Ultrasound-Mediated S100A6 Gene Therapy Ameliorates Myocardial Ischemia/Reperfusion (I/R) Injury

by

Azadeh Mofid

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Science
University of Toronto

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Doctor of Philosophy

Institute of Medical Science
University of Toronto
2016

Abstract

New therapies targeting myocardial ischemia/reperfusion (I/R) injury are key to averting the adverse remodeling process and subsequent heart failure (HF) post myocardial infarction (MI). S100A6 is a member of the superfamily of EF-hand Ca\(^{2+}\)-binding proteins that modulate many key pathways involved in myocardial I/R injury and adverse left ventricular remodeling including cardiomyocyte apoptosis and hypertrophy.

Methods and Results

S100A6 overexpression improved calcium transients and protected against apoptosis induced by hypoxia-reoxygenation via enhanced calcineurin activity, while knockdown of S100A6 had detrimental effects in rat neonatal cardiomyocytes, \textit{in vitro}. Moreover, S100A6
overexpressing HUVECs show enhanced tube formation and augmented migration as markers of angiogenesis in vitro.

In a rat model of myocardial I/R, S100A6 expression is up-regulated in the infarct and peri-infarct regions of the left ventricle (LV) following myocardial I/R injury but occurs simultaneous or just after the peak of apoptosis and LV functional deterioration.

Ultrasound-targeted microbubble destruction (UTMD) delivery of hS100A6-plasmid prior to I/R yields a survival advantage, improves LV systolic function and myocardial perfusion, attenuates cardiac hypertrophy and reduces infarct size through prevention of apoptosis and necrosis and enhancement of calcium handling post myocardial I/R injury, in vivo.

Finally, UTMD delivery of hS100A6-minicircle (MC) immediately after I/R injury yields similar therapeutic benefits on reduction of infarct size, improved LV systolic function and myocardial perfusion, as compared to control and empty minicircle UTMD.

Conclusion

The present study is the first to demonstrate the therapeutic benefits of targeted hS100A6 gene delivery in the setting of cardiac I/R injury, resulting in a significant improvement in LV function, a reduction in infarct size and prevention of adverse LV remodeling. Gene therapy by UTMD of S100A6 holds promise as adjunctive therapy to primary percutaneous coronary intervention to help ameliorate ischemia/reperfusion injury.
Everyone has been made for some particular work, and the desire for that work has been put in every heart.

Rumi
Acknowledgments

First, I would like to thank my parents for their never-ending love and ongoing support. I also would like to acknowledge my sisters for their words of encouragement and my brother-in-laws for the inspiration and guidance. I want to thank my lovely nephew and niece (Shayan and Ava) whose significant presence in my life has made it more beautiful and meaningful.

In particular, my deepest gratitude goes to my supervisor, Dr. Howard Leong-Poi who has been a wonderful mentor. He not only provided me with the scientific support, motivation and guidance necessary for my PhD research project, but also with intellectual inputs. He has been instilling moral values in his lab members, teaching us by great examples of teamwork and resilience. I also want to thank Dr. Howard Leong-Poi’s lab manager, Michael Kuliszewski, who has been very supportive during these 5 years.

The contribution of the members of my program advisory committee, Dr. Thomas Parker and Dr. Kim Connelly was essential in improvement of this research project. During program advisory committee meetings, they offered insightful advice and criticisms, which were deeply appreciated and completely applied.

I would like to extend my gratitude to the examination committee members, Dr. Phyllis Billia and Dr. David Hess, for providing me with constructive feedback and attending my Ph.D. dissertation defense session. I would also like to thank Dr. Anthony Gramolini for his priceless help in calcium transient studies.
Contributions

- I would like to acknowledge the following contributions from members of Dr. Leong-Poi’s lab at Keenan Research Centre for Biomedical Science, Li Ka Shing Knowledge Institute, St. Michael’s Hospital, University of Toronto:

  - **Paul J.H. Lee M.Sc.** for assistance in immunohistochemistry (IHC) and TUNEL staining as well as echocardiography imaging
  - **Pratiek N. Matkar M.Sc.** for assistance in Western Blotting and Matrigel assay
  - **Wei J. Cao B.Sc.** for assistance in Western Blotting and cell culture
  - **Dmitriy Rudenko B.Sc.** for assistance in PCR, Minicircle production and UTMD gene delivery
  - **Nadav S. Newman B.Sc.** for assistance in surgical procedures and UTMD gene delivery
  - **Christine Liao M.Sc.** for assistance in contrast enhanced perfusion imaging
  - **Hao H. Chen M.Sc.** for assistance in tissue cryosectioning, H&E and MTC staining
  - **Michael A. Kuliszewski B.Sc.** for assistance in confocal microscopic imaging and graphic design
  - **Kolsoom Afrasiabi M.D.** for assistance in surgical procedures, UTMD mediated gene delivery and echocardiography imaging
  - **Saleh A. Alghadeer M.D.** for assistance in surgical procedures and animal preparation
I would like to acknowledge the following contribution from a member of Dr. Anthony O. Gramolini’s lab at Department of Physiology, University of Toronto:

Cynthia Abbasi M.Sc. for assistance in calcium transient imaging

I would like to acknowledge the following contributions from members of Dr. Thomas Parker’s lab at Keenan Research Centre for Biomedical Science, Li Ka Shing Knowledge Institute, St. Michael’s Hospital, University of Toronto:

James N. Tsoporis Ph.D. for assistance in IHC staining

J. F. Desjardins B.Sc. for assistance in surgical procedures
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<td>Lymphoid Enhancer-Binding Factor</td>
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<tr>
<td>PCI</td>
<td>Percutaneous Coronary Intervention</td>
</tr>
</tbody>
</table>
**PDGF**  Platelet-Derived Growth Factor

**PESDA**  Perfluorocarbon-Exposed Sonicated Dextrose Albumin

**PG-E2**  Prostaglandin E2

**PI3K**  Phosphoinositide 3-kinase

**PKC**  Protein Kinase C

**PLB**  Phospholamban

**PSLA**  Para Sternal Long Axis

**RAGE**  Receptor of Advanced Glycation End-Products

**RAAS**  Renin-Angiotensin-Aldosterone System

**ROS**  Reactive Oxygen Species

**RT-PCR**  Real-Time Polymerase Chain Reaction

**RyR**  Ryanodine Receptors

**SAX**  Short Axis

**SCF**  Stem Cell Factor

**SDF1**  Stromal Cell-Derived Factor-1

**SDS-PAGE**  Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

**SEM**  Standard Error of Mean
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase</td>
</tr>
<tr>
<td>SIAH</td>
<td>Seven In Absentia Homologue</td>
</tr>
<tr>
<td>SIP</td>
<td>SIAH-1 Interacting Protein</td>
</tr>
<tr>
<td>skACT</td>
<td>Skeletal α-Actin</td>
</tr>
<tr>
<td>Skp1</td>
<td>S-phase Kinase-Associated Protein 1</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic Reticulum</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal Transducers and Activators of Transcription 1</td>
</tr>
<tr>
<td>STEMI</td>
<td>ST-Segment Elevation Myocardial Infarction</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour-Associated Macrophages</td>
</tr>
<tr>
<td>t-BID</td>
<td>Truncated BID</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA Box Binding Protein</td>
</tr>
<tr>
<td>TCF</td>
<td>T Cell Factor</td>
</tr>
<tr>
<td>TGF-b</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue Inhibitors of Metalloproteinase</td>
</tr>
<tr>
<td>Tm</td>
<td>Tropomyosin</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TnT</td>
<td>Troponin-T</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-Related Apoptosis-Inducing Ligand</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotide Transferase-Mediated dUTP Nick-End Labeling</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase-Type Plasminogen Activator Receptor</td>
</tr>
<tr>
<td>UTMD</td>
<td>Ultrasound-Targeted Microbubble Destruction</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>β2-AR</td>
<td>β2-Adrenergic Receptor</td>
</tr>
<tr>
<td>βARKct</td>
<td>Beta Adrenergic Receptor Kinase Carboxyl-Terminus</td>
</tr>
<tr>
<td>β-MHC</td>
<td>Beta Myosin Heavy Chain</td>
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Chapter 1

Introduction and Literature Review
1. Heart Disease

In 2007, 1.3 million Canadians were reported to have heart disease, accounting for 4.8% of Canadians (PHAC, 2009). Additionally, heart disease is the leading cause of death in the United States regardless of the ethnicity or gender. Every year approximately 600,000 people die of heart disease in the United States, meaning one in every four deaths are heart disease related (Murphy, Xu, and Kochanek 2013; Heron 2012). The most significant risk factors for heart diseases are smoking, high blood pressure, diabetes and high cholesterol level. Some lifestyle choices such as poor diet, physical inactivity, excessive alcohol consumption and medical conditions including obesity also contribute towards heart diseases ("Million hearts: strategies to reduce the prevalence of leading cardiovascular disease risk factors--United States, 2011" 2011). The most common type of heart disease is coronary artery disease which costs the United States 109.9 billion dollars every year including healthcare services, prescription medications and loss of productivity ("Heart Disease and Stroke Statistics-2014 Update: A Report from the American Heart Association" 2014; Heidenreich et al. 2011).

1.1 Coronary Artery Disease

Coronary atherosclerosis or coronary artery disease (CAD) is the leading cause of 7,254,000 deaths worldwide according to the WHO, which is 12.8% of all deaths (Hausenloy and Yellon 2013). Atherosclerosis is one of the most significant and prevalent causes of
disability and death in the United States and throughout the world, with 25 million Americans suffering from at least one clinical manifestation of atherosclerosis. In many patients, atherosclerosis remains silent until a significant cardiovascular event occurs (Faxon et al. 2004). CAD was previously considered a cholesterol storage disease but its complex nature has now been revealed. It has been shown that the interaction of multiple elements including cellular components of the arterial wall, blood flow and inflammatory pathways eventually lead to atherosclerotic plaque formation (Libby and Theroux 2005).

1.2 Acute Coronary Syndrome

An acute coronary syndrome (ACS) is a condition in which blood flow to the myocardium is suddenly reduced, and occurs via several mechanisms. One mechanism which is responsible for the majority of ACS cases is rupture of the atherosclerotic plaque’s protective fibrous cap leading to coronary thrombosis (Davies 1996). Other mechanisms are superficial erosion in the atherosclerotic plaque, erosion of calcific plaque and intra-plaque hemorrhage. These account for the less common cases of ACS (Falk, Shah, and Fuster 1995; Virmani et al. 2002).

1.3 Myocardial Ischemia

Myocardial ischemia occurs when any significant myocardial zone does not receive enough oxygen via coronary blood flow to meet metabolic demands. Under normal conditions
the myocardial oxygen demand is met by tightly regulated coronary blood flow. Regulatory mechanisms include release of adenosine, production of relaxing and constricting factors by the platelets and endothelium derived relaxing factor by the vascular endothelium. When an atherosclerotic plaque ruptures or the endothelium of the coronary arteries is damaged, its sub-endothelial layers get exposed to blood and circulatory platelets. As a result, vasoconstrictor substances such as serotonin and thromboxane get released. The vasoconstrictors plus acetylcholine overcome the regulatory mechanisms and along with the thrombotic occlusion, contribute to decreased blood flow and oxygen to the myocardium and activation of the ischemic cascade (Detry 1996).

The ischemic cascade occurs in a known sequence beginning with perfusion abnormalities, which causes metabolic imbalance and the production of lactate, instead of its normal consumption, and potassium loss. This causes diastolic abnormalities and impairment of ventricular relaxation. Systolic abnormalities then occurs in rapid succession, which are characterized by reduced myocardial contractility. The final steps are ST-segment changes on electrocardiography (ECG) and clinical manifestations such as angina pectoris (Detry 1996).

If the interruption is brief (less than 30 minutes), restoration of blood flow reverses most of these abnormalities. However, if the interruption persists and there is no collateral blood flow, then myocardial necrosis starts to occur (Detry 1996).
1.4 Myocardial Stunning

If the blood flow is restored in less than 30 minutes following ACS, myocardial infarction (MI) does not occur. Instead, short-term myocardial dysfunction occurs which recovers late after the acute ischemic insult. This phenomenon is called myocardial stunning, which can be detected by echocardiography (Detry 1996) and other modalities that evaluate LV systolic function.

1.5 Myocardial Ischemia/Reperfusion (I/R) Injury

Early reperfusion (restoration of coronary blood flow) by thrombolytic therapy or percutaneous coronary intervention (PCI) limits infarct size and reduces mortality in patients presenting with acute ST-segment elevation MI (STEMI) (O'Gara et al. 2013). While outcomes have improved, morbidity and mortality still remain significant, with 1-year rates of major adverse cardiovascular events ranging from 8 to 17% (Sanidas et al. 2014). While essential, the acute restoration of blood flow within the epicardial coronary artery can lead to metabolic and structural events that result in a further loss of cardiomyocytes through multiple mechanisms, termed as myocardial I/R injury (Yellon and Hausenloy 2007; Eltzschig and Eckle 2011) (Figure 1). As a consequence, the development of new therapies to further reduce infarct size and preserve left ventricular (LV) systolic function after acute STEMI remains a priority (Hausenloy and Yellon 2013; Sanada, Komuro, and Kitakaze 2011).
Myocardial I/R injury is a complex, multi-faceted process that has been extensively studied, with important contributing factors including oxidative stress, calcium overload, immune responses, inflammation and impaired metabolism, which lead to myocyte and endothelial cell (EC) loss through programmed cell death (apoptosis), non-programmed cell death (necrosis) and autophagy (Yellon and Hausenloy 2007; Hausenloy and Yellon 2013). Thus, the ability to specifically target multiple underlying biological processes involved in reperfusion injury would be a key aspect in the development of novel therapeutic strategies to address myocardial I/R injury (Anversa and Nadal-Ginard 2002; Hausenloy and Yellon 2013; Sutton and Sharpe 2000).
**Figure 1.** Myocardial ischemia vs. Ischemia/Reperfusion (I/R) Injury.

