(1-7) in cardiac function and remodeling after myocardial infarction. A single bolus of $3.0x10^8$ transfection units of lenti-Ang-(1-7) was injected into the left ventricle chamber of the heart in 5-day-old male Sprague-Dawley rats. Six weeks after viral administration, either a left anterior descending coronary artery ligation (CAL) or sham surgery was performed. Four weeks after the surgery, echocardiography and hemodynamic parameters were measured to assess the cardiac function. Heart tissues were collected to histological assessment and subsequent mRNA determination. Lenti-viral transduction efficiency was determined by measure the viral vector expression in the rat heart using PCR after reverse transcriptin of RNA and SYBR

Green Realtime PCR. A parrellel i*n vitro* hypoxia experiment was also carried out to test the effects of Ang-(1-7) on rat neonatal cardiac myocytes (RNCM) exposed to 1 h hypoxia. Myocytes were isolated from 5-day-old Sprague-Dawley rat heart ventricles.

RESULTS: Lenti-viral vector significantly increased transgene expression in the rat heart tissues. Four weeks after the myocardial infarction a significant decrease in ejection fraction, deterioration of left ventricular systolic pressure, reduction in dp/dt, and an increase in left ventricular end diastolic pressure (LVEDP) and ventricular hypertrophy was oberved. Overexpression of Ang-(1-7) attenuated these impairments to a non-significant level, markedly illustrated by a significant reduction in LVEDP and ventricular hypertrophy. Overexpression of Ang-(1-7) prevented myocardial wall thinning and tended to decrease the infarction area. Furthermore, myocardial infarction caused an up-regulation of ACE mRNA expression but tended to downregulate of ACE2 mRNA expression. Overexpression of Ang-(1-7) prevented these changes and also up-regulated Bradykinin B2 receptor, a speculated cardiac protective factor. In the *in vitro* study, Ang-(1-7) protected RNCM from hypoxia induced cell death and mediated up-regulation of speculated protective factors (ACE2, BKR2 and IL-10) and downregulation of inflammatory cytokines (TNF-α and IL-6).

CONCLUSIONS: Cardiac overexpression of Ang-(1-7) exerts protective influence on the heart function post myocardial infarction injury by persevering cardiac function, and attenuating cardiac remodeling post-MI. The effects of Ang-(1-7) may be mediated through restoring the ACE/ ACE2 balance and upregulating protective factors (BKR2 and IL10).

Introduction

It has well been established that components of the RAS plays a detrimental role in progression of heart failure. Pharmacotherapeutic interventions in patients as well as in experimental animal models provide support for this concept. Clinical treatment of cardiovascular diseases with either angiotensin-converting enzyme inhibitors (ACEi) (Anonymous 1993) or Angiotensin type 1 receptor blockers (ARB) (Thurmann et al., 1998) provide significant protection for patients from myocardial infarction and heart failure. Animal studies have also demonstrated that ACEi and ARB prevent cardiovascular injury (Jalil et al., 1991) (Stier et al., 1991). The ACEi not only reduces the formation and actions of Ang II, but also causes a significant elevation of angiotensin-(1-7) (Ang-(1-7)) levels (Keidar et al., 2007) .

Ang-(1-7) has been implicated in protecting cardiac functions and preventing pathological cardiac remodeling (Santos et al., 2004). Several investigators have demonstrated various cardioprotective actions of Ang-(1-7). For instance, Ang-(1-7) reduces the incidence and the duration of postischemic reperfusion arrhythmias in isolated rat heart (Ferreira et al., 2001) (De Mello, 2004). Ang-(1-7) has also been shown to improve contractile function in rat hearts after intravenous infusion of Ang-(1-7) (Sampaio et al., 2003). Chronic infusion of Ang-(1-7) has been shown to preserve cardiac function, and improves coronary perfusion and endothelial function in a rat model for heart failure (Loot et al., 2002). AVE0991, a nonpeptide agonist for Mas receptor, has been shown to significantly improve cardiac function in isolated perfused rat hearts (Ferreira et al., 2007). Using an Ang-(1-7) transgenic rat model that overproduces Ang-(1-7), Ang-(1-7) reduced cardiac hypertrophy induced by isoproterenol (Santos et al., 2004). Ang-(1-7) significantly attenuated myocyte

hypertrophy and interstitial fibrosis induced by Ang II (Grobe et al., 2007b). In the DOCA–salt hypertensive rat model, chronic administration of Ang-(1-7) effectively prevented the myocardial and perivascular fibrosis (Grobe et al., 2006). Tallant et al. (Tallant et al., 2005) presented evidence for Mas-mediated antihypertrophic effect of Ang-(1-7) in rat cardiomyocytes.

We and others (Ferrario et al., 1997) (Ferreira et al., 2007) have proposed that there are two axes of the RAS, the ACE-AngII-AT1R axis and ACE2-Ang-(1-7)-Mas axis with opposing actions. The ACE2-Ang-(1-7)-Mas axis has been shown to be involved in vasodilatory, anti-hypertrophic and antiproliferative effects, in contrast to the vasoconstrictive, hypertrophic effects mediated by the ACE-AngII-ATIR axis. Preservation of cardiac function requires maintenance of this balance between these two axes.

In the present study we used a gene therapy approach in an attempt to overexpress cardiac Ang-(1-7) to evaluate any cardioprotective effects of this peptide in a rodent model of myocardial infarction. The present study was also designed to to evaluate potential cardioprotective mechanisms modulated by Ang-(1-7). We hypothesized that Ang-(1-7) may restore the balance between the ACE-AngII-AT1R axis and the ACE2-Ang-(1-7)-Mas axis in the RAS, and possibly have anti-inflammatory actions to thereby produce cardioprotection in response to myocardial infarction injury.

Material and Methods

Characterization of Ang-(1-7) in Lentiviral Vector

Lenti-viral vectors were created as previously described (Coleman et al., 2003) . Lenti-viral vectors containing Ang-(1-7) was driven by elongation factor promoter (EF1α-Ang-(1-7), Lenti-Ang-(1-7)). The Lenti-Ang-(1-7) vectors were tittered using the HIV-1

p24 antigen ELISA assay (Beckman Coulter) kit following the manufacturer's instructions.

Lenti-Ang-(1-7) Administration

All of the animal protocols were approved by the institutional animal care and use committee (IACUC) and conducted according to National Institutes of Health guidelines. Five-day–old male Sprague-Dawley rats received a single intraventricular injection of $3.5x10^8$ transfection units of lenti-Ang-(1-7) in 30 µL 1x PBS, as described previously (Metcalfe et al., 2004a) . This method of gene transfer by our lentiviral vector provides a close to 100% animal survival rate and has been established to produce efficient and long-term transduction of the heart. After viral administration, animals were returned to their mothers until weaning.

Myocardial Infarction

At 6 weeks of age, rats were separated into 4 experimental groups (control sham, MI, Ang-(1-7), and MI+Ang-(1-7); N=4-7 per group) and subjected to either coronary artery ligation surgery or mock surgery. Myocardial infarction was induced by ligation of the left anterior descending coronary artery. At the time of operation, rats were anesthetized with isoflurane (2.0–2.5% in oxygen), after which rats were intubated with an 18-gauge intravenous catheter and mechanically ventilated with this isoflurane-oxygen mixture using a Harvard ventilator (model 683, Harvard Apparatus, Holliston, Mass). After the chest was cleaned and shaved, rats were underwent a left-lateral thoracotomy. The thorax was entered via the left fourth intercostal space and the pericardium incised to expose the heart. The heart was exposed, and ligated at the proximal left anterior descending coronary artery 2–3 mm from its origin between the pulmonary artery conus and the left atrium with a 7–0 polypropylene suture. Successful

cessation of blood flow was indicated by elevation of ST segment on electrocardiogram and cyanosis of anterior LV wall; if necessary the procedure was repeated by placement of a second or third ligature. The heart was returned to its normal position, and the thorax evacuated of fluid and air and sutured closed. All of the animals received buprenorphine hydrochloride (Buprenex, 0.02 mg/kg q12 IM, Reckitt and Colman Pharmaceuticals) and were closely monitored for signs of discomfort. Sham operated rats underwent the identical surgical procedure as described above except that the suture was not tightened around the coronary artery. In the present study, the operation-related mortality was approximately 25% 24 h after operation.

Echocardiography

Cardiac function was evaluated using a Hewlett Packard Sonos Model 5500 with a 12-Hz transducer at 4 weeks after coronary artery ligation surgery. Rats were anesthetized with isofluorane for echocardiographic examinations. Images were obtained from the parasternal short axis. All measurements were based on the average of three consecutive cardiac cycles. Measurements obtained by echocardiography include left ventricular end diastolic diameter (LVED), left ventricular end systolic diameter (LVES), and left ventricular posterior wall thickness (LVPW). The ejection fraction was calculated as follows: (LVEDv-LVESv)/LVEDv x 1/100.

Hemodynamic Measurements

Rats were anesthetized with ketamine/xylazine/ acepromazine rodent cocktail (30 mg/6 mg/1mg/kg, i.m.). The rats were placed in supine position and the body temperature was maintained at 37^{o}C by a heated pad throughout the experiment. The left ventricular function was measured using a 22 Gauge needle filled with heparin saline (20 IU/ml) inserted into the left ventricular chamber. The data was recorded after

stabilization of the tracing using a liquid pressure transducer, which was interfaced to a PowerLab (ADInstruments, Colorado Springs, CO, USA) signal transduction unit. Data were analyzed by using the Chart program that was supplied with the PowerLab system.The parameters measured include a peak systolic pressure of left ventricle (LVSP), a maximal positive and negative rate of rise in left ventricular pressure (dP/dt_{max} and dP/dt_{min}), heart rate (HR), and left ventricular end diastolic pressure(LVEDP).

Histological Analysis

Following the hemodynamic measurement, the hearts were harvested. The ventricles were separated from atria and rinsed in PBS. The ventricles were weighed and cut into 3 thick sections made perpendicular to the long axis. The basal and apex section was snap frozen in liquid nitrogen and stored at –80°C for subsequent Quantitative Real-time PCR measurements. The middle section was used to measure infarct size and cardiac remodeling parameters. Cardiac remodeling was determined by ventricular hypertrophy and cardiac fibrosis. Ventricular hypertrophy was determined by measuring wet weights of rat heart ventricles normalized to body mass. Cross sections of the ventricles were then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm. Ventricular sections were stained with H & E (hematoxylin and eosin) to determine myocyte diameter or with Picro-Sirius Red to measure interstitial fibrosis. Myocyte diameter was determined at 40 x magnification, using the ImageJ program from National Institutes of Health as previously described (Grobe et al., 2006). Quantification of cardiomyocyte diameters was carried out by an individual who was blinded to the treatments. 20 separate images from different (nonoverlapping) regions of the left ventricle free wall only were examined. Left ventricular (LV) wall thickness was

also measured by the ImageJ program and represented as a percentage of control LV wall thickness.

Rat Neonatal Cardiac Myocytes: Isolation and Culture

Rat Neonatal Cardiac Myocytes (RNCM) and were isolated from the ventricles of 5-day-old Sprague-Dawley rats according to the method adapted from Zhang et al (Zhang et al., 2001) . Briefly, rat ventricles were dissociated by mechanical disaggregating and enzymatic digestion with 1% collagenase II (Worthington Biochem. Corp., Freehold, New Jersey). Next, cells were pre-plated in the presence of 5% fetal bovine serum (FBS) in order to separate RNCM from non-cardiomyocytes. After 1 hour of pre-plating, the suspended cells, comprising mostly of RNCM, were removed from the attached non-cardiomyocytes, counted by hemocytometer, diluted to 2×10^6 viable cells/ml in culture medium with 10%FBS and plated in gelatin-coated culture plates. RNCM were grown in DMEM (Dulbecco's modified Eagle's medium)/F-12 supplemented with 1% penicillin/streptomycin, 10 mM HEPES ,10 µg/ml insulin, 10 µg/ml transferrin and 10% (v/v) FBS (fetal bovine serum). Cells were plated in gelatin [0.1% v/v]-coated culture dishes containing the same media/sera, and were grown in a 5% CO2/95% air humidified incubator 100 µM bromodeoxyuridine was also added to the media in order to inhibit fibroblast growth. Spontaneous beating was observed in over 95% of cells after 2 days in culture, indicating that the isolated cells were indeed myocytes. This was further confirmed by positive staining of these cells for immunoreactive alpha-sarcomeric actin antibody (Figure 3-1).

Hypoxia/re-oxygenation model and LDH viability assay

Culture medium to grow RNCM was changed every 24 hours. 48 h later after culturing, Ang-(1-7) was added into the medium to reach 10µm final concentration. Ang-

(1-7) was added 1 h before exposing cells to a 95% N_2/5% CO_2 mixture (or normoxic control). Following hypoxic exposure to 1 h, cells were returned to normoxic conditions. 24 h later, culture media was collected to detect lactate dehydrogenase (LDH) level and cells were collected to detect mRNA levels for different genes. LDH level was analyzed according to the manufacturer's specifications (Roche Applied Science, Indianapolis,USA). The assay is based on the measurement of LDH released into the culture media when the integrity of the cell membrane is lost.

RNA Isolation and PCR

Tissues or cells were homogenized and total RNA was isolated using RNAqueous 4 polymerase chain reaction (PCR) kit (Ambion, Foster City, CA, USA) according to the manufacturer's instructions. 200ng RNA was reverse transcribed with iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA).

Transducing efficacy of Lenti-Ang-(1-7) in rat myocardium was examined by testing the expression of lenti-viral vector. PCR and SYBR green realtime RT PCR was used to quantify the lenti-viral vector expression. For lenti-Ang-(1-7), the forward primer (5'-CATCACCCATCGAGAAACC-3') was located in IgG fragment and the reverse primer (5'-GGACCAAGCCTGGCCATGTCC-3') was located in the human prorenin fragment of the transgene. GAPDH primers were: forward primer: (5′-GCCAGCCTCGTCTCATAGACA-3′), reverse primer: (3′- GTCCGATACGGCCAAATCC -5′). A 450-bp fragment was amplified by PCR using 59°C as annealing temperature and 30 cycles. The SYBR green real-time PCR assays for each target gene were performed on cDNA samples.

The AT1R, AT2R, Collagen I, Collagen III, TGF-beta, TNF-alpha, ACE2, ACE, IL-1, IL-10, and IL-6 were analyzed by quantitative real-time PCR using Taqman probe

(Applied Biosystems). Real-time PCR was run using ABI Prism 7000 sequence detection system. All cDNA samples were assayed in triplicate. Data were normalized to GAPDH mRNA level.

Statistical Analysis

Results are expressed as mean±SE. Data were analyzed by one way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. Values of $P<0.05$ were considered statistically significant. All of the data were analyzed using GraphPad Prism 5 software (Graphpad Prism Institute Inc).

Results

Lenti-viral Vector Mediated Overexpression of Ang-(1-7) in Rat Hearts

Figure 2-1a summarized the experimental protocol. The Lenti-Ang-(1-7) was injected into the left ventricular chamber of 5-day-old SD rats. 6 weeks after gene delivery, the chest was opened and a suture was placed around the left anterior descending coronary artery (CAL). Myocardial infarction was confirmed by the ST-segment elevation as determined by electrocardiogram (ECG), and a representing tracing is shown in Figure 2-1b. Lenti-viral gene expression was determined by PCR and Realtime RT PCR for all groups. Lenti-viral vector mRNA level was significantly increased for Lenti-Ang-(1-7) and MI+Lenti-Ang-(1-7) groups, and undetectable for control and MI, as shown in figure 2-2.

Effects of Ang-(1-7) Overexpression on Cardiac Function Post-MI

Echocardiographic analyses performed at 4 weeks post myocardial infarction surgery. Myocardial infarction caused a significant reduction in Ejection Fraction in the MI group relative to control, Lenti-Ang-(1-7). Ang-(1-7) overexpression was able to attenuate the decrease in ejection fraction induced by myocardial infarction, as shown in

Figure 2-3. Left ventricular systolic pressure, maximum dp/dt, and minimum dp/dt were significantly decreased in MI group, relative to control and Lenti-Ang-(1-7) (Table 2-1). Left ventricular end diastolic pressure was significantly increased in the MI group compared to control and Lenti-Ang-(1-7). Neonatal Ang-(1-7) treatment was able to attenuate these changes (Table 2-1). Heart rate was not significantly different among groups, although there was a trend for an increase in the untreated MI group (Table 2-1).

Effects of Ang-(1-7) Overexpression on Ventricular Remodeling Post-MI

Lenti-viral vector mediated Ang-(1-7) overexpression attenuated MI induced cardiac hypertrophy as evaluated by both the ventricular weight to body weight ratio and measurement of cardiomyocyte diameter, as shown in Figure 2-4. Body weights were not significantly different among the groups (Figure 2-4c). Wall thinning and fibrosis were observed in MI group. Ang-(1-7) overexpression prevented the wall thinning and tended to decrease infarction area, as shown in Figure 2-5.

Ang-(1-7)-mediated protection post-MI is associated with restoration balance between ACE-AngII-AT1R axis and ACE2-Ang-(1-7)-Mas axis.

To determine possible cardio-protective mechanism mediated by Ang-(1-7) overexpression, the left ventricular tissue was harvested to measure the genes involving in the RAS and cardiac remodeling. As showing in Table 2-2, ACE level was significantly increased in MI group compared to control and Lenti-Ang-(1-7) groups. The overexpression of Ang-(1-7) prevented this increase in the MI+ Lenti-Ang-(1-7) group. In contrast, ACE2 and Mas receptor levels tended to decrease in MI group compared to control and Lenti-Ang-(1-7) treated animals; however, overexpression Ang-(1-7) in the MI group led to an increase in cardiac ACE2 and Mas receptor levels after MI.

Overexpression of Ang-(1-7) also increased bradykinin receptor B2 (BKR) in MI+Lenti-Ang-(1-7) group. Ang-(1-7) overexpression tended to reduce the increase in the expression of AT1R, TGF-β, Collagen I, and Collagen III expression mediated by MI. IL-1, IL-6, IL-10, and AT2R mRNA level were very low and undetectable (data not shown in the Table).

Ang-(1-7) Increases the Viability of RNCM after Hypoxia Exposure.

Lactase dehydrogenase (LDH) level in the culture medium, a marker for cell death, is increased when cell membrane is damaged. When cells undergo necrosis or apoptosis, the cell membrane is damaged and leaks. Thereby, LDH is released from intracellular compartment to extracellular media. LDH level in the media is used as an indicator for cell viability. LDH level in the media is negatively correlated with the cell viability. Rat neonatal cardiac myocyte (RNCM) cells were exposed to hypoxia for 1 h and then cultured under normoxia conditions.24 h after the hypoxia exposure, LDH level in the RNCM culture medium was significantly increased following hypoxia exposure and Ang-(1-7) treatment was able to completely prevent the LDH release, indicating Ang-(1-7) provides some protection to RNCM cells from hypoxia induced cell death (Figure 2-6). Twenty-four h after the hypoxic exposure, gene expression was analyzed in all cell cultures. Ang-(1-7) treatment mediated significant increase in AT2R, ACE2, IL-10 expression levels (Table 2-3). Inflammatory cytokines (TNF-α and IL-6) were significantly increased in the hypoxic RNCM. Ang-(1-7) prevented hypoxia-induced increase.

Discussion

In the present study, the effects of Ang-(1-7) overexpression mediated by lentiviral vector on cardiac function and ventricular remodeling were examined in a rat coronary artery ligation model. We found that overexpression of Ang-(1-7) provides significant protection against left ventricular dysfunction caused by myocardial infarction, as shown by preserving ejection fraction, and preventing the dysfunctional changes in dp/dt and left ventricular end diastolic pressure. Moreover, Ang-(1-7) significantly attenuated ventricular hypertrophy and tended to prevent wall thinning and ventricular fibrosis.

Left ventricular remodeling characterized by hypertrophy and fibrosis are risk factors for heart failure following myocardial infarction. The cardioprotective effects (antihypertrophy and antifibrosis) of Ang-(1-7) are consistent with previous findings by us and others. Ang-(1-7) transgenic animals showed less ventricular hypertrophy and fibrosis in the AngII hypertensive rat model (Mercure et al., 2008). Ang-(1-7) also prevents ventricular hypertrophy induced isoproterenol in Ang-(1-7) transgenic animals (Santos et al., 2004). Ang-(1-7) administered via osmotic minipumps also was effective in preventing the myocardial and perivascular fibrosis in the DOCA–salt hypertensive rat model (Grobe et al., 2006).

Ang-(1-7) has been speculated to mediate part of the cardioprotective effects attributed to ACEi and ARB, since Ang-(1-7) levels are elevated with these two treatments (Ferrario et al., 2005). The Ang-(1-7) elevation in the plasma can be explained by an increase in ACE2 levels after ACEi and/or ARB treatment and ACE 2 exhibits a high catalytic efficiency for the generation of Ang-(1–7) from Ang II (Vickers et al., 2002b). ACEi is reported to increase cardiac ACE2 mRNA level (Ferrario et al.,

2005). ARB treatment has been shown to result in a significant upregulation of ACE2 mRNA level in the viable myocardium in the myocardial infarction model (Ishiyama et al., 2004b). ARB treatment also has been reported to increase cardaic ACE2 mRNA level and cardiac ACE2 activity; and a combination of ARB and ACEi exerted similar effects (Ferrario et al., 2005). Previous studies from our lab have shown that overexpression of ACE2 renders protective effects against ischemia-induced left ventricular dysfunction (Der Sarkissian et al., 2008). Results from the current study elucidate possible mechanisms for the cardioprotective effects of Ang-(1-7). After myocardial infarction, ACE expression level was significantly upregulated in the heart and AT1R tended to upregulate, but ACE2 and Mas expression was downregulated. All these changes following myocardial infarction indicate the disrupted balance between ACE-AngII-AT1R axis and ACE2-Ang-(1-7)-Mas axis for the RAS in the heart. The activity of the ACE-AngII-AT1R axis is increased and the activity of the ACE2-Ang-(1-7)-Mas axis is decreased. Ang-(1-7) expression restores the balance by decreasing the ACE-AngII-AT1R axis by preventing the upregulation of AT1R induced by myocardial infarction, and by activating ACE2-Ang-(1-7)-Mas axis through upregulating ACE2 and Mas receptor.

Myocardial infarction causes local hypoxia in the infarct zone. Cardiac myocytes die during the hypoxic phase of a myocardial infarction. The *in vitro* hypoxia and re-oxygenation mimics this hypoxic phase of myocardial infarction. The *in vitro* hypoxia study may provide some evidence to support a cardioprotective role of Ang-(1-7) for the cardiac myocytes. In the *in vitro* experiment, Ang-(1-7) treatment protected cardiac myocytes from hypoxia induced cell death. The protective effects of Ang-(1-7) treatment

was associated with an upregulation of ACE2, AT2R, IL-10 (anti-inflammatory cytokine), and down-regulation of inflammatory cytokines (IL-6 and TNF-α) in the RNCM. The cardioprotective effects of Ang-(1-7) may be explained by the alteration of these mediators. The ACE2 upregulation is consistent with our *in vivo* findings. AT2R upregulation provides supports for the cardioprotective role for AT2R and possible interaction among ACE2, Ang-(1-7), and AT2R. Zisman et al (Zisman et al., 2003a) has shown a direct correlation between Ang-(1-7) forming activity and AT2R density in the hearts from primary pulmonary hypertensive patients. Evidence for cardioprotective role of AT2R from the current study is consistent with others too. Direct AT2R stimulation improves post-myocardial infarction systolic and diastolic function (Kaschina et al., 2008), and AT2R have been resported to functionally interact with Ang-(1-7) through its Mas receptor (Castro et al., 2005).

Proinflammatory cytokines (e.g., tumor necrosis factor [TNF]-α and IL-6), anti-inflammatory cytokine (e.g. IL-10), and cytokines having pro- and anti- inflammatory activities (e.g. transforming growth factor-β1 [TGF- β1]) play a critical role in mediating homeostasis within the heart in response to cardiac injury. IL-6 and TNF-α are bio-marker for heart failure. IL-6 plays an important role in the pathophysiology of congestive heart failure patients, as IL-6 has been shown to increase in the plasma of these patients (Tsutamoto et al., 1998). TNF-α RNA and protein is also elevated in patients and in animal models with advanced heart failure (Testa et al., 1996) (Torre-Amione et al., 1996). ACEi significantly attenuated MI-induced increase in the expression of cardiac cytokines (TNF-α and TGF-β1) (Blais et al., 2002). Anti-inflammatory therapy is also being investigated for the treatment of myocardial

infarction. A recent report has shown that treatment with rhIL-10 significantly improved LV function in rats with heart failure after experimental MI (Stumpf et al., 2008). Consistent with these reports, IL-6 and TNF-α were increased in the myocytes exposed to hypoxia in this study. Ang-(1-7) treatment was observed to elevate IL-10 levels and to prevent the increase in IL-6 and TNF-α in hypoxic RNCM. However, observations summarized from *in vitro* study were not exactly the same for the *in vivo* study. For the *in vivo* study, IL-6 and TNF-α were not different and IL-10 and AT2R expression in the hearts were undetectable. However, for the *in vitro* study, Ang-(1-7) upregulated IL-10 and AT2R and prevented the increase in IL-6 and TNF-α in the RNCM exposed to hypoxia. The inconsistency between *in vivo* and *in vitro* results could be explained as total RNA was isolated from several cell types in the heart (including cardiomyocytes, cardio-fibroblasts, and endothelial cells). It is possible that the changes observed in cardiac myocytes were masked by the contributions from the non-cardiomyocyte cells.

The half life for Ang-(1-7) is very short and dependent on species. In rodents, Ang-(1–7) has a very short half-life (approximately 20 s) following intravenously administration (Iusuf et al., 2008). In humans, Ang-(1-7) half life varies by the route of administration. Following intravenously administration, the half life for Ang-(1–7) was approximately 30 min in humans (Kono et al., 1986). Upon repeated subcutaneous administration, the half-life was 29 min (Rodgers et al., 2006). Considering the short half life for Ang-(1-7), it is quite difficult to maintain a therapeutic level for Ang-(1-7) when Ang-(1-7) peptide is administrated into patients or rodents by intravenous infusion or subcutaneous injection. Without achieving certain level of Ang-(1-7) in the plasma or tissues, Ang-(1-7) may not exert its cardioprotective effects. Therefore a gene therapy

approach, like the one described here, could be used to mediate long-term Ang-(1-7) production after single administration of viral vector containing Ang-(1-7). Viral vector can elicit efficient transduction and long-term expression. Therefore, daily regimens can be eliminated. Moreover, viral vectors can be directed to specific target tissues, which could reduce unwanted side effects. We have recently demonstrated that use of an rAAV9 serotype is not only cardiospecific but target preferentially the myocytes (Qi et al., 2009). Utilization with such a specific viral vector to target the heart may even enhance the effects observed in the present study. However in the current study, we cannot specify if there is any cell specificity with the lenti-viral vector.

In summary, our data demonstrate that overexpression of Ang-(1-7) preserves cardiac function and attenuates cardiac remodeling post myocardial infarction. These beneficial effects involve restoration of the RAS balance and upregulation of anti-inflammatory cytokine. Taken together, all these studies indicate that targeting of the ACE2-Ang-(1-7)-Mas axis could hold novel therapeutic strategy in the treatment of myocardial infarction and its associated complications.