During myocardial ischemia, hypoxia induces anaerobic metabolism, accumulation of lactic acid and acidification of the cytosol. This leads to intracellular Na\(^+\) and Ca\(^{2+}\) overload, inhibition of myofibrils contraction and prevention of MPTP opening (top). Post reperfusion, lactic acid is washed out and physiologic pH is restored. This will lead to MPTP opening and myofibril hypercontracture in presence of intracellular Ca\(^{2+}\) overload (bottom).
1.6 Cardiomyocyte Death

Cardiomyocyte death is a common characteristic of MI, myocardial I/R injury and HF (Whelan, Kaplinskiy, and Kitsis 2010). The three main cellular pathways leading to cardiomyocyte death are autophagy, necrosis and apoptosis (Chiong et al. 2011).

1.6.1 Autophagy

Autophagy is a process in which intracellular components such as mitochondria and macromolecules are delivered by auto-phagosomes to lysosomes for digestion (Levine and Kroemer 2008). During myocardial ischemia, induction of autophagy via the AMP-inducible protein kinase pathway is a pro-survival mechanism to restore energy (Matsui et al. 2007; Yan et al. 2005; Hamacher-Brady et al. 2007; Russell et al. 2004). Following reperfusion, although delivery of oxygen and nutrients to the myocardium is restored, auto-phagosome clearance is impaired due to formation of reactive oxygen species (ROS), which up regulates BECLIN-1 and reduces Lysosome Associated Membrane Protein-2 (LAMP2) that is necessary for auto-phagosome-lysosome fusion. This in turn will lead to more cardiomyocyte death (Matsui et al. 2007; Ma et al. 2012).

Any therapy modulating autophagy may help prevent heart failure following acute MI and myocardial I/R injury. Although metformin, an AMPK activator, and Rapamycin, an mTOR
inhibitor, can regulate autophagy, the lack of evidence and complexity of this cell death mechanism make it more challenging for clinical application (Chiong et al. 2011) (Figure 2).

Figure 2. Autophagy Pathway.
Atg5 (autophagy related protein-5) and Beclin-1 are key proteins with significant role in induction of autophagy during periods of cell stress. mTOR, an atypical serine/threonine kinase, is the main suppressor of autophagy which is regulated by Raptor and suppressed by Rapamycin.

1.6.2 Apoptosis

Apoptosis or programmed cell death occurs through two different pathways: extrinsic and intrinsic pathways (Whelan, Kaplinskiy, and Kitsis 2010). Although apoptosis rarely occurs in healthy myocardium (apoptotic index: 0.01-0.001%), these two pathways contribute to cardiomyocyte death following myocardial ischemia, I/R injury and heart failure (apoptotic index: 0.12%- 0.70%) (Whelan, Kaplinskiy, and Kitsis 2010). Considering low levels of
cardiomyocyte proliferation, even an apoptotic index of 0.1% can lead to profound myocardial loss (37%) over a year (van Empel et al. 2005; Wencker et al. 2003). Myocardial ischemia induced-apoptosis peaks at 4.5 hours post MI, which precedes necrotic cell death, which peaks at 24 hours (Kajstura et al. 1996). Myocardial ischemia induced-apoptosis is accelerated if myocardial ischemia is followed by reperfusion (Fliss and Gattinger 1996).

Tumor necrosis factor alpha (TNF-α), FAS ligands and TNF-related apoptosis-inducing ligand (TRAIL) stimulate the extrinsic pathway whereas in the intrinsic pathway the key player is the mitochondria (Whelan, Kaplinskiy, and Kitsis 2010; Lee and Gustafsson 2009). Mitochondria not only produce required energy (ATP) for nonstop contraction of cardiomyocytes, but also play a key role in stress-induced cardiomyocyte apoptosis through caspase activation (caspase dependent pathway) or Apoptosis Inducing Factor (AIF)(caspase independent pathway)(Holly et al. 1999; Bahi et al. 2006; Bae et al. 2010). The final step of the intrinsic pathway is fragmentation of mitochondria and release of cytochrome-c (Parra et al. 2008). This mechanism of cell death is highly regulated in the myocardium through the actions of pro-apoptotic and anti-apoptotic proteins such as the BAX and BCL-2 family of proteins, respectively (Condorelli et al. 1999; Hochhauser et al. 2007; Chen et al. 2001). The extrinsic pathway can also activate the intrinsic pathway. Caspase-8 activation by the extrinsic pathway cleaves BCL-2 interacting protein, BH3 interacting-domain (BID), and this produces truncated
Bid (t-BID), which activates the intrinsic pathway (Whelan, Kaplinskiy, and Kitsis 2010) (Figure 3). Although anti-apoptotic therapies have been studied in heart failure, clinical trials on the application of anti-apoptotic agents for AMI or I/R injury are scant (Balakumar and Singh 2006; Bozkurt et al. 2001; Chiong et al. 2011).

**Figure 3.** Apoptotic Pathway in cardiomyocytes. Extrinsic and intrinsic apoptotic pathways are depicted in this diagram. Extrinsic pathway gets activated by interaction of death ligands with the death receptors which will activate caspase-8 and caspase-3 and will eventually activate apoptosis. The extrinsic pathway is linked to the intrinsic pathway through activation of BID and release of Cytochrome-c from mitochondria which will form apoptosomes and activate caspase-9 and caspase-3. XIAP is an endogenous caspase inhibitor which is abundant in cardiomyocytes making them resistant to apoptosis (Chiong et al. 2011)
1.6.3 Necrosis

Necrosis is a cell death pathway that is characterized by cellular and organelle swelling, loss of ATP and plasma membrane rupture, leading to cell degradation and release of cytoplasmic contents. Furthermore, this phenomenon also activates inflammatory responses (Whelan, Kaplinskiy, and Kitsis 2010). Although it was previously believed that necrosis is unplanned cell death in response to stress, recent studies have shown some regulatory mechanisms involved in this process such as death receptors activation, Ca$^{2+}$ transients, ROS formation, and opening of mitochondrial permeability transition pores (MPTP) (Vanlangenakker et al. 2008; Henriquez et al. 2008; Kroemer, Galluzzi, and Brenner 2007).

Following myocardial ischemia, lack of oxygen activates the anaerobic glycolysis pathway as a source of energy. Activation of this pathway results in production and accumulation of hydrogen ion (H$^+$) and acidosis. Ion pumps exchange H$^+$ for Na$^+$ and this subsequently increases the amount of intracellular Na$^+$. This further weakens the function of Na$^+$/Ca$^{2+}$ exchanger and results in calcium accumulation within the cell. The excess calcium then enters the mitochondria and activates calcium dependent hydrogenase to form ROS. Reperfusion provides oxygen required for ATP production and restores mitochondrial membrane potential. This increases entry of calcium into the mitochondria, opening of MPTP, mitochondrial swelling and necrotic cell death (Whelan, Kaplinskiy, and Kitsis 2010). MPTP opening is a major
mechanism responsible for cell necrosis following reperfusion (Baines et al. 2005) (Figure 4).

Necrosis is also a major contributor to cell death in chronic heart failure and its role is more significant than apoptosis. Calcium overload and adrenergic system stimulation are the leading mechanisms underlying necrosis in the failing heart (Chiong et al. 2011).

Recent studies aimed at targeting MPTP opening have shown promising results for treatment of myocardial I/R injury. Cyclosporine as a MPTP inhibitor and its targets, Calcineurin and nitric oxide synthase (NOS), has therapeutic potential (Piot et al. 2008; Chiong et al. 2011).

**Figure 4.** Necrotic Pathway in cardiomyocytes post I/R.
During I/R injury, lack of ATP influences membrane pumps’ function leading to Na\(^+\), H\(^+\) and Ca\(^{2+}\) accumulation inside the cell. Water gets absorbed in the cell and organelles which will result in swelling and rupture of lysosomes and mitochondrions. Rupture of lysosomes will release proteases and lead to necrosis. On the other hand, activation of death receptors will trigger ROS formation which will result in MPTP opening and necrosis.
1.7 Ventricular Remodeling

A physiologic or pathologic cascade of events by which ventricular size, shape, and function are regulated is called ventricular remodeling. These events are influenced by mechanical, neurohormonal, and genetic factors influencing all three major components of the myocardium including myocytes, the extracellular matrix, and the microcirculation (Pfeffer and Braunwald 1990; Rouleau et al. 1993; Weber 1997; Erlebacher et al. 1984). While physiologic remodeling occurs during normal growth, pathologic remodeling occurs under many stress situations, including post-MI, long-standing hypertension, severe valvular heart disease or cardiomyopathy (Sutton and Sharpe 2000).

Early after myocardial infarction, this cascade of biochemical intracellular signaling starts to attenuate cardiac dysfunction induced by early loss of functioning cardiomyocytes (apoptosis and necrosis) and later hemodynamic pressure and volume overloading (Mann 1999) (Figure 5). The goal of remodeling is to preserve cardiac output, which is favorable initially, but continues for weeks or months where it becomes detrimental, producing dilatation, hypertrophy, and collagen scars. These changes eventually lead to progressive cardiac dysfunction and heart failure in the long term. Pathologic post-infarction remodeling can be divided to early (first 72 hours) and late (after 72 hours) phases (Swynghedauw 1999; Pfeffer and Braunwald 1990; Warren et al. 1988).
Figure 5. Ventricular Remodeling.
Ventricular remodeling starts early after myocardial infarction and I/R injury. Myocyte death results in neutrophil infiltration and activation of MMPs which will lead to collagen degradation and LV dilatation, an early step in the remodeling process. Cardiomyocyte loss impairs pump function and decreases cardiac output which results in activation of the sympathetic nervous system and RAAS leading to myocyte hypertrophy. The final step of the remodeling process is fibrotic tissue formation which occurs due to macrophage and fibroblast chemotaxis and transformation, activation of tissue inhibitor of metalloproteinases (TIMPs) and subsequent collagen synthesis.

1.7.1 Early Remodeling

Early remodeling starts with migration of macrophages, monocytes, and neutrophils into the infarct zone, which in turn initiates intracellular signaling and neuro-hormonal activation to localize the inflammatory response. Neutrophils also release matrix metalloproteinase (MMPs), which degrade inter-myocyte collagen, leading to expansion of the infarct area. This infarct
expansion causes left ventricular wall thinning and chamber dilatation, which increases wall stress during systole and diastole. Acute increased wall stress augments shortening via Frank/Starling relations and induces perturbations in circulatory hemodynamics, which in turn activates the sympathetic adrenergic system. Activation of sympathetic adrenergic system leads to activation of renin-angiotensin-aldosterone system (RAAS), and production of atrial and brain natriuretic peptides (ANP and BNP). ANP/BNP reduces intravascular volume and systemic vascular resistance and improves ventricular filling and pump function temporarily. The early remodeling process triggers the changes leading to late remodeling (Warren et al. 1988; Cleutjens et al. 1995; Sadoshima et al. 1992; Lew et al. 1985; Sutton and Sharpe 2000).

1.7.2 Late Remodeling

Late remodeling involves myocyte hypertrophy and structural changes triggered by mechanical inputs, which are transduced into biochemical events that eventually modify protein synthesis by influencing transcription initiation, transcription elongation, and translation. Therefore, these signaling pathways with the help of activated transcription factors reactivate the fetal cardiac gene program, which produces proteins involved in contractility, calcium handling, and myocardial energetics. This is a beneficial adaptation to stress primarily, but abnormal expression of these genes can also lead to maladaptive changes over the long term (Miyata et al. 2000; Palermo J 1995; Swynghedauw 1999).
The most significant examples are the genes encoding fetal isoforms of contractile muscle proteins such as skeletal $\alpha$-actin (skACT) and $\beta$-myosin heavy chain (MHC) that are re-expressed following ischemic injury while the expression of their counterparts, cardiac $\alpha$-actin and $\alpha$-MHC, are repressed. This phenomenon results in myocardial hypertrophy, one of the most significant myocardial changes during LV remodeling (Chien et al. 1993; Parker 1993).

1.7.3 Cardiomyocyte Hypertrophy

Cardiomyocyte hypertrophy involves microscopic enlargement of each individual myocyte (up to 70%) plus replication of sarcomeres (Anversa et al. 1985). Even though it helps normalize wall tension during the early stages, hypertrophy is considered a maladaptive process that can eventually lead to HF (Esposito G 2002). Four distinct signaling pathways involved in the myocardial hypertrophic response are as follows (van Empel and De Windt 2004):

The gp130-receptor signaling pathway, which activates genes involved in hypertrophy (c-fos, ANP), cell survival [B-cell lymphoma-extra-large (BCL-xL), mitochondrial antioxidant manganese superoxide dismutase (MnSOD)] and angiogenesis [vascular endothelial growth factor (VEGF)] (van Empel and De Windt 2004).

IGF-1-PI3K-Akt signaling pathway, which activates genes involved in hypertrophy (mTOR-P70S6K) and anti-apoptosis [B-cell lymphoma 2, (Bcl-2)] while inhibits pro-apoptotic genes (van Empel and De Windt 2004).
Calcium-dependent Calcineurin-mediated signaling pathway, which activates genes involved in hypertrophy [CyclinT-Cyclin-dependent kinase 9 (cdk9)] and cell survival [Nuclear Factor of Activated T-cells (NFAT)] (van Empel and De Windt 2004).

Nuclear Factor Kappa from B Cells (NF-kB) signaling pathway, which activates genes involved in hypertrophy [G protein–coupled receptors (GPCRs)] and regulates expression of both anti-apoptotic [cIAPs, Bcl-2, FLICE, FLICE-like inhibitory protein (FLIP)], and pro-apoptotic (Fas, FasL, caspase-8, caspase-11, TNF-α) genes (van Empel and De Windt 2004).

Both hypertrophy and apoptosis are involved in the pathogenesis of heart failure, and were previously thought to be separate mechanisms. Recently, it has been shown that these processes involve multiple parallel and congregating signals, whereby hypertrophy can promote cell survival. The balance between the hypertrophic (survival) and apoptotic signals determines the overall outcome; compensated hypertrophy or decompensated heart failure (Figure 6).

A favorable therapy is the one which can suppress adverse signaling pathways that induce abrupt hypertrophic responses while enhancing the beneficial ones, by increasing cell survival and preventing apoptosis (Hayakawa et al. 2003).
Figure 6. Compensated Myocardial Hypertrophy and Heart Failure.
Two major adaptive responses involved in heart failure are myocardial hypertrophy and cardiomyocyte apoptosis. Although they were previously perceived as separate entities, they influence each other. Thus, the final outcome depends on the balance between these two signaling pathways.

1.7.4 Contractile Function of the Heart and Calcium Handling

Excitation-contraction coupling (EC-coupling) directs the contractile function of the heart. Two major calcium-sensing apparatuses, dihydropyridine and ryanodine receptors (RyR), play a significant role in the excitation phase of excitation-contraction coupling. The primary phase of EC-coupling is generated through the spread of action potentials down the T-tubule portion of the plasma membrane. Following depolarization of the membrane, an influx of calcium through L-type and T-type calcium channels occurs (Katoh, Schlotthauer, and Bers 2000; Bers 2001). Then the RyR senses the increase in calcium content of the junctional
sarcoplasmic reticulum (SR) and releases additional calcium from the SR through a phenomenon called calcium induced calcium release (CICR) (Katoh, Schlotthauer, and Bers 2000; Bers 2001).

Calcium is the main activator of the myofilaments. Increased concentration of intercellular calcium in the myofilament matrix leads to calcium binding to the C component of the troponin complex (TnC). This binding stimulates a conformational change in troponin I (TnI), Troponin-T (TnT) and Tropomyosin (Tm), which are major proteins of the thin filaments. This conformational change moves the position of tropomyosin on the thin filament, which permits myosin binding to the actin filament, eventually leading to the shortening of myofilaments and force creation (Kobayashi, Jin, and de Tombe 2008).

To start relaxation, the calcium ion concentration has to be reduced in the myofilaments matrix. This occurs via reuptake of calcium. Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pump and the sodium calcium exchanger (NCX) direct the reuptake of calcium. SERCA is a calcium-ATPase pump functioning through interaction with phospholamban (PLB) to pump intracellular calcium ions into the SR. PLB works as a switch for the activation of SERCA. Phosphorylation of PLB removes its inhibitory effects on SERCA and SERCA pumps calcium back into the SR (Masaki et al. 1997; Shannon, Pogwizd, and Bers 2003). During early cardiac remodeling, relaxation is significantly distorted. This occurs due to direct alterations in the expression levels of proteins involved in calcium extrusion and reuptake.
such as SERCA, PLB, and NCX (Zarain-Herzberg, MacLennan, and Periasamy 1990; Grover and Khan 1992).

### 1.7.5 Scar Formation and Fibrosis

Shortly after MI and I/R injury, damaged myocytes release cytokines such as transforming growth factor beta (TGF-β) into the infarct zone, which stimulates chemotaxis and proliferation of macrophages and fibroblasts (Desmouliere et al. 1993). On the other hand, γ-interferon activates macrophages, which release nitric oxide. Released nitric oxide increases the vascular permeability and subsequent cellular inflammatory responses (Desmouliere et al. 1993; Sigusch, Campbell, and Weber 1996; Guarda et al. 1993).

TGF-β induces the transformation of interstitial fibroblasts to myofibroblasts, which adhere to the fibrin-fibronectin matrix and produce pro-collagen types III and I (A A Knowlton 1992; Desmouliere et al. 1993). Collagen types III and I get deposited predominantly in the infarct zone but to a lesser extent in the non-infarct zone. Expression of genes encoding collagen Type III significantly increases by day 2 post MI and remains high for 3 weeks while expression of gene encoding collagen type I rises later at day four and remains high for a longer period of time (3 months) (Cleutjens et al. 1995).

Although detection of collagen debris in the infarcted region is microscopically possible at day 7, at day 28, most of the infarcted region is replaced by the collagen debris and necrotic
tissue. Then the collagen formation decreases and most fibroblasts get degraded by apoptosis (Cleutjens et al. 1995; Sutton and Sharpe 2000). Several factors regulate synthesis of collagen types III and I such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), ANP, bradykinin-mediated prostaglandin E2 (PG-E2), nitric oxide (NO) and AngII–related mechanical deformation (Weber 1997). Amount of collagen cross-linking and the strength of the mature scar are determined by mechanical forces (McCormick et al. 1994).

1.7.6 Angiogenesis

Angiogenesis, the development of new capillary blood vessels from existing microvessels, is a core event in myocardial repair following infarction. Impaired angiogenesis leads to immature scar tissue formation and eventually cardiac rupture (Barandon et al. 2004). Many studies have confirmed the beneficial effects of angiogenesis stimulation on ischemic myocardium (Ahn et al. 2008; Boodhwani et al. 2006; Nelson, Passeri, and Frishman 2000; Pandya, Dhalla, and Santani 2006).

Angiogenesis starts following degradation of the basement membrane, which increases vessel permeability and induces proteolysis of the extracellular matrix in the vicinity of the damaged vessels. This event, in the presence of activated pro-angiogenic factors, promotes migration, proliferation and assembly of ECs and eventually leads to neovessel formation. There are several regulatory factors for angiogenesis. A family of growth factors, the VEGFs, is the
most significant group of proteins involved in angiogenesis regulation. Among this family, VEGF-A and its receptors on vascular ECs (VEGF-R1 and VEGF-R2) are the key players in the vascularization (Ferrara and Gerber 2001; Janet R. McColm 2004; Ria et al. 2003; Neufeld et al. 1999; Cochain, Channon, and Silvestre 2013; Carmeliet and Jain 2011).

Hypoxia-inducible transcription factors (HIF1-a, HIF2-a) are involved in the regulation of vascularization by targeting pro-angiogenic genes such as VEGF. The expression of HIF proteins in cardiomyocytes, ECs and inflammatory cells increases early after MI, and remains elevated up to 4 weeks after MI in rats (Pugh and Ratcliffe 2003; Jurgensen et al. 2004). The nitric oxide synthase (NOS) enzymes are other regulatory proteins which are involved in angiogenesis in part through HIF1-α and VEGF-dependent pathways (Landmesser et al. 2004).

It has been shown in a rat MI model that angiogenesis starts at the border zones first and extends into the infarcted regions of the myocardium. The peak of neovessel formation in the infarcted myocardium was seen at day 7 which declined at day 14 out to day 28 post-MI. Therefore most of the angiogenesis occurs at early stages post myocardial infarction (Tieqiang Zhao 2010).

Tieqiang Zhao et al. (2010) have also shown that VEGF-A is a significant trigger for angiogenesis in the border zone but is not essential for the delayed angiogenesis in the infarct area following myocardial ischemia (Tieqiang Zhao 2010).
1.7.7 Cardiomyocyte Replication

Despite the previous notion that cardiomyocytes are terminally differentiated, it has recently been shown that a fraction of cardiomyocytes (15-20%) can retain the capacity to divide (Kajstura J1 2000; Leri A1 2000). Re-entry of adult cardiomyocytes into the cell cycle or replication has been recently suggested as one of the aspects of ventricular remodeling following ischemia. Previous studies have shown high levels of DNA replication as well as expression of growth-related genes soon after MI in the adult mammalian heart and human end-stage cardiac failure (Anversa P1 1998; Kajstura et al. 1998; Beltrami AP1 2001).

The extent of cardiomyocyte division is time dependent and is significantly higher shortly after MI than at later stages of terminal ischemic cardiomyopathy (Kajstura et al. 1998; Beltrami AP1 2001). Cardiomyocyte division is mostly observed in the peri-infarct region of myocardium. In the infarct region, cardiomyocyte division is minimal because cells become non-viable during the first few hours following ischemia and the vasculature is severely damaged (Hirzel et al. 1976).

1.8 New Therapies

In the acute phase, restoration of blood flow to the infarct region (reperfusion) by thrombolysis or primary percutaneous coronary intervention (PCI) is of proven value and still the first leading therapeutic option. It can limit the size of the infarcted area; prevent its expansion
and transmurality, salvage endocardial tissue and restore function to stunned myocardium in the infarct border zones. Ischemic/re-perfused myocardium with characteristic contraction-band necrosis has greater tensile strength and fewer tendencies to expansion than ischemic myocardium with transmural necrosis (Jeremy et al. 1987; Pirolo, Hutchins, and Moore 1986; Sutton and Sharpe 2000).

In the chronic phase, when remodeling is in progress, the patency of arteries, loading conditions, neurohormonal activation and presence of local tissue growth factors are very influential. Therefore, conventional therapies, such as ACE inhibition and beta blockade, have focused on these influential factors. (Sutton and Sharpe 2000). These conventional therapies cannot completely recover cardiac function or prevent heart failure, therefore novel therapeutic strategies are still needed (Shah and Mann 2011).

New therapeutic strategies should address remodeling by regulating molecular and cellular factors and pathways involved in tissue repair, hypertrophy, fibrosis, and angiogenesis. Studies aiming at prevention of extracellular collagen breakdown, blockade of TGF-β1, enhancement of intracellular calcium handling, improvement of contractile function, prevention of apoptosis and facilitation of cardiomyocytes regeneration are examples of avenues of research for newer therapeutic strategies (Sutton and Sharpe 2000).
1.8.1 Gene Therapy for Ischemic Heart Disease

The expanding application of genetic manipulations and increasing amount of data on the genes linked to myocardial remodeling have motivated scientists to study the expression of potential target genes in the myocardium post-MI and focus on the responsible pathways in order to find an efficient target gene for the therapeutic purposes. These investigations have elicited promising results as overexpression or knockdown of specific target genes have led to improvement in cardiac function and attenuation of the remodeling process (Table 1).

1.8.2 Delivery Methods

Various myocardial gene delivery techniques have been tested so far. Most of these methods have shown limited efficacy and did not produce a significant therapeutic response (Bernecker, del Monte, and Hajjar 2003; Simons et al. 2000). Gene delivery techniques can be classified based on the approach (direct injection, intravascular injection/infusion, ex-vivo delivery), delivery site (epicardial, endocardial or intramuscular injection) (Katz et al. 2011).

1.8.3 Direct Gene Delivery

Direct gene delivery is performed by injection into the myocardium, pericardium or the endocardium. They can be done either invasively during thoracotomy (open chest) or non-invasively via a transcutaneous approach (Katz et al. 2011). Although direct injection of the desired gene is a very effective technique that can deliver genes to a specific location (around the
injection site) but have limitations, including its invasive nature, non-repeatable and non-specificity for cell types (Kornowski et al. 2000; Kobulnik et al. 2009). Intra-pericardial injection showed significant transduction in the pericardium and limited transduction in the myocardium. It also showed significant expression of the target gene in hepatic tissue (Zhang et al. 1999).

Catheter-based endocardial injections have shown promising results as well but despite its simplicity, they have been associated with complications such as ventricular perforation, cardiac tamponade, endocardial thrombosis and intra-myocardial hematoma especially when used on thinned ventricular myocardium following chronic myocardial infarction (Gwon et al. 2001). Furthermore, the most effective location of injection (infarct area or border-zone), the frequency of ideal and accurate injections and the dose of injection has not been fully defined (Sanborn et al. 2001).

Intra-myocardial injection has been tried using plasmid-DNA and adenoviral vectors. When plasmid DNA was injected directly to the myocardium, the efficacy was dramatically low but the duration of transgene expression was long (up to 6 months) (Acsadi et al. 1991; Lin et al. 1990). Because of low efficacy, intra-myocardial plasmid injection may not produce clinically significant effects (R J Guzman 1993).
Application of adenoviral vectors improved the transfection efficacy of the myocardial injection technique but was hampered by its short duration and side effects. The expression of the transgene lasts only for one week and triggered an inflammatory response due to needle induced trauma and reaction to adenovirus antigens (R J Guzman 1993; Barr et al. 1994). One study in a canine model showed that transgene expression following intra-myocardial injection of adenoviral vector peaks at day 2 and decreases dramatically by day 14. The off-target effects were not significant in this model (Magovern et al. 1996). Intra-myocardial injection has been successfully used in rodent models for overexpression of β2-adrenergic receptor (β2-AR) and β-adrenergic receptor kinase carboxyl-terminus (βARKct) genes in order to treat heart failure following cardiomyopathy and myocardial ischemia, respectively (Tomiyasu et al. 2000; Rengo et al. 2009).

1.8.4 Systemic or Trans-Vascular Gene Delivery

Trans-vascular gene delivery with cardiac catheterization is divided to three categories: intra-ventricular, anterograde and retrograde intra-coronary and intravenous delivery. Since these techniques can diffusely deliver the transgene to all four chambers of heart, it is more suitable for gene delivery to address heart failure (Hajjar et al. 2000; Donahue et al. 1997; Baker 2002; Logeart et al. 2001; Hayase et al. 2005).
1.8.5 Intracoronary Delivery of Recombinant Adenovirus

There are many factors influencing the efficacy of gene transfer by intracoronary delivery of recombinant adenoviruses, such as coronary flow rate, blood pressure, total amount of virus, duration of adenovirus presence in the coronary circulation, composition of perfusion fluid, temperature and capillary permeability (Donahue et al. 1997; Donahue et al. 1998). Intracoronary administration of recombinant adenovirus for gene delivery to the rabbit myocardium has been shown to be an ineffective technique at physiologic perfusion pressures (Wright et al. 2001; Kornowski et al. 2000). To overcome short residence of viral vector in the coronary circulation and to increase vascular permeability, transient occlusion of the upstream artery has been performed (Logeart et al. 2000).