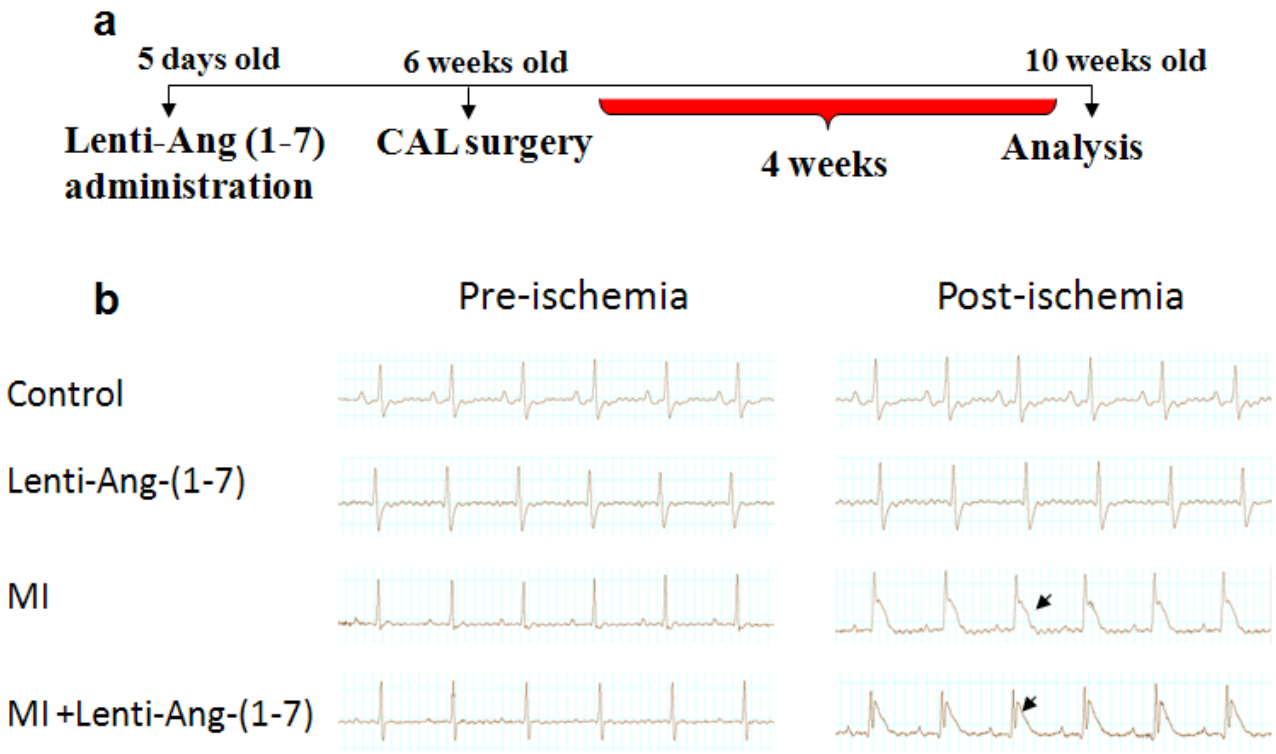

Figure 2-1. Schematic representation of study protocol. (a) Illustration of the experimental protocol for myocardial infarction. Lenti-(Ang1-7) was administered into left ventricular chamber of 5-day-age SD pups. Coronary artery ligation (CAL) was performed when SD rats were 6 weeks old. 4 weeks after the surgery, animals were subjected to echocardiography followed by hemodynamic heart function assessment and tissues harvest ;N=4-7 per group. (b) Representative electrocardiogram (ECG) at basal showing normal tracing and showing an elevation in ST-segment following CAL, as indicated by arrow. n=4-7/ group

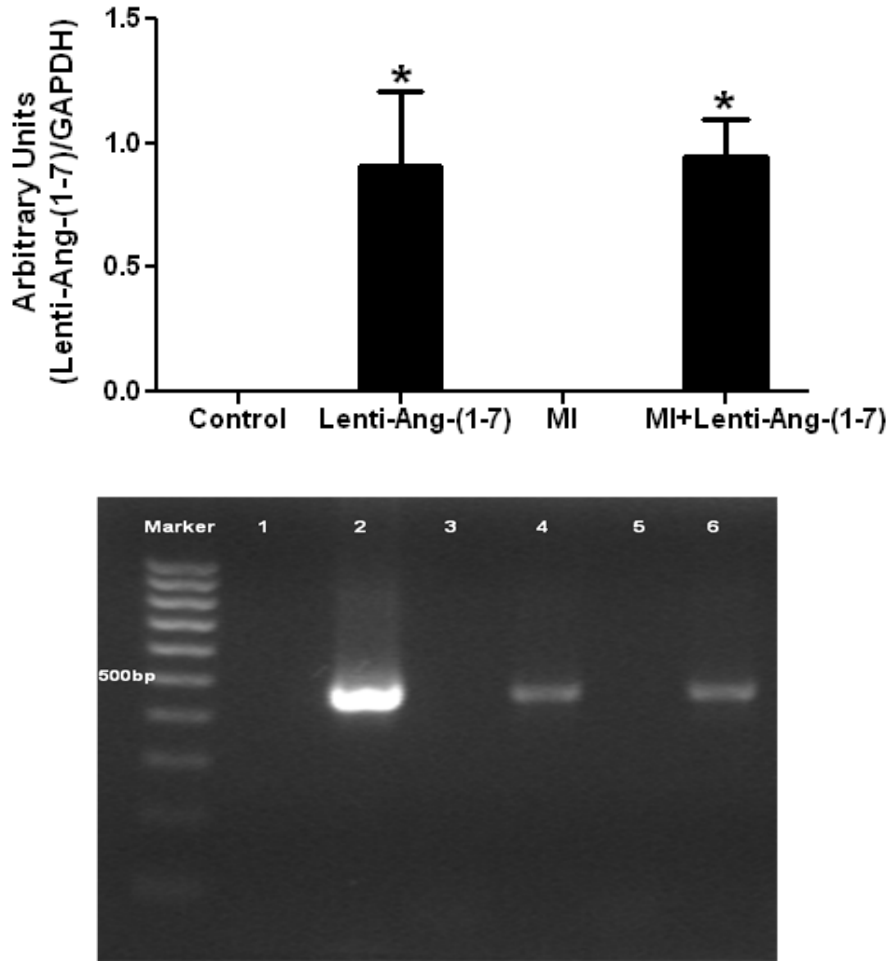

Figure 2-2. Transduction efficiency of Lenti-Ang-(1-7) in rat hearts (N=4-7 per group). Graph shows semi-quantitative Real-Time RT-PCR to detect the expression Lenti-Ang-(1-7) viral vector. Lenti-viral vector expression in the heart was also detected by agarose gel electrophoresis. Lane1: negative control, lane 2: positive control, lane 3: control sham, lane 4: lenti-Ang-(1-7), lane 5: MI, lane 6: MI+lenti-Ang-(1-7). * P<0.001 Lenti-Ang-(1-7) and MI+Lenti-Ang-(1-7) vs control and MI; n=4-7/ group.

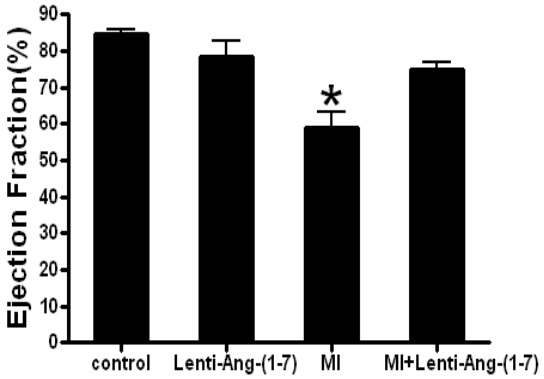

Figure 2-3. Effect of Ang-(1-7) gene transfer and myocardial infarction on ventricular function (N=4-7 per group) (A) Echocardiogrphic analyses of rat myocardium 4 weeks after the CAL demonstrates significant improvement in ejection fraction in Lenti-Ang(1-7) treated animals as compared to MI group.(* MI vs. all other groups, $p<0.05$, n=4-7/ group)

Table 2-1. Hemodynamic data 4 weeks after CAL surgery

	Control	Lenti-Ang-(1-7)	MI	MI+Lenti-Ang-(1-7)
LVSP	108.6 ± 4.2	91.2 ± 2.9	83.6 ± 6.9*	110.7 ± 4.6
HR	260.8 ± 15.3	247 ± 14.8	336.1 ± 29.6	262.9 ± 11.3
dp/dt max	8417.1 ± 337.4	7188.1 ± 1329.0	4479.4 ± 1419.3*	7154.5 ± 1369.2
dp/dt min	5166.2 ± 453.9	4768.0 ± 1260.0	3115.6 ± 441.9*	4690.5 ± 816.8
LVMP	35.6 ± 2.4	36.9 ± 4.6	45.2 ± 2.1	43.6 ± 0.6
LVEDP	4.0 ± 1.3	0.8 ± 1.3	12.5 ± 2.7*	4.9 ± 0.7

HR(heart rate), beats per minute; LVSP (left ventricular systolic pressure) , millimeters of mercury; Max dp/dt and Min dp/dt (maximal and minimal peak rate of left ventricular pressure), millimeters of mercury per second; LVMP(left ventricle mean pressure), millimeters of mercury; LVEDP(left ventricle end-diastolic pressure), millimeters of mercury. (* MI vs all other groups; N=4-7 per group).

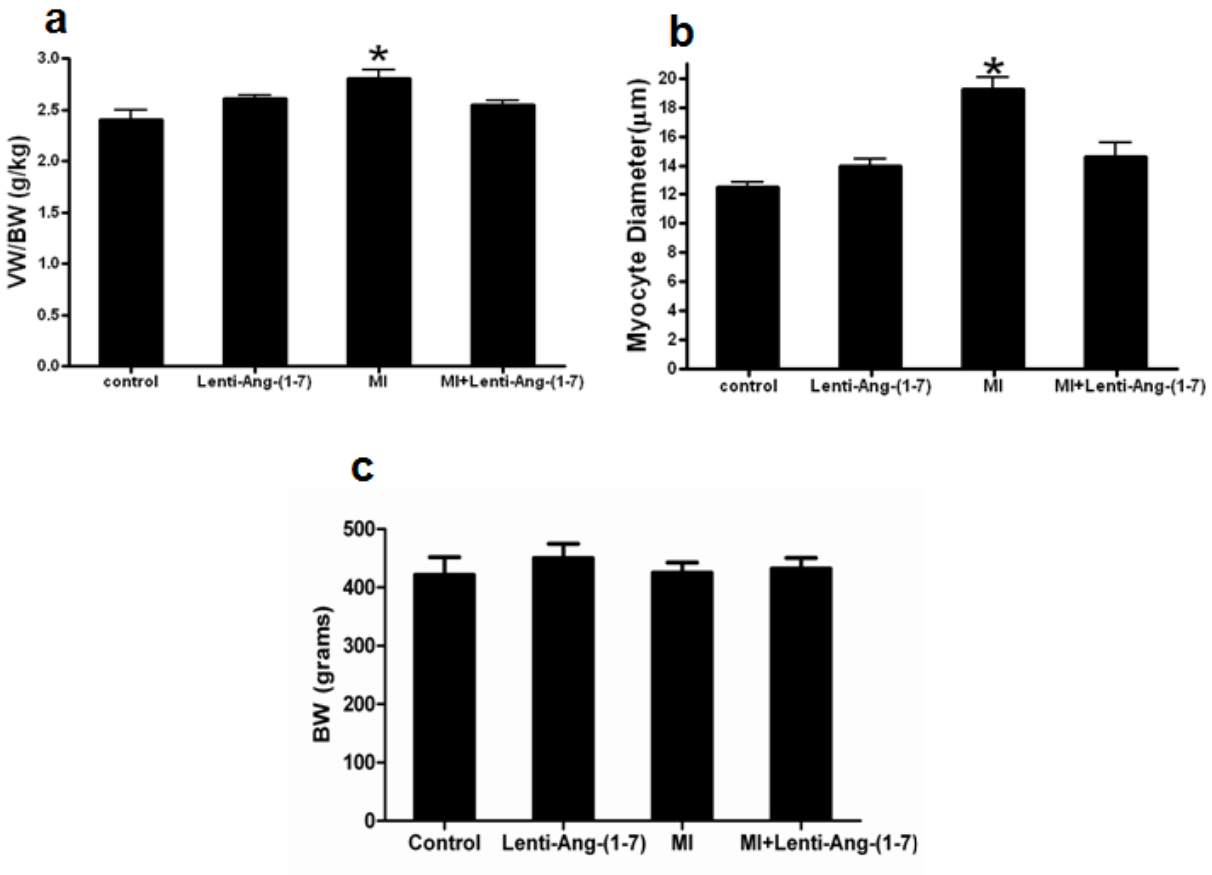

Figure 2-4. Effect of Ang-(1-7) gene transfer on ventricular hypertrophy (N=4-7 per group). Lentiviral delivery of Ang-(1-7) attenuates MI induced cardiac hypertrophy as evaluated by the ventricular weight(g) to body weight(Kg) ratio (a) , measurement of myocyte diameter in the LV free wall peri-infarct area (b). Body weight was not significantly different among groups(c). n=4-7/ group

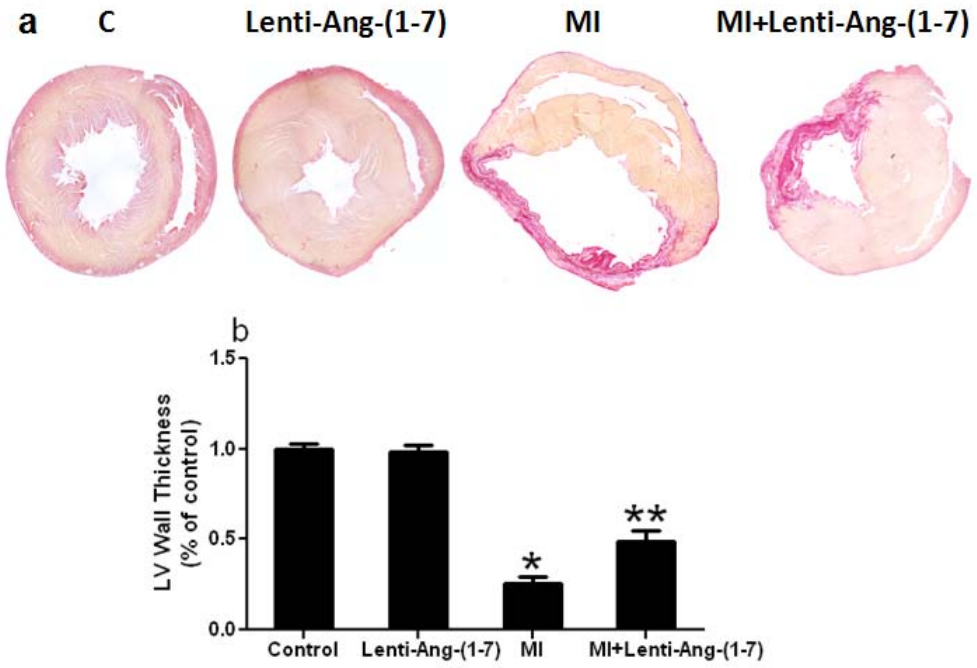

Figure 2-5. Effects of Ang-(1-7) gene transfer on ventricular fibrosis and LV wall thickness (N=4-7 per group). (a) Picro-Sirius Red staining shows significant anterior wall thinning and collagen deposition at 4 weeks after CAL surgery in MI animals. Ang-(1-7) overexpression tended to attenuate fibrosis induced by MI. (b) Quantification of the LV wall thickness as a percentage of control left ventricular wall thickness. MI significantly induced a reduction in LV wall thickness and Ang-(1-7) attenuated the LV wall thinning. (* MI vs control, Lenti-Ang-(1-7), and MI+Lenti-Ang-(1-7); ** MI+Lenti-Ang-(1-7) vs control, Lenti-Ang-(1-7)); n=4-7/ group.

Table 2-2. Quantitative Real Time PCR data

	Control (N=7)	Lenti-Ang-(1-7) (N=4)	MI (N=6)	MI+ Lenti-Ang-(1-7) (N=6)
AT1R	1.00 ± 0.33	0.73 ± 0.10	1.6 ± 0.21	0.81 ±0.10
TGF-β	0.59 ± 0.21	0.57 ± 0.24	0.98 ± 0.01	0.83 ± 0.04
BKR	0.33 ± 0.08	0.33 ± 0.07	0.28 ± 0.02	0.74 ± 0.11*
ACE2	0.76 ± 0.03	0.56 ± 0.05	0.43 ± 0.21	1.17 ± 0.22 #
ACE	1.64 ± 0.29	0.98 ± 0.07	3.48 ± 0.62&	1.62 ± 0.34
MAS	0.99 ± 0.40	1.06 ± 0.12	0.29 ± 0.04	0.85 ± 0.31
TNF-α	0.64 ± 0.09	0.82 ± 0.06	0.67 ± 0.09	1.00 ± 0.08
COLI	0.27 ± 0.08	0.39 ± 0.17	0.95 ± 0.22**	0.53 ± 0.24
COLIII	0.45 ± 0.08	0.88 ± 0.06	1.13 ± 0.17**	0.71 ± 0.07

P<0.05, # MI+lenti-Ang-(1-7) vs Lenti-Ang-(1-7) and MI , * MI+lenti-Ang-(1-7) vs all other groups, & MI vs all other groups; ** MI vs control. Data are depicted as mRNA fold changes relative to GAPDH mRNA calculated using the expression $2^{\wedge -\Delta\Delta Ct}$ and expressed as a mean fold change ±SEM.

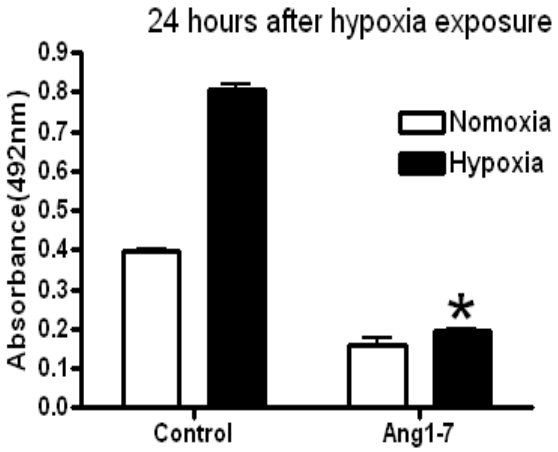

Figure 2-6. Ang-(1-7) effects in cell viability assay. 10µm Ang-(1-7) was added 1hour before hypoxia study. Cell viability was tested by LDH kit. Bar graphs are means ± S.E.M., N = 6. * $P<0.05$, Ang-(1-7) treated hypoxia cells vs corresponding control; n=4-7/ group.

Table 2-3. Quantitative Real Time PCR data for *in vitro* experiment

	Normoxia	Hypoxia	Nomorxia+Ang1-7	Hypoxia+Ang1-7
AT1R	1.19 ± 0.26	1.41 ± 0.32	0.83 ± 0.18	1.04 ± 0.20
AT2R	0.89 ± 0.10	1.17 ± 0.07	0.97 ± 0.24	1.42 ± 0.18#
ACE	0.97± 0.05	1.00± 0.04	0.96 ± 0.08	0.99 ± 0.05
ACE2	0.42 ± 0.13	0.29 ± 0.10	0.28 ± 0.16	0.95 ± 0.20*
MAS	0.91 ± 0.05	1.23 ± 0.04	1.36 ± 0.24	1.12 ± 0.04
BKR	0.76 ± 0.13	0.92 ± 0.14	0.52 ± 0.10	0.51 ± 0.12
TGF-β	1.34 ± 0.27	1.21 ± 0.14	1.16 ± 0.27	1.24 ± 0.24
COLI	0.99 ± 0.03	1.16 ± 0.08	1.28 ± 0.13	1.12 ± 0.03
COLIII	0.96 ±0.05	0.91 ± 0.02	0.67 ± 0.09	0.88 ± 0.05
IL-10	0.03 ± 0.004	0.01 ± 0.001	1.05 ± 0.14**	1.30 ± 0.23**
TNF-α	0.44 ± 0.08	1.67 ± 0.26 &	0.18 ± 0.05	0.40 ± 0.05
IL-6	0.97 ± 0.04	1.35 ± 0.04 &	0.75 ± 0.12	0.88 ± 0.05

P<0.05, # Hypoxia+Ang-(1-7) vs Normoxia and Normoxia+Ang-(1-7), * Hypoxia+Ang-(1-7) vs all other groups; ** Nomorxia+Ang-(1-7) and Hypoxia+Ang-(1-7) vs corresponding controls; & hypoxia vs all other groups; IL-1 level was undetectable and not shown in this table. N = 6. Data are depicted as mRNA fold changes relative to GAPDH mRNA calculated using the expression $2^{\wedge -\Delta\Delta Ct}$ and expressed as a mean fold change ±SEM.

CHAPTER 3
SELECTIVE TROPISM OF THE RECOMBINANT ADENO-ASSOCIATED VIRUS 9 SEROTYPE FOR RAT CARDIAC TISSUE

Note: This chapter has already been published in the Journal of Gene Medicine: Qi YF, Liu X, Li HW, Shenoy V, Li QH, William W, Sumners C and Katovich MJ. Selective tropism of the recombinant adeno-associated virus 9 serotype for rat cardiac tissue. Journal of Gene Medicine. [Epub ahead of print], 2009.

Abstract

Cardiac gene transfer may serve as a novel therapeutic approach for heart disease. Numerous serotypes of rAAV have been identified with variable tropisms to cardiac tissue. Both *in vitro* and *in vivo* experiments were undertaken to compare cardiac tropisms of rAAV-2, 5, 7, 8, and 9. For the *in vitro* studies, 10^7 vector genome (vg) of rAAV-2, 5, 7, 8, or 9 were used to transduce both rat neonatal cardiac myocytes (RNCM) and fibroblasts (RNCF). For the *in vivo* studies, $4x10^{10}$ vg of rAAV-2, 5, 7, 8, or 9, and $4x10^{11}$ vg of rAAV8 or 9 were administered in 5-day-old rats via a relatively non-invasive intracardiac injection. 1 and 2 months post administration, GFP expression in tissues was visualized and GFP mRNA was quantified by Real-Time PCR. At 3 days post viral transduction, rAAV9 and rAAV2 produced the highest transducing efficiency in RNCM. Only AAV2 elicited any transduction in the RNCF. The *in vivo* results indicated that the order for transduction efficiency in the heart was: rAAV9>rAAV8>rAAV7>rAAV2=rAAV5. The transduction efficiency order in the liver was: rAAV2>rAAV5>rAAV7>rAAV8>AAV9. Injection of a higher dose ($4x10^{11}$ vg) of rAAV9 provided more widespread and highly cardiac-selective GFP expression in the heart than rAAV8. Zero to minimal expression of GFP was found in the lung and kidney for both doses of all rAAV serotypes utilized. Collectively, these results suggest that rAAV9 provide the most selective and stable transduction efficiency in cardiac tissue, and this expression was primarily exhibited in the cardiac myocytes.

Introduction

Cardiac gene therapy is considered as a promising therapeutic tool for the treatment of cardiac diseases (Yla-Herttuala and Alitalo, 2003) (Markkanen et al., 2005). However, development of vectors that produce efficient and optimal gene transfer in the heart has not yet met expectations, as several clinical trials related to cardiac gene therapy have failed. Most failures have been attributed to the lack of clinical and physiological efficacy, which could be due to the low efficiency of myocardial gene transfer and/or limited duration of transgene expression (Bekeredjian and Shohet, 2004) (Muller et al., 2007). Thus, in order to facilitate long-term gene transduction of the heart in both animal studies and clinical trials, selection of vector type and route of vector delivery are crucial factors influencing the success of cardiac gene therapy.

Recombinant adeno-associated virus (rAAV), derived from members of *Parvoviridae* family of non-pathogenic viruses, are emerging as one of the most promising vector systems for cardiac gene transfer. rAAV has exhibited highly efficient transduction and capacity with long-term gene expression , and is associated with low immunogenicity (Wright et al., 2001) (Vandendriessche et al., 2007), and lack of apparent cytotoxicity in tissues such as skeletal muscle (Arruda et al., 2005), liver (Snyder et al., 1997), heart (Woo et al., 2005a) (Chu et al., 2003) (Vassalli et al., 2003) and arteries (Vassalli et al., 2003).

At least 12 different rAAV serotypes have been identified, including rAAV1 through rAAV12 (Romano, 2005). Different rAAV serotypes have variable transduction efficiency and differential tropism to various tissues in the body. The available data concerning transduction efficiency of rAAV serotypes in cardiac tissue are divergent and difficult to interpret, due to differences in viral vector doses, promoters composed within the viral

vectors, animal species, and/or route of administration. Among the currently used rAAV serotypes, rAAV2 is the best-characterized and best-documented AAV serotype in research studies and clinical trials. rAAV2 has been reported to elicit high levels of myocardial transduction when compared to rAAV1, 3, 4 and 5, after intramyocardially injection into the left ventricular wall in adult Balb/C mice (Du et al., 2004). Similar results were observed with intravenous administration via the tail vein in adult mice (Muller et al., 2006); or with intracoronary perfusion in adult Sprague–Dawley rats (Muller et al., 2006). However, these observations are not universally consistent. When AAV5 was compared to rAAV2, the rAAV5 demonstrated dramatically enhanced transduction efficiency *in vitro* in differentiated myocytes (>500 fold) (Duan et al., 2001). A more recent study revealed that rAAV8, along with rAAV1 and 6, showed preferable tropism for transducing adult rat myocardium, compared to rAAV2, 3, 4, 5, and 7, when AAV1 though 8 were intramyocardially administered into the left ventricular apex of adult Sprague-Dawley rats (Palomeque et al., 2007). Subsequent studies demonstrated that both rAAV7 and rAAV9 exerted rapid-onset and high transgene expression in cardiac tissue, when intravenously injected via tail vein in adult mice (Zincarelli et al., 2008). When compared to rAAV8, rAAV9 provided widespread myocardium transduction in adult mice, following administration of the virus by the same intravenous route (Vandendriessche et al., 2007). When rAAV1, 6, 7, 8 and 9 were compared together, rAAV9 provided global cardiac gene transfer both in the mouse after intrapericardial injection into neonatal mice, and high transduction in the rat heart after direct intramyocardial injection into adult myocardium (Bish et al., 2008). Therefore,rAAV2,rAAV5,rAAV7,rAAV8 and rAAV9 can all serve as a possible vector

candidate for targeting the rat heart and should be comprehensively compared, side by side.

The route of rAAV vector administration may substantially determine transduction efficiency and viral tropisms *in vivo*, since rAAV vectors can non-specifically infect a variety of tissues. The ideal route of administration of viral vectors to animals should have two important characteristics: providing transduction exclusively localized to the target organ and being easy to apply *in vivo*. An intramyocardial injection directly into the myocardium can mediate localized transgene expression with much less possibility of transducing other vital organs such as the liver, lung, and kidney. However, this method required thoracotomy surgery and transduction inside the myocardium would also be very limited to the area surrounding the injection sites. An intravenous injection is another commonly applied option to administer viral vectors, but it requires larger viral vector dose and transduction is not limited to the target organ. Intracoronary artery perfusion is another choice to deliver a transgene to the heart, but it is also a difficult technique. Considering, the pros and cons of possible administration routes, injecting rAAV vector directly into left ventricle cavity of rat neonatal pups could be the most applicable and least technically difficult method for delivering a transgene into myocardium. This is a novel and not extensively used method in comparing AAVs tropism studies. The hearts of neonatal rats are nearly visible under their transparent skin and the injection into the left ventricular cavity would only require a single injection and small volume. Rat pups recover within minutes after the injection and, with experience; nearly 100% of them survive the process (Falcon et al., 2004). The viral

dose used in neonatal rat pups would also be considerably lower than that used in adult rats.

In the present study, we performed a comprehensive side-by-side in vitro and in vivo analysis using rAAV2, rAAV5, rAAV7, rAAV8 and rAAV9 to characterize their tissue tropism, especially cardiac transduction, in the rat. For the in vitro studies neonatal cardiac myocytes and fibroblasts were utilized. For the in vivo experiments, rAAV serotypes 2,5,7,8 and 9 were injected into the left cardiac ventricular cavity of 5-day-old SD neonatal rats, in order to maximize the transgene expression in the rat heart. Transgene expression in the tissues was examined at one-month and two-month post-viral injection both by observing GFP under fluorescence microscope and by quantifying GFP mRNA through quantitative Real-Time PCR. We also sought to identify the dose required for global transgene delivery in rats. Histological and molecular analyses were performed to reveal the tissue tropism for each rAAV serotypes.

Material and Methods

Rat Neonatal Cardiac Myocytes and Rat Neonatal Cardiac Fibroblasts: Isolation and Culture

Rat Neonatal Cardiac Myocytes (RNCM) and Rat Neonatal Cardiac Fibroblasts (RNCF) were isolated from the ventricles of 5-day-old Sprague-Dawley rats according to the method adapted from Zhang et al (Zhang et al., 2001). Briefly, rat ventricles were dissociated by mechanical disaggregating and enzymatic digestion with 1% collagenase II (Worthington Biochem. Corp., Freehold, New Jersey). Next, cells were pre-plated in the presence of 5% fetal bovine serum (FBS) in order to separate RNCM from non-cardiomyocytes. After 1 hour of pre-plating, the suspended cells, comprising mostly of RNCM, were removed from the attached non-cardiomyocytes, counted by

hemocytometer, diluted to 2 x 106 viable cells/ml in culture medium with 10%FBS and plated in gelatin-coated culture plates. RNCM were grown in DMEM (Dulbecco's modified Eagle's medium)/F-12 supplemented with 1% penicillin/streptomycin, 10 mM HEPES ,10 µg/ml insulin, 10 µg/ml transferrin and 10% (v/v) FBS (fetal bovine serum). Cells were plated in gelatin [0.1% v/v]-coated culture dishes containing the same media/sera, and were grown in a 5% CO2/95% air humidified incubator. 100 µM bromodeoxyuridine was also added to the media in order to inhibit fibroblast growth. Spontaneous beating was observed in over 95% of cells after 2 days in culture, indicating that the isolated cells were indeed myocytes. This was further confirmed by positive staining of these cells for immunoreactive alpha-sarcomeric actin antibody (Figure1).

The attached cells on the pre-plating dish were mainly RNCF, which were cultured in 10% FBS medium [DMEM supplemented with 10% (v/v) FBS, 1% penicillin/streptomycin and 50 µg/ml ascorbic acid]. Greater than 95% of cultured cells were RNCF, as determined by positive immuno-staining for vimentin (Grobe et al., 2007a). Fibroblasts cultures were produced by dissociating RNCF cultures using trypsin/EDTA (this also eliminate any residual myocytes) and placing the dissociated cells in fibroblast culturing medium for 24 hours before their use. All animal procedures were approved by the University of Florida Institutional Care and Use Committee.

Immunostaining

RNCM cultures were washed three times with 1x PBS and fixed in 4% (v/v) paraformaldehyde (5 minutes, room temperature). Following permeabilization with 0.3% (v/v) Triton X-100 and 0.1% NP40 (10 min, room temperature), non-specific binding was blocked with 1% (w/v) bovine serum albumin (BSA) in 0.3% (v/v) Triton X-100 (10 min,

room temperature). Antibodies against alpha sarcomeric actin (ab28052, Abcam, MA, US) were diluted (1:100) in 1% (w/v) BSA and incubated with RNCM at 4°C overnight in a humidified chamber. RNCM were then washed three times in 1x PBS. A goat anti-mouse secondary antibody (A11032, Invitrogen, Carlsbad, CA,US) was incubated for 1 hour at room temperature. After three successive washing with 1x PBS, the cells were mounted using fluorescent mounting medium containing DAPI (H-1200, Vector Laboratories, CA, US) and viewed by fluorescence microscopy.

In Vitro Transduction of RNCM and RNCF

RNCM and RNCF were plated in 24-well plates at a density of $2x10^5$ cells per well and grown for 48 hours. After the culture media was removed and replaced with fresh media containing 10^7 vg of either rAAV2, rAAV5, rAAV7, rAAV8 or rAAV9. At 3 days post-transduction, GFP expression in RNCM and RNCF was visualized using a fluorescence microscope. Total RNA was extracted from these cells to quantify GFP gene expression using real time RT-PCR. For *in vitro* transduction efficiency evaluation, four separate experiments for each rAAV serotype and each experiments was tested in quadruplicate for same time point on both cell types.

In Vivo Transduction of rAAV in Rats

rAAV 5, 7, 8, and 9 were pseudotyped and cross-packaged vectors, containing the AAV2 inverted terminal repeats (ITRs) and the capsid genes of AAV5, 7, 8 and 9. Pseudotyped vectors containing AAV2-ITR was used because of the existence of the safety profile of AAV2- ITR in animal models and humans. The rAAV2, 5, 7, 8 and 9 constructs were engineered to carry a ubiquitous chicken-β-actin promoter and green fluorescence protein (GFP) as the reporter gene. All of the rAAV constructs were made by the Viral Vector Core at University of Florida. The rAAV vectors production, harvest,

purification, and testing were carried out as previously described (Zolotukhin et al., 2002). rAAV vectors were purified by idodixanol gradient centrifugation and anion-exchange (Q-sepharose) chromatography. The purity of viral vector was tested by silver staining following electrophoresis on 10% SDS-polyacrylamide gels on representative preparations. The physical titer of rAAV vector genome was determined by Real Time polymerase chain reaction (PCR). The production and purification methods generated 99% pure vector stocks with titers of 1x 10^{12} to 1x 10^{13} vector genome/ml. Wild-type AAV and replication-competent AAV contamination was at an undetectable level in the vector stock prepared using this method.

Five-day-old male Sprague Dawley rats (n=36) were delivered by pregnant rats purchased from Charles River Laboratories (MA, US). At 5 days of age, they were lightly anesthetized with isoflurane (Pittman-Moore, Washington Crossing, NJ, US), and injected intracardiacally with 4x10^{10} or 4x10^{11} vector genome (vg) of rAAVs-CBA-GFP (30ul) or the same volume of 1x DPBS as a control, as described previously (Du et al., 2004) (Qing et al., 1999). In previous studies using a lenti-viral vector we have shown that this method of gene transfer results in 100% survival rate (Falcon et al., 2004). After viral administration, the pups were returned to their mother until weaning and were maintained under specific pathogen-free (SPF) conditions. After weaning, rats were housed 2 rats/cage under SPF conditions and used for experiments at either 1 month of age (~170 g body wt) or 2 months of age (~280 g body wt). All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

At either four weeks or eight weeks of age, rats were injected intraperitoneally with 400 IU heparin, and deeply anesthetized with a rodent cocktail containing ketamine,

xylazine, and acepromazine (30, 6, and 1mg/kg, respectively, subcutaneously). Chests were opened to expose the heart and a 16-gauge needle was inserted into the cavity of the left ventricle in order to perfuse the whole body with 1x PBS containing 2IU/ml heparin for 20 min. Heparin, an anticoagulant, was used in this report was to prevent clot formation and to help remove blood cells from the tissues during perfusion. Any excess of blood cells left in the tissues can generate artificial fluorescence. Even though heparin has been reported to inhibit AAV2 transduction by competing with AAV2 to bind its main receptor on cell surface (heparan sulfate proteoglycan) (Summerford and Samulski, 1998), in the current study it was only used at the termination of the study, four or eight weeks post viral administration. Thus it is highly unlikely that the use of heparin in the current protocol would interfere with the transduction of AAV2 or any other AAV viral vector *in vivo*. Samples of liver, lung, kidney and heart were collected respectively. A portion of each tissues was dissected and was preserved in 4% Para-formaldehyde and then embedded in Sakura Tissue-Tek Oct Compound (4583 O.C.T. Compound, Sakura Finetek U.S.A. Inc.). Embedded tissues were cut on 10μm thick sections for the determination of GFP expression in each tissue by fluorescence microscopy. The remaining tissues were snap-frozen for the analysis of GFP mRNA.

Visualization of GFP

GFP fluorescence in 10μm thick sections of heart, liver, lung, and kidney were directly imaged on an Olympus Model BX41 fluorescent microscope. Images were captured and imported into a DP controller. Images from the heart were captured at two different magnifications (2x and 10x) while all other tissues were captured only at 10x magnification. All fluorescent images were collected using an identical exposure time. Quantification of GFP positive cells was carried out by two individuals who were blinded

to the treatments. GFP positive cells were counted in 35 different regions of the heart and liver sections using the ImageJ program. The results for each animal were then averaged for subsequent statistical analysis.

Total RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analyses

Total RNA was isolated using RNAqueous-4PCR kits (AM1914, Ambion, Texas, USA). RNA concentration was quantified by UV spectrum at 260nm, and reverse transcribed using iScript cDNA synthesis kits (Bio-Rad) according to the manufacturer's instructions. Synthesized cDNA corresponding to 100ng of total RNA, was used for real-time PCR. Specific primers for the GFP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; internal control) genes were designed using Primer Express Software (PE Applied Biosystems), for use in real time reverse transcription-polymerase chain reaction (RT-PCR) analysis. All primers were purchased from IDTDNA (Integrated DNA technologies, USA). The primers used were as follows: GAPDH, forward primer: 5′- GCCAGCCTCGTCTCATAGACA -3′, reverse primer: 3′-GTCCGATACGGCCAAATCC -5′; GFP, forward primer: 5′-CCTCTCCGCTGAGAGAAAATTT -3′, reverse primer: 3′-TGGTCCCAATTCTCGTGGAA -5′. The SYBR green real-time PCR assays for each target gene were performed on cDNA samples using an ABI Prism 7000 Detection system (PE Applied Biosystems). GAPDH assays were run in parallel for each sample. Amplication was carried out in optical 96-well reaction-plates (Applied Biosystems) with each well containing 1.5µl cDNA template, 0.5µl sense primer (0.15µM), 0.5µl antisense primer (0.15µM), 5ul SYBR Green PCR Master Mix (Invitrogen) and DEPC-treated

water 2.5µl. The PCR conditions for two-step PCR were one cycle of 95^{o}C for 10min, followed by 40 cycles of 60^{o}C for 1min and then 95^{o}C for 15s.

Statistical Analysis

Experimental values are expressed as mean ± standard error (SE). One way and two way ANOVA were used to compare the mean values between experimental and control groups, depending on the specific study. A Bonferroni's multiple comparison test was used to determine the significant differences between groups. A p value of <0.05 was considered statistically significant.

Results

Evaluation of *In Vitro* Gene Transfer by rAAV Serotypes in RNCM and RNCF

RNCM and RNCF were transduced with 10^{7} vector genome (vg) of either rAAV2, rAAV5, rAAV7, rAAV8, or rAAV9, and 72 hours later GFP expression was analyzed by visualizing green fluorescence. All serotypes of rAAV were capable of producing gene transfer to RNCM as demonstrated by fluorescence imaging (Figure 2a). Quantitative real time-PCR (Figure 2b) demonstrated that rAAV2 and rAAV9 were the most effective serotypes for transducing RNCM. rAAV7 induced moderate levels of GFP gene transfer compared to rAAV2 and rAAV9, and GFP expression following rAAV5 and rAAV8 treatment was relatively low. With the exception of rAAV2, none of the vectors produced significant GFP expression in RNCF (Figure 3).

Evaluation of *In Vivo* Gene Transfer Elicited by rAAV Serotypes in Rat at One Month Post Injection

A total of $4x10^{10}$vg of rAAV2, rAAV5, rAAV7, rAAV8, or rAAV9, each in 30µl 1x DPBS, was injected into the left ventricular cavity of 5-day-old SD rat pups (3 animals for each AAV serotype) in order to analyze the transducing capacity and tissue-tropism

of these viral serotypes *in vivo*. Three control animals received an equivalent volume of 1x DPBS. One month post-injection, animals were sacrificed to evaluate GFP transduction and gene expression using fluorescence microscopy and real-time RT-PCR. As shown in Figures 4a and summarized in 4b, rAAV9 produced the highest level of cardiac transduction of the GFP gene. In contrast, rAAV2 provided a high level gene transfer in the rat liver, while transduction capacity elicited by the other rAAV serotypes was significantly lower (Figures 4a and 4c). Transgene mRNA expression in the heart and liver (Figure 4d) corresponded with the histological results (Figure 4a, b and c). GFP expression in the lung and kidney was barely detectable as tested by Real-time RT-PCR (Figure 4d).

Evaluation of *In Vivo* Gene Transfer by rAAV Serotypes in Rat at Two Months Post Injection

To determine if rAAV9 would continue to provide a high level of cardiac-specific gene transfer, rats were treated with the same doses of rAAV vectors as described above (3 animals per AAV serotype), and sacrificed at two months post-viral injection. The rAAV9 serotype continued to provide highly efficient, global transgene expression to the rat heart (Figures 5a, 5b, and 5d), while expression was significantly lower with the other AAV serotypes. The transgene expression level in the liver was minimal for all other serotypes (Figure 5a and 5c), while rAAV2 serotype still provided some level of transduction (Figures 5a, 5c and 5d) in the liver. Transgene expression in the lungs and kidney was still barely detectable (Figure 5d). Transgene expression in the rat heart (Figure 6a) and liver (Figure 6b) was not statistically different between one month and two months post-transduction, although there was a trend for an increase expression in the heart, and a decrease in expression in the liver, over time.

Dose Response and Biodistribution of Transgene Expression Following rAAV8 and rAAV9 Injection in Rat

Since rAAV8 and rAAV9 were the most cardiac-selective rAAVs amongst the 5 serotypes used, we intracardially injected a higher dose ($4x10^{11}$vg) of rAAV8 and rAAV9 into 5 day old rat pups in order to determine the biodistribution of these two serotypes. Rats were sacrificed at 1 month of age and biodistribution analysis was performed to compare the extent of GFP expression in non-cardiac tissues in the high dose group treated with rAAV8 and rAAV9. rAAV9 treatment elicited widespread gene transfer in rat hearts at both doses, and GFP expression was much lower in the hearts of rats treated with rAAV8 (Figure 7). When the GFP positive cells in the rat hearts were checked under high magnification, these GFP cells exhibited cross striations, characteristic of cardiomyocytes (Figure 8a). A minimal number of GFP positive cells were detected in the liver, and expression was virtually absent in other tissues examined (Figure 8b), further suggesting that these serotypes, with our method of in vivo administration, is primarily cardioselective.

Discussion

The aim of the present study was to compare five serotypes of rAAV with respect to gene transfer efficiency and relative cardiac tropisms using both an *in vitro* approach and a relatively non-invasive *in vivo* approach (administering the rAAV into left cardiac ventricular cavity of rat neonatal pups). To our knowledge, we are the first group to perform comprehensive comparisons of rAAV9 with other serotypes using both an intracardiac viral administration in the rat and two *in vitro* cardiac cell types. The ultimate goal of this study is to develop a clinically relevant gene therapy approach for specifically targeting the heart with rAAV, without the use of a tissue specific promoter.

In vitro screening of the rAAV serotype (pseudotype) that is most suitable for cardiac gene therapy is desirable. It can allow for the subsequent studies detailing the molecular mechanisms responsible for tissue-tropism of each rAAV serotype. Different serotypes of rAAV have their own distinct tissue tropism, which is determined by whether the rAAV is able to enter cells and affect different intracellular molecular mechanisms. Viral receptors at the target cell surface play a key role in the transduction process since they represent the first biological barrier to be overcome during viral infection. For rAAV2, its transduction efficiency and tissue tropism are dependent on a primary receptor [heparin sulfate progetoglycan] (Summerford and Samulski, 1998) (Summerford et al., 1999) and on co-receptors [αVβ5 integrin and human fibroblast growth factor receptor] (Qing et al., 1999) (Summerford et al., 1999). rAAV5 utilizes a sialic acid (Kaludov et al., 2001) and platelet-derived growth factor receptor (Di Pasquale et al., 2003) for cellular transduction. Lamini receptors may also mediate the actions of rAAV2, 8, and 9 (Akache et al., 2006). The tropism and receptors used by rAAV7, 8 and 9 are unknown or not well-understood. Based on our *in vitro* studies,

rAAV2, 5, 7, 8 and 9 are all capable of transducing primary rat neonatal cardiac myocytes (RNCM), suggesting the RNCM may have receptors to bind to all five serotypes of rAAV. Both rAAV2 and rAAV9 mediate a significantly higher transgene expression in primary RNCM, compared to AAV5, 7 and 8. However, there is a distinct difference observed between cardiac fibroblasts and myocytes. Only AAV2 efficiently transduced primary rat neonatal cardiac fibroblasts (RNCF). The fibroblast growth factor receptor in RNCF may provide AAV2 with the ability to transfect RNCF, in comparison to rAAV5, 7, 8 and 9. Although the data obtained in the current study can allow for further characterization of the intracellular mechanisms among the various serotypes of rAAV, it was not the primary goal of this investigation. We were more focused on which of the various serotypes would be the most selective for subsequent gene delivery experiments to target genes for the cardiac myocytes.

The rat models for studying cardiac-vascular diseases are well established in the literature, such as models of myocardial infarction induced by coronary artery ligation or aortic banding induced pressure overload cardiomyopathy. These models offer the opportunity to assess potentially therapeutic genes in established disease models. However, rodent cardiac tropism of rAAV serotypes has been mostly determined by comparing the effects of rAAV vectors in mice. Only few papers report comparing the cardiac tropisms of rAAV vectors in rats, by using intramyocardial injection (Palomeque et al., 2007) (Bish et al., 2008) or ex vivo perfusion (Miyagi et al., 2008). In rat hearts, it is extremely important to find out which rAAV serotype mediates efficient cardiac tropic transgene expression by using a relative non-invasive route of administration. However, based on the size of the animal, the rat model requires more viral vector to be delivered

compared with the mouse model, in order to achieve a therapeutic level of transgene expression. Administration of a large amount of viral vector at one time could cause cytotoxicity due to non-specific transgene expression in untargeted tissues or cells. Thus, the use of a cardiac-selective rAAV and a single injection of a small volume of rAAV into neonatal SD rats may be an answer for this dilemma, without having to develop a more tissue specific promoter.

Our *in vivo* results suggest that rAAV9 provides highly efficient and long lasting gene transfer to rat hearts following a single intracardiac injection of 30 µl of rAAV into the left ventricular chamber of 5-day-old SD rats. Both fluorescent and mRNA determination demonstrated that transgene (GFP) expression in the rat hearts was generally restricted to the heart, maintained for at least two months post viral administration and tended to increase with time. rAAV9 mediated the most efficient transgene expression in the rat heart, which is consistent with results found in mice after systemic vector administration (Vandendriessche et al., 2007) (Zincarelli et al., 2008) (Bish et al., 2008) (Pacak et al., 2006) (Inagaki et al., 2006) (Fechner et al., 2008) (Yang et al., 2009) and in the rat after direct intra-myocardial injection (Palomeque et al., 2007) (Bish et al., 2008), or after coronary artery *ex vivo* perfusion (Miyagi et al., 2008), or after intravenous injection (Suckau et al., 2009). Moreover, unlike intravenous administration of rAAV9 in mice (Inagaki et al., 2006) or in rats (Suckau et al., 2009), the rAAV9 in this current study produced very low GFP expression in the rat liver. Even when high dose of rAAV9 was administered, GFP expression was not substantially increased in the rat liver. GFP expression in the rat heart was widespread and increased compared to GFP expression at low dose administration. These results prove

that our viral administration method selectively mediates widespread transgene expression in the rat heart and limits transduction in the rat liver or other organs. Furthermore; our viral administration method is simple and less damaging to the heart tissue, as it only required a single injection of small volume of viral vectors when compared to other methods. Also, the route of administration applied in the current paper does not require any extensive surgical maneuvers and could be viewed relatively non-invasive, compared to coronary artery *ex vivo* perfusion (Miyagi et al., 2008)or multiple intramyocardial injections (Bish et al., 2008).

For both rAAV8 and rAAV9, *in vivo* results show that GFP positive cells in the rat heart exhibit striations, a characteristic of cardiac myocytes. This shows consistency with the *in vitro* results, in which both rAAV8 and rAAV9 preferably transduce rat neonatal cardiac myocytes, without any transduction in the cardiac fibroblast. rAAV9 mediated more efficient GFP expression than rAAV8 with same titer of viral vector. Different ability to transduce cardiac myocytes may be attributed to different receptor affinities on the cell membrane, differential viral internalization and nuclear uncoating (Sipo et al., 2007). Further investigation is necessary to determine whether the rAAV9 receptors and the molecular mechanisms beyond the receptors are responsible for producing a cardiac-selective transgene expression by rAAV9. rAAV2 mediated more efficient transgene expression in the liver than either rAAV5, 7, 8 or 9. However, rAAV2 was less efficient in transducing the heart tissue. Our findings on the transduction ability of rAAV2, 7 and 9 in liver tissue were not consistent with the findings of other studies (Zincarelli et al., 2008), as rAAV7 and 9 have been reported to mediate more efficient transgene expression in the mouse liver than AAV2 after systemic

administration. This disparity may be due to species differences or the different kind of promoters used in the two studies.

Collectively, our results suggest that rAAV9 preferably transduces cardiac myocytes *in vitro* and efficiently transduces the rat cardiac myocytes *in vivo* and as such, may represent an important viral vector for cardiac gene therapy. rAAV9 appears to be the most cardiotropic serotype in the rat and may be used in investigations for cardiac gene therapy. These *in vitro* and *in vivo* studies suggest that systemic administration of rAVV9 would be selective for the heart, and more specific for the cardiac myocytes than the fibroblasts, and thus experiments targeting gene transfer to myocytes would be best attained by using rAAV9 over other serotypes. Cardiac selectivity of rAAV9 in the rodents can be further enhanced by retargeting the rAAV capsids (Li et al., 2008), or transcriptional and/or transductional targeting of vectors (Muller et al., 2007) (Muller et al., 2006).

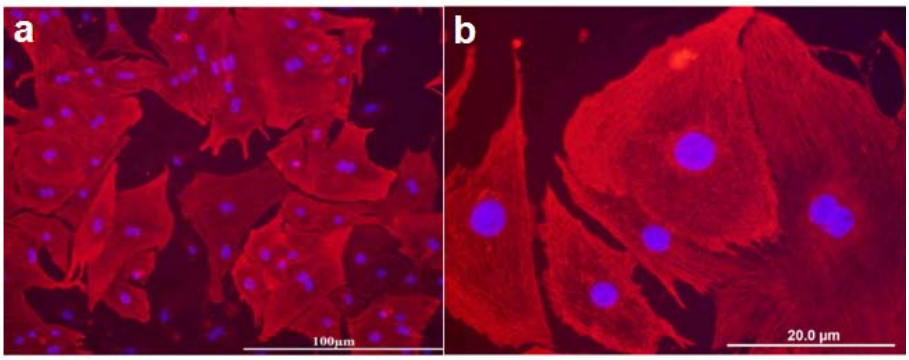

Figure 3-1. Immunostaining of Rat Neonatal Cardiac Myocytes (RNCM) with alpha-sarcomeric actin. Fluorescence micrographs showing alpha sarcomeric actin immunostaining in cultured RNCM (red fluorescence). Nuclei were counterstained with DAPI (blue fluorescence). a: RNCM at 10x magnification. Scale bar=100µm. b: RNCM at 40x magnification. Scale bar=20µm

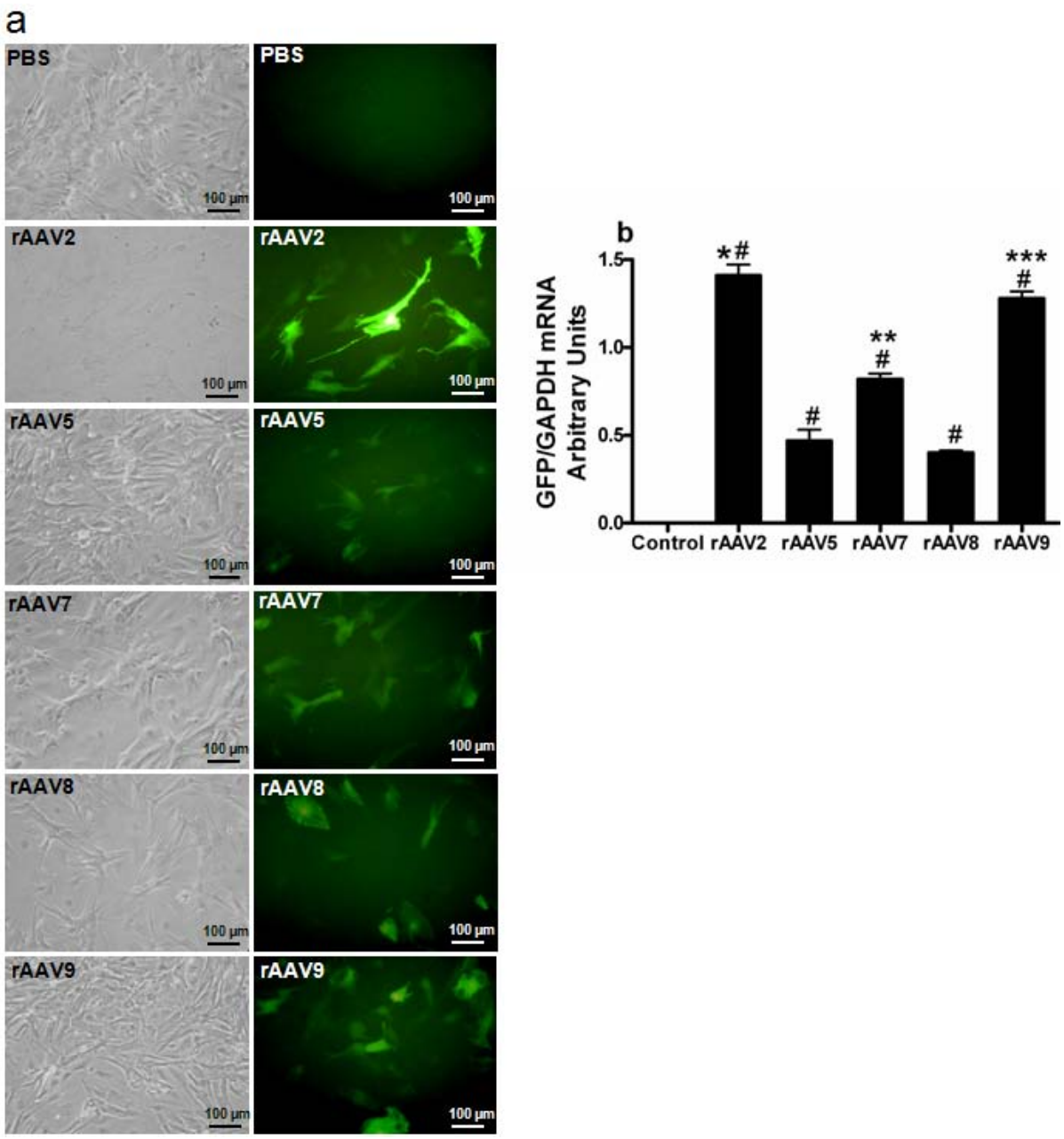

Figure 3-2. rAAVs-mediated gene transfer into RNCM in vitro. RNCM were grown in tissue culture plates and incubated with 10^7vg of rAAV2, rAAV5, rAAV7, rAAV8 or rAAV9 for 72 hours. a: Representative phase and the corresponding fluorescence micrographs showing GFP fluorescence under each treatment condition. b: Level of GFP mRNA in the RNCM was quantified by Real-time PCR and normalized to GAPDH mRNA. Bar graphs are means ± S.E.M., N = 4. # P<0.001 (rAAV2, 5, 7, 8 and 9 vs control); * P < 0.05 and *** P <0.05 (rAAV2 and rAAV9 > rAAV5, rAAV7 and rAAV8); ** P < 0.05 (rAAV7 > rAAV5 and rAAV8).