1.8.6 Intravenous (IV) Gene Delivery

Intravenous gene delivery of DNA expression plasmids especially when mediated by cationic liposomes has shown promising results. For instance, IV administration of a pure DNA expression plasmid containing the human tissue Kallikrein gene produced a sustained reduction in blood pressure for 6 weeks in rats with spontaneous hypertension (Wang, Chao, and Chao 1995). Cationic liposome-mediated, IV gene delivery has been shown to produce significant levels of exogenous transgene expression in all harvested tissues (N Zhu 1993). IV delivery of
the wild type p53 gene using cationic liposomes has also produced significant anti-tumor effects in nude mice with human tumors but lacking p53 expression (Lesoon-Wood et al. 1995).

1.8.7 Ultrasound Targeted Microbubble Destruction (UTMD)

Three important characteristics desired for a safe and efficient gene delivery technique are 1) non-invasive nature, 2) target-tissue specificity and 3) ease of application (Kobulnik et al. 2009). Contrast ultrasound-mediated gene delivery (UMGD) or ultrasound-targeted microbubble destruction (UTMD) has been proven as a novel and safe technique for site-specific gene therapy (Xie et al. 2012).

Microbubble carrier agents are composed of a gas-filled core (nitrogen or other inert gas) with an outer shell (lipid, synthetic biopolymer, or albumin) with a mean diameter of 1-3 µm. These microbubbles have similar properties as red blood cells, and are able to freely circulate through the pulmonary and systemic microcirculation after an intravenous injection (Lindner et al. 2002). For UMGD, microbubble-DNA complexes are typically administered as a slow continuous infusion that allows a stable concentration within the blood during external ultrasound application. At high acoustic power, non-linear vibrations of the microbubbles at their resonant frequency leads to the destruction of the microbubbles. The resultant bioeffects produce transfection via several mechanisms including sonoporation and endocytosis (Christiansen et al.
Three methods for microbubble-gene delivery have been used for UMGD:

1) Microbubbles generated with the genes incorporated into the shell

2) Charge-coupling or attachment after microbubble generation

3) Passive co-administration of DNA with free microbubbles

Out of these three methods, our lab has experience with charge coupling, whereby negatively charged plasmid DNA is charge-coupled to cationic microbubbles. Previous studies demonstrated approximately 6700 plasmids attaching to each cationic microbubble (Christiansen et al. 2003).

For UMGD, the microbubble-DNA complexes or suspension is given intravenously as a slow bolus or continuous infusion. Due to limited ultrasound beam width, the ultrasound transducer should be scanned across the target organ or tissue to maximize delivery. Studies have shown that in order to maximize gene transfection, parameters should be set for 1) triggered ultrasound at set intervals, 2) lower transmit frequency, and 3) greater acoustic power (Chen, Shohet, et al. 2003). This technique has been successfully applied for gene transfection of skeletal and cardiac muscles (Christiansen et al. 2003; Shohet et al. 2000; Leong-Poi et al. 2007). When UMGD is applied for local gene delivery to the LV myocardium, expression of transgene
is higher in the anterior LV wall as compared to the posterior wall due to the proximity of the anterior wall to the ultrasound probe and attenuation of the posterior wall from the high microbubble concentration within the left ventricular cavity (Chen, Shohet, et al. 2003).

The summary of pre-clinical studies of ultrasound-mediated gene delivery (UMGD) for cardiac diseases is presented in Table 1. From our lab, Paul Lee et al. successfully studied UMGD of Survivin plasmid DNA to prevent apoptosis and attenuate left ventricular (LV) systolic dysfunction in a rat model of heart failure induced by doxorubicin (DOX). He showed that Survivin gene therapy by UMGD resulted in reduced cardiomyocyte apoptosis, less interstitial fibrosis, and attenuated LV systolic dysfunction, as compared to untreated control animals and animals treated with UMGD of empty plasmid (Lee et al. 2014).
<table>
<thead>
<tr>
<th>Author</th>
<th>Model</th>
<th>Target Gene</th>
<th>Carrier Microbubble</th>
<th>Main Study Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mukherjee et al. 2000)</td>
<td>Wistar-Kyoto rats</td>
<td>VEGF</td>
<td>PESDA $^1$</td>
<td>Increased endothelial and smooth muscle cell proliferation</td>
</tr>
<tr>
<td>(Vannan et al. 2002)</td>
<td>Canine model</td>
<td>CAT $^2$</td>
<td>Neutral and cationic bubbles containing Perfluorobutane gas</td>
<td>Greater expression of CAT in myocardium Systemic expression of CAT in remote tissue</td>
</tr>
<tr>
<td>(Erikson, Freeman, and Chandrasekar 2003)</td>
<td>Rat I/R model</td>
<td>Antisense TNFα</td>
<td>PESDA $^1$</td>
<td>Down regulation of interleukin-1 beta, and Intercellular Adhesion Molecule-1 (ICAM-1)</td>
</tr>
<tr>
<td>(Kondo et al. 2004)</td>
<td>Rat MI model</td>
<td>HGF $^3$</td>
<td>Optison</td>
<td>Improved cardiac function and inotropic reserve Reduced scar formation, increased capillary density</td>
</tr>
<tr>
<td>(Korpanty G1 2005)</td>
<td>Rat normal myocardium</td>
<td>VEGF</td>
<td>Cationic liposomes containing Perfluoropropane gas</td>
<td>Enhanced angiogenesis, increased capillary and arteriolar density</td>
</tr>
<tr>
<td>(Fujii et al. 2009)</td>
<td>Rat MI model</td>
<td>SCF $^4$ and VEGF</td>
<td>Definity</td>
<td>Enhanced progenitor cell recruitment Increased vascular density and myocardial perfusion Functional improvement of left ventricle</td>
</tr>
<tr>
<td>(Fujii H1 2011)</td>
<td>Rat MI model</td>
<td>SCF, SDF-1a</td>
<td>Definity</td>
<td>Enhanced progenitor cell/myofibroblast recruitment, Improved ejection fraction (EF)</td>
</tr>
<tr>
<td>(Yuan et al. 2012)</td>
<td>Canine MI model</td>
<td>HGF</td>
<td>Lipid and palmitic acid glycerol Perfluoropropan gas</td>
<td>Increased capillary density Enhanced regional blood flow</td>
</tr>
<tr>
<td>(Sun L1 2013)</td>
<td>Rat I/R model</td>
<td>Human AKT</td>
<td>HSPC and DOTMA with Octafluoropropane gas</td>
<td>Decreased apoptosis and infarct size, Increased LV wall thickness, Improved vascularity, perfusion and cardiac function</td>
</tr>
</tbody>
</table>

**Table 1. UTMD Mediated Gene Delivery for Heart Disease**

PESDA: Perfluorocarbon-Exposed Sonicated Dextrose Albumin, CAT: Chloramphenicol Acetyltransferase, VEGF: Vascular Endothelial Growth Factor

HGF: Hepatocyte Growth Factor, Akt: A serine–threonine kinase known as protein kinase B, SCF: Stem Cell Factor, SDF-1a: Stromal Cell-Derived Factor 1a
1.8.8 Vectors

Plasmid DNA or p-DNA is a non-viral vector that does not integrate into the host genome. They are safer and less expensive than viral vectors and can be manipulated easily (Faurez et al. 2010). However, there are few disadvantages regarding the application of these vectors, including their low transfection efficiency and suboptimal expression of the transgene. Expression of the transgene cassette in plasmid DNA vector decreases gradually over a very short period of time. This is due to formation of transcriptionally repressive proteins, which get deposited on extra-genomic DNA on the DNA backbone. This eventually leads to chromatization of backbone genes and silencing of the transgene (Chen, He, et al. 2003; Chen et al. 2004; Gracey Maniar et al. 2013).

Minicircle (MC) DNA is similar to plasmid DNA. They both have the transgene cassette, which is expressed very quickly after gene delivery (Darquet et al. 1997). But MC lacks prokaryotic sequences in the backbone such as the replication region and antibiotic resistance gene sequence. These sequences are removed before episomal DNA isolation by site-specific recombination in Escherichia coli (Chen, He, and Kay 2005; Kay, He, and Chen 2010) (Figure 7). The small size of MC compared to pDNA results in higher transfection efficiency and a more sustained expression of the transgene over a period of weeks as compared to plasmids where expression wanes after 7 days (Molnar et al. 2004). The avoidance of using antibiotic resistance
genes as selection markers for plasmid production eliminates the safety concerns of potential allergic immunogenic reactions due to the residual antibiotic. The combination of improved transfection efficiency and improved safety profile make MC vectors highly attractive alternatives to conventional plasmid gene vectors for clinical applications.

**Figure 7** Production of Minicircle DNA from parental plasmid.
Addition of Arabinose will activate endonuclease and integrase which will remove the bacterial backbone including antibiotic resistance gene and origin of replication. The bacterial backbone gets degraded. The remaining segment which consists of the transgene and its promoter makes the Minicircle.

1.9 S100 Family of EF Hand Calcium Binding Proteins

Among all the potential target therapeutic genes to prevent post myocardial infarction remodeling, there is a family of EF-hand Ca$^{2+}$ binding proteins known as S100, which comprises 19 members that are differentially expressed in a large number of cell types. Members of this
protein family have been implicated in the $\text{Ca}^{2+}$ dependent and in some cases, $\text{Zn}^{2+}$ or $\text{Cu}^{2+}$ dependent regulation of a variety of intracellular activities such as protein phosphorylation, enzyme activities, cell proliferation (including neoplastic transformation) and differentiation, the dynamics of cytoskeleton constituents, the structural organization of membranes, intracellular $\text{Ca}^{2+}$ homeostasis, inflammation and protection from oxidative cell damage. Some S100 members are released or secreted into the extracellular space and exert trophic or toxic effects on neighboring cells mainly through RAGE receptors depending on their concentration (Donato 2001).

Among all members of S100 family of proteins, S100A1, S100B and recently, S100A6 have attracted the most attention in cardiovascular research because of their proposed or established roles in the regulation of cardiomyocyte contractility, hypertrophy and apoptosis and are considered as potential therapeutic targets to prevent post infarct or I/R ventricular remodeling and heart failure.

1.9.1 Interaction Between Members of S100 Family

Members of the S100 family have the ability to form homo-dimers and hetero-dimers with each other. Among these members, the hetero-dimerization between S100B-S100A1 and S100B-S100A6 has been demonstrated recently. S100B-S100A6 heterodimer may play a regulatory role in the growth of melanoma cells (Yang et al. 1999). S100A1-S100B hetero-
dimerization can inhibit the $\alpha_1$-adrenergic-mediated induction of the S100B (Tsoporis et al. 2003). There is no data on the hetero-dimerization of S100A6-S100A1.

1.9.2 S100A6 (Calcyclin)

S100A6 is a member of a superfamily of EF-hand Ca$^{2+}$-binding proteins that regulate a wide array of cellular and molecular functions, including cell proliferation, differentiation and survival, as well as Ca$^{2+}$ dynamics, cardiomyocyte contractility, hypertrophy and apoptosis (Nowotny et al. 2000; Brinks et al. 2011).

1.9.2.1 Expression of S100A6

S100A6 (Calcyclin), a 10-kDa protein from this family of EF-hand calcium binding proteins, is less well-studied and data on its expressional regulation and function in myocardium under normal or pathological conditions are scarce. It is known that under normal conditions S100A6 is repressed in the myocardium but following myocardial ischemia in vivo, it is upregulated in the myocardium adjacent to the infarct area (day 35)(Tsoporis, Marks, Haddad, O'Hanlon, et al. 2005). Furthermore, increased levels of S100A6 in the peri-infarct regions of myocardium 24 hours following myocardial I/R have been demonstrated before by Cai et al. (Cai XY 2011). S100A6 can also be induced in cardiomyocytes by inflammatory cytokines and growth factors in vitro (Tsoporis, Izhar, and Parker 2008b). The time course and anatomical distribution of these expressional changes following myocardial ischemia or I/R injury has not
been fully elucidated. S100A6 is also found in epithelial cells and fibroblasts, which are two major cell types in the myocardium (Kuznicki et al. 1992).

1.9.2.2 S100A6 and Receptor of Advanced Glycation End-products (RAGE)

S100A6 extracellular function is mainly through interaction with the V and C2 domains of the Receptor of Advanced Glycation End-products (RAGE) (Figure 8). RAGE binding to extracellular S100A6 causes translocation of intracellular S100A6 from the cytoplasmic location to the peri-nuclear region but the consequence of this translocation is not known. Like other members of this protein family, some extracellular S100A6 gets back to the cells by active internalization through vesicle formation (Leclerc et al. 2007; Hsieh et al. 2002; Leclerc et al. 2009; Hsieh HL1 2004). S100A6 bound to the C2 domain of RAGE is specific to this member of the family. Most other S100 proteins bind the V and/or C1 domains. Therefore, S100A6 may have discordant function compared to others (Leclerc et al. 2007; Donato 2007). Binding of S100 proteins to the tetramerization (TET) domain of p53 is a general property of this family. In contrast, binding to the negative regulatory domain (NRD) of p53 is not common to all S100 proteins. S100A1 has a strong binding affinity for NRD, but S100A6 shows no binding or very weak binding affinity (Tsoporis et al. 2014).
Figure 8 Binding Domains of RAGE Receptors and Their Potential Ligands.
S100A1, S100B and S100A6 bind to the V domain of extracellular segment of RAGE receptor which is called soluble RAGE. S100A6 binds to C2 domain too which is specific to this member of EF-Ca^{2+} binding protein.

1.9.2.3 S100A6 and Apoptosis

It has been suggested that S100A6 has differential effects on apoptosis depending on cell type, its concentration and location (intracellular or extracellular). For instance, the human liver cell line (Hep3B) overexpressing S100A6 was more susceptible to apoptosis induced by calcium ionophore. This was explained by increased activity of caspase-3 in these S100A6 overexpressing cells (Joo et al. 2008). Extracellular S100A6 triggers the JNK and consequently the Caspase 3/7 pathways, causing apoptosis in neuroblastoma cells (Donato 2007). In cardiomyocytes, S100A6 overexpression prevented TNF-α induced apoptosis in vitro. The proposed mechanism is suggested to be interference with p53 phosphorylation, especially at
serine 15 residue. This interaction is both physical (Ca\textsuperscript{2+} dependent binding to the tetramerization domain of p53) and non-physical (through some unknown pathways) (Tsoporis, Izhar, and Parker 2008b; Fernandez-Fernandez, Rutherford, and Fersht 2008). James Tsoporis et al. have recently shown that conditional overexpression of S100A6 in transgenic mice, results in less myocardial apoptosis, 35 days post MI (Tsoporis et al. 2014).