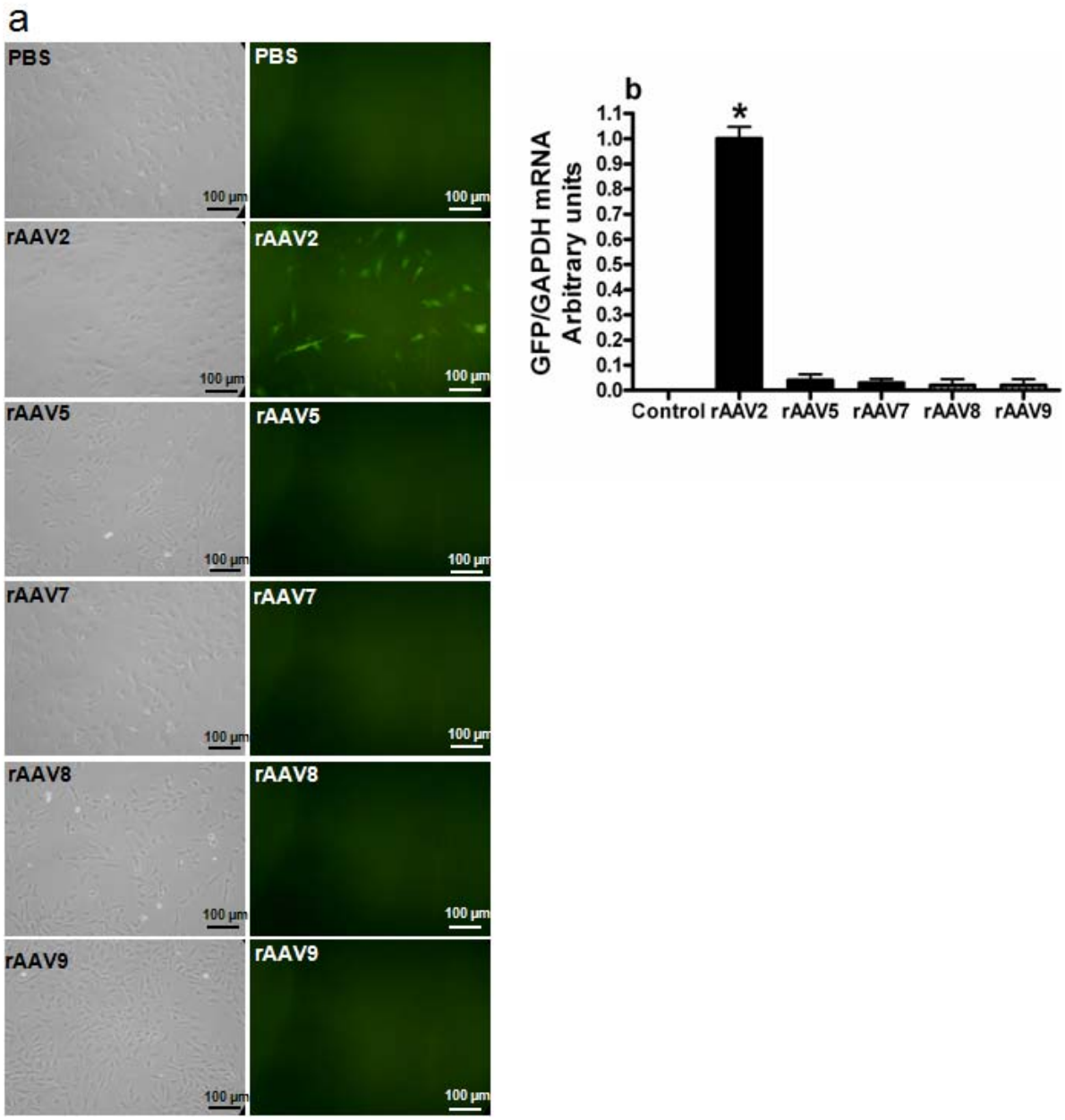

Figure 3-3. rAAVs-mediated gene transfer into RNCF in vitro. RNCF were grown in tissue culture plates and incubated with 10^7vg of rAAV2, rAAV5, rAAV7, rAAV8 or rAAV9 for 72 hours. a: Representative phase and the corresponding fluorescence micrographs showing GFP fluorescence under each treatment condition. b: Level of GFP mRNA in the RNCF was quantified by Real-time PCR and normalized to GAPDH mRNA. Bar graphs are means ± S.E.M., N = 4. * $P < 0.05$ (rAAV2 showed higher GFP level in RNCF than controls and other serotypes of rAAVs.)

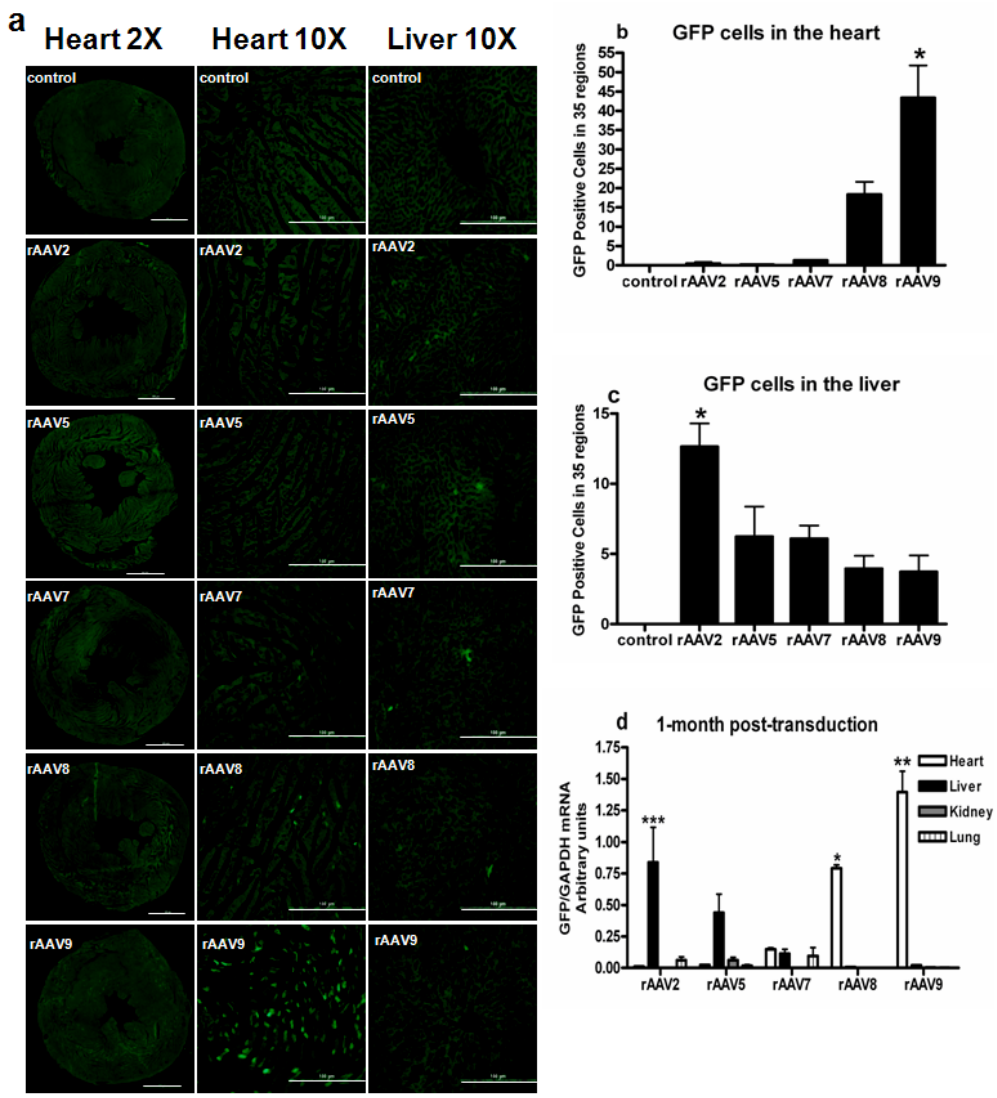

Figure 3-4. GFP expression in rat tissues at 1-month post injection. a: Representative fluorescence micrographs of sections taken from rat heart (at 2x magnification scale bar =500µm and 10x magnification scale bar = 100µm) and liver (at 10x magnification, scale bar =100µm) 1 month following intracardiac injection of 30µl 1xDPBS containing $4x10^{10}$vg of rAAV-CBA-GFP of the indicated serotype. b: Quantification of the number of GFP positive cells from hearts following each treatment condition (Means ± SE ,N=3). * P<0.001(rAAV9>rAAV2,rAAV5,rAAV7 and rAAV8) c: Quantification of the number of GFP positive cells from livers following each treatment condition (Means ± SE ,N=3). * P<0.01(rAAV2> control, rAAV8 and rAAV9). d: Quantification of GFP mRNA expression level in the heart, liver, kidney and lung tissues at one month post-transduction, normalized to GAPDH (Mean ± SE, N=3). *P<0.05 (rAAV8 >rAAV2, and 7 , but <rAAV9); ** P<0.001(rAAV9>rAAV2, 5,7 and 8) ; *** P<0.001(rAAV2 >rAAV5, 7, 8 and 9).

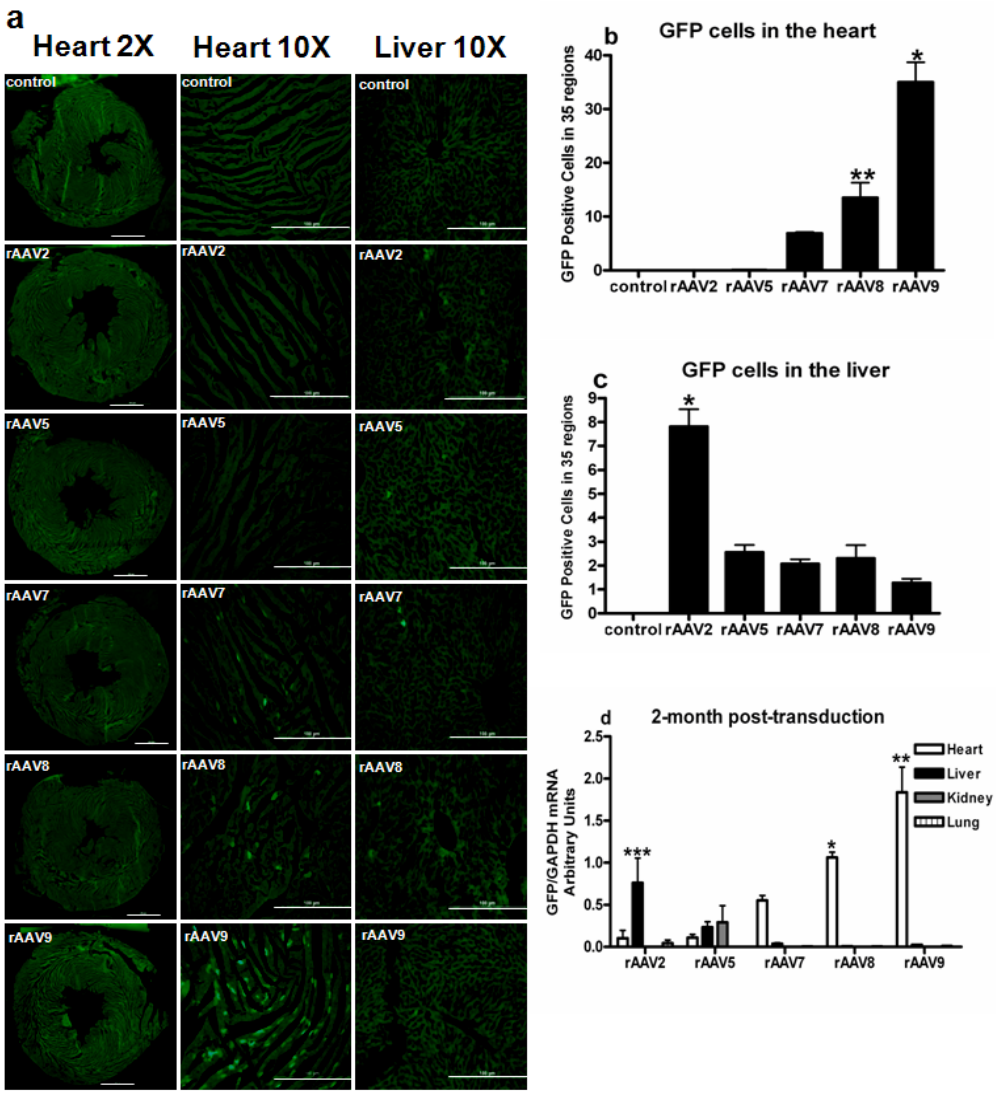

Figure 3-5. GFP expression in rat tissues at 2-month post injection.a: Representative fluorescence micrographs of sections taken from rat heart (at 2x magnification scale bar:500µm and 10x magnification scale bar: 100µm) and liver (at 10x magnification, scale bar: 100µm) 2 month following intracardiac injection of 30µl 1xDPBS containing $4x10^{10}$vg of rAAV-CBA-GFP of the indicated serotype. b: Quantification of the number of GFP positive cells from hearts following each treatment condition (Means ± SE ,N=3). * P<0.001(rAAV9>control, rAAV2, 5,7 and 8), ** P<0.01 (rAAV8> control, rAAV2 and rAAV5, <AAV9) c: Quantification of the number of GFP positive cells from livers following each treatment condition (Means ± SE ,N=3). * P<0.001 (rAAV2>control, rAAV5,7, 8 and 9). d: Quantification of GFP mRNA expression level in the heart, liver, kidney and lung at 2 month post-transduction ,normalized to GAPDH (Mean ± SE, N=3). *P<0.05(rAAV8 >rAAV2 and 5, but <rAAV9); ** P<0.001(rAAV9>rAAV2,5,7 and 8). *** P<0.05(rAAV2>rAAV5,7,8 and 9.)

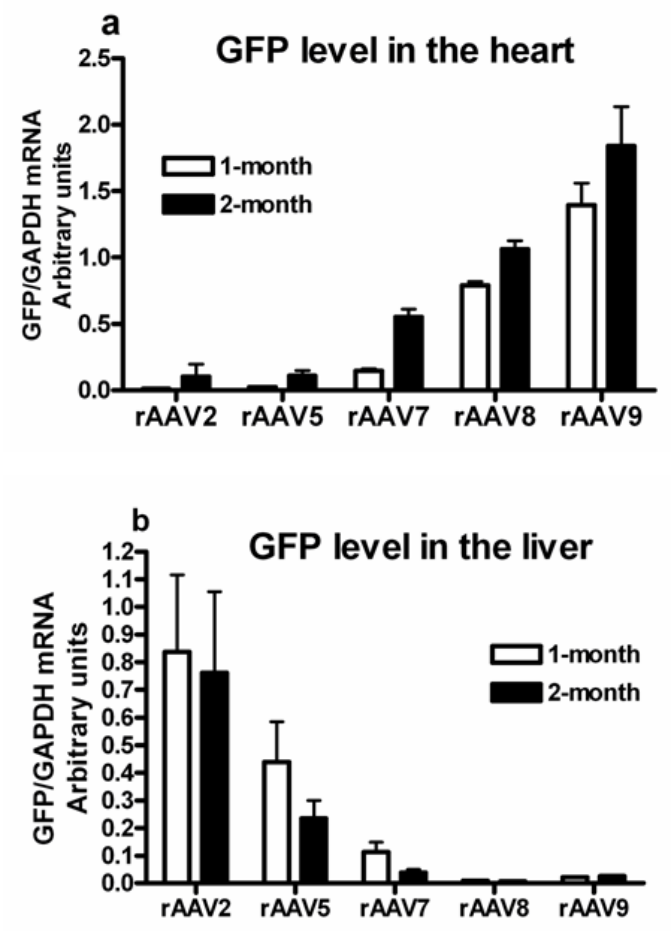

Figure 3-6. Quantitative analyses of GFP mRNA expression in rat tissues.a: Comparison of GFP mRNA expression level in the heart between one month and two months post injection. The GFP level in the heart is not statistically different between one month and two months. b: Comparison of GFP gene level in the liver between one month and two months post injection. The GFP level in the liver is not statistically different between one month and two months.

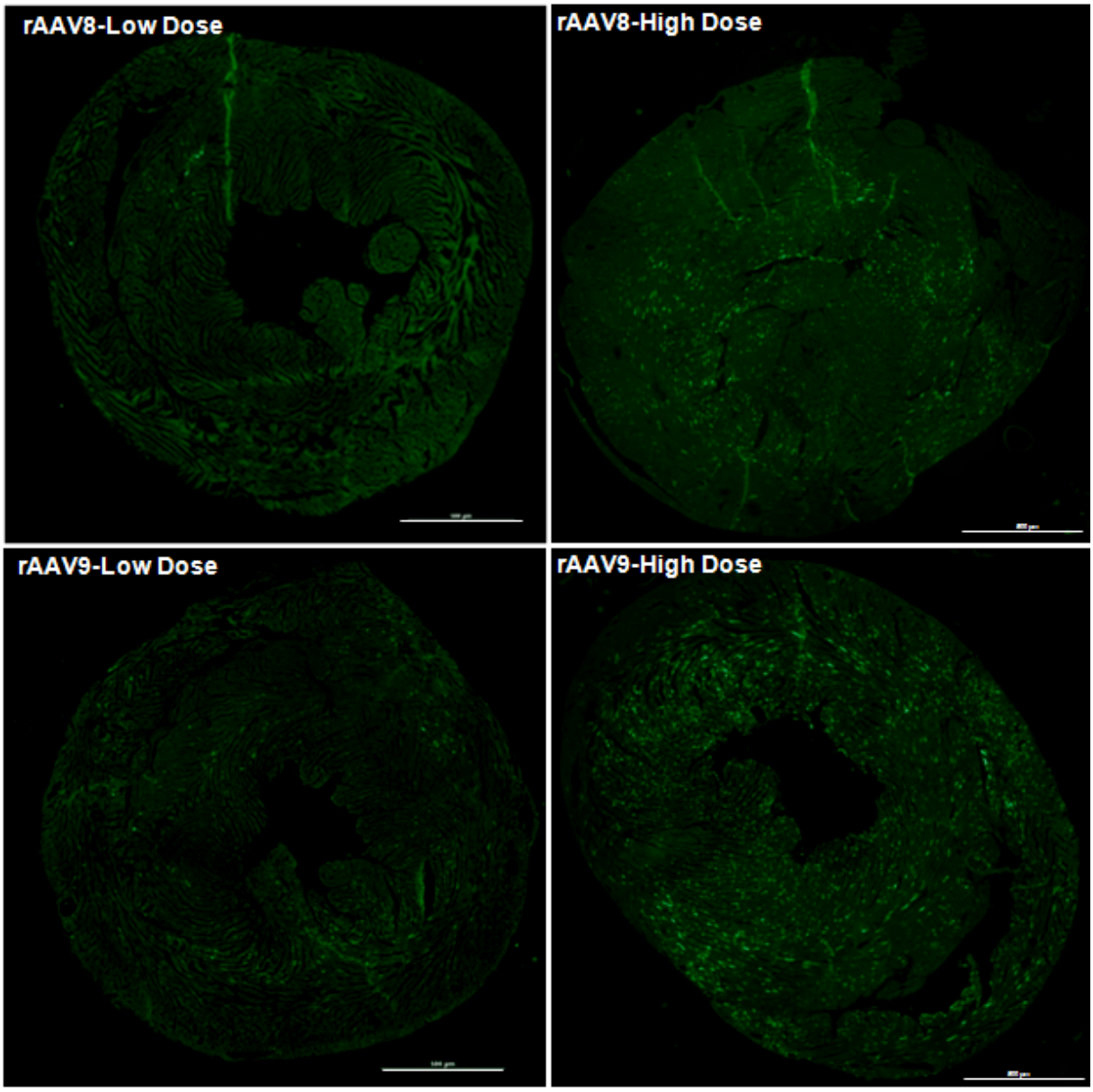

Figure 3-7. Dose responses of transgene expression following rAAV8 and rAAV9 transduction. GFP expression in rat hearts was examined at one month post injection of $4x10^{10}$ vg (low dose) or $4x10^{11}$ vg (high dose) of rAAV8- or rAAV9-CBA-GFP.Scale bar: 500μm. Images were taken at 2x magnification and merged using Photoshop11.0. The upper row shows GFP expression in rat hearts following rAAV8 injection. The Lower row shows GFP expression in rat hearts following rAAV9 injection.

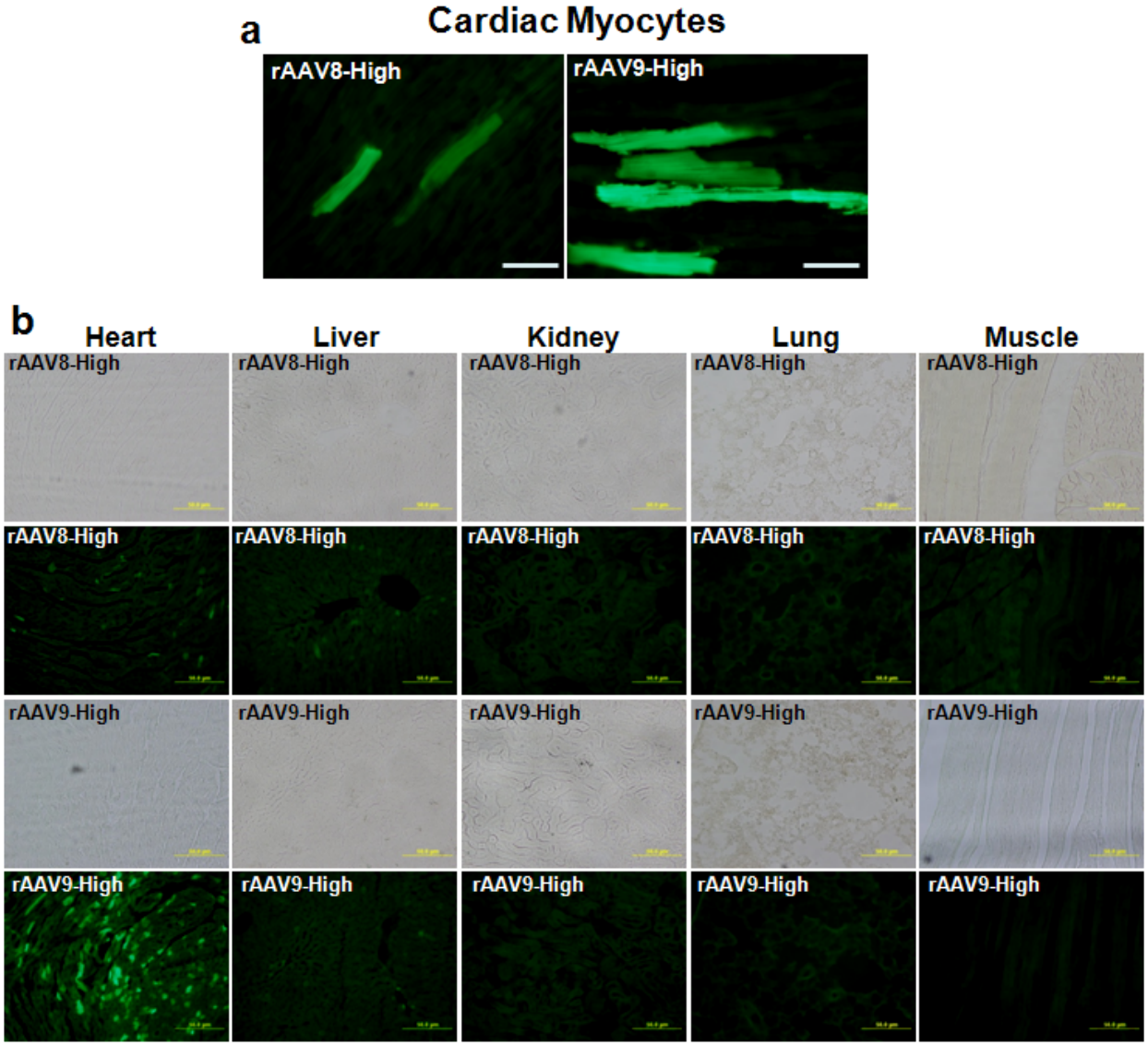

Figure 3-8. Biodistribution of transgene expression following rAAV8 and rAAV9 transduction. a: GFP expression in rat cardiac myocytes was examined at one month after intracardiacly administrating high dose of rAAV8 or rAAV9. Representative images were taken at high magnification. Scale bar: 50μm. b: GFP expression in heart, liver, kidney, lung and skeletal muscle were examined at one month post intracardiac injection of $4x10^{11}$ vg (high dose) of either rAAV8 or rAAV9. First and third rows are images taken in Bright Field and the second and fourth rows are corresponding fluorescence micrographs. Each column represents one type of tissue. rAAV8-High (high dose) is shown in the first and second rows and rAAV9-High(high dose) in the third and fourth rows. Scale bar: 50μm.

CHAPTER 4
CARDIAC-SELECTIVE OVEREXPRESSION OF ANGIOTENSIN TYPE 2 RECEPTOR IMPROVES CARDIAC FUNCTION AND ATTENUATES LEFT VENTRICULAR REMODELING

Abstract

OBJECTIVES: The aim of this study was to examine the effect of cardiac-selective overexpression angiotensin type 2 receptor (AT2R) on left ventricular (LV) dysfunction and remodeling in a rat coronary artery ligation (CAL) model.

BACKGROUND: Myocardial ischemia can result in significant myocardial damage, including myocyte death, fibrosis, and wall thinning, leading to impaired ventricular function and cardiac failure. The renin-angiotensin system (RAS) plays an important role in cardiac remodeling post-myocardial infarction.

METHODS: Both prevention and reversal studies were performed. For the prevention study, $4x10^{10}$ vector genome (vg) of rAAV9-CBA-AT2R was injected into the left ventricle chamber of the heart in 5-day-old rats. Six weeks after viral administration, the left anterior descending coronary arteries were ligated. For the reversal study, $4x10^{10}$ vg of rAAV9-CBA-AT2R was administrated to the periphery of the infracted myocardium area immediately after coronary artery ligation (CAL) surgery. In both studies, hemodynamic measurements were performed via echocardiography and intracardiac catheter 4 weeks after CAL surgery for Control (n=6), MI (n=6), rAAV9-GFP (n=3), MI+rAAV9-AT2R (prevention, n=4) and MI+rAAV9-AT2R (reversal, n=4) groups. Cardiac tissues were analyzed for inflammation and cardiac remodeling markers with real-time reverse transcription PCR.

RESULTS: Myocardial infarction resulted in a significant decrease in ejection fraction, deterioration of left ventricular systolic pressure, dp/dt, and increase in left

ventricular end diastolic pressure (LVEDP) and ventricular hypertrophy. Overexpression of AT2R attenuated this impairment to a non-significant level, markedly illustrated by a significant reduction in LVEDP and ventricular hypertrophy. Overexpression of AT2R also prevented myocardial wall thinning and tended to decrease the infarction area. Furthermore, myocardial infarction caused a an up-regulation of AT1R, TGF-β, ACE, Collagen I , and Collagen III mRNA expression. The AT2R overexpression prevented these changes and also up-regulated the other speculated cardiac protective factor-Mas receptors and Bradykinin B2 receptor levels.

CONCLUSIONS: Cardiac-selective overexpression of AT2R exerts beneficial effects on the heart function post-myocardial infarction by preserving cardiac function and attenuating cardiac remodeling post MI. The protective effects of AT2R may be attributed to its antihypertrophic and antifibrotic effects.

Introduction

Myocardial infarction (MI), commonly known as a heart attack occurs when the blood supply to a part of the heart is interrupted. The resulting ischemia causes irreversible damage to the heart tissue (Williams and Benjamin, 2000). The damage after myocardial infarction results in left ventricular (LV) remodeling, representing by molecular, cellular and interstitial changes. LV remodeling is manifested as adverse alterations in the size, shape and function of the ventricle, often leading to left ventricular dysfunction, dilated cardiomyopathy and heart failure (Tiyyagura and Pinney, 2006) (Pfeffer and Braunwald, 1990). Evidence shows that the adverse alteration in the heart after a MI is associated with a marked increase in cardiovascular morbidity and mortality (Anavekar and Solomon, 2005).

Growing evidence implies that the Renin Angiotensin System (RAS) contributes to the progression of myocardial infarction. It is well documented that angiotensin II (AngII) plays a critical role in the development of post MI LV remodeling (Anavekar and Solomon, 2005) . AngII has two major receptor subtypes, type 1 (AT1R) and type 2 receptors (AT2R), both of which are expressed in the heart (Ozono et al., 2000) and play a crucial role in cardiovascular physiology and disease. Numerous experimental findings have demonstrated that the AT2R is upregulated under pathological conditions like heart failure (Tsutsumi et al., 1998) (Regitz-Zagrosek et al., 1995). AT1R signaling contributes to the deterioration of MI by mediating vasoconstriction, cardiomyocyte hypertrophy, fibroblast proliferation, and interstitial collagen deposition (Matsubara, 1998) (Weber and Brilla, 1991). In contrast, the AT2R is generally thought to exert an opposing effect to AT1R in the cardiovascular system. It is also suggested that part of protective effects of Angiotensin receptor blockers (ARBs) are possibly mediated through AT2R (Matsubara, 1998), since unbounded AngII stimulates AT2R.

However, some investigators have reported that the AT2R may cause hypertrophy (D'Amore et al., 2005) , dilated cardiomyopathy and heart failure in transgenic mice (Yan et al., 2003), and chronic AT2R expression depresses myocardial contractility in TG mice with overexpression of AT2R (Nakayama et al., 2005). Therefore the effects of the AT2R on cardiac remodeling is controversial and yet to be resolved. This study intends to help clarify the functions of AT2R post MI in protecting the heart from further damage and restoring part of cardiac function. To date, there are no published reports studying effects of AT2R in myocardial infarction model with the use of viral vectors.