1.9.2.4 S100A6 and Fibrotic Tissue Formation

The existing data on the effect of S100A6 overexpression on myocardial necrosis and fibrotic scar formation is limited to one study (171). Tsoporis et al. recently showed that conditional overexpression of S100A6 in transgenic mice, results in less myocardial fibrosis, 35 days post MI (Tsoporis et al. 2014).

1.9.2.5 S100A6 and Cell Cycle Regulation and Proliferation

S100A6 expression is cell cycle dependent. S100A6 is mostly expressed in G0 and S phases (Calabretta et al. 1986). Its role in cell cycle regulation, proliferation, differentiation and migration has been well-studied and proven in different tissues, especially regarding its role in tumorigenesis and metastasis (Breen and Tang 2003). Various pathways have been suggested to be involved in this context.

The pathway involved in hepatocellular carcinoma cell proliferation is PI3K/AKT pathway (Li et al. 2014). Through this pathway, S100A6 overexpression increases expression
and availability of β-catenin. Free β-catenin gets translocated to the nucleus and activates β-catenin/TCF-mediated transcription of target genes such as u-PAR, c-MYC, and Cyclin D, which leads to increased proliferation and invasiveness of HepG2.2.15 tumor cells (Li et al. 2014; Lee et al. 2008; Fuchs et al. 2005).

The suggested pathway in neonatal cardiomyocytes is through Calcyclin-binding protein (CacyBP). CacyBP is the S100A6 interacting partner (Filipek and Kuznicki 1998). Both S100A6 (Calcyclin) and its interacting partner (CacyBP) get up-regulated following MI. CacyBP/SIP is also up regulated during the differentiation of cardiomyoblasts (Au et al. 2006). Siah-1 interacting protein (SIP) is a human homolog of CacyBP. CacyBP/SIP interact with Siah-1 and Skp1, forming Siah-1-CacyBP/SIP-Skp1 complex. This complex binds to β-catenin, which regulates the activity of Tcf/Lef-family transcription factors. This family of transcription factors induces expression of target genes such as cyclin D1, c-myc and promotes cell proliferation (Shtutman et al. 1999; Matsuzawa and Reed 2001).

S100A6 can stimulate proliferation and migration of colorectal cancer cells through ERK and p38 mitogen-activated protein kinase (MAPK) activation pathways (Duan et al. 2014). In fibroblasts and epithelial cells, S100A6 is involved in cell cycle progression but it does not significantly influence their viability (Slomnicki and Lesniak 2010).
1.9.2.6 S100A6 and Angiogenesis

In the ECs, S100A6 interacts with heat-shock proteins (HSP70/HSP90), STAT1 and p53, which stimulate expression of ECs-cycle progression genes such as CDK1, Cyclin A1 (CCNA1) and Cyclin B1 (CCNB1). Cell-cycle progression proteins activate serine/threonine protein kinase activity, which stimulates cell proliferation (Bao et al. 2012; Riabowol et al. 1989).

1.9.2.7 S100A6 and Myocardial Hypertrophy

S100A6 inhibits the induction of the cardiac fetal gene promoters in cultured neonatal rat cardiomyocytes (Tsoporis, Marks, Haddad, O'Hanlon, et al. 2005), suggesting a role in modulation of cardiac hypertrophy. James Tsoporis and his colleagues have recently shown that conditional overexpression of S100A6 in transgenic mice, results in attenuation of myocardial hypertrophy, 35 days post MI (Tsoporis et al. 2014).

1.9.2.8 S100A6 and Calcium Handling and Calcineurin Activity

The existing data on the role of S100A6 in calcium handling and contractility of cardiomyocytes is limited. Only recently, a study investigated the effect of S100A6 overexpression on Ca$^{2+}$ transient and contractility in rat adult cardiomyocytes. They investigated the effect of S100A6 and S100A2 overexpression on myocyte contraction amplitude and relaxation speed as well as Ca$^{2+}$ release amplitude and speed following field stimulation. Their results did not show any significant effect on calcium transient for S100A6 during stimulated...
action potential. However, they did not study the effect of S100A6 gene overexpression on spontaneous action potentials (Wang et al. 2014).

Calcineurin (CaN) is a Ca2+/Calmodulin dependent serine/threonine-specific phosphatase (Stewart et al. 1982). CaN has been reported as a critical mediator for cardiac hypertrophy and cardiomyocytes apoptosis through regulation of Bel-2-associated death promoter (BAD) phosphorylation and localization (Wang HG1 1999). CaN activity increases following myocardial I/R injury in rat hearts following cleavage through calpain-mediated proteolysis (Sharma 2005). There is no data on the effect of S100A6 overexpression on the expression of Calcineurin and its phosphatase activity.

1.9.3 S100A1

1.9.3.1 Expression of S100A1

S100A1 is highly expressed in skeletal and cardiac muscle under normal conditions and its expression is markedly down-regulated in rat myocardium following experimental left ventricular MI or pressure overload (at day 35). It is suggested that their expressional changes are related to their different functions in normal and diseased states (Tsoporis et al. 1997, 1998).
1.9.3.2 S100A1 and Receptor of Advanced Glycation End-products (RAGE)

It is suggested that S100A1 binds to the V domain of RAGE receptor for extracellular function.

1.9.3.3 S100A1 and Apoptosis

Extracellular S100A1 protein gets endocytosed into cardiomyocytes through Ca$^{2+}$ dependent Clathrin-mediated process and prevents apoptosis through ERK1/2 pathway (Most et al. 2003).

1.9.3.4 S100A1 and Fibrotic Tissue Formation

David Rodhe et al. demonstrated the anti-fibrotic effects of S100A1. They showed that S100A1 is released following MI in mice, gets internalized by neighboring cardiac fibroblasts (CFs) and assumes immune-modulatory and anti-fibrotic roles. CFs show enhanced intercellular adhesion molecule-1 (ICAM-1) and decreased collagen levels (Rohde et al. 2014).

1.9.3.5 S100A1 and Cell Cycle Regulation and Proliferation

In PC12 cells (cell line derived from a pheochromocytoma of the rat adrenal medulla), S100A1 regulates cell cycle through regulation of cytoskeleton compartments such as tubulin/microtubule levels and neurite organization (Zimmer et al. 1998).
1.9.3.6 S100A1 and Angiogenesis

Patrick Most and his colleagues showed that S100A1 gets down regulated in patients during chronic limb ischemia. They hypothesized that S100A1 is critical for ECs function in ischemic angiogenesis. Since S100A1 has stimulatory effect on eNOS, its deficiency impairs eNOS activity in ECs. On the other hand, S100A1 deficiency leads to PKC hyperactivation. PKC inhibits eNOS by phosphorylation and enhances VEGF-receptor 2 (VEGFR2) degradation, which attenuate VEGF signaling (Most et al. 2013).

1.9.3.7 S100A1 and Myocardial Hypertrophy

S100A1 can also inhibit the α1-adrenergic-mediated induction of the S100B, skACT, and β-MHC promoters in vitro (Tsoporis et al. 2003), which inhibit myocardial hypertrophy.

1.9.3.8 S100A1, Calcium Handling and Calcineurin Activity

S100A1 effect on calcium handling depends on its subcellular location. Intracellular S100A1 works on SR calcium regulatory proteins while extracellular S100A1 mediates sarcolemmal calcium regulation after endocytosis (Most et al. 2005). Endogenous S100A1 mainly controls Ca^{2+} homeostasis by directly affecting the RYR and stimulating CICR (Donato 2001). In large animal models treatment with adeno-associated virus serotype 9 (AAV9)–S100A1 two weeks after MI could prevent and reverse progressive deterioration of cardiac...
performance and LV remodeling by normalizing cardiomyocyte Ca\textsuperscript{2+} cycling, SR calcium handling, and energy homeostasis (Pleger et al. 2011).

1.9.4 S100B

1.9.4.1 Expression of S100B

S100B is normally absent in cardiomyocytes and is induced in hypertrophic myocardium, 35 days following MI or \(\alpha1\)-adrenergic stimulation (Tsoporis et al. 1997, 1998).

1.9.4.2 S100B and Receptor of Advanced Glycation End-products (RAGE)

S100B interacts with V-domain of RAGE. Although RAGE is the most significant receptor of S100B and plays an important role in cell signaling, recent studies in myoblasts suggest that RAGE is not the sole receptor for S100B (Adami C1 2004; Sorci et al. 2003, 2004).

1.9.4.3 S100B and Apoptosis

_in vitro_ studies have shown that extracellular S100B at concentrations >50 nmol/L induces myocyte apoptosis by interacting with RAGE receptors and activating ERK1/2 and p53 signaling. Its induced expression in peri-infarct regions of myocardium following ischemia and elevated plasma levels have been in accordance with the progressive rise in myocardial apoptosis in the peri-infarct areas of myocardium, _in vivo_ (Tsoporis et al. 2010).
1.9.4.4 S100B and Calcium Handling and Calcineurin Activity

Cultured cerebellar astrocytes derived from S100B-null mice showed altered stimulus-evoked calcium transients (Xiong et al. 2000). S100B can also increase the activity of purified and cytoskeletal Calcineurin in a Ca\(^{2+}\) dependent manner. Co-localization of S100B and Calcineurin in the astrocyte cytoskeleton has also been reported (Leal et al. 2004).

1.9.4.5 S100B and Cell Cycle Regulation and Proliferation

Overexpression of S100B in PC12 neuronal cells increased their proliferation through activating the PI3-K/Akt pathway (Arcuri et al. 2005). Overexpression of S100B in myoblasts also resulted in increased proliferation and reduced differentiation (Heizmann, Fritz, and Schafer 2002).

1.9.4.6 S100B and Angiogenesis

It has been shown that S100B up-regulation can increase angiogenesis in glial tumors (Wang H1 2013). S100B even at low levels can modulate the activity of tumor-associated macrophages (TAM) by activating Stat3 (Zhang et al. 2011). TAMs release angiogenic factors such as VEGF-A in response to hypoxia. VEGF-A stimulates chemotaxis of ECs and promotes angiogenesis (Bianchi et al. 2011; Zhai, Heppner, and Tsirka 2011; Du et al. 2008). Another suggested mechanism is through S100B-RAGE interaction. During MI, α\(_1\) adrenergic stimulation induces expression of S100B in surviving but damaged cardiomyocytes that leak
S100B to the extracellular space, activating RAGE in ECs and in VEGF-releasing epithelial cells (Tsoporis, Marks, Haddad, Dawood, et al. 2005; Ma et al. 2007).

1.9.4.7 S100B and Myocardial Hypertrophy

*In vitro* data show that S100B could inhibit hypertrophic response in cardiomyocytes following α1-adrenergic stimulation (Tsoporis et al. 1997). It has also been suggested that S100B plays a role as an intrinsic negative regulator of the myocardial hypertrophic response following MI (Tsoporis et al. 1998).
Chapter 2

Hypotheses and Objectives
As discussed in the first chapter, S100A6 overexpression has anti-apoptotic effects in various cell types including cardiomyocytes. Although the effect of S100A6 overexpression on TNF-α induced apoptosis has been studied before, its effect on hypoxia/re-oxygenation induced apoptosis in cardiomyocytes has not been studied as yet. We believe that hypoxia/re-oxygenation of neonatal rat cardiomyocytes can be a representative in vitro model for myocardial ischemia/reperfusion injury in vivo.

1. **Hypothesis:**

S100A6 gene overexpression will abrogate TNF-α induced apoptosis in H9c2 cells and hypoxia/re-oxygenation induced apoptosis in isolated neonatal rat cardiomyocytes, in vitro.

**Objective:**

To determine the effect of S100A6 gene overexpression on the TNF-α induced apoptosis in H9c2 cells and hypoxia/re-oxygenation induced apoptosis in neonatal rat cardiomyocytes, in vitro.

Up-regulation of S100A6 has been shown post myocardial ischemia but the course of S100A6 gene expression during 28 days post myocardial ischemia/reperfusion injury has not been described yet. On the other hand, interactions between various members of EF-hand Ca^{2+}
binding protein family have been shown previously. Therefore we decided to test the following hypothesis:

2. **Hypothesis:**

S100A6 gets up regulated in the infarct and peri-infarct regions of LV in the rat model of myocardial I/R injury, *in vivo*. The course of S100A6 expression is different from other members of this family including S100A1 and S100B.

**Objective:**

To determine the course of S100A6 gene expression during 28 days following myocardial I/R injury in relation to the expression of other members of S100 family of proteins (S100A1 and S100B), cardiomyocyte apoptosis and LV functional changes, *in vivo*.

Effect of S100A1 gene expression on the calcium transients in cardiomyocytes and its pro-angiogenic influence on endothelial cells has been previously studied. Given the fact that S100A6 shares many similarities with this member of EF-hand Ca^{2+} binding protein family (S100A1), we hypothesized that:

3. **Hypothesis:**
S100A6 overexpression impacts calcium transients and Calcineurin activity in rat neonatal cardiomyocytes, *in vitro*. S100A6 overexpression influences tubule formation and migration of human umbilical vein endothelial cells *in vitro*, indicating potential pro-angiogenic properties.

**Objective:**

To assess the effect of S100A6 overexpression on calcium transients and Calcineurin activity in rat neonatal cardiomyocytes, *in vitro*.

To measure the effect of S100A6 overexpression on tubule formation capacity and migration potential of human umbilical vein endothelial cells *in vitro* as markers of angiogenesis.