The present study was designed to determine whether cardiac selective over-expression of AT2R prevents cardiac dysfunction in rat myocardial infarction model, and to evaluate potential cardioprotective mechanisms modulated by AT2R.

Materails and Methods

Characterization of rAAV9-GFP and rAAV9-AT2R Viral Vectors

Production and characterization and rAAV9-GFP and rAAV9-AT2R were performed according to same methods, as described in methods section in chapter 3.

Coronary Artery Ligation

Myocardial infarction was induced by ligation of the left anterior descending coronary artery. At the time of operation, rats were anesthetized with isoflurane (2.0–2.5% in oxygen), after which rats were intubated with an 18-gauge intravenous catheter and mechanically ventilated with this isoflurane-oxygen mixture using a Harvard ventilator (model 683, Harvard Apparatus, Holliston, Mass). After the chest was cleaned and shaved, rats were underwent a left-lateral thoracotomy. The thorax was entered via the left fourth intercostal space and the pericardium incised to expose the heart. The heart was exposed, and ligated at the proximal left anterior descending coronary artery 2–3 mm from its origin between the pulmonary artery conus and the left atrium with a 7–0 polypropylene suture. Successful cessation of blood flow was indicated by elevation of ST segment on electrocardiogram and cyanosis of anterior LV wall; if necessary the procedure was repeated by placement of a second or third ligature. The heart was returned to its normal position, and the thorax was evacuated of fluid and air and closed. All of the animals received buprenorphine hydrochloride (Buprenex, 0.02 mg/kg q12 IM, Reckitt and Colman Pharmaceuticals) and were closely monitored for signs of discomfort. Sham operated rats underwent the identical surgical procedure as

described above except that the suture was not tightened around the coronary artery. In the present study, the operation-related mortality was approximately 25% 24 h after operation.

rAAV9-GFP and rAAV9-AT2R Administration

All of the animal protocols were approved by the institutional animal care and use committee (IACUC) and conducted according to National Institutes of Health guidelines.

For the reversal study, $4x10^{10}$ vg of rAAV9 vectors in 100 µL 1x DPBS was injected into multiple sites of myocardium around infarct area immediately after coronary artery ligation surgery using a 0.5 ml insulin syringe, before the chest cavity was sutured closed.

For the prevention study, five-day–old male Sprague-Dawley rats received a single intraventricular injection of $4x10^{10}$ vector genome of rAAV 9-GFP or rAAV9-AT2R viral vectors in 30 µL 1x DPBS, as described in chapter 3. This method of gene transfer by rAAV vector provides a 100% animal survival rate and has been established to produce efficient, cardiac-selective and long-term transduction of the heart. After viral administration, animals were returned to their mothers until weaning. At 6 weeks of age, rats were separated into 4 experimental groups (control sham, MI, rAAV9-GFP, and MI+rAAV-AT2R; N=4-7 per group) and subjected to either coronary artery ligation surgery or mock surgery.

Echocardiography

Cardiac function was evaluated using a Hewlett Packard Sonos Model 5500 with a 12-Hz transducer at 4 weeks after coronary artery ligation surgery. Rats were anesthetized with isofluorane for echocardiographic examinations. Images were

obtained from the parasternal short axis. All measurements were based on the average of three consecutive cardiac cycles. Measurements obtained by echocardiography include left ventricular end diastolic diameter (LVED), left ventricular end systolic diameter (LVES), and left ventricular posterior wall thickness (LVPW). The ejection fraction was calculated as follows: (LVEDv-LVESv)/LVEDv x 1/100.

Hemodynamic Measurements

Rats were anesthetized with ketamine/xylazine/ acepromazine rodent cocktail (30 mg/6 mg/1mg/kg, i.m.). The rats were placed in supine position and the body temperature was maintained at 37 ^{o}C by a heated pad throughout the experiment. The left ventricular function was measured using a 22 Gauge needle filled with heparin saline (20 IU/ml) inserted into the left ventricle chamber. The data was recorded after stabilization of the tracing using a liquid pressure transducer, which was interfaced to a PowerLab (ADInstruments, Colorado Springs, CO, USA) signal transduction unit. Data were analyzed by using the Chart program that was supplied with the PowerLab system.The parameters include a peak systolic pressure of left ventricle (LVSP), a maximal positive and negative rate of rise in left ventricular pressure (±LVdP/dtmax), HR, and mean artery pressure (MAP).

Histological Analysis

Following the hemodynamic measurement, the hearts were harvested. Hearts were removed, and the ventricles were separated from atria and rinsed in PBS. The ventricles were weighed and cut into 3 thick sections made perpendicular to the long axis. The basal and apex section was snap frozen in liquid nitrogen and stored at –80°C to do western-blot analysis and Quantitative Real-time PCR. The middle section was used to measure infarct size and cardiac remodeling parameters. Cardiac remodeling

was determined by ventricular hypertrophy and cardiac fibrosis. Ventricular hypertrophy was determined by measuring wet weights of rat heart ventricles normalized to tibial length. Cardiac hypertrophy can be more accurately quanified by relative heart weight to tibial length than to body weight , as tibial length is a reliable reference to normalize heart weight in conditions in which body weight changes (Yin et al., 1982).Cross sections of the ventricles were then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm. Ventricular sections were stained with H & E (hematoxylin and eosin) to determine myocyte diameter or with Picro-Sirius Red to mesure interstitial fibrosis. Myocyte diameter was determined at 40x magnification, using the ImageJ program from National Institutes of Health as previously described (Grobe et al., 2007c). 20 separate images from different (nonoverlapping) regions of the left ventricle free wall only were examined. Left ventricular (LV) wall thickness was examined using ImageJ program too. Quantification of myocyte diameters and left ventricular wall thickness were carried out by an individual who was blinded to the treatments.

Quantification mRNA Levels

Tissues was homogenized and total RNA was isolated using RNAquous 4 polymerase chain reaction (PCR) kit (Ambion, Foster City, CA, USA) according to the manufacturer's instructions. 200ng RNA was reverse transcribed with iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The AT1R, AT2R, Collagen I, Collagen III, TGF-beta, TNF-alpha, BKR-B2, ACE2, ACE, IL-10 and IL-6 were analyzed by quantitative real-time PCR using Taqman probe (Applied Biosystems). Real-time PCR was run using ABI Prism 7000 sequence detection system. All cDNA samples were assayed in triplicate. Data were normalized to GAPDH RNA.

Statistical Analysis

Results are expressed as mean±SE. Data were analyzed by ANOVA with Bonferroni correction for multiple comparisons. Values of $P<0.05$ were considered statistically significant. All of the data were analyzed using GraphPad Prism 5 software (Graphpad Prism Institute Inc).

Results

rAAV9 Mediated Cardiac-Selective Overexpression of AT2R in Rat Hearts

For the reversal and prevention studies, left anterior descending coronary artery ligation (CAL) was performed on 6-week-age rats. Myocardial infarction was confirmed by the ST-segement elevation as determined by electrocardiogram (ECG), and a representing tracing is shown in Figure 4-1. rAAV9-AT2R mediated overexpression of AT2R was determined by semi-quantitative Realtime PCR. AT2R expression level was significantly increased in both the reversal study and prevention study, but the AT2R level in the reversal study was lower than the prevention study (Figure 4-2).

Effects of AT2R Overexpression on Cardiac Function Post-MI

Echocardiographic analyses were performed at 4 weeks post myocardial infarction surgery. Myocardial infarction caused a significant reduction in ejection fraction in the MI group relative to the control and rAAV9-GFP. AT2R overexpression was able to restore ejection fraction, as shown in Figure 4-3. Minimum dp/dt was significantly decreased in MI group, relative to other groups (Table 4-1). Left ventricular end diastolic pressure was significantly increased in the MI group compared to control and rAAV9-GFP (Table 4-1). In both the reversal and prevention studies, overexpression of AT2R was able to attenuate these changes (Table 4-1). Heart rate was not significantly different among

groups (Table 4-1), although there was a tendency for the MI group to display a higher heart rate.

Effects of AT2R Overexpression on Ventricular Remodeling Post-MI

AT2R overexpression mediated by rAAV9 attenuated MI induced cardiac hypertrophy as evaluated by both the ventricular weight to tibial length ratio and measurement of myocyte diameter, as shown in Figure 4-4. MI induced significantly decrease in the left ventricular wall thickness. AT2R overexpression significantly attenuated left ventricular wall thinning caused by myocardial infarction. AT2R overexpression also tended to decrease infarction area and significantly attenuated the thining of the ventricular wall, as shown in Figure 4-5.

AT2R-mediated Protective Mechanism Post-MI

To determine the mechanism of cardioprotection by AT2R overexpression, the left ventricular tissue was harvested and measured the levels of genes involving in the RAS and cardiac remodeling. As shown in Table 4-2, there was a significant increase in levels of components of the ACE-AngII-AT1R axis, e.g. upregulation of ACE and AT1R in the MI group. Additionally, in the MI group, TGF-beta, Collagen I, and Collagen III level were also upregulated. Overexpression of AT2R was able to decrease these changes. Overexpression of AT2R also induced an upregulation of Bradykinin B2 receptor and Mas receptor levels. AT2R-mediated protection post-myocardial infarction is associated with restoration of the balance in the RAS and a decrease in mediators of cardiac fibrosis. It is of interest to note that these positive effects of AT2R overexpression were similar whether the overexpression of the transgene occurred in the neonatal animals well before CAL or immediately after the CAL procedure.

Discussion

In the present study, the effects of cardiac selective overexpression of AT2R mediated by rAAV9 vector on the cardiac function and ventricular remodeling were examined in a rat coronary artery ligation model. We found that cardiac selective overexpression of AT2R provides significant protection against left ventricular dysfunction caused by myocardial infarction, as shown by a preserved ejection fraction and dp/dt, and a reduction in left ventricular end diastolic pressure back towards control values. Moreover, ventricular hypertrophy and wall thinning was attenuated by cardiac-selective overexpression of AT2R.

The majority of the myocardial infarction studies performed with either AT2R-knockout animals or transgenic overexpressions of AT2R in animals demonstrated that AT2R has antihypertrophic effects, or mediates part of the protective effects of AT1R antagonists. Yet, several investigators reported that transgenic overexpression of AT2R in cardiomyocytes *in vivo* resulted in enhanced hypertrophy and dilated cardiomyopathy. Part of these discrepancies may be due to both compensatory mechanisms *in vivo* and difficulty in controlling expression level of trans-gene in the transgenic AT2R knockout or overexpression mouse model. We have recently reported (chapter 3 data) that use of rAAV9 to transduce our transgene, results in significant and selective transduction into the heart during the time course reported in the current study. Unfortunately we did not assess the time course for incorporation of the transgene for the ‘reversal’ study. Previous reports suggest that transgene expression can occur as early as 1 day after the direct intracardiac injection (Su et al., 2006) and as long as 1 year after after the intracardiac injection by a transdiaphragmatic approach (Woo et al., 2005b). Thus in the current study we have demonstrated that, in contrast to transgenic

models of AT2R over/under expression, selective delivery of AT2R *in vivo* by rAAV9 viral vector may avoid some of the potential discrepancies reported in the literature, and may be a better tool for investigating the more precise roles of AT2R in LV cardiac remodeling that occurs after MI. It is also not established how AT2R mediates its protective effects. Most of the studies have been done on transgenic animals in which the AT2R has already been overexpressed at birth before myocardial infarction, and as the AT2R has been implicated in the developmental process, does this early overexpression lead to yet to be determined compensatory changes that cloud the interpretation of the findings. It is intriguing to investigate whether AT2R mediated effects are working through preventing and/or reversing the adverse remodeling process.

AT2R is re-expressed and/or upregulated under pathophysiological conditions such as MI (Nio et al., 1995) and mechanical injury (Tiyyagura and Pinney, 2006). This re-expression of AT2R may act to offset the trophic/proliferative effects of Ang II via AT1R (Stroth and Unger, 1999). Numerous experimental findings demonstrate that an increase in cardiac AT2R in heart failure may provide beneficial effects in the heart. All these studies indicate that AT2R requires being upregulated in the heart following myocardial infarction. The endogenous level of upregulation of AT2R may not be adequate to exert any significant beneficial effects. This study used recombinant adeno associated viral vector serotype 9 to deliver AT2R into the myocardium. Our group has reported that administration of rAAV9 into rat heart mediates efficient and cardiac-selective expression in the rats. The collected results demonstrate that an overexpression of AT2R in the heart, shortly after birth or immediately after the cardiac

ischemic insult, does provide beneficial effects on cardiac function and structure. Yet to be determined is to what level of expression of AT2R is a threshold to mediate these changes or if any greater increases in receptor number would produce more pronounced protective effects.

Angiotensin receptor blockers (ARBs) reduce cardiovascular mortality and morbidity in patients with heart failure after MI (Jugdutt and Menon, 2004). Selectively blockade of AT1R with ARBs results in an elevation of the levels of circulating AngII which can then stimulate the unopposed AT2R. Thus, it is hypothesized that the beneficial effects of ARBs may be mediated, at least in part, through AT2R activation. Furthermore, administration of ARB is associated with an upregulation of cardiac ACE2 and Ang-(1-7) levels (Ishiyama et al., 2004a) (Trask and Ferrario, 2007). ACE2 and Ang-(1-7) each have reported cardioprotective effects against heart failure and cardiac hypertrophy. Cardiac overexpression of ACE2 mediated by Lenti-viral vector preserved cardiac function and attenuated left ventricular wall thinning following myocardial infarction (Der Sarkissian et al., 2008). Chronic Ang-(1-7) treatment not only attenuated the development of heart failure in the MI model (Ishiyama et al., 2004a) but also prevented cardiac hypertrophy and fibrosis in rats (Iwata et al., 2005) (Wang et al., 2005) (Grobe et al., 2007c). ACE2 activity or Ang-(1-7) forming activity has been shown to be directly correlated with AT2R density (Zisman et al., 2003a) . Also as shown in chapter 2, Ang-(1-7) overexpression preserved cardiac function and upregulated both ACE2 and AT2R expression, which provided evidence for the interaction among ACE2, Ang-(1-7) , and AT2R. Taken together, it is hypothesized that AT2R exerts its protective effects on the heart post MI through interacting with ACE2- Ang-(1-7)-Mas axis. In the

current study, Mas receptor level was significantly upregulated. AT2R overexpression and also reduced the upregulated AT1R induced by myocardial infarction, in both the prevention and reversal studies. All these results support the hypothesis that AT2R exerts its protective effects on the heart following MI possibly through antagonizing ACE-AngII-AT1R and stimulating the ACE2-Ang-(1-7)-mas axis.

In the MI group, Collagen I, Collagen III, and TGF- β1 were significantly increased in the heart tissure post myocardial infarction. Cardiac-selective overexpression of AT2R prevents this increase and brings back towards control values. In cardiac hypertrophy and heart failure, expression of TGF-β1 in the myocardium is reported to be increased (Weber, 1997). This increase in TGF- β1 expression is consistent with the result from the current study. This increase in TGF- β1 expression may directly participate in the progressive remodeling process in heart failure. Treatment with ARBs also markedly decreased TGF-β levels in infracted hearts (Ju et al., 1997), suggesting that TGF-β induction in the remodeling myocardium is in part mediated through AngII signaling. TGF-β appears to be a crucial mediator in the pathogenesis of post MI remodeling, because of its important role in regulating fibrous tissue deposition, composition of the extracellular matrix, and cardiac hypertrophy (Lim and Zhu, 2006). Thus, it is possible that AT2R reduces LV cardiac remodeling post MI by interacting with TGF-β signaling pathways.

In summary, our data demonstrate that overexpression of AT2R preserves cardiac function and attenuates cardiac remodeling post myocardial infarction. These beneficial effects involve restoration of the RAS balance and prevention of the upregulation of fibrotic factors (TGF-β, Collagen I , and Collagen III). Taken together, all these studies

indicate that targeting of the AT2R could hold novel therapeutic strategy in the treatment of myocardial infarction and its associated complications.

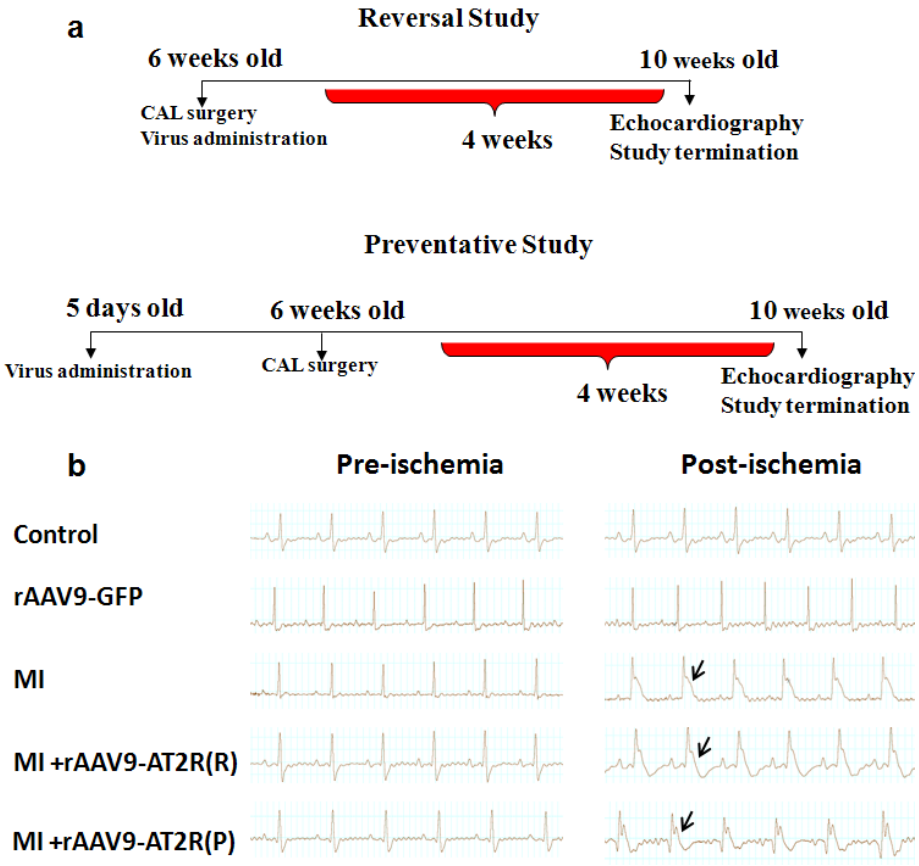

Figure 4-1. Schematic representation of study protocol. (a) Illustration of the experimental protocol. For the preventative study, rAAV9-AT2R was administered into left ventricular chamber of 5-day-age SD pups. Coronary artery ligation (CAL) was performed when SD rats were 6 weeks old. For the reversal study, rAAV9-AT2R was administrated onto the left ventricular myocardium around infarction area right after the CAL surgery. 4 weeks after the surgery, animals were subjected to echocardiography followed by hemodynamic heart function assessment and tissues harvest. (b) Representative electrocardiogram (ECG) at basal showing normal tracing and showing an elevation in ST-segment following CAL, as indicated by arrow.

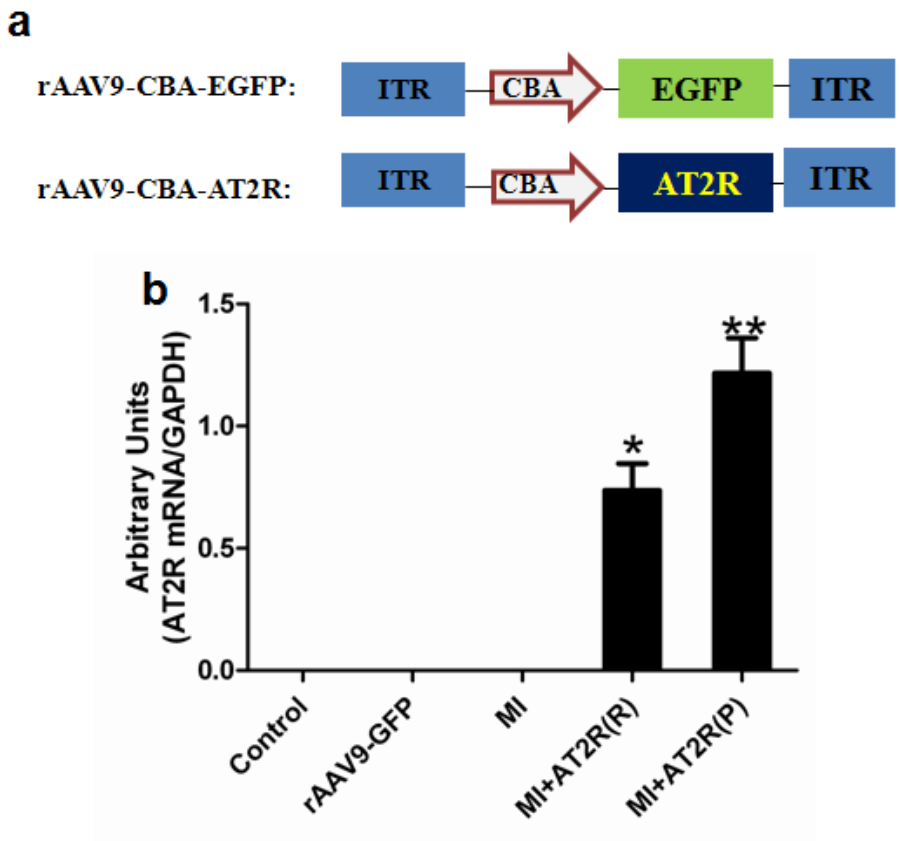

Figure 4-2. (a) Schematic representation of structural components of the rAAV9-GFP or rAAV9-AT2R viral vector. (b) Semi-quantitative Real-Time RT-PCR detection of AT2R mRNA level presented in heart tissues. (* $p<0.05$ MI+rAAV9-AT2R(R) and MI+rAAV9-AT2R(P) vs control and rAAV9-GFP, ** $p<0.05$ MI+rAAV-AT2R(P) vs MI+rAAV9-AT2R(R); N=4-6/group).

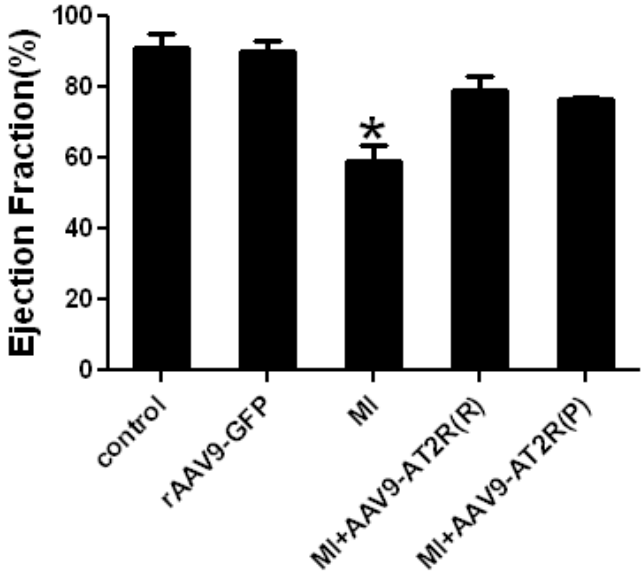

Figure 4-3. Effect of AT2R gene transfer and myocardial infarction on ventricular function. Echocardiography analyses of rat myocardium 4 weeks after the CAL demonstrates significant improvement in ejection fraction in rAAV9-AT2R treated animals as compared to MI group. (* MI vs. control, rAAV9-GFP, MI+rAVV9-AT2R(R), and MI + rAAV9-AT2R(P); N=4-6/group).

Table 4-1. Hemodynamic data at 4 weeks post MI

	Control	rAAV9-GFP	MI	MI+rAAV9-AT2R(R)	MI+rAAV9-AT2R(P)
LVSP	112.6 ± 4.5	108.1 ± 5.3	89.5 ± 8.8	105.5 ± 8.1	109.5 ± 10.0
HR	279.9 ± 18.0	324.3 ± 20.4	359.2 ± 28.8	333.4 ± 54.6	353.4 ± 48.6
dp/dt max	4194.0 ± 179.1	3867.8 ± 167.0	3220.5 ± 486.6	3614.3 ± 140.5	4398.9 ± 168.9
dp/dt min	3765.3 ± 204.4	3394.2 ± 138.8	3023.3 ± 19.3 *	3275.0 ± 149.9	3848.2 ± 90.5
LVMP	41.2 ± 2.8	45.2 ± 2.2	45.2 ± 2.1	51.9 ± 0.8	47.1 ± 3.4
LVEDP	4.0 ± 1.5	1.6 ± 1.1	12.5 ± 2.7**	3.9 ± 0.1	4.2 ± 2.3

HR(heart rate), beats per minute; LVSP (left ventricular systolic pressure) , millimeters of mercury; +dp/dt and –dp/dt (maximal and minimal peak rate of left ventricular pressure), millimeters of mercury per second; LVMP(left ventricle mean pressure), millimeters of mercury; LVEDP(left ventricle end-diastolic pressure), millimeters of mercury. (* MI vs. contro, rAAV9-GFP, and MI + rAAV9-AT2R(P), ** MI vs all other groups; N=4-6/group).

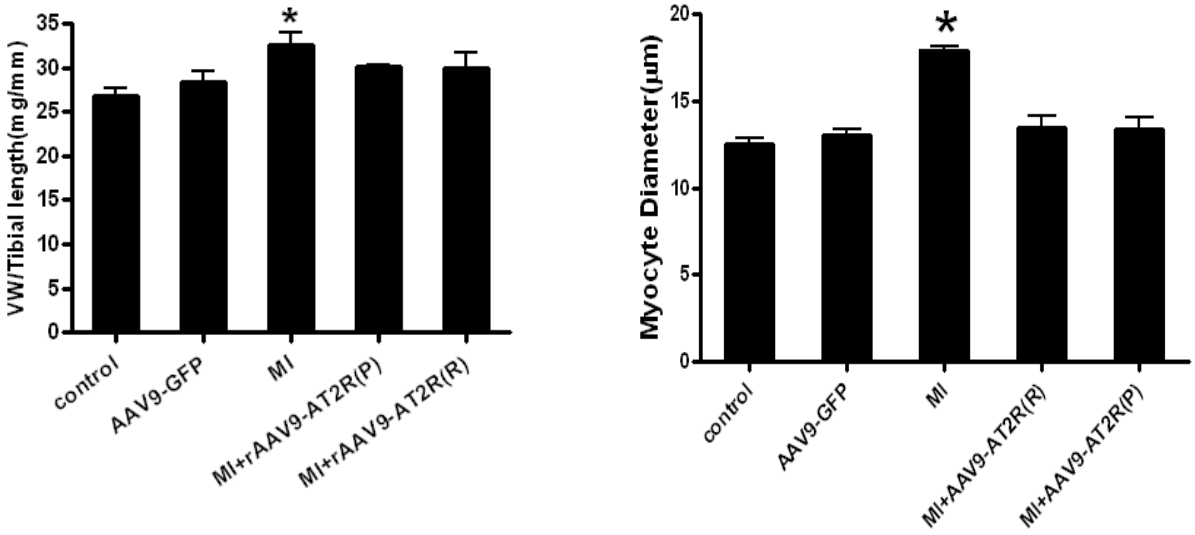

Figure 4-4. Effects of AT2R on ventricular hypertrophy. Overexpression of AT2R attenuates MI induced cardiac hypertrophy as evaluated by the ventricular weight(g) to tibia length(mm) ratio (A) and measurement of myocyte diameter (B). (* MI vs. control, rAAV9-GFP, MI+rAVV9-AT2R(R), and MI + rAAV9-AT2R(P); N=4-6/group).