Considering our preliminary *in vitro* data, which confirmed the anti-apoptotic property of S100A6 overexpression in neonatal rat cardiomyocytes post hypoxia/re-oxygenation and our *in vivo* results that showed a delay in up-regulation of S100A6 post myocardial I/R injury compared to the peak of apoptosis and necrosis, we hypothesized that:

4. **Hypothesis:**

UTMD delivery of S100A6-plasmids prior to the myocardial I/R injury will attenuate cardiomyocyte apoptosis, reduce infarct size and improve LV function and myocardial perfusion, and limit ventricular hypertrophy, *in vivo*.

**Objective:**
To assess the effect of UTMD mediated delivery of S100A6-plasmid prior to the myocardial I/R injury on cardiomyocyte apoptosis, infarct size and LV function, myocardial perfusion and ventricular hypertrophy, *in vivo*.

It has been shown that Minicircle DNA is more efficient transduction vector than conventional plasmids and can lead to faster and longer lasting expressional modifications because of its shorter sequence. Our data also confirmed therapeutic benefits for S100A6 plasmid delivery if done 2 days prior to the myocardial I/R injury, but this approach is not translatable to the clinic. We hypothesized that:

5. **Hypothesis:**

UTMD mediated S100A6-minicircle delivery immediately after myocardial I/R injury attenuates cardiomyocyte apoptosis, reduces infarct size and improves LV function and myocardial perfusion, *in vivo*.

**Objective:**

To assess the effect of UTMD of S100A6-minicircle immediately after myocardial I/R on cardiomyocytes apoptosis, infarct size and LV function, myocardial perfusion, *in vivo*.
Chapter 3
Myocardial Ischemia/Reperfusion Injury: Time Course of S100A6 Gene Expression in Relation to Expression of S100A1 and S100B Genes, LV Dimensional/Functional Alterations and Apoptosis
Abstract

**Introduction:** Acute myocardial infarction (MI) can lead to chronic cardiac dysfunction and heart failure (HF) due to the remodeling process. With increasing number of patients surviving MI following successful reperfusion therapy, new treatments aimed at prevention of adverse remodeling are a focus of much research. S100A6, a member of EF-hand Ca$^{2+}$-binding proteins, has anti-remodeling effects that may play a role in the prevention of I/R myocardial injury. Although up regulation of endogenous S100A6 gene expression following myocardial ischemia has been reported before, the course of S100A6 gene expression compared to other S100 proteins, S100A1 and S100B, and its relation to cardiomyocyte apoptosis and LV functional deterioration following myocardial I/R injury has not been fully elucidated.

**Material and methods:** Fifty male Fischer rats underwent acute I/R induction surgery by a 30-minute ligation of the left anterior descending coronary artery (LAD) followed by reperfusion. Animals were followed by serial echocardiography and blood sampling at various time points (6 hours, 1, 2, 3, 7, 14 and 28 days) with subgroups of animals sacrificed at each time point to harvest tissue.

**Results:** A significant increase in LV dimensions and reduction in LV systolic function were detected early (6 hours) after myocardial I/R injury that remained relatively stable until day 28. S100A6 expression progressively increased in the infarct region, reached a significant peak
at day 3 and remained high out to day 28. Up regulation of S100B gene expression was delayed to S100A6 whereas S100A1 expression was down regulated (lowest level at day 3) following myocardial I/R injury. Apoptosis occurred in the infarct (highest during first 24 hours) and peri-infarct regions of myocardium (peak between day 3 and 7) post I/R injury.

**Conclusions:** The up-regulation S100A6 gene expression following myocardial I/R injury is not early enough to prevent the peak of cardiomyocyte apoptosis and LV functional deterioration. Considering the anti-apoptotic effects of S100A6 protein, early delivery of S100A6 gene to the LV myocardium may ameliorate LV systolic dysfunction and prevent adverse remodeling. The opposite time courses of S100A1 and S100A6 expressional changes following myocardial I/R are suggestive of a potential counteraction between these two members of S100 family proteins.
3.1 Introduction

Among all the potential therapeutic genes targeting myocardial I/R injury, there is a family of EF-hand Ca\(^{2+}\) binding proteins comprising 19 members that are differentially expressed in a large number of cell types. Members of this protein family have been implicated in the Ca\(^{2+}\) dependent and in some cases, Zn\(^{2+}\) or Cu\(^{2+}\) dependent regulation of a variety of intracellular activities such as protein phosphorylation, enzymes activation, cell proliferation/differentiation, cytoskeleton constituents’ dynamics, structural organization of membranes, intracellular Ca\(^{2+}\) homeostasis, inflammation, and oxidative stress protection.

Among all members of S100 family of proteins, S100A1, S100B and recently, S100A6 have attracted most of the attention in cardiovascular research because of their proposed role in regulation of cardiomyocyte contractility, hypertrophy and apoptosis. S100A1 and S100B, 20-kDa Ca\(^{2+}\)-binding homodimers, have been extensively studied, specifically as potential therapeutic targets to prevent adverse ventricular remodeling and HF. In contrast to the aforementioned two S100 proteins, S100A6 (Calcyclin), a 10-kDa protein from this family is not as well-studied, and as such data on its expressional regulation and function in the myocardium under normal or pathological conditions are scarce. Based on the existing data, S100A6 extracellular function is mainly through interaction with the V and C2 domains of the Receptor of Advanced Glycation End-products (RAGE). RAGE binding to extracellular S100A6 can
cause translocation of intracellular S100A6 from the cytoplasmic area to the peri-nuclear region but the consequence of this translocation is not known. Like other members of this protein family, extracellular S100A6 is able to get back into cells by active internalization through vesicle formation (Leclerc et al. 2007; Hsieh et al. 2002; Leclerc et al. 2009; Hsieh et al. 2004). S100A6 can also be induced in cardiomyocytes by inflammatory cytokines and growth factors in vitro (Tsoporis, Izhar, and Parker 2008b). It is known that under normal conditions S100A6 gene expression is repressed in the myocardium but following myocardial ischemia, its expression is regulated in the area adjacent to the infarcted myocardium (day 35) (Tsoporis, Marks, Haddad, O'Hanlon, et al. 2005).

Considering all the missing parts of this puzzle, we aimed to determine the time course of S100A6 gene expression in various regions of the LV following myocardial I/R injury in rats, and compare it to expression of S100A1 and S100B genes. At the same time, we intended to assess the course of LV functional and dimensional alterations as well as cardiomyocyte apoptosis during this period, in order to gain deeper insights into S100A6 effects on the remodeling process post myocardial I/R injury and shed light on the potential therapeutic application of S100A6-gene delivery to LV myocardium to prevent HF following I/R injury.
3.2 Methods and Experimental Protocol

3.2.1 In vitro Protocol

3.2.1.1 Cell Cultures, S100A6 Transfection and TNF-α Treatment

The in vitro study was conducted on H9c2 cells (rat cardiomyoblasts; ATCC microbiology). For in vitro overexpression of S100A6, we used a non-liposomal multicomponent transfection reagent FUGENE 6 (Promega Corporation, Catalogue Number: E2691). The cultures were incubated with the mixture containing the transfection reagent, DMEM (ATCC 30-2002) and GFP tagged human S100A6 plasmid for 48 hours. Subgroup of cell cultures was treated with TNF-α (10ng/ml) for 48 hours (Figure 9) to induce apoptosis.

**Figure 9** In vitro Experimental Protocol
3.2.1.2 Terminal Deoxynucleotide Transferase-Mediated dUTP Nick-End Labeling (TUNEL)

Cardiomyoblasts were grown on two chamber slides, transfected with GFP tagged human S100A6 plasmid and treated with TNF-α as described above. Non-transfected cardio-myoblasts and vehicle (PBS) treated cells were included as control groups. Apoptotic cell labeling was performed using Apoptag Red in situ Apoptosis Detection kit (Millipore, Catalogue#2189979) that detects apoptosis by the indirect TUNEL method, utilizing an anti-digoxigenin antibody with a rhodamine fluorochrome. The nuclei were counterstained with DAPI containing Vectashield mounting medium (Vector Laboratories Inc, Catalogue#H1200). The transfected cells expressing GFP fused human S100A6 protein were distinguishable by green fluorescence, apoptotic nuclei were detectable by a red fluorescence and all nuclei were blue.

3.2.1.3 Caspase 3/7 Activity Assay

Caspase (the cysteine aspartic acid-specific protease family) is a key effector of apoptosis in mammalian cells. The Caspase-Glo 3/7 Assay was used to measure caspase-3 and-7 activities in cultured cardiomyoblasts as a marker for apoptosis. The assay provides a luminogenic caspase-3/7 substrate, which contains the amino acid sequence Asp-Glu-Val-Asp (DEVD). Adding Caspase-Glo 3/7 reagent results in cell lysis, followed by caspase cleavage of the DEVD substrate and generating a glow-type luminescent signal, produced by luciferase. The
luminescence signals of the samples are read using the SpectraMax Luminescence Micro-Plate Reader and are compared to the values of controls.

In brief, cardiomyoblasts were grown in 96-well plates, transfected with GFP tagged human-S100A6 and treated with TNF-α as described above. Non-transfected cardio-myoblasts and vehicle (PBS) treated cells were grown is separate wells as control groups. We added 100µl of Caspase-Glo® 3/7 Reagent to each well of a white-walled 96-well plate containing treated cells and controls in culture medium. We gently mixed contents of wells using a plate shaker at 300–500rpm for 40 seconds. Then, we incubated the plates at room temperature for 1 hour. The luminescence of each sample was measured in a plate-reading luminometer.

3.2.2. In vivo Protocol

3.2.2.1 Animal Preparation

The study protocol (ACC Protocol # 236-2R) was approved by the Animal Care and Use Committee at the Keenan Research Centre for Biomedical Science, Li Ka Shing Knowledge Institute, St Michael's Hospital, University of Toronto, in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

3.2.2.2 Animal Experimental Protocol, I/R Induction Surgery

Myocardial I/R was induced by ligating the LAD coronary artery as previously described (Mahmoud et al. 2014). Animals were anaesthetized with inhaled 2-3% Isoflurane in 1.5 L/min
oxygen, intubated and volume controlled ventilated (85/min with tidal volume of 1.25 mL) with 1.5-2% Isoflurane in 1.5-2 L/min oxygen using a rodent respirator (model 683, Harvard Instruments). Buprenorphine (0.02 mg/kg SC) and Anafen (5 mg/kg SC) were administered prior to the surgery. After preparing the surgical site with ethanol and povidone-iodine, under sterile conditions the rat’s chest was opened. An incision was made in the skin on the left side of the chest, and the pectoral muscles were gently retracted to expose the ribs. An incision was made through the fourth intercostal space and the ribs were gently spread to expose the heart.

Myocardial I/R was induced by ligation of LAD with a 6-0 Prolene ligature. A curved needle was passed through the myocardium beneath the LAD coronary artery just below the atrial appendage. The suture was then routed into a clamping device, which compressed the artery to induce a reversible occlusion. The procedure was considered successful if the myocardium changed color from a red hue to a pale white. After 30 minutes the clamp was released and the ligature removed. Reperfusion was confirmed by observing the change from cyanotic to pink in the myocardium. The chest was compressed to expel intra-pleural air. The ribs, muscle and skin were sutured and saline was administered (10 mL/kg, SC). After recovery, the rat was extubated and placed on a warming blanket. Successful I/R injury was confirmed by echocardiography post I/R induction surgery (Mahmoud et al. 2014; Oleg Tarnavski 2004).
3.2.2.3 Echocardiography and Cardiac Catheterization

Under inhaled anesthesia (1.5-2% isoflurane in 1.5-2 L/min oxygen), echocardiography was performed using the Vevo 2100 system (Visual Sonics) with MS250 transducer (13–24MHz) at baseline and various time points post myocardial I/R induction surgery (6 hours, 1 day, 2 days, 3 days, 7 days, 14 days and 28 days) (Figure 10). A parasternal long-axis (PLAX) view was recorded, ensuring that the mitral valve, aortic valve and the apex were visualized. A parasternal short-axis (PSAX) view was recorded at the level of mid papillary muscles. Both 2-dimensional and M-mode (MM) views were recorded at the same level. LV end-diastolic dimension (EDD) and end-systolic dimension (ESD) were measured from SAX view and fractional shortening (FS, %) was calculated using the following equation: (LV EDD-LV ESD) / LV EDD) ×100.

*Echocardiography and sacrificing subgroups of animals to collect tissue

**Figure 10 In vivo Experimental Protocol, Myocardial I/R Injury Model**
Fractional area change (FAC) was measured by manually tracing end-systolic and end-diastolic LV cavity areas on 2D-echo images in SAX views. Ejection fraction (EF, %) was measured in PLAX view using mono-plane Simpson’s technique. All values were averaged over 3 consecutive cycles.

3.2.2.4 Blood Sampling

Blood sampling was performed upon anaesthetized animals (after undergoing echocardiography). Around 0.5 ml of venous blood was collected from the tail vein, immediately put on ice and the plasma was separated by centrifugation (2500 rpm, 15 min, 4 °C). Plasma samples were tested by enzyme-linked immunosorbent assay (ELISA) to measure the level of circulating rat S100A6 protein post myocardial I/R injury compared to controls.

3.2.2.5 Postmortem Analysis

Terminal Deoxynucleotide Transferase-Mediated dUTP Nick-End Labeling (TUNEL)

Analysis of apoptosis was performed in one LV section obtained from the sample that showed maximal infarct size. Using the ApopTag Peroxidase In Situ Apoptosis Detection Kit, apoptotic cardio-myocytes were detected by labeling and detecting DNA strand breaks using Terminal Deoxynucleotide Transferase-Mediated dUTP nick-end labeling (TUNEL). The apoptotic index (FITC⁺ nuclei×100/DAPI⁺ nuclei) was calculated in various regions of LV sections (infarct, peri-infarct and non-infarct regions) using fluorescent microscopy with a
magnification of 40x. The density of cardiomyocytes’ nuclei was counted in four representative microscopic fields in each region of interest. The cardiomyocyte origin of the apoptotic cells was confirmed by immunohistochemistry (IHC) staining for anti-sarcomeric alpha actinin antibody (ab9465). The assay was standardized to the positive control sections treated with DNase I (1 U/ml for 30 min at 37°C) to induce formation of DNA strand breaks.