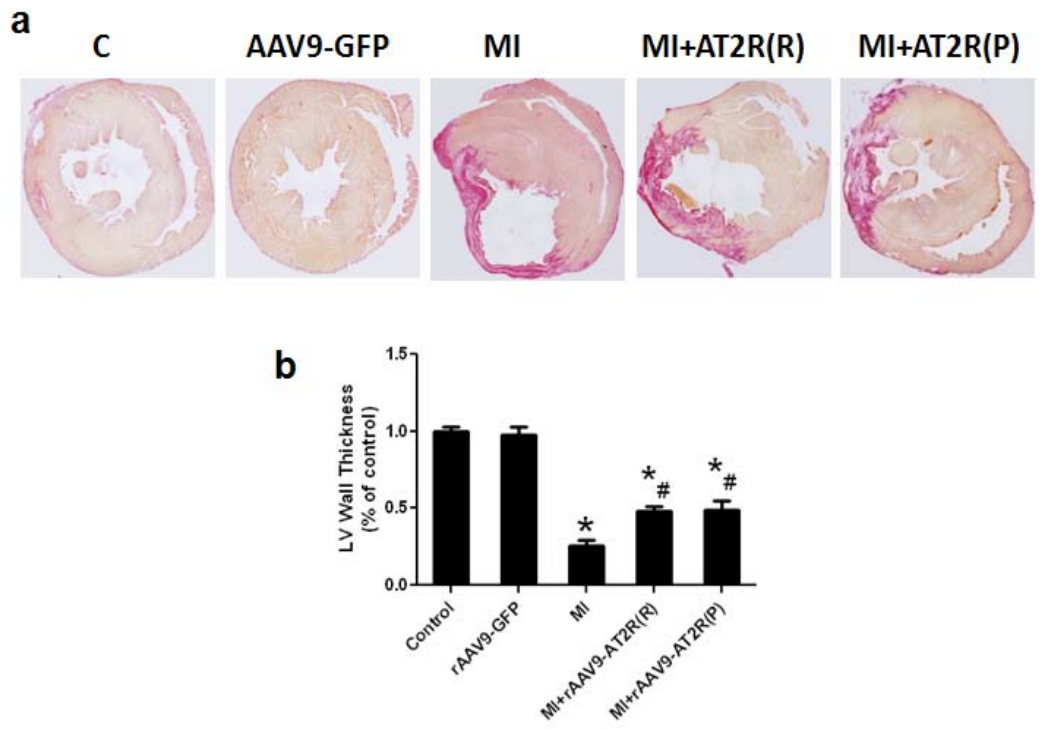

Figure 4-5. Effect of AT2R gene transfer on ventricular fibrosis and left ventricular wall thinning. (a) Picro-Sirius Red staining shows anterior wall thinning and collagen deposition in MI, MI+AT2R(R), and MI+AT2R(P) animals. (b) Quantification of the left ventricular wall thickness as a percentage of left ventricular wall thickness of control. MI significantly decreased left ventricular wall thickness. In both reversal and prevention studies, overexpression of AT2R attenuated the thinning of the left ventricular wall. * MI, MI+rAAV9-AT2R(R), MI+rAAV9-AT2R(P) vs control and rAAV9-GFP; # MI+rAAV9-AT2R(R) and MI+rAAV9-AT2R(P) vs MI, N=4-6/group.

Table 4-2. Quantitative Real Time PCR data

	Control (N=6)	rAAV9-GFP (N=4)	MI (N=6)	MI+rAAV9-AT2R(R) (N=5)	MI+rAAV9-AT2R(P) (N=4)
AT1R	0.42 ± 0.16	0.42 ± 0.08	1.78 ± 0.22&	0.63 ±0.18	0.65 ± 0.17
TGF-β	0.47 ± 0.15	0.29 ± 0.08	1.19 ± 0.23 &	0.70 ± 0.08	0.43 ± 0.04
ACE	1.19 ± 0.35	1.31 ± 0.19	3.00 ± 0.36 &	1.15 ± 0.22	1.23 ± 0.15
COLI	0.22 ± 0.10	0.13 ± 0.06	1.31 ± 0.39 &	0.53 ± 0.15	0.44 ± 0.04
COLIII	0.32 ± 0.09	0.32 ± 0.08	0.95 ± 0.08 &	0.39 ± 0.03	0.46 ± 0.15
BKR	0.23 ± 0.07	0.45 ± 0.08	0.28 ± 0.03	0.99 ± 0.28 **	1.16 ± 0.08 **
ACE2	0.76 ± 0.03	0.75 ± 0.13	0.43 ± 0.21	0.82 ± 0.21	1.11 ± 0.22
MAS	0.30 ± 0.08	0.41 ± 0.07	0.34 ± 0.07	0.88 ± 0.16 **	1.03 ± 0.23 **

& MI vs all other groups; ** MI+rAAV9-AT2R(R) and MI+rAAV9-AT2R(P) vs control, rAVV9-GFP, and MI ; $ MI+rAAV9-AT2R(R) vs control ; # MI+rAAV9-AT2R(P) vs all other groups. Data are depicted as mRNA fold changes relative to GAPDH mRNA calculated using the expression $2^{\wedge -\Delta\Delta Ct}$ and expressed as a mean fold change ±SEM.

CHAPTER 5
OVERALL DISCUSSIONS AND CONCLUSIONS

Results presented here indicate that Ang-(1-7) and AT2R play a cardioprotective role in cardiovascular diseases and these components of the RAS may interact with each other to contribute to the observed cardioprotective effects. (1) Lentiviral-mediated Ang-(1-7) overexpression preserves cardiac function and prevents the development of cardiac hypertrophy in a rat myocardial infarction model. (2) rAAV9 mediates efficient cardiac-selective transgene expression in the rat heart compared to other recombinant adeno associated viral vectors. (3) rAAV9-AT2R viral vectors mediate cardiac-selective AT2R overexpression in the heart when the viral vector were administered before CAL (prevention study) and at the time of CAL (the reversal study). Cardiac-selective overexpression AT2R preserves cardiac function and prevents the development of cardiac remodeling in rat myocardial infarction model, for both the reversal study and the prevention study.

1. Ang-(1-7) Preserves Cardiac Function and Prevents Cardiac Remodeling

The study presented in Chapter 2 provides strong evidence that Ang-(1-7) overexpression preserves the cardiac function and attenuates ventricular remodeling in a rat model of coronary occlusion. Overexpression of Ang-(1-7) provides significant protection against left ventricular dysfunction caused by myocardial infarction, as shown by preservation of ejection fraction and restoration of dp/dt, and left ventricular end diastolic pressure. Moreover, ventricular hypertrophy, and wall thinning tended to be attenuated by overexpression of Ang-(1-7). Ang-(1-7) overexpression appears to restore the balance between the ACE-AngII-AT1R axis and the ACE2-AngII-Mas of the RAS, by preventing the upregulation of ACE and AT1R following the MI, and increasing ACE2

and Mas receptor expression that were downregulated by myocardial infarction. Ang-(1-7) also resulted in an upregulation of the bradykinin B2 receptor in the heart. The finding that Ang-(1-7) treatment increased AT2R level in the rat neonatal cardiac myocyte cells after hypoxia exposure suggests evidence for possible interaction between ACE2, Ang-(1-7), and AT2R. These observations parallel the association between ACE2 activity and AT2R levels in hearts of pulmonary hypertension patients reported by Zisman (2004).

The possible pitfalls and future experiments for the Ang-(1-7) project

1) It is quite difficult to achieve therapeutic concentration by infusion Ang-(1-7) peptide into the body; because of the short half-life for Ang-(1-7). Using viral vector to deliver Ang-(1-7) can elevate Ang-(1-7) level to reach therapeutic concentrations. It would be better to deliver the viral vector containing Ang-(1-7) to patients at high risk of heart attack prior to the occurance of a heart attack. The ideal viral vector would mediate conditional and cardiac-selective overexpression of therapeutic gene. Lenti-viral vector containing Ang-(1-7) was the only availabe resource to use when I started this project. **2)** A-779 is a potent and selective Ang-(1-7) antagonist. A-779 antagonizes several action of Ang-(1-7). The cardioprotective effects of Ang-(1-7) could be further confirmed by using its antagonist (A779) to block the protective effects in the myocardial infarction model. Two more groups should be added to this project: one group using only A779 and the other group using A779 and Lenti-Ang-(1-7) to overexpress Ang-(1-7) at same time. **3)** In the current project, possible mediators for the cardioprotective effects of Ang-(1-7) are only examined by measuring changes in mRNA level. ACE2 activity and protein level for ACE2, Mas, AT1R, AT2R, and BKR should be examined to

further confirm the changes observed by Realtime PCR. **4)** Cardiac functions are examined by echocardiography and catheterization. Both of these two techniques have their drawbacks. Echocardiography is a noninvasive examination technique, but it requires experienced technician to perform and it has large marginal error for the examination of cardiac function. Catheterization is done by inserting a needle into left ventricle chamber to measure the pressure changes. It is an invasive method and the needle puncture itself could induce changes in cardiac functions. Magnetic resonance imaging (MRI) is a noninvasive technique to examine the structure and function of the heart. MRI provides better spatial resolution than echocardiography. **5)** Ang-(1-7) level in the serum and in the heart tissue are not measured. The future experiments will be designed to quantify the Ang-(1-7) level in the heart tissues and serum by a commercially available ELISA kit.

2. rAAV9 Mediated Cardiac Selective Transgene Expression In The Rat Heart

The study presented in Chapter 3 provides strong evidence that rAAV9 produces the most selective and stable transduction efficiency in cardiac tissue, and this expression was primarily exhibited in the cardiac myocytes. This study was to compare five serotypes of rAAV with respect to gene transfer efficiency and relative cardiac tropisms using both an *in vitro* approach and a relatively non-invasive *in vivo* approach (administering the rAAV into left cardiac ventricular cavity of rat neonatal pups). The ultimate goal of this study was to develop a clinically relevant gene therapy approach for specifically targeting the heart with rAAV, without the use of a tissue specific promoter.

The possible pitfalls and future experiments for the rAAVs project

1) The viral vector administration method in this project is relatively non-invasive. It requires experienced hands to perform the injection into the the heart. **2)** In this rAAVs project, a time course study was not performed to determine when the viral vectors first start to mediate transgene expression and how long the transduction would last. Also, transduction efficiency *in vivo* was examined by histological methods after the animal were sacrificed. There is an *in vivo* live imaging system available at the University of Florida. Live imaging system could provide time-course information for the viral vector transduction in the animals.

3. AT2R Preserves Cardiac Function and Attenuates Cardiac Remodeling

In Chapter 3, rAAV9 was confirmed to mediate more cardiac-selective transgene expression than rAAV2, 5, 7, and 8. Chapter 4 provided strong evidence that the cardiac selective overexpression AT2R mediated by rAAV9 vector can preserve cardiac function and attenuate ventricular remodeling in a rat coronary artery ligation model. Cardiac selective overexpression of AT2R provides significant protection against left ventricular dysfunction caused by myocardial infarction, as shown by preserving ejection fraction and dp/dt, and reducing left ventricular end diastolic pressure back to control levels. Moreover, ventricular hypertrophy and wall thinning tended to decreased with cardiac-selective overexpression of AT2R. Overexpression of the AT2R significantly upregulated the bradykinin B2 receptor and Mas receptor level in both the prevention and reversal studies. Overexpression of AT2R also prevented the MI-induced gene upregulation, including AT1R, TGF-β, ACE, Collagen I, and Collagen III. All these results support the hypothesis that AT2R exerts its protective effects on the

heart following a MI, by antagonizing ACE-AngII-AT1R axis and by increasing the ACE2-Ang-(1-7)-Mas axis and restoring balance in the RAS.

The possible pitfalls and future experiments for the AT2R project

1) AT2R protein level was not examined by western-blot because there was no good and commercially available AT2R antibody to use. Also the change in AT2R levels following myocardial infarction was not examined in a time course manner. It has been reported that AT2R expression is upregulated following myocardial infarction. No one has performed any experiments to trace the change for AT2R expression; or how long the re-expresssion or upregulation would last. **2)** The cardioprotective effects of AT2R could be further confirmed by using an AT2R selective antagonist (PD123319). Two more groups should be added to this project: one group using only PD123319 and the other group using PD123319 and rAAV9-AT2R to overexpress AT2R at same time. **3)** In the current project, possible mediators for the cardioprotective effects of AT2R were only examined by measuring changes in mRNA level. Cytokine levels, ACE2 activity and protein level for ACE2, Mas, AT1R, and BKR should be examined by ELISA, activity assay or western-blot to further confirm the changes observed by Realtime PCR. **4)** Cardiac functions would be better evaluated by MRI as stated above. **4)** The effects of AT2R overexpression in the rat neonatal cardiac myocytes (RNCM) were tested (data not shown in this chapter). Overexpression of AT2R mediated by adeno viral vector induced apotosis in RNCM. To study this AT2R induced apoptosis in RNCM, a range of AT2R expression (from low expression to high expression) in RNCM was done. Expression of AT2R at low level in RNCM did not induce apoptosis. But

experiments desgined to test the protective effects of AT2R at low expression level in the hypoxic RNCM still needs to be determined.

LIST OF REFERENCES

Effect of ramipril on mortality and morbidity of survivors of acute myocardial infarction with clinical evidence of heart failure. The Acute Infarction Ramipril Efficacy (AIRE) Study Investigators. 1993. Lancet 342, 821-828.

Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. The SOLVD Investigators. 1991. N. Engl. J. Med. 325, 293-302.

Akache, B., Grimm, D., Pandey, K., Yant, S.R., Xu, H., Kay, M.A., 2006. The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. J. Virol. 80, 9831-9836 doi: 10.1128/JVI.00878-06.

Alexander, L.D., Alagarsamy, S., Douglas, J.G., 2004. Cyclic stretch-induced cPLA2 mediates ERK 1/2 signaling in rabbit proximal tubule cells. Kidney Int. 65, 551-563 doi: 10.1111/j.1523-1755.2004.00405.x.

Anavekar, N.S., Solomon, S.D., 2005. Angiotensin II receptor blockade and ventricular remodelling. J. Renin Angiotensin Aldosterone Syst. 6, 43-48 doi: 10.3317/jraas.2005.006.

Angelis, E., Tse, M.Y., Adams, M.A., Pang, S.C., 2006. Effect of AT2 blockade on cardiac hypertrophy as induced by high dietary salt in the proatrial natriuretic peptide (ANP) gene-disrupted mouse. Can. J. Physiol. Pharmacol. 84, 625-634 doi: 10.1139/y06-016.

Arruda, V.R., Stedman, H.H., Nichols, T.C., Haskins, M.E., Nicholson, M., Herzog, R.W., Couto, L.B., High, K.A., 2005. Regional intravascular delivery of AAV-2-F.IX to skeletal muscle achieves long-term correction of hemophilia B in a large animal model. Blood 105, 3458-3464 doi: 10.1182/blood-2004-07-2908.

Baker, K.M., Chernin, M.I., Schreiber, T., Sanghi, S., Haiderzaidi, S., Booz, G.W., Dostal, D.E., Kumar, R., 2004. Evidence of a novel intracrine mechanism in angiotensin II-induced cardiac hypertrophy. Regul. Pept. 120, 5-13 doi: 10.1016/j.regpep.2004.04.004.

Barber, M.N., Sampey, D.B., Widdop, R.E., 1999. AT(2) receptor stimulation enhances antihypertensive effect of AT(1) receptor antagonist in hypertensive rats. Hypertension 34, 1112-1116.

Basso, N., Terragno, N.A., 2001. History about the discovery of the renin-angiotensin system. Hypertension 38, 1246-1249.

Bautista, R., Sanchez, A., Hernandez, J., Oyekan, A., Escalante, B., 2001. Angiotensin II type AT(2) receptor mRNA expression and renal vasodilatation are increased in renal failure. Hypertension 38, 669-673.

Bedecs, K., Elbaz, N., Sutren, M., Masson, M., Susini, C., Strosberg, A.D., Nahmias, C., 1997. Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase. Biochem. J. 325 (Pt 2), 449-454.

Bekeredjian, R., Shohet, R.V., 2004. Cardiovascular gene therapy: angiogenesis and beyond. Am. J. Med. Sci. 327, 139-148.

Bish, L.T., Morine, K., Sleeper, M.M., Sanmiguel, J., Wu, D., Gao, G., Wilson, J.M., Sweeney, L., 2008. AAV9 Provides Global Cardiac Gene Transfer Superior to AAV1, AAV6, AAV7, and AAV8 in the Mouse and Rat. Hum. Gene Ther. doi: 10.1089/hgt.2008.123.

Blais, C.,Jr, Lapointe, N., Rouleau, J.L., Clement, R., Bachvarov, D.R., Adam, A., 2002. Effects of captopril and omapatrilat on early post-myocardial infarction survival and cardiac hemodynamics in rats: interaction with cardiac cytokine expression. Can. J. Physiol. Pharmacol. 80, 48-58.

Bozkurt, B., Kribbs, S.B., Clubb, F.J.,Jr, Michael, L.H., Didenko, V.V., Hornsby, P.J., Seta, Y., Oral, H., Spinale, F.G., Mann, D.L., 1998. Pathophysiologically relevant concentrations of tumor necrosis factor-alpha promote progressive left ventricular dysfunction and remodeling in rats. Circulation 97, 1382-1391.

Brede, M., Roell, W., Ritter, O., Wiesmann, F., Jahns, R., Haase, A., Fleischmann, B.K., Hein, L., 2003. Cardiac hypertrophy is associated with decreased eNOS expression in angiotensin AT2 receptor-deficient mice. Hypertension 42, 1177-1182 doi: 10.1161/01.HYP.0000100445.80029.8E.

Brilla, C.G., Janicki, J.S., Weber, K.T., 1991. Impaired diastolic function and coronary reserve in genetic hypertension. Role of interstitial fibrosis and medial thickening of intramyocardial coronary arteries. Circ. Res. 69, 107-115.

Brogelli, L., Parenti, A., Ledda, F., 2002. Inhibition of vascular smooth muscle cell growth by angiotensin type 2 receptor stimulation for in vitro organ culture model. J. Cardiovasc. Pharmacol. 39, 739-745.

Bryant, D., Becker, L., Richardson, J., Shelton, J., Franco, F., Peshock, R., Thompson, M., Giroir, B., 1998. Cardiac failure in transgenic mice with myocardial expression of tumor necrosis factor-alpha. Circulation 97, 1375-1381.

Buisson, B., Bottari, S.P., de Gasparo, M., Gallo-Payet, N., Payet, M.D., 1992. The angiotensin AT2 receptor modulates T-type calcium current in non-differentiated NG108-15 cells. FEBS Lett. 309, 161-164.

Bujak, M., Dobaczewski, M., Chatila, K., Mendoza, L.H., Li, N., Reddy, A., Frangogiannis, N.G., 2008. Interleukin-1 receptor type I signaling critically regulates infarct healing and cardiac remodeling. Am. J. Pathol. 173, 57-67 doi: 10.2353/ajpath.2008.070974.

Cardini, J.F., Santos, R.A., Martins, A.S., Machado, R.P., Alzamora, F., 1988. Site of entry of kininase II into renal tubular fluid. Hypertension 11, I66-8.

Carey, R.M., Siragy, H.M., 2003. Newly recognized components of the renin-angiotensin system: potential roles in cardiovascular and renal regulation. Endocr. Rev. 24, 261-271.

Carey, R.M., Wang, Z.Q., Siragy, H.M., 2000. Role of the angiotensin type 2 receptor in the regulation of blood pressure and renal function. Hypertension 35, 155-163.

Castro, C.H., Santos, R.A., Ferreira, A.J., Bader, M., Alenina, N., Almeida, A.P., 2006. Effects of genetic deletion of angiotensin-(1-7) receptor Mas on cardiac function during ischemia/reperfusion in the isolated perfused mouse heart. Life Sci. 80, 264-268 doi: 10.1016/j.lfs.2006.09.007.

Castro, C.H., Santos, R.A., Ferreira, A.J., Bader, M., Alenina, N., Almeida, A.P., 2005. Evidence for a functional interaction of the angiotensin-(1-7) receptor Mas with AT1 and AT2 receptors in the mouse heart. Hypertension 46, 937-942 doi: 10.1161/01.HYP.0000175813.04375.8a.

Chang, R.S., Lotti, V.J., 1991. Angiotensin receptor subtypes in rat, rabbit and monkey tissues: relative distribution and species dependency. Life Sci. 49, 1485-1490.

Chu, D., Sullivan, C.C., Weitzman, M.D., Du, L., Wolf, P.L., Jamieson, S.W., Thistlethwaite, P.A., 2003. Direct comparison of efficiency and stability of gene transfer into the mammalian heart using adeno-associated virus versus adenovirus vectors. J. Thorac. Cardiovasc. Surg. 126, 671-679.

Cintron, G., Johnson, G., Francis, G., Cobb, F., Cohn, J.N., 1993. Prognostic significance of serial changes in left ventricular ejection fraction in patients with congestive heart failure. The V-HeFT VA Cooperative Studies Group. Circulation 87, VI17-23.

Clark, M.A., Tallant, E.A., Diz, D.I., 2001. Downregulation of the AT1A receptor by pharmacologic concentrations of Angiotensin-(1-7). J. Cardiovasc. Pharmacol. 37, 437-448.

Cohn, J.N., Ferrari, R., Sharpe, N., 2000. Cardiac remodeling--concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling. J. Am. Coll. Cardiol. 35, 569-582.

Cohn, J.N., Johnson, G., Ziesche, S., Cobb, F., Francis, G., Tristani, F., Smith, R., Dunkman, W.B., Loeb, H., Wong, M., 1991. A comparison of enalapril with hydralazine-isosorbide dinitrate in the treatment of chronic congestive heart failure. N. Engl. J. Med. 325, 303-310.

Coleman, J.E., Huentelman, M.J., Kasparov, S., Metcalfe, B.L., Paton, J.F., Katovich, M.J., Semple-Rowland, S.L., Raizada, M.K., 2003. Efficient large-scale production and concentration of HIV-1-based lentiviral vectors for use in vivo. Physiol. Genomics 12, 221-228 doi: 10.1152/physiolgenomics.00135.2002.

D'Amore, A., Black, M.J., Thomas, W.G., 2005. The angiotensin II type 2 receptor causes constitutive growth of cardiomyocytes and does not antagonize angiotensin II type 1 receptor-mediated hypertrophy. Hypertension 46, 1347-1354 doi: 10.1161/01.HYP.0000193504.51489.cf.

de Gasparo, M., Catt, K.J., Inagami, T., Wright, J.W., Unger, T., 2000. International union of pharmacology. XXIII. The angiotensin II receptors. Pharmacol. Rev. 52, 415-472.

De Mello, W.C., 2004. Angiotensin (1-7) re-establishes impulse conduction in cardiac muscle during ischaemia-reperfusion. The role of the sodium pump. J. Renin Angiotensin Aldosterone Syst. 5, 203-208 doi: 10.3317/jraas.2004.041.

Der Sarkissian, S., Grobe, J.L., Yuan, L., Narielwala, D.R., Walter, G.A., Katovich, M.J., Raizada, M.K., 2008. Cardiac overexpression of angiotensin converting enzyme 2 protects the heart from ischemia-induced pathophysiology. Hypertension 51, 712-718 doi: 10.1161/HYPERTENSIONAHA.107.100693.

Deten, A., Holzl, A., Leicht, M., Barth, W., Zimmer, H.G., 2001. Changes in extracellular matrix and in transforming growth factor beta isoforms after coronary artery ligation in rats. J. Mol. Cell. Cardiol. 33, 1191-1207 doi: 10.1006/jmcc.2001.1383.

Di Pasquale, G., Davidson, B.L., Stein, C.S., Martins, I., Scudiero, D., Monks, A., Chiorini, J.A., 2003. Identification of PDGFR as a receptor for AAV-5 transduction. Nat. Med. 9, 1306-1312 doi: 10.1038/nm929.

Dimmeler, S., Rippmann, V., Weiland, U., Haendeler, J., Zeiher, A.M., 1997. Angiotensin II induces apoptosis of human endothelial cells. Protective effect of nitric oxide. Circ. Res. 81, 970-976.

Dobruch, J., Paczwa, P., Lon, S., Khosla, M.C., Szczepanska-Sadowska, E., 2003. Hypotensive function of the brain angiotensin-(1-7) in Sprague Dawley and renin transgenic rats. J. Physiol. Pharmacol. 54, 371-381.

Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N., Donovan, M., Woolf, B., Robison, K., Jeyaseelan, R., Breitbart, R.E., Acton, S., 2000. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circ. Res. 87, E1-9.

Du, L., Kido, M., Lee, D.V., Rabinowitz, J.E., Samulski, R.J., Jamieson, S.W., Weitzman, M.D., Thistlethwaite, P.A., 2004. Differential myocardial gene delivery by recombinant serotype-specific adeno-associated viral vectors. Mol. Ther. 10, 604-608 doi: 10.1016/j.ymthe.2004.06.110.

Duan, D., Yan, Z., Yue, Y., Ding, W., Engelhardt, J.F., 2001. Enhancement of muscle gene delivery with pseudotyped adeno-associated virus type 5 correlates with myoblast differentiation. J. Virol. 75, 7662-7671 doi: 10.1128/JVI.75.16.7662-7671.2001.

Dudley, D.T., Hubbell, S.E., Summerfelt, R.M., 1991. Characterization of angiotensin II (AT2) binding sites in R3T3 cells. Mol. Pharmacol. 40, 360-367.

Dulin, N.O., Alexander, L.D., Harwalkar, S., Falck, J.R., Douglas, J.G., 1998. Phospholipase A2-mediated activation of mitogen-activated protein kinase by angiotensin II. Proc. Natl. Acad. Sci. U. S. A. 95, 8098-8102.

Falcon, B.L., Stewart, J.M., Bourassa, E., Katovich, M.J., Walter, G., Speth, R.C., Sumners, C., Raizada, M.K., 2004. Angiotensin II type 2 receptor gene transfer elicits cardioprotective effects in an angiotensin II infusion rat model of hypertension. Physiol. Genomics 19, 255-261 doi: 10.1152/physiolgenomics.00170.2004.

Fechner, H., Sipo, I., Westermann, D., Pinkert, S., Wang, X., Suckau, L., Kurreck, J., Zeichhardt, H., Muller, O., Vetter, R., Erdmann, V., Tschope, C., Poller, W., 2008. Cardiac-targeted RNA interference mediated by an AAV9 vector improves cardiac function in coxsackievirus B3 cardiomyopathy. J. Mol. Med. 86, 987-997 doi: 10.1007/s00109-008-0363-x.

Ferrario, C.M., Jessup, J., Chappell, M.C., Averill, D.B., Brosnihan, K.B., Tallant, E.A., Diz, D.I., Gallagher, P.E., 2005. Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. Circulation 111, 2605-2610 doi: 10.1161/CIRCULATIONAHA.104.510461.

Ferrario, C.M., Chappell, M.C., Tallant, E.A., Brosnihan, K.B., Diz, D.I., 1997. Counterregulatory actions of angiotensin-(1-7). Hypertension 30, 535-541.

Ferreira, A.J., Jacoby, B.A., Araujo, C.A., Macedo, F.A., Silva, G.A., Almeida, A.P., Caliari, M.V., Santos, R.A., 2007. The nonpeptide angiotensin-(1-7) receptor Mas agonist AVE-0991 attenuates heart failure induced by myocardial infarction. Am. J. Physiol. Heart Circ. Physiol. 292, H1113-9 doi: 10.1152/ajpheart.00828.2006.

Ferreira, A.J., Santos, R.A., Almeida, A.P., 2001. Angiotensin-(1-7): cardioprotective effect in myocardial ischemia/reperfusion. Hypertension 38, 665-668.

Ford, W.R., Clanachan, A.S., Jugdutt, B.I., 2000. Characterization of cardioprotection mediated by AT2 receptor antagonism after ischemia-reperfusion in isolated working rat hearts. J. Cardiovasc. Pharmacol. Ther. 5, 211-221.

Fraga-Silva, R.A., Pinheiro, S.V., Goncalves, A.C., Alenina, N., Bader, M., Santos, R.A., 2008. The antithrombotic effect of angiotensin-(1-7) involves mas-mediated NO release from platelets. Mol. Med. 14, 28-35 doi: 10.2119/2007-00073.Fraga-Silva.

Francis, G.S., Benedict, C., Johnstone, D.E., Kirlin, P.C., Nicklas, J., Liang, C.S., Kubo, S.H., Rudin-Toretsky, E., Yusuf, S., 1990. Comparison of neuroendocrine activation in patients with left ventricular dysfunction with and without congestive heart failure. A substudy of the Studies of Left Ventricular Dysfunction (SOLVD). Circulation 82, 1724-1729.

Frangogiannis, N.G., Mendoza, L.H., Lindsey, M.L., Ballantyne, C.M., Michael, L.H., Smith, C.W., Entman, M.L., 2000. IL-10 is induced in the reperfused myocardium and may modulate the reaction to injury. J. Immunol. 165, 2798-2808.

Frantz, S., Hu, K., Adamek, A., Wolf, J., Sallam, A., Maier, S.K., Lonning, S., Ling, H., Ertl, G., Bauersachs, J., 2008. Transforming growth factor beta inhibition increases mortality and left ventricular dilatation after myocardial infarction. Basic Res. Cardiol. 103, 485-492 doi: 10.1007/s00395-008-0739-7.

Gallinat, S., Busche, S., Schutze, S., Kronke, M., Unger, T., 1999. AT2 receptor stimulation induces generation of ceramides in PC12W cells. FEBS Lett. 443, 75-79.

Gallinat, S., Yu, M., Dorst, A., Unger, T., Herdegen, T., 1998. Sciatic nerve transection evokes lasting up-regulation of angiotensin AT2 and AT1 receptor mRNA in adult rat dorsal root ganglia and sciatic nerves. Brain Res. Mol. Brain Res. 57, 111-122.

Giani, J.F., Gironacci, M.M., Munoz, M.C., Pena, C., Turyn, D., Dominici, F.P., 2007. Angiotensin-(1 7) stimulates the phosphorylation of JAK2, IRS-1 and Akt in rat heart in vivo: role of the AT1 and Mas receptors. Am. J. Physiol. Heart Circ. Physiol. 293, H1154-63 doi: 10.1152/ajpheart.01395.2006.

Gironacci, M.M., Coba, M.P., Pena, C., 1999. Angiotensin-(1-7) binds at the type 1 angiotensin II receptors in rat renal cortex. Regul. Pept. 84, 51-54.

Gohlke, P., Pees, C., Unger, T., 1998. AT2 receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism. Hypertension 31, 349-355.

Grady, E.F., Sechi, L.A., Griffin, C.A., Schambelan, M., Kalinyak, J.E., 1991. Expression of AT2 receptors in the developing rat fetus. J. Clin. Invest. 88, 921-933 doi: 10.1172/JCI115395.

Granger, C.B., McMurray, J.J., Yusuf, S., Held, P., Michelson, E.L., Olofsson, B., Ostergren, J., Pfeffer, M.A., Swedberg, K., CHARM Investigators and Committees, 2003. Effects of candesartan in patients with chronic heart failure and reduced left-ventricular systolic function intolerant to angiotensin-converting-enzyme inhibitors: the CHARM-Alternative trial. Lancet 362, 772-776 doi: 10.1016/S0140-6736(03)14284-5.

Greenberg, B., Quinones, M.A., Koilpillai, C., Limacher, M., Shindler, D., Benedict, C., Shelton, B., 1995. Effects of long-term enalapril therapy on cardiac structure and function in patients with left ventricular dysfunction. Results of the SOLVD echocardiography substudy. Circulation 91, 2573-2581.

Grobe, J.L., Der Sarkissian, S., Stewart, J.M., Meszaros, J.G., Raizada, M.K., Katovich, M.J., 2007a. ACE2 overexpression inhibits hypoxia-induced collagen production by cardiac fibroblasts. Clin. Sci. (Lond) 113, 357-364 doi: 10.1042/CS20070160.

Grobe, J.L., Mecca, A.P., Lingis, M., Shenoy, V., Bolton, T.A., Machado, J.M., Speth, R.C., Raizada, M.K., Katovich, M.J., 2007b. Prevention of angiotensin II-induced cardiac remodeling by angiotensin-(1-7). Am. J. Physiol. Heart Circ. Physiol. 292, H736-42 doi: 10.1152/ajpheart.00937.2006.

Grobe, J.L., Mecca, A.P., Lingis, M., Shenoy, V., Bolton, T.A., Machado, J.M., Speth, R.C., Raizada, M.K., Katovich, M.J., 2007c. Prevention of angiotensin II-induced cardiac remodeling by angiotensin-(1-7). Am. J. Physiol. Heart Circ. Physiol. 292, H736-42 doi: 10.1152/ajpheart.00937.2006.

Grobe, J.L., Mecca, A.P., Mao, H., Katovich, M.J., 2006. Chronic angiotensin-(1-7) prevents cardiac fibrosis in DOCA-salt model of hypertension. Am. J. Physiol. Heart Circ. Physiol. 290, H2417-23 doi: 10.1152/ajpheart.01170.2005.

Guillen, I., Blanes, M., Gomez-Lechon, M.J., Castell, J.V., 1995. Cytokine signaling during myocardial infarction: sequential appearance of IL-1 beta and IL-6. Am. J. Physiol. 269, R229-35.

Haller, H., Lindschau, C., Erdmann, B., Quass, P., Luft, F.C., 1996. Effects of intracellular angiotensin II in vascular smooth muscle cells. Circ. Res. 79, 765-772.

Han, R.O., Ray, P.E., Baughman, K.L., Feldman, A.M., 1991. Detection of interleukin and interleukin-receptor mRNA in human heart by polymerase chain reaction. Biochem. Biophys. Res. Commun. 181, 520-523.

Hasdai, D., Scheinowitz, M., Leibovitz, E., Sclarovsky, S., Eldar, M., Barak, V., 1996. Increased serum concentrations of interleukin-1 beta in patients with coronary artery disease. Heart 76, 24-28.

He, J., Ogden, L.G., Bazzano, L.A., Vupputuri, S., Loria, C., Whelton, P.K., 2001. Risk factors for congestive heart failure in US men and women: NHANES I epidemiologic follow-up study. Arch. Intern. Med. 161, 996-1002.

Hein, L., Barsh, G.S., Pratt, R.E., Dzau, V.J., Kobilka, B.K., 1995. Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. Nature 377, 744-747 doi: 10.1038/377744a0.

Hoffmann, A., Cool, D.R., 2003. Angiotensin II receptor types 1A, 1B, and 2 in murine neuroblastoma Neuro-2a cells. J. Recept. Signal Transduct. Res. 23, 111-121 doi: 10.1081/RRS-120018764.

Horiuchi, M., Akishita, M., Dzau, V.J., 1999. Recent progress in angiotensin II type 2 receptor research in the cardiovascular system. Hypertension 33, 613-621.

Huang, X.C., Richards, E.M., Sumners, C., 1996. Mitogen-activated protein kinases in rat brain neuronal cultures are activated by angiotensin II type 1 receptors and inhibited by angiotensin II type 2 receptors. J. Biol. Chem. 271, 15635-15641.

Ichihara, A., Hayashi, M., Hirota, N., Okada, H., Koura, Y., Tada, Y., Kaneshiro, Y., Tsuganezawa, H., Saruta, T., 2003. Angiotensin II type 2 receptor inhibits prorenin processing in juxtaglomerular cells. Hypertens. Res. 26, 915-921.

Ichiki, T., Labosky, P.A., Shiota, C., Okuyama, S., Imagawa, Y., Fogo, A., Niimura, F., Ichikawa, I., Hogan, B.L., Inagami, T., 1995. Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. Nature 377, 748-750 doi: 10.1038/377748a0.

Inagaki, K., Fuess, S., Storm, T.A., Gibson, G.A., Mctiernan, C.F., Kay, M.A., Nakai, H., 2006. Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. Mol. Ther. 14, 45-53 doi: 10.1016/j.ymthe.2006.03.014.

Ishiyama, Y., Gallagher, P.E., Averill, D.B., Tallant, E.A., Brosnihan, K.B., Ferrario, C.M., 2004a. Upregulation of angiotensin-converting enzyme 2 after myocardial infarction by blockade of angiotensin II receptors. Hypertension 43, 970-976 doi: 10.1161/01.HYP.0000124667.34652.1a.

Ishiyama, Y., Gallagher, P.E., Averill, D.B., Tallant, E.A., Brosnihan, K.B., Ferrario, C.M., 2004b. Upregulation of angiotensin-converting enzyme 2 after myocardial infarction by blockade of angiotensin II receptors. Hypertension 43, 970-976 doi: 10.1161/01.HYP.0000124667.34652.1a.

Iusuf, D., Henning, R.H., van Gilst, W.H., Roks, A.J., 2008. Angiotensin-(1-7): pharmacological properties and pharmacotherapeutic perspectives. Eur. J. Pharmacol. 585, 303-312 doi: 10.1016/j.ejphar.2008.02.090.

Iwata, M., Cowling, R.T., Gurantz, D., Moore, C., Zhang, S., Yuan, J.X., Greenberg, B.H., 2005. Angiotensin-(1-7) binds to specific receptors on cardiac fibroblasts to initiate antifibrotic and antitrophic effects. Am. J. Physiol. Heart Circ. Physiol. 289, H2356-63 doi: 10.1152/ajpheart.00317.2005.

Jaiswal, N., Tallant, E.A., Jaiswal, R.K., Diz, D.I., Ferrario, C.M., 1993. Differential regulation of prostaglandin synthesis by angiotensin peptides in porcine aortic smooth muscle cells: subtypes of angiotensin receptors involved. J. Pharmacol. Exp. Ther. 265, 664-673.

Jaiswal, N., Diz, D.I., Chappell, M.C., Khosla, M.C., Ferrario, C.M., 1992. Stimulation of endothelial cell prostaglandin production by angiotensin peptides. Characterization of receptors. Hypertension 19, II49-55.

Jalil, J.E., Janicki, J.S., Pick, R., Weber, K.T., 1991. Coronary vascular remodeling and myocardial fibrosis in the rat with renovascular hypertension. Response to captopril. Am. J. Hypertens. 4, 51-55.

Ju, H., Zhao, S., Jassal, D.S., Dixon, I.M., 1997. Effect of AT1 receptor blockade on cardiac collagen remodeling after myocardial infarction. Cardiovasc. Res. 35, 223-232.

Jugdutt, B.I., Menon, V., 2004. AT1 receptor blockade limits myocardial injury and upregulates AT2 receptors during reperfused myocardial infarction. Mol. Cell. Biochem. 260, 111-118.

Kaludov, N., Brown, K.E., Walters, R.W., Zabner, J., Chiorini, J.A., 2001. Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. J. Virol. 75, 6884-6893 doi: 10.1128/JVI.75.15.6884-6893.2001.

Kaschina, E., Grzesiak, A., Li, J., Foryst-Ludwig, A., Timm, M., Rompe, F., Sommerfeld, M., Kemnitz, U.R., Curato, C., Namsolleck, P., Tschope, C., Hallberg, A., Alterman, M., Hucko, T., Paetsch, I., Dietrich, T., Schnackenburg, B., Graf, K., Dahlof, B., Kintscher, U., Unger, T., Steckelings, U.M., 2008. Angiotensin II type 2 receptor stimulation: a novel option of therapeutic interference with the renin-angiotensin system in myocardial infarction? Circulation 118, 2523-2532 doi: 10.1161/CIRCULATIONAHA.108.784868.

Katada, J., Meguro, T., Saito, H., Ohashi, A., Anzai, T., Ogawa, S., Yoshikawa, T., 2005. Persistent cardiac aldosterone synthesis in angiotensin II type 1A receptor-knockout mice after myocardial infarction. Circulation 111, 2157-2164 doi: 10.1161/01.CIR.0000163562.82134.8E.

Keidar, S., Kaplan, M., Gamliel-Lazarovich, A., 2007. ACE2 of the heart: From angiotensin I to angiotensin (1-7). Cardiovasc. Res. 73, 463-469 doi: 10.1016/j.cardiores.2006.09.006.

Kono, T., Taniguchi, A., Imura, H., Oseko, F., Khosla, M.C., 1986. Biological activities of angiotensin II-(1-6)-hexapeptide and angiotensin II-(1-7)-heptapeptide in man. Life Sci. 38, 1515-1519.

Konstam, M.A., Kronenberg, M.W., Rousseau, M.F., Udelson, J.E., Melin, J., Stewart, D., Dolan, N., Edens, T.R., Ahn, S., Kinan, D., 1993. Effects of the angiotensin converting enzyme inhibitor enalapril on the long-term progression of left ventricular dilatation in patients with asymptomatic systolic dysfunction. SOLVD (Studies of Left Ventricular Dysfunction) Investigators. Circulation 88, 2277-2283.

Konstam, M.A., Rousseau, M.F., Kronenberg, M.W., Udelson, J.E., Melin, J., Stewart, D., Dolan, N., Edens, T.R., Ahn, S., Kinan, D., 1992. Effects of the angiotensin converting enzyme inhibitor enalapril on the long-term progression of left ventricular dysfunction in patients with heart failure. SOLVD Investigators. Circulation 86, 431-438.

Kubota, T., McTiernan, C.F., Frye, C.S., Slawson, S.E., Lemster, B.H., Koretsky, A.P., Demetris, A.J., Feldman, A.M., 1997. Dilated cardiomyopathy in transgenic mice with cardiac-specific overexpression of tumor necrosis factor-alpha. Circ. Res. 81, 627-635.

Kucharewicz, I., Pawlak, R., Matys, T., Pawlak, D., Buczko, W., 2002. Antithrombotic effect of captopril and losartan is mediated by angiotensin-(1-7). Hypertension 40, 774-779.

Kurisu, S., Ozono, R., Oshima, T., Kambe, M., Ishida, T., Sugino, H., Matsuura, H., Chayama, K., Teranishi, Y., Iba, O., Amano, K., Matsubara, H., 2003. Cardiac angiotensin II type 2 receptor activates the kinin/NO system and inhibits fibrosis. Hypertension 41, 99-107.

Kuwahara, F., Kai, H., Tokuda, K., Kai, M., Takeshita, A., Egashira, K., Imaizumi, T., 2002. Transforming growth factor-beta function blocking prevents myocardial fibrosis and diastolic dysfunction in pressure-overloaded rats. Circulation 106, 130-135.

Lacraz, S., Nicod, L.P., Chicheportiche, R., Welgus, H.G., Dayer, J.M., 1995. IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. J. Clin. Invest. 96, 2304-2310 doi: 10.1172/JCI118286.

Lai, Z.C., Rushton, E., Bate, M., Rubin, G.M., 1993. Loss of function of the Drosophila zfh-1 gene results in abnormal development of mesodermally derived tissues. Proc. Natl. Acad. Sci. U. S. A. 90, 4122-4126.

le Tran, Y., Forster, C., 1997. Angiotensin-(1-7) and the rat aorta: modulation by the endothelium. J. Cardiovasc. Pharmacol. 30, 676-682.

Lefer, A.M., Ma, X.L., Weyrich, A.S., Scalia, R., 1993. Mechanism of the cardioprotective effect of transforming growth factor beta 1 in feline myocardial ischemia and reperfusion. Proc. Natl. Acad. Sci. U. S. A. 90, 1018-1022.

Lefer, A.M., Tsao, P., Aoki, N., Palladino, M.A.,Jr, 1990. Mediation of cardioprotection by transforming growth factor-beta. Science 249, 61-64.

Lehtonen, J.Y., Horiuchi, M., Daviet, L., Akishita, M., Dzau, V.J., 1999. Activation of the de novo biosynthesis of sphingolipids mediates angiotensin II type 2 receptor-induced apoptosis. J. Biol. Chem. 274, 16901-16906.

Leung, P.S., 2007. The physiology of a local renin-angiotensin system in the pancreas. J. Physiol. 580, 31-37 doi: 10.1113/jphysiol.2006.126193.

Levy, D., Garrison, R.J., Savage, D.D., Kannel, W.B., Castelli, W.P., 1990. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. N. Engl. J. Med. 322, 1561-1566.

Li, H., Gao, Y., Grobe, J.L., Raizada, M.K., Katovich, M.J., Sumners, C., 2007. Potentiation of the antihypertensive action of losartan by peripheral overexpression of the ANG II type 2 receptor. Am. J. Physiol. Heart Circ. Physiol. 292, H727-35 doi: 10.1152/ajpheart.00938.2006.

Li, W., Asokan, A., Wu, Z., Van Dyke, T., DiPrimio, N., Johnson, J.S., Govindaswamy, L., Agbandje-McKenna, M., Leichtle, S., Redmond, D.E.,Jr, McCown, T.J., Petermann, K.B., Sharpless, N.E., Samulski, R.J., 2008. Engineering and selection of shuffled AAV genomes: a new strategy for producing targeted biological nanoparticles. Mol. Ther. 16, 1252-1260 doi: 10.1038/mt.2008.100.

Lim, H., Zhu, Y.Z., 2006. Role of transforming growth factor-beta in the progression of heart failure. Cell Mol. Life Sci. 63, 2584-2596 doi: 10.1007/s00018-006-6085-8.

Lloyd-Jones, D., Adams, R., Carnethon, M., De Simone, G., Ferguson, T.B., Flegal, K., Ford, E., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S., Ho, M., Howard, V., Kissela, B., Kittner, S., Lackland, D., Lisabeth, L., Marelli, A., McDermott, M., Meigs, J., Mozaffarian, D., Nichol, G., O'Donnell, C., Roger, V., Rosamond, W., Sacco, R., Sorlie, P., Stafford, R., Steinberger, J., Thom, T., Wasserthiel-Smoller, S., Wong, N., Wylie-Rosett, J., Hong, Y., American Heart Association Statistics Committee and Stroke Statistics Subcommittee, 2009. Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 119, 480-486 doi: 10.1161/CIRCULATIONAHA.108.191259.

Loot, A.E., Roks, A.J., Henning, R.H., Tio, R.A., Suurmeijer, A.J., Boomsma, F., van Gilst, W.H., 2002. Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. Circulation 105, 1548-1550.

Maggioni, A.P., Anand, I., Gottlieb, S.O., Latini, R., Tognoni, G., Cohn, J.N., Val-HeFT Investigators (Valsartan Heart Failure Trial), 2002. Effects of valsartan on morbidity and mortality in patients with heart failure not receiving angiotensin-converting enzyme inhibitors. J. Am. Coll. Cardiol. 40, 1414-1421.

Markkanen, J.E., Rissanen, T.T., Kivela, A., Yla-Herttuala, S., 2005. Growth factor-induced therapeutic angiogenesis and arteriogenesis in the heart--gene therapy. Cardiovasc. Res. 65, 656-664 doi: 10.1016/j.cardiores.2004.10.030.

Matrougui, K., Loufrani, L., Heymes, C., Levy, B.I., Henrion, D., 1999. Activation of AT(2) receptors by endogenous angiotensin II is involved in flow-induced dilation in rat resistance arteries. Hypertension 34, 659-665.

Matsubara, H., 1998. Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. Circ. Res. 83, 1182-1191.

McKay, R.G., Pfeffer, M.A., Pasternak, R.C., Markis, J.E., Come, P.C., Nakao, S., Alderman, J.D., Ferguson, J.J., Safian, R.D., Grossman, W., 1986. Left ventricular remodeling after myocardial infarction: a corollary to infarct expansion. Circulation 74, 693-702.

McMullen, J.R., Gibson, K.J., Lumbers, E.R., Burrell, J.H., Wu, J., 1999. Interactions between AT1 and AT2 receptors in uterine arteries from pregnant ewes. Eur. J. Pharmacol. 378, 195-202.

Mercure, C., Yogi, A., Callera, G.E., Aranha, A.B., Bader, M., Ferreira, A.J., Santos, R.A., Walther, T., Touyz, R.M., Reudelhuber, T.L., 2008. Angiotensin(1-7) blunts hypertensive cardiac remodeling by a direct effect on the heart. Circ. Res. 103, 1319-1326 doi: 10.1161/CIRCRESAHA.108.184911.

Metcalfe, B.L., Huentelman, M.J., Parilak, L.D., Taylor, D.G., Katovich, M.J., Knot, H.J., Sumners, C., Raizada, M.K., 2004a. Prevention of cardiac hypertrophy by angiotensin II type-2 receptor gene transfer. Hypertension 43, 1233-1238 doi: 10.1161/01.HYP.0000127563.14064.FD.

Metcalfe, B.L., Huentelman, M.J., Parilak, L.D., Taylor, D.G., Katovich, M.J., Knot, H.J., Sumners, C., Raizada, M.K., 2004b. Prevention of cardiac hypertrophy by angiotensin II type-2 receptor gene transfer. Hypertension 43, 1233-1238 doi: 10.1161/01.HYP.0000127563.14064.FD.

Metra, M., Nodari, S., Parrinello, G., Giubbini, R., Manca, C., Dei Cas, L., 2003. Marked improvement in left ventricular ejection fraction during long-term beta-blockade in patients with chronic heart failure: clinical correlates and prognostic significance. Am. Heart J. 145, 292-299 doi: 10.1067/mhj.2003.105.

Miyagi, N., Rao, V.P., Ricci, D., Du, Z., Byrne, G.W., Bailey, K.R., Nakai, H., Russell, S.J., McGregor, C.G., 2008. Efficient and durable gene transfer to transplanted heart using adeno-associated virus 9 vector. J. Heart Lung Transplant. 27, 554-560 doi: 10.1016/j.healun.2008.01.025.

Moores, C.A., Hekmat-Nejad, M., Sakowicz, R., Milligan, R.A., 2003. Regulation of KinI kinesin ATPase activity by binding to the microtubule lattice. J. Cell Biol. 163, 963-971 doi: 10.1083/jcb.200304034.

Muller, O.J., Katus, H.A., Bekeredjian, R., 2007. Targeting the heart with gene therapy-optimized gene delivery methods. Cardiovasc. Res. 73, 453-462 doi: 10.1016/j.cardiores.2006.09.021.

Muller, O.J., Leuchs, B., Pleger, S.T., Grimm, D., Franz, W.M., Katus, H.A., Kleinschmidt, J.A., 2006. Improved cardiac gene transfer by transcriptional and transductional targeting of adeno-associated viral vectors. Cardiovasc. Res. 70, 70-78 doi: 10.1016/j.cardiores.2005.12.017.

Muthalif, M.M., Benter, I.F., Uddin, M.R., Harper, J.L., Malik, K.U., 1998. Signal transduction mechanisms involved in angiotensin-(1-7)-stimulated arachidonic acid release and prostanoid synthesis in rabbit aortic smooth muscle cells. J. Pharmacol. Exp. Ther. 284, 388-398.

Nakayama, M., Yan, X., Price, R.L., Borg, T.K., Ito, K., Sanbe, A., Robbins, J., Lorell, B.H., 2005. Chronic ventricular myocyte-specific overexpression of angiotensin II type 2 receptor results in intrinsic myocyte contractile dysfunction. Am. J. Physiol. Heart Circ. Physiol. 288, H317-27 doi: 10.1152/ajpheart.00957.2003.

Neves, L.A., Santos, R.A., Khosla, M.C., Milsted, A., 2000. Angiotensin-(1-7) regulates the levels of angiotensin II receptor subtype AT1 mRNA differentially in a strain-specific fashion. Regul. Pept. 95, 99-107.

Nio, Y., Matsubara, H., Murasawa, S., Kanasaki, M., Inada, M., 1995. Regulation of gene transcription of angiotensin II receptor subtypes in myocardial infarction. J. Clin. Invest. 95, 46-54 doi: 10.1172/JCI117675.

Ohkubo, N., Matsubara, H., Nozawa, Y., Mori, Y., Murasawa, S., Kijima, K., Maruyama, K., Masaki, H., Tsutumi, Y., Shibazaki, Y., Iwasaka, T., Inada, M., 1997. Angiotensin type 2 receptors are reexpressed by cardiac fibroblasts from failing myopathic hamster hearts and inhibit cell growth and fibrillar collagen metabolism. Circulation 96, 3954-3962.

Oishi, Y., Ozono, R., Yano, Y., Teranishi, Y., Akishita, M., Horiuchi, M., Oshima, T., Kambe, M., 2003. Cardioprotective role of AT2 receptor in postinfarction left ventricular remodeling. Hypertension 41, 814-818 doi: 10.1161/01.HYP.0000048340.53100.43.

Ozono, R., Matsumoto, T., Shingu, T., Oshima, T., Teranishi, Y., Kambe, M., Matsuura, H., Kajiyama, G., Wang, Z.Q., Moore, A.F., Carey, R.M., 2000. Expression and localization of angiotensin subtype receptor proteins in the hypertensive rat heart. Am. J. Physiol. Regul. Integr. Comp. Physiol. 278, R781-9.

Ozono, R., Wang, Z.Q., Moore, A.F., Inagami, T., Siragy, H.M., Carey, R.M., 1997. Expression of the subtype 2 angiotensin (AT2) receptor protein in rat kidney. Hypertension 30, 1238-1246.

Pacak, C.A., Mah, C.S., Thattaliyath, B.D., Conlon, T.J., Lewis, M.A., Cloutier, D.E., Zolotukhin, I., Tarantal, A.F., Byrne, B.J., 2006. Recombinant adeno-associated virus serotype 9 leads to preferential cardiac transduction in vivo. Circ. Res. 99, e3-9 doi: 10.1161/01.RES.0000237661.18885.f6.

Palomeque, J., Chemaly, E.R., Colosi, P., Wellman, J.A., Zhou, S., Del Monte, F., Hajjar, R.J., 2007. Efficiency of eight different AAV serotypes in transducing rat myocardium in vivo. Gene Ther. 14, 989-997 doi: 10.1038/sj.gt.3302895.

Patten, R.D., Udelson, J.E., Konstam, M.A., 1998. Ventricular remodeling and its prevention in the treatment of heart failure. Curr. Opin. Cardiol. 13, 162-167.

Paul, M., Poyan Mehr, A., Kreutz, R., 2006. Physiology of local renin-angiotensin systems. Physiol. Rev. 86, 747-803 doi: 10.1152/physrev.00036.2005.

Pfeffer, J.M., Pfeffer, M.A., Braunwald, E., 1985. Influence of chronic captopril therapy on the infarcted left ventricle of the rat. Circ. Res. 57, 84-95.

Pfeffer, M.A., Braunwald, E., 1990. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation 81, 1161-1172.

Phillips, M.I., Schmidt-Ott, K.M., 1999. The Discovery of Renin 100 Years Ago. News Physiol. Sci. 14, 271-274.

Pinheiro, S.V., Simoes e Silva, A.C., Sampaio, W.O., de Paula, R.D., Mendes, E.P., Bontempo, E.D., Pesquero, J.B., Walther, T., Alenina, N., Bader, M., Bleich, M., Santos, R.A., 2004. Nonpeptide AVE 0991 is an angiotensin-(1-7) receptor Mas agonist in the mouse kidney. Hypertension 44, 490-496 doi: 10.1161/01.HYP.0000141438.64887.42.

Qi, Y., Liu, X., Li, H., Shenoy, V., Li, Q., Hauswirth, W.W., Sumners, C., Katovich, M.J., 2009. Selective tropism of the recombinant adeno-associated virus 9 serotype for rat cardiac tissue. J. Gene Med. doi: 10.1002/jgm.1404.

Qing, K., Mah, C., Hansen, J., Zhou, S., Dwarki, V., Srivastava, A., 1999. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. Nat. Med. 5, 71-77 doi: 10.1038/4758.

Re, R.N., Cook, J.L., 2006. The intracrine hypothesis: an update. Regul. Pept. 133, 1-9 doi: 10.1016/j.regpep.2005.09.012.

Regitz-Zagrosek, V., Friedel, N., Heymann, A., Bauer, P., Neuss, M., Rolfs, A., Steffen, C., Hildebrandt, A., Hetzer, R., Fleck, E., 1995. Regulation, chamber localization, and subtype distribution of angiotensin II receptors in human hearts. Circulation 91, 1461-1471.

Reudelhuber, T.L., 2006. A place in our hearts for the lowly angiotensin 1-7 peptide? Hypertension 47, 811-815 doi: 10.1161/01.HYP.0000209020.69734.73.

Rice, G.I., Thomas, D.A., Grant, P.J., Turner, A.J., Hooper, N.M., 2004. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. Biochem. J. 383, 45-51 doi: 10.1042/BJ20040634.

Rodgers, K.E., Ellefson, D.D., Espinoza, T., Hsu, Y.H., diZerega, G.S., Mehrian-Shai, R., 2006. Expression of intracellular filament, collagen, and collagenase genes in diabetic and normal skin after injury. Wound Repair Regen. 14, 298-305 doi: 10.1111/j.1743-6109.2006.00124.x.