**Caspase 3/7 Activity Assay**

The Caspase-Glo 3/7 Assay was used to measure caspase-3 and -7 activities in protein extracts from the tissue collected from various regions of LV (infarct, peri-infarct and non-infarct regions). Tissue samples were homogenized and protein concentrations were determined according to the method of Bradford protein assay. We added 100µl of Caspase-Glo® 3/7 Reagent to each well of a white-walled 96-well plate containing 100µl of the protein extracts (50µg/100µl in PBS). We gently mixed contents of wells using a plate shaker at 300–500 rpm for 40 seconds. Then we incubated the plates at room temperature for 1 hour. The luminescence of each sample was measured in a plate-reading luminometer.

**Immunohistochemistry (IHC) staining**

In order to determine the origin of apoptotic nuclei, IHC staining for specific cell markers were carried out on TUNEL stained sections. Briefly, OCT-embedded tissue sections were fixed in 2% paraformaldehyde and permeabilized with Triton (0.1% in PBS) for 10 minutes. The
sections were blocked by normal goat serum (10%, Life technologies; catalogue # 50-062Z) for an hour. Antibodies for mouse anti-sarcomeric-alpha actinin (1:100, Abcam; ab9465) and goat polyclonal secondary antibody to mouse IgG conjugated to Cy3 (1:500, Abcam: ab97035) were used in conjunction with the TUNEL kit for double fluorophore detection. The nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Vector). The images were captured using a confocal microscope.

Plasma Isolation and ELISA Assay

To measure the level of S100A6 protein in plasma, Rat S100 Calcium Binding Protein A6 (S100A6) ELISA kit (MyBioSource, Cat#MBS725304) was used. This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for S100A6 has been pre-coated onto a micro-plate. Standards and samples were pipetted into the wells where the immobilized antibody bound to the existing S100A6 proteins. After removing unbound substances, a biotin-conjugated antibody specific for S100A6 was added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells. Color develops in proportion to the amount of S100A6 bound in the initial step. The color development was stopped and the intensity of the color was measured in comparison to the standard curve using the VersaMax Absorbance Micro-plate Reader.
Real-Time PCR

Total mRNA was extracted from various regions of the LV myocardium (infarct, peri-infarct and remote non-infarct regions) using Trizol reagent, and reverse-transcribed into cDNA applying a reverse transcription system (iScript™ Reverse Transcription Supermix for RT-qPCR). Amplification was performed with Power SYBR Green PCR Master Mix in a StepOne format. Gene expression levels were normalized to housekeeping TATA box binding protein (TBP) gene, and data were analyzed with StepOne software v2.1.

The primers’ sequences were as follows:

Rats S100A6 primer:

5'-GGCCATCGGCCTTCTCGTGG-3' (forward)
5'-TGCTCAGGGTGTGCTTGTCACC-3' (reverse)

Rat TBP primer:

5'-CCATTGCCAGGCACCACCCC-3' (forward)
5'-AGGCTGGTGTGGCAGGAGTGA-3' (reverse)

Rat S100B primer:

5'-GGAGCTCATCAACAACGAGC-3' (forward)
5'-GAAGTCACACTCCCCATCC-3' (reverse)

Rat S100A1 primer:
5′-CAATGTGTTCCATGCCCACTC-3′ (forward)
5′-AGCAGGTCTTTTCAGCTCCTTC-3′ (reverse)

Human/Rat S100A6 primer:
5′-GACAAGCACACCCCTGAGCAA-3′ (forward)
5′-CAGCCTTTGCAATTTCAGCATCC-3′ (reverse)

Western Blotting

Tissue samples were homogenized and protein concentrations were determined according to the method of Bradford protein assay. Proteins of equal loading were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with rabbit Anti-rat S100A6 primary antibody (Abcam, Cat#ab134149) and Goat Anti-Rabbit IgG (HRP) (Abcam, Cat#ab6721). Blots of protein were probed with mouse anti-Glyceraldehyde-3-Phosphate Dehydrogenase primary antibody (Anti-GAPDH, Abcam, Cat#ab8245) and Rabbit Anti-Mouse IgG (HRP) (Abcam, Cat#ab97046) for internal reference. Secondary antibodies were detected using Electrochemi-luminescence (ECL) system. Band intensity was quantified by scanning densitometry using VersaDoc Imaging System.
3.2.2.6 Statistical Methods

Continuous variables were presented as mean+/−standard error of the mean (SEM). Normal distribution was evaluated with the Kolmogorov–Smirnov test, and logarithmic or square root transformations were performed on the continuous variables of non-normal distribution. Differences among groups are analyzed by one-way analysis of variance (ANOVA) followed by post hoc analysis (Bonferroni’s correction). GLM Univariate Analysis of variance (two-way ANOVA) followed by Bonferroni correction was applied for multiple comparisons when there were more than two factors influencing a variable. All analyses used 2-sided tests with an overall significance level of alpha=0.05, and were performed with SPSS 15.0 for Mac.

3.3 Results

3.3.1 In vitro Results

In the cardiomyoblast cultures, the amount of TNF-α induced apoptosis was significantly lower in the S100A6 transfected samples (1% vs. 7.5% in TUNEL stained slides, P<0.05, N=7). Furthermore, the ratio of caspase 3/7 activity in S100A6 transfected cultures over non-transfected controls was 22% (N=7).
3.3.2 *In vivo* Results

3.3.2.1 LV Function and Dimensions

Proximal LAD ligation for 30 minutes followed by reperfusion created a non-transmural infarction. After 28 days, scar formation was complete and infarct area was distinguishable with obvious central compact necrosis characterized by localized deposition of collagen and prominent contraction bands in HE stained sections (Figure 11).

**Figure 11** Representative image of H&E stained section of the LV.

It demonstrates the characteristic central compact necrosis in the infarct region of LV at the level of the papillary muscles 28 days post myocardial I/R injury. The magnified image (×10) shows the haemorrhage and inflammatory response in the necrotic tissue.

The circumferential extent of the akinetic segment of the LV, which represents the extent of LV ischemia/infarction, was measured in the PSAX view by repeated echocardiographic assessments at various time points (6 hours, 1 day, 2 days, 3 days, 7 days, 14 days, 28 days, N=50) (Figure 12).

**Figure 12** Representative echocardiographic images of LV. PSAX (left) and M-mode (right), in a healthy control rat (top) and a rat post myocardial I/R injury at day 28 (bottom)
Ventricular EDD and ESD were measured in the PSAX view at the mid papillary muscle level using two-dimensional echocardiography at various time points (6 hours, 1 day, 2 days, 3 days, 7 days, 14 days, 28 days, N=50). A significant rise in the LV EDD (baseline EDD: 6.2±0.8mm, 6 hours EDD: 7.9±0.7mm) and LV ESD (baseline ESD: 3.8±0.8mm, 6 hours ESD: 6.3±0.7mm) occurred early after myocardial I/R injury (6 hours) that remained unchanged over time during 28 days (Figure 13A). LV FS and EF ratios demonstrated a significant decrease by 6 hours after myocardial I/R and remained relatively constant over 28 days (baseline FS: 47.6±5.3%, 6 hours FS: 20.90±3.5%) (Baseline EF: 77.9±5.5%, 6 hours EF: 46.78±5.7%) (Figure 13B). LAD ligation for 30 minutes followed by reperfusion resulted in akinesis of 32.2±2.3% of the LV circumference, which remained stable during 28 days post myocardial I/R injury (Figure 13C).
Figure 13 LV dimensions, LVEF and circumferential extent of LV akinesis.

LVEDD and LVESD recorded by 2D-echocardiography in PSAX view at various time points (6 hours, 1 day, 2 days, 3 days, 7 days, 14 days, 28 days) following myocardial I/R injury in rats (Mean+/−SEM, P<0.05 vs. baseline, N=50) (A), LVEF and LVFS measured by 2D-echocardiography in PSAX view at various time points (6 hours, 1 day, 2 days, 3 days, 7 days, 14 days, 28 days) following myocardial I/R injury in rats (Mean+/−SEM, P<0.05 vs. baseline, N=50) (B), Circumferential extent of LV akinesis measured by 2D-echocardiography in the PSAX view at various time points (6 hours, 1 day, 2 days, 3 days, 7 days, 14 days, 28 days) following myocardial I/R injury in rats (Mean+/−SEM, N=50) (C).
3.3.2.2 Expression of Rat-S100A6 in LV Myocardium

S100A6 mRNA was measured in various regions of the LV (infarct, peri-infarct and remote non-infarct regions) in 42 rats at various time points (6 hours, 1 day, 2 days, 3 days, 7 days, 14 days and 28 days) over 28 days. In the infarct area, S100A6 expression progressively increased and reached a significant peak (5.2±0.9 fold increase compared to non-ischemic controls) by day 3 then decreased slightly, but remained elevated (3.3±0.9 fold increase compared to non-ischemic controls) out to day 28. In the peri-infarct region, S100A6 expression showed a similar trend but to a lesser extent. In remote non-ischemic region, the changes in S100A6 expression were not statistically significant (Figure 14).

**Figure 14** S100A6 gene expression in LV myocardium, after I/R injury.
Amount of S100A6 mRNA (measured by qRT-PCR) in the infarct, peri-infarct and non-infarct regions of rat myocardium (compared to normal myocardium) at various time points (6 hours, 1 day, 3 days, 7 days, 14 days, 28 days) following myocardial I/R injury (Mean+/−SEM, P<0.05 vs. normal LV and 6 hours, total N= 42, each group N=7).
3.3.2.3 Rat-S100A6 Protein Levels in Plasma and LV Myocardium

S100A6 protein level in plasma of 50 rats was measured using sandwich ELISA assay at various time points (baseline, 3 days, 7 days, 14 days, 28 days) post myocardial I/R injury. The S100A6 protein did not show any significant rise in plasma after myocardial I/R injury and remained steady during 28 days (baseline level: 1.39±0.6 ng/µl, day 28 level: 1.44±0.4 ng/µl) (Figure 15A).

Level of S100A6 protein was measured by western blotting in various regions of the LV myocardium in 42 rats at various time points (6 hours, 1 day, 3 days, 7 days, 14 days, and 28 days). In the infarct region of the LV, S100A6 protein levels showed an early peak at day 3 (1.97±0.2 fold increase over normal myocardium) and remained elevated out to day 28 (2.3±0.3 fold increase over normal myocardium). In the peri-infarct and non-infarct regions of LV, S100A6 protein levels showed similar trends but to a lesser extent (Figure 15B, C).
**Figure 15** S100A6 protein level in various regions of the LV myocardium and plasma post I/R. S100A6 protein level in the plasma of rats at various time points (baseline, 3 days, 7 days, 14 days, 28 days) post myocardial I/R injury (Mean+/−SEM, P>0.05, Total N=50, each group N=7-9) (A), A representative image of western blot membrane shows the bands for S100A6 protein in the infarct, peri-infarct and remote non-infarct regions of rat myocardium at day 3 and 28 following I/R injury. GAPDH is used as the house keeping protein and protein extract from normal non-ischemic rat myocardium is control (B), Amount of S100A6 protein in the infarct, peri-infarct and remote non-infarct regions of rat myocardium at various time points (6 hours, 1 day, 3 days, 7 days, 14 days, 28 days) following I/R injury in comparison to normal myocardium measured by western blot (Mean+/−SEM, P<0.05 vs. normal LV and 6 hours, Total N= 42, each group N=7) (C).
3.3.2.4 Expression of Rat-S100A1 in LV Myocardium

S100A1 mRNA was measured in various regions of the LV (infarct, peri-infarct and remote non-infarct regions) in 42 rats at various time points (6 hours, 1 day, 3 days, 7 days, 14 days and 28 days) over 28 days. In the infarct area, S100A1 expression gradually decreased and reached its nadir at day 3 (0.28±0.06 fold change over normal myocardium) then progressively returned to baseline levels by day 28. In the peri-infarct and remote non-infarct regions, expression of S100A1 follows the same trend (Figure 16).

**Figure 16** S100A1-gene expression in the LV myocardium.
Amount of S100A1 mRNA (measured by qRT-PCR) in the infarct, peri-infarct and non-infarct regions of rat myocardium (compared to normal myocardium) at various time points (6 hours, 1 day, 3 days, 7 days, 14 days and 28 days) following myocardial I/R injury on 42 rats (Mean+/−SEM, P<0.05 vs. 6H and healthy Control, N=4-11 at each time point).
3.3.2.5 Expression of Rat-S100B in the LV Myocardium

S100B mRNA was measured in various regions of the LV (infarct, peri-infarct and remote non-infarct regions) in 42 rats at various time points (6 hours, 1 day, 3 days, 7 days, 14 days and 28 days) over 28 days. In the infarct area, S100B expression progressively increased after day 7 with a significant peak (6.0±1.2 fold increase compared to healthy myocardium) at day 28. In peri-infarct and remote non-infarct regions, the changes in S100B expression followed a similar pattern but were not statistically significant (Figure 17).

![Figure 17 S100B gene expression in the LV myocardium.](image)

S100B mRNA level in various regions of the LV (infarct, peri-infarct and remote non-infarct) during 28 days post myocardial I/R injury measured by RT-PCR (Mean+/–SEM, P<0.05 vs. normal LV, 6 hours, day1, day 3 and day 7, Total N=42, each group N=7).
3.3.2.6 Rate of Apoptosis in the LV Myocardium

Apoptosis was measured by TUNEL staining in the infarct, peri-infarct and remote non-infarct regions of the LV myocardium at different time points (6 hours, 1 day, 2 days, 3 days, 7 days, 14 days, 28 days) in 38 rats. In the infarct region of the LV, apoptosis was significantly increased during 24 hours post myocardial I/R injury peaking at 6 hours (the apoptotic index at 6 hours: 8.17±3.40% and 24 hours: 5.67±2.37%). The TUNEL results are less reliable for detecting apoptosis after 24 hours in the infarct region because of the high number of FITC positive nuclei in the early stage of necrosis (Figure 18B).