Roks, A.J., Nijholt, J., van Buiten, A., van Gilst, W.H., de Zeeuw, D., Henning, R.H., 2004. Low sodium diet inhibits the local counter-regulator effect of angiotensin-(1-7) on angiotensin II. J. Hypertens. 22, 2355-2361.

Romano, G., 2005. Current development of adeno-associated viral vectors. Drug News. Perspect. 18, 311-316 doi: 10.1358/dnp.2005.18.5.917326.

Rosamond, W., Flegal, K., Friday, G., Furie, K., Go, A., Greenlund, K., Haase, N., Ho, M., Howard, V., Kissela, B., Kittner, S., Lloyd-Jones, D., McDermott, M., Meigs, J., Moy, C., Nichol, G., O'Donnell, C.J., Roger, V., Rumsfeld, J., Sorlie, P., Steinberger, J., Thom, T., Wasserthiel-Smoller, S., Hong, Y., American Heart Association Statistics Committee and Stroke Statistics Subcommittee, 2007. Heart disease and stroke statistics--2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 115, e69-171 doi: 10.1161/CIRCULATIONAHA.106.179918.

Rosenkranz, S., 2004. TGF-beta1 and angiotensin networking in cardiac remodeling. Cardiovasc. Res. 63, 423-432 doi: 10.1016/j.cardiores.2004.04.030.

Rowe, B.P., Saylor, D.L., Speth, R.C., Absher, D.R., 1995. Angiotensin-(1-7) binding at angiotensin II receptors in the rat brain. Regul. Pept. 56, 139-146.

Sadoshima, J., Xu, Y., Slayter, H.S., Izumo, S., 1993. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. Cell 75, 977-984.

Sampaio, W.O., Nascimento, A.A., Santos, R.A., 2003. Systemic and regional hemodynamic effects of angiotensin-(1-7) in rats. Am. J. Physiol. Heart Circ. Physiol. 284, H1985-94 doi: 10.1152/ajpheart.01145.2002.

Santos, R.A., Castro, C.H., Gava, E., Pinheiro, S.V., Almeida, A.P., Paula, R.D., Cruz, J.S., Ramos, A.S., Rosa, K.T., Irigoyen, M.C., Bader, M., Alenina, N., Kitten, G.T., Ferreira, A.J., 2006. Impairment of in vitro and in vivo heart function in angiotensin-(1-7) receptor MAS knockout mice. Hypertension 47, 996-1002 doi: 10.1161/01.HYP.0000215289.51180.5c.

Santos, R.A., Ferreira, A.J., Nadu, A.P., Braga, A.N., de Almeida, A.P., Campagnole-Santos, M.J., Baltatu, O., Iliescu, R., Reudelhuber, T.L., Bader, M., 2004. Expression of an angiotensin-(1-7)-producing fusion protein produces cardioprotective effects in rats. Physiol. Genomics 17, 292-299 doi: 10.1152/physiolgenomics.00227.2003.

Santos, R.A., Simoes e Silva, A.C., Maric, C., Silva, D.M., Machado, R.P., de Buhr, I., Heringer-Walther, S., Pinheiro, S.V., Lopes, M.T., Bader, M., Mendes, E.P., Lemos, V.S., Campagnole-Santos, M.J., Schultheiss, H.P., Speth, R., Walther, T., 2003. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc. Natl. Acad. Sci. U. S. A. 100, 8258-8263 doi: 10.1073/pnas.1432869100.

Santos, R.A., Campagnole-Santos, M.J., Andrade, S.P., 2000. Angiotensin-(1-7): an update. Regul. Pept. 91, 45-62.

Santos, R.A., Campagnole-Santos, M.J., Baracho, N.C., Fontes, M.A., Silva, L.C., Neves, L.A., Oliveira, D.R., Caligiorne, S.M., Rodrigues, A.R., Gropen Junior, C., 1994. Characterization of a new angiotensin antagonist selective for angiotensin-(1-7): evidence that the actions of angiotensin-(1-7) are mediated by specific angiotensin receptors. Brain Res. Bull. 35, 293-298.

Santos, R.A., Brosnihan, K.B., Jacobsen, D.W., DiCorleto, P.E., Ferrario, C.M., 1992. Production of angiotensin-(1-7) by human vascular endothelium. Hypertension 19, II56-61.

Schiavone, M.T., Santos, R.A., Brosnihan, K.B., Khosla, M.C., Ferrario, C.M., 1988. Release of vasopressin from the rat hypothalamo-neurohypophysial system by angiotensin-(1-7) heptapeptide. Proc. Natl. Acad. Sci. U. S. A. 85, 4095-4098.

Schmaier, A.H., 2003. The kallikrein-kinin and the renin-angiotensin systems have a multilayered interaction. Am. J. Physiol. Regul. Integr. Comp. Physiol. 285, R1-13 doi: 10.1152/ajpregu.00535.2002.

Schuijt, M.P., Basdew, M., van Veghel, R., de Vries, R., Saxena, P.R., Schoemaker, R.G., Danser, A.H., 2001. AT(2) receptor-mediated vasodilation in the heart: effect of myocardial infarction. Am. J. Physiol. Heart Circ. Physiol. 281, H2590-6.

Seghaye, M., Duchateau, J., Bruniaux, J., Demontoux, S., Bosson, C., Serraf, A., Lecronier, G., Mokhfi, E., Planche, C., 1996. Interleukin-10 release related to cardiopulmonary bypass in infants undergoing cardiac operations. J. Thorac. Cardiovasc. Surg. 111, 545-553.

Semple, P.F., Boyd, A.S., Dawes, P.M., Morton, J.J., 1976a. Angiotensin II and its heptapeptide (2-8), hexapeptide (3-8), and pentapeptide (4-8) metabolites in arterial and venous blood of man. Circ. Res. 39, 671-678.

Semple, P.F., Brown, J.J., Lever, A.F., MacGregor, J., Morton, J.J., Powell-Jackson, J.D., Robertson, J.I., 1976b. Renin, angiotensin II and III in acute renal failure: note on the measurement of of angiotensin II and III in rat blood. Kidney Int. Suppl. 6, S169-76.

Semple, P.F., Morton, J.J., 1976. Angiotensin II and angiotensin III in rat blood. Circ. Res. 38, 122-126.

Senbonmatsu, T., Saito, T., Landon, E.J., Watanabe, O., Price, E.,Jr, Roberts, R.L., Imboden, H., Fitzgerald, T.G., Gaffney, F.A., Inagami, T., 2003. A novel angiotensin II type 2 receptor signaling pathway: possible role in cardiac hypertrophy. EMBO J. 22, 6471-6482 doi: 10.1093/emboj/cdg637.

Senbonmatsu, T., Ichihara, S., Price, E.,Jr, Gaffney, F.A., Inagami, T., 2000. Evidence for angiotensin II type 2 receptor-mediated cardiac myocyte enlargement during in vivo pressure overload. J. Clin. Invest. 106, R1-5.

Shibata, M., Endo, S., Inada, K., Kuriki, S., Harada, M., Takino, T., Sato, N., Arakawa, N., Suzuki, T., Aoki, H., Suzuki, T., Hiramori, K., 1997. Elevated plasma levels of interleukin-1 receptor antagonist and interleukin-10 in patients with acute myocardial infarction. J. Interferon Cytokine Res. 17, 145-150.

Silva, D.M., Vianna, H.R., Cortes, S.F., Campagnole-Santos, M.J., Santos, R.A., Lemos, V.S., 2007. Evidence for a new angiotensin-(1-7) receptor subtype in the aorta of Sprague-Dawley rats. Peptides 28, 702-707 doi: 10.1016/j.peptides.2006.10.007.

Silva-Antonialli, M.M., Tostes, R.C., Fernandes, L., Fior-Chadi, D.R., Akamine, E.H., Carvalho, M.H., Fortes, Z.B., Nigro, D., 2004. A lower ratio of AT1/AT2 receptors of angiotensin II is found in female than in male spontaneously hypertensive rats. Cardiovasc. Res. 62, 587-593 doi: 10.1016/j.cardiores.2004.01.020.

Sipo, I., Fechner, H., Pinkert, S., Suckau, L., Wang, X., Weger, S., Poller, W., 2007. Differential internalization and nuclear uncoating of self-complementary adeno-associated virus pseudotype vectors as determinants of cardiac cell transduction. Gene Ther. 14, 1319-1329 doi: 10.1038/sj.gt.3302987.

Siragy, H.M., Xue, C., Abadir, P., Carey, R.M., 2005. Angiotensin subtype-2 receptors inhibit renin biosynthesis and angiotensin II formation. Hypertension 45, 133-137 doi: 10.1161/01.HYP.0000149105.75125.2a.

Snyder, R.O., Miao, C.H., Patijn, G.A., Spratt, S.K., Danos, O., Nagy, D., Gown, A.M., Winther, B., Meuse, L., Cohen, L.K., Thompson, A.R., Kay, M.A., 1997. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nat. Genet. 16, 270-276 doi: 10.1038/ng0797-270.

Speth, R.C., Kim, K.H., 1990. Discrimination of two angiotensin II receptor subtypes with a selective agonist analogue of angiotensin II, p-aminophenylalanine6 angiotensin II. Biochem. Biophys. Res. Commun. 169, 997-1006.

Steckelings, U.M., Kaschina, E., Unger, T., 2005. The AT2 receptor--a matter of love and hate. Peptides 26, 1401-1409 doi: 10.1016/j.peptides.2005.03.010.

Stier, C.T.,Jr, Chander, P., Gutstein, W.H., Levine, S., Itskovitz, H.D., 1991. Therapeutic benefit of captopril in salt-loaded stroke-prone spontaneously hypertensive rats is independent of hypotensive effect. Am. J. Hypertens. 4, 680-687.

Stoll, M., Hahn, A.W., Jonas, U., Zhao, Y., Schieffer, B., Fischer, J.W., Unger, T., 2002. Identification of a zinc finger homoeodomain enhancer protein after AT(2) receptor stimulation by differential mRNA display. Arterioscler. Thromb. Vasc. Biol. 22, 231-237.

Stroth, U., Unger, T., 1999. The renin-angiotensin system and its receptors. J. Cardiovasc. Pharmacol. 33 Suppl 1, S21-8; discussion S41-3.

Stumpf, C., Seybold, K., Petzi, S., Wasmeier, G., Raaz, D., Yilmaz, A., Anger, T., Daniel, W.G., Garlichs, C.D., 2008. Interleukin-10 improves left ventricular function in rats with heart failure subsequent to myocardial infarction. Eur. J. Heart Fail. 10, 733-739 doi: 10.1016/j.ejheart.2008.06.007.

Su, H., Huang, Y., Takagawa, J., Barcena, A., Arakawa-Hoyt, J., Ye, J., Grossman, W., Kan, Y.W., 2006. AAV serotype-1 mediates early onset of gene expression in mouse hearts and results in better therapeutic effect. Gene Ther. 13, 1495-1502 doi: 10.1038/sj.gt.3302787.

Suckau, L., Fechner, H., Chemaly, E., Krohn, S., Hadri, L., Kockskamper, J., Westermann, D., Bisping, E., Ly, H., Wang, X., Kawase, Y., Chen, J., Liang, L., Sipo, I., Vetter, R., Weger, S., Kurreck, J., Erdmann, V., Tschope, C., Pieske, B., Lebeche, D., Schultheiss, H.P., Hajjar, R.J., Poller, W.C., 2009. Long-Term Cardiac-Targeted RNA Interference for the Treatment of Heart Failure Restores Cardiac Function and Reduces Pathological Hypertrophy. Circulation doi: 10.1161/CIRCULATIONAHA.108.783852.

Sugino, H., Ozono, R., Kurisu, S., Matsuura, H., Ishida, M., Oshima, T., Kambe, M., Teranishi, Y., Masaki, H., Matsubara, H., 2001. Apoptosis is not increased in myocardium overexpressing type 2 angiotensin II receptor in transgenic mice. Hypertension 37, 1394-1398.

Summerford, C., Bartlett, J.S., Samulski, R.J., 1999. AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. Nat. Med. 5, 78-82 doi: 10.1038/4768.

Summerford, C., Samulski, R.J., 1998. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J. Virol. 72, 1438-1445.

Tallant, E.A., Ferrario, C.M., Gallagher, P.E., 2005. Angiotensin-(1-7) inhibits growth of cardiac myocytes through activation of the mas receptor. Am. J. Physiol. Heart Circ. Physiol. 289, H1560-6 doi: 10.1152/ajpheart.00941.2004.

Tallant, E.A., Lu, X., Weiss, R.B., Chappell, M.C., Ferrario, C.M., 1997. Bovine aortic endothelial cells contain an angiotensin-(1-7) receptor. Hypertension 29, 388-393.

Tan, F., Morris, P.W., Skidgel, R.A., Erdos, E.G., 1993. Sequencing and cloning of human prolylcarboxypeptidase (angiotensinase C). Similarity to both serine carboxypeptidase and prolylendopeptidase families. J. Biol. Chem. 268, 16631-16638.

Testa, M., Yeh, M., Lee, P., Fanelli, R., Loperfido, F., Berman, J.W., LeJemtel, T.H., 1996. Circulating levels of cytokines and their endogenous modulators in patients with mild to severe congestive heart failure due to coronary artery disease or hypertension. J. Am. Coll. Cardiol. 28, 964-971.

Thom, T., Haase, N., Rosamond, W., Howard, V.J., Rumsfeld, J., Manolio, T., Zheng, Z.J., Flegal, K., O'Donnell, C., Kittner, S., Lloyd-Jones, D., Goff, D.C.,Jr, Hong, Y., Adams, R., Friday, G., Furie, K., Gorelick, P., Kissela, B., Marler, J., Meigs, J., Roger, V., Sidney, S., Sorlie, P., Steinberger, J., Wasserthiel-Smoller, S., Wilson, M., Wolf, P., American Heart Association Statistics Committee and Stroke Statistics Subcommittee, 2006. Heart disease and stroke statistics--2006 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 113, e85-151 doi: 10.1161/CIRCULATIONAHA.105.171600.

Thurmann, P.A., Kenedi, P., Schmidt, A., Harder, S., Rietbrock, N., 1998. Influence of the angiotensin II antagonist valsartan on left ventricular hypertrophy in patients with essential hypertension. Circulation 98, 2037-2042.

Tipnis, S.R., Hooper, N.M., Hyde, R., Karran, E., Christie, G., Turner, A.J., 2000. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. J. Biol. Chem. 275, 33238-33243 doi: 10.1074/jbc.M002615200.

Tiyyagura, S.R., Pinney, S.P., 2006. Left ventricular remodeling after myocardial infarction: past, present, and future. Mt. Sinai J. Med. 73, 840-851.

Torre-Amione, G., Kapadia, S., Benedict, C., Oral, H., Young, J.B., Mann, D.L., 1996. Proinflammatory cytokine levels in patients with depressed left ventricular ejection fraction: a report from the Studies of Left Ventricular Dysfunction (SOLVD). J. Am. Coll. Cardiol. 27, 1201-1206 doi: 10.1016/0735-1097(95)00589-7.

Touyz, R.M., Endemann, D., He, G., Li, J.S., Schiffrin, E.L., 1999. Role of AT2 receptors in angiotensin II-stimulated contraction of small mesenteric arteries in young SHR. Hypertension 33, 366-372.

Trask, A.J., Averill, D.B., Ganten, D., Chappell, M.C., Ferrario, C.M., 2007. Primary role of angiotensin-converting enzyme-2 in cardiac production of angiotensin-(1-7) in transgenic Ren-2 hypertensive rats. Am. J. Physiol. Heart Circ. Physiol. 292, H3019-24 doi: 10.1152/ajpheart.01198.2006.

Trask, A.J., Ferrario, C.M., 2007. Angiotensin-(1-7): pharmacology and new perspectives in cardiovascular treatments. Cardiovasc. Drug Rev. 25, 162-174 doi: 10.1111/j.1527-3466.2007.00012.x.

Tsutamoto, T., Hisanaga, T., Wada, A., Maeda, K., Ohnishi, M., Fukai, D., Mabuchi, N., Sawaki, M., Kinoshita, M., 1998. Interleukin-6 spillover in the peripheral circulation increases with the severity of heart failure, and the high plasma level of interleukin-6 is an important prognostic predictor in patients with congestive heart failure. J. Am. Coll. Cardiol. 31, 391-398.

Tsutsumi, Y., Matsubara, H., Masaki, H., Kurihara, H., Murasawa, S., Takai, S., Miyazaki, M., Nozawa, Y., Ozono, R., Nakagawa, K., Miwa, T., Kawada, N., Mori, Y., Shibasaki, Y., Tanaka, Y., Fujiyama, S., Koyama, Y., Fujiyama, A., Takahashi, H., Iwasaka, T., 1999. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. J. Clin. Invest. 104, 925-935 doi: 10.1172/JCI7886.

Tsutsumi, Y., Matsubara, H., Ohkubo, N., Mori, Y., Nozawa, Y., Murasawa, S., Kijima, K., Maruyama, K., Masaki, H., Moriguchi, Y., Shibasaki, Y., Kamihata, H., Inada, M., Iwasaka, T., 1998. Angiotensin II type 2 receptor is upregulated in human heart with interstitial fibrosis, and cardiac fibroblasts are the major cell type for its expression. Circ. Res. 83, 1035-1046.

Tsuzuki, S., Matoba, T., Eguchi, S., Inagami, T., 1996. Angiotensin II type 2 receptor inhibits cell proliferation and activates tyrosine phosphatase. Hypertension 28, 916-918.

Utsunomiya, H., Nakamura, M., Kakudo, K., Inagami, T., Tamura, M., 2005. Angiotensin II AT2 receptor localization in cardiovascular tissues by its antibody developed in AT2 gene-deleted mice. Regul. Pept. 126, 155-161 doi: 10.1016/j.regpep.2004.09.004.

Vandendriessche, T., Thorrez, L., Acosta-Sanchez, A., Petrus, I., Wang, L., Ma, L., DE Waele, L., Iwasaki, Y., Gillijns, V., Wilson, J.M., Collen, D., Chuah, M.K., 2007. Efficacy and safety of adeno-associated viral vectors based on serotype 8 and 9 vs. lentiviral vectors for hemophilia B gene therapy. J. Thromb. Haemost. 5, 16-24 doi: 10.1111/j.1538-7836.2006.02220.x.

Vassalli, G., Bueler, H., Dudler, J., von Segesser, L.K., Kappenberger, L., 2003. Adeno-associated virus (AAV) vectors achieve prolonged transgene expression in mouse myocardium and arteries in vivo: a comparative study with adenovirus vectors. Int. J. Cardiol. 90, 229-238.

Vickers, C., Hales, P., Kaushik, V., Dick, L., Gavin, J., Tang, J., Godbout, K., Parsons, T., Baronas, E., Hsieh, F., Acton, S., Patane, M., Nichols, A., Tummino, P., 2002a. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J. Biol. Chem. 277, 14838-14843 doi: 10.1074/jbc.M200581200.

Vickers, C., Hales, P., Kaushik, V., Dick, L., Gavin, J., Tang, J., Godbout, K., Parsons, T., Baronas, E., Hsieh, F., Acton, S., Patane, M., Nichols, A., Tummino, P., 2002b. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J. Biol. Chem. 277, 14838-14843 doi: 10.1074/jbc.M200581200.

Villarreal, F.J., Kim, N.N., Ungab, G.D., Printz, M.P., Dillmann, W.H., 1993. Identification of functional angiotensin II receptors on rat cardiac fibroblasts. Circulation 88, 2849-2861.

Voros, S., Yang, Z., Bove, C.M., Gilson, W.D., Epstein, F.H., French, B.A., Berr, S.S., Bishop, S.P., Conaway, M.R., Matsubara, H., Carey, R.M., Kramer, C.M., 2006. Interaction between AT1 and AT2 receptors during postinfarction left ventricular remodeling. Am. J. Physiol. Heart Circ. Physiol. 290, H1004-10 doi: 10.1152/ajpheart.00886.2005.

Wang, H., Gallinat, S., Li, H.W., Sumners, C., Raizada, M.K., Katovich, M.J., 2004. Elevated blood pressure in normotensive rats produced by 'knockdown' of the angiotensin type 2 receptor. Exp. Physiol. 89, 313-322 doi: 10.1113/expphysiol.2004.027359.

Wang, L.J., He, J.G., Ma, H., Cai, Y.M., Liao, X.X., Zeng, W.T., Liu, J., Wang, L.C., 2005. Chronic administration of angiotensin-(1-7) attenuates pressure-overload left ventricular hypertrophy and fibrosis in rats. Di Yi Jun Yi Da Xue Xue Bao 25, 481-487.

Wang, Z.Q., Moore, A.F., Ozono, R., Siragy, H.M., Carey, R.M., 1998. Immunolocalization of subtype 2 angiotensin II (AT2) receptor protein in rat heart. Hypertension 32, 78-83.

Weber, K.T., 2000. Fibrosis and hypertensive heart disease. Curr. Opin. Cardiol. 15, 264-272.

Weber, K.T., 1997. Extracellular matrix remodeling in heart failure: a role for de novo angiotensin II generation. Circulation 96, 4065-4082.

Weber, K.T., Brilla, C.G., 1992. Factors associated with reactive and reparative fibrosis of the myocardium. Basic Res. Cardiol. 87 Suppl 1, 291-301.

Weber, K.T., Brilla, C.G., 1991. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. Circulation 83, 1849-1865.

Widdop, R.E., Gardiner, S.M., Kemp, P.A., Bennett, T., 1992. Inhibition of the haemodynamic effects of angiotensin II in conscious rats by AT2-receptor antagonists given after the AT1-receptor antagonist, EXP 3174. Br. J. Pharmacol. 107, 873-880.

Wiemer, G., Dobrucki, L.W., Louka, F.R., Malinski, T., Heitsch, H., 2002. AVE 0991, a nonpeptide mimic of the effects of angiotensin-(1-7) on the endothelium. Hypertension 40, 847-852.

Williams, R.S., Benjamin, I.J., 2000. Protective responses in the ischemic myocardium. J. Clin. Invest. 106, 813-818 doi: 10.1172/JCI11205.

Wong, M., Staszewsky, L., Latini, R., Barlera, S., Glazer, R., Aknay, N., Hester, A., Anand, I., Cohn, J.N., 2004. Severity of left ventricular remodeling defines outcomes and response to therapy in heart failure: Valsartan heart failure trial (Val-HeFT) echocardiographic data. J. Am. Coll. Cardiol. 43, 2022-2027 doi: 10.1016/j.jacc.2003.12.053.

Woo, Y.J., Zhang, J.C., Taylor, M.D., Cohen, J.E., Hsu, V.M., Sweeney, H.L., 2005a. One year transgene expression with adeno-associated virus cardiac gene transfer. Int. J. Cardiol. 100, 421-426 doi: 10.1016/j.ijcard.2004.09.003.

Woo, Y.J., Zhang, J.C., Taylor, M.D., Cohen, J.E., Hsu, V.M., Sweeney, H.L., 2005b. One year transgene expression with adeno-associated virus cardiac gene transfer. Int. J. Cardiol. 100, 421-426 doi: 10.1016/j.ijcard.2004.09.003.

Wright, M.J., Wightman, L.M., Lilley, C., de Alwis, M., Hart, S.L., Miller, A., Coffin, R.S., Thrasher, A., Latchman, D.S., Marber, M.S., 2001. In vivo myocardial gene transfer: optimization, evaluation and direct comparison of gene transfer vectors. Basic Res. Cardiol. 96, 227-236.

Yamada, T., Horiuchi, M., Dzau, V.J., 1996. Angiotensin II type 2 receptor mediates programmed cell death. Proc. Natl. Acad. Sci. U. S. A. 93, 156-160.

Yamamoto, K., Chappell, M.C., Brosnihan, K.B., Ferrario, C.M., 1992. In vivo metabolism of angiotensin I by neutral endopeptidase (EC 3.4.24.11) in spontaneously hypertensive rats. Hypertension 19, 692-696.

Yan, X., Price, R.L., Nakayama, M., Ito, K., Schuldt, A.J., Manning, W.J., Sanbe, A., Borg, T.K., Robbins, J., Lorell, B.H., 2003. Ventricular-specific expression of angiotensin II type 2 receptors causes dilated cardiomyopathy and heart failure in transgenic mice. Am. J. Physiol. Heart Circ. Physiol. 285, H2179-87 doi: 10.1152/ajpheart.00361.2003.

Yang, L., Jiang, J., Drouin, L.M., Agbandje-McKenna, M., Chen, C., Qiao, C., Pu, D., Hu, X., Wang, D.Z., Li, J., Xiao, X., 2009. A myocardium tropic adeno-associated virus (AAV) evolved by DNA shuffling and in vivo selection. Proc. Natl. Acad. Sci. U. S. A. doi: 10.1073/pnas.0813207106.

Yang, Z., Bove, C.M., French, B.A., Epstein, F.H., Berr, S.S., DiMaria, J.M., Gibson, J.J., Carey, R.M., Kramer, C.M., 2002. Angiotensin II type 2 receptor overexpression preserves left ventricular function after myocardial infarction. Circulation 106, 106-111.

Yang, Z., Zingarelli, B., Szabo, C., 2000. Crucial role of endogenous interleukin-10 production in myocardial ischemia/reperfusion injury. Circulation 101, 1019-1026.

Yao, L., Huang, K., Huang, D., Wang, J., Guo, H., Liao, Y., 2008. Acute myocardial infarction induced increases in plasma tumor necrosis factor-alpha and interleukin-10 are associated with the activation of poly(ADP-ribose) polymerase of circulating mononuclear cell. Int. J. Cardiol. 123, 366-368 doi: 10.1016/j.ijcard.2007.06.069.

Yin, F.C., Spurgeon, H.A., Rakusan, K., Weisfeldt, M.L., Lakatta, E.G., 1982. Use of tibial length to quantify cardiac hypertrophy: application in the aging rat. Am. J. Physiol. 243, H941-7.

Yla-Herttuala, S., Alitalo, K., 2003. Gene transfer as a tool to induce therapeutic vascular growth. Nat. Med. 9, 694-701 doi: 10.1038/nm0603-694.

Zhang, X., Azhar, G., Nagano, K., Wei, J.Y., 2001. Differential vulnerability to oxidative stress in rat cardiac myocytes versus fibroblasts. J. Am. Coll. Cardiol. 38, 2055-2062.

Zincarelli, C., Soltys, S., Rengo, G., Rabinowitz, J.E., 2008. Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection. Mol. Ther. 16, 1073-1080 doi: 10.1038/mt.2008.76.

Zisman, L.S., Keller, R.S., Weaver, B., Lin, Q., Speth, R., Bristow, M.R., Canver, C.C., 2003a. Increased angiotensin-(1-7)-forming activity in failing human heart ventricles: evidence for upregulation of the angiotensin-converting enzyme Homologue ACE2. Circulation 108, 1707-1712 doi: 10.1161/01.CIR.0000094734.67990.99.

Zisman, L.S., Meixell, G.E., Bristow, M.R., Canver, C.C., 2003b. Angiotensin-(1-7) formation in the intact human heart: in vivo dependence on angiotensin II as substrate. Circulation 108, 1679-1681 doi: 10.1161/01.CIR.0000094733.61689.D4.

Zolotukhin, S., Potter, M., Zolotukhin, I., Sakai, Y., Loiler, S., Fraites, T.J.,Jr, Chiodo, V.A., Phillipsberg, T., Muzyczka, N., Hauswirth, W.W., Flotte, T.R., Byrne, B.J., Snyder, R.O., 2002. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods 28, 158-167.

BIOGRAPHICAL SKETCH

Yanfei Qi was born and grew up in the small town of Guizhou province, China. Yanfei received her Doctor of Medicine at Guiyang Medical College in July 2002. During the internship in the hospital, Yanfei discovered her interest in biomedical research. She decided to pursue her science career at Guiyang Medical College. Under the guidance of Dr. Xilin Ren, she worked on a project to study an association between Alzheimer's disease and gene polymorphism of the alpha 4 nicotinic receptor in Alzheimer patients. During the process of studying the project, Dr. Ren advised her to pursue her interest in biomedical research and apply to graduate school at the University of Florida in the United States. In 2005, Yanfei began her graduate school in the graduate program in Department of Pharmacodynamics at the University of Florida. Under the supervision of Dr. Michael J. Katovich, she has been studying the role of angiotension type 2 receptor and Ang-(1-7) in cardiovascular function.