In the peri-infarct region the apoptosis peaked at day 3 (the apoptotic index at day 3: 2.04±0.4%) then decreased significantly by day 28 (the apoptotic index at day 28: 0.19±0.06%). In the remote non-infarct the same trend of changes in the amount of apoptosis was observed but it was not statistically significant (Figure 18C).
**Figure 18** Apoptosis in the LV myocardium post I/R (TUNEL).

Representative image of IHC stained sections of LV myocardium at the level of the papillary muscles. Green color shows the FITC\(^+\) apoptotic nuclei, blue represents all nuclei and red shows myocardial sarcomeres. Remote non-infarct (left) and infarct regions (right) of the rat myocardium are shown 6 hours following myocardial I/R injury (A), Apoptosis in infarct region of rat myocardium during 24 hours following myocardial I/R injury measured in TUNEL stain sections (Mean+/-SEM, P<0.05 vs. Baseline, Total N=14, each group N=7,) (B), Apoptosis in peri-infarct and remote non-infarct regions of rat myocardium at various time points (6 hours, 1 day, 2 days, 3 days, 7 days, 14 days and 28 days) following myocardial I/R injury measured in TUNEL stained sections (Mean+/-SEM, P<0.05 vs. baseline and 6 hours, Total N=42, each group N= 4-8) (C).
3.3.2.7 Caspase 3/7 Activity in LV Myocardium

Caspase 3/7 activity was measured in infarct, peri-infarct and remote non-infarct regions of the myocardium in 37 rats at various time points (6 hours, 1 day, 2 days, 3 days, 7 days, 14 days and 28 days) following myocardial I/R injury compared to normal non-ischemic rats. Caspase 3/7 activity significantly increased after day 2 post myocardial I/R injury and reached the peak by day 7 (Caspase 3/7 activity: 4.50±0.71 fold increase over control non–ischemic myocardium), then decreased gradually and significantly by day 28 (Caspase 3/7 activity: 2.07±1.40 fold increase over control non–ischemic myocardium). There was a slight rise in caspase 3/7 activity in the peri-infarct region on day 3 but it was not statistically significant. In the remote non-infarct region, caspase 3/7 activity didn’t change during 28 days post myocardial I/R injury (Figure 19).
**Figure 19** Apoptosis in the LV myocardium post I/R (caspase 3/7 activity).
Caspase 3/7 activity in the infarct, peri-infarct and remote non-infarct regions of the LV myocardium at various time points (6 hours, 2 days, 3 days, 7 days, 14 days and 28 days) following myocardial I/R injury compared to normal non-ischemic myocardium (Mean+/−SEM, P<0.05 vs. 6 hours and day2, Total N=37, N=4-8 in each group).

### 3.4 Discussion

Previous studies have shown up-regulation of S100A6 and S100B genes but down regulation of S100A1 gene expression in the myocardium following infarction (Tsoporis et al. 1997; Tsoporis et al. 2003; Tsoporis, Marks, Haddad, O'Hanlon, et al. 2005; Cai XY 2011). Our present study confirms previous data, and extends them by providing more detailed analysis over the time course of expressional changes of these three important S100 genes within the first 28 days post myocardial I/R injury. S100A6 up regulation was highest within the infarcted regions
of the LV compared to the peri-infarct areas. Furthermore, expression of S100A6 in remote non-infarcted regions of the LV showed no significant change during the first 28 days post myocardial I/R injury. This suggests that S100A6 gene expression is influenced by the severity of ischemic injury or hypoxic stress. Longer follow up may be required to study the expression of S100A6-gene in the remote non-infarct region of LV at later time points when adverse remodeling and heart failure occurs. Using Paul J.H. Lee’s tissue samples I checked the level of S100A6 gene expression in a rat model of the doxorubicin-induced heart failure, which showed a significant up regulation of S100A6-gene expression in this model of dilated cardiomyopathy. On the other hand, our analysis on the human LV myocardial samples showed that S100A6-gene expression is up regulated in the setting of chronic ischemic cardiomyopathy. Thus, cumulative data suggests that myocardial infarction and I/R injury are associated with up regulation in S100A6-gene expression. On the contrary, S100A1 expression is down regulated in the myocardium post I/R injury.

Various interactions have been reported between the members of the S100 protein family and they have been able to trans-repress or trans-activate each other through protein-protein interaction such as inhibition of $\alpha_1$-adrenergic-mediated induction of the S100B by S100A1 in cultured neonatal rat cardiomyocytes (Tsoporis et al. 2003). It has also been demonstrated that S100A6 inhibits induction of S100B promoter by phenylephrine in cultured neonatal rat
cardiomyocytes (Tsoporis, Marks, Haddad, O'Hanlon, et al. 2005). No significant interaction has been reported between S100A1 and S100A6 so far. In this study, the contradictory expression of S100A1 and S100A6 post myocardial I/R injury suggests a possible trans-repression effect for S100A6 and S100A1 genes, however this needs to be explored in future studies by immunoprecipitation technique as well as colocalization assessment.

Although S100A6 mRNA and S100A6 protein levels both increase post myocardial I/R injury, the incongruity between these two values suggest the presence of post-transcriptional and translational regulatory mechanisms, such as participation of microRNAs in S100A6 expression regulation following hypoxic stress. This can be further investigated by application of microRNA array analysis.

The delayed peak of caspase 3/7-activity compared to the maximum apoptosis detected by TUNEL staining confirms the high sensitivity and low specificity of TUNEL staining and the fact that TUNEL staining is not able to differentiate between DNA disintegration during early phases of necrosis and DNA fragmentation throughout apoptosis (Duan et al. 2003). It also suggests a possible contribution of caspase-independent programmed cell death (CI-PCD) pathways during the first week post myocardial I/R injury. Based on our TUNEL staining and caspase 3/7-activity assay results, the peak of S100A6 expression is simultaneous or occurs just after the peak of cardiomyocyte apoptosis. Considering our in vitro data, which demonstrate anti-
apoptotic effects for S100A6-gene overexpression on cardiomyoblasts, one can hypothesize that over expression of S100A6 at earlier time points may result in reduced apoptosis and improved LV function over time, after I/R injury. To explore the effect of S100A6 overexpression on the myocardial remodeling process, comprehensive studies are required to investigate the influence of S100A6 on cardiomyocytes apoptosis, LV functional parameters and geometry.

3.5 Conclusion

S100A6 expression gradually increases following I/R mostly in the infarct and peri-infarct regions of myocardium, and occurs simultaneous with or just after the peak of cardiomyocytes apoptosis and well after LV functional deterioration. Forced overexpression of S100A6 gene at earlier time points may prevent early cardiomyocyte apoptosis and improve LV geometry and function.
Chapter 4

UTMD Mediated S100A6-Plasmid Delivery Prior to the Myocardial I/R Injury Attenuates Cardiomyocyte Apoptosis, Reduces Infarct Size, Improves LV Function, Recovers Myocardial Perfusion and Prevents Left Ventricular Hypertrophy
Abstract

**Background:** Overexpression of S100A6 gene, a member of the family of EF-hand calcium-binding proteins, can inhibit cardiomyocytes apoptosis through interference with P53 phosphorylation. We have previously shown that S100A6 gene expression is up-regulated post myocardial I/R injury but occurs simultaneous with or just after the peak of cardiomyocyte apoptosis and LV functional deterioration. Existing data demonstrates that cardiomyocyte-specific transgenic mice overexpressing S100A6 develop less cardiac hypertrophy, interstitial fibrosis and myocyte apoptosis after permanent coronary ligation, findings that support S100A6 as a potential therapeutic target for MI.

**Objectives:** To investigate the effect of UTMD delivery of S100A6-plasmid prior to I/R on cardiomyocyte apoptosis, infarct size, LV function, myocardial perfusion and ventricular hypertrophy post myocardial I/R injury.

**Methods:** *In vitro* studies examined the effects of S100A6-overexpression and S100A6-knockdown on hypoxia/re-oxygenation induced apoptosis in neonatal cardiomyocytes as well as calcium transients and calcineurin activity. In addition, tubule formation and cell migration was assessed in S100A6 over-expressing and S100A6-knockdown HUVECs. GFP-tagged human-S100A6 plasmid or empty plasmid was delivered to the left ventricular (LV) myocardium by ultrasound-targeted microbubble destruction (UTMD) in Fischer-344 rats, 2 days prior to 30-
minute ligation of the LAD coronary artery followed by reperfusion. Control animals received no UTMD. 2D-echocardiography was used to assess LV systolic function and extent of ventricular wall akinesia. Contrast-enhanced ultrasound perfusion imaging was used to measure myocardial perfusion. Post-mortem tissue analysis was performed to assess the size of non-viable myocardium at day 1 as well as collagen rich necrotic scar size and LV hypertrophy at day 28.

**Results:** S100A6 overexpression in neonatal cardiomyocytes improved calcium transients and protected against apoptosis induced by hypoxia-reoxygenation via enhanced Calcineurin activity, while knockdown of S100A6 had detrimental effects. S100A6 overexpression in HUVECs *in vitro* enhanced tube formation. UTMD mediated S100A6 gene therapy prior to myocardial I/R yielded a survival advantage compared to empty plasmid and non-treated controls. S100A6 pre-treated animals had reduced LV extent of akinesia by echocardiography, smaller infarct size after 24 hours and lesser collagen rich scar formation after 28 days post myocardial I/R injury. They also had improved LV systolic function and greater perfusion in the infarct territory, with less myocyte apoptosis and attenuated cardiac hypertrophy.

**Conclusion:** S100A6 overexpression by UTMD helps ameliorate myocardial I/R injury, resulting in lower mortality and improved LV systolic function post myocardial I/R injury, via attenuation of apoptosis, enhanced perfusion, and reduced cardiac hypertrophy and infarct size. S100A6 is a potential therapeutic target for acute myocardial I/R injury.
4.1 Introduction

S100A6, a member of the superfamily of EF-hand Ca\textsuperscript{2+}-binding proteins, regulates a wide array of cellular and molecular functions, including cell proliferation, differentiation and survival, as well as Ca\textsuperscript{2+} dynamics, cardiomyocyte contractility, hypertrophy and apoptosis (Nowotny et al. 2000; Brinks et al. 2011). Earlier studies have demonstrated up regulation of S100A6 gene expression in the ischemic myocardium (Cai et al. 2011). Our previous data confirmed up regulation of S100A6 in the infarct and peri-infarct regions of myocardium during 28 days post I/R injury.

On the other hand, the existing data had shown that S100A6 has differential effects on apoptosis depending on specific cell types and S100A6 concentration. S100A6 overexpression prevented TNF-α induced cardiomyocyte apoptosis \textit{in vitro}. The proposed mechanism is suggested to be the interference with p53 phosphorylation at serine 15. This interaction is both physical, Ca\textsuperscript{+} dependent binding to the tetramerization domain of p53, and non-physical through unknown pathways (Tsoporis, Izhar, and Parker 2008b; Fernandez-Fernandez, Rutherford, and Fersht 2008; Tsoporis, Izhar, and Parker 2008a). S100A6 gene overexpression also inhibited the induction of the cardiac fetal gene promoters in cultured neonatal rat cardiomyocytes \textit{in vitro} (Tsoporis, Marks, Haddad, O'Hanlon, et al. 2005), suggesting a role in modulating cardiac hypertrophy. Most recently, cardiomyocyte-specific transgenic mice overexpressing S100A6
showed attenuation of cardiac hypertrophy, less interstitial fibrosis and reduced myocyte apoptosis compared to control animals in a coronary ligation model of MI (Tsoporis et al. 2013).

Given that S100A6 has the potential to modulate several key pathways involved in I/R injury, including myocyte apoptosis and Ca\(^{2+}\) dynamics, as well as positively influence cardiac hypertrophy, a key element in the remodeling process post-MI, we hypothesized that S100A6-gene transfer prior to myocardial I/R injury would ameliorate adverse LV remodeling and attenuate LV systolic dysfunction in a rat model of myocardial I/R injury.
4.2 Methods and Experimental Protocols

4.2.1 *In vitro* Protocol

Neonatal cardiomyocytes isolated from 2-3 days old neonatal rats were transduced by adenovirus containing GFP tagged human-S100A6 plasmid or S100A6-shRNA for S100A6 overexpression and S100A6-knockdown, respectively. Cultured cells were incubated in a hypoxic chamber for 24 hours followed by re-oxygenation and apoptosis was measured using TUNEL staining and caspase 3/7-activity assay after 24 hours of re-oxygenation. Furthermore, calcium transient and Calcineurin activity were measured in the S100A6-overexpressing and S100A6-knockdown cells compared to controls (Figure 20A). Adenoviral transduced S100A6-overexpressing and S100A6-knockdown HUVECs were tested for tubule formation capacity and migration potential compared to controls (Figure 20B).
**Figure 20** *In vitro* Experimental Protocol on Neonatal Cardiomyocytes (A) and HUVECs (B)

**A. Neonatal Rat Cardiomyocytes**

1. **Hypoxia/Re-oxygenation** for 24 hours
2. Fluorescent Imaging, RT-PCR, Western Blotting
3. Calcium Transients and Calcineurin Measurement Activity
4. Caspase 3/7 Activity Assay and TUNEL Staining for Apoptosis Assessment

**B. Human Umbilical Vein Endothelial Cell (HUVECs)**

1. **500µL** of HUVEC suspension in Boyden companion plates
2. VEGF (50 ng/ml), SDF-1 (100 ng/mL)
3. And 8 µm insert

**Boyden chamber insert washed, adherent cells fixed and stained using DiffQuik and quantified**

**Cells Plated on the surface of the Matrigel and stimulated with VEGF (50 ng/ml)**

**Capillary-like Network Formation photographed and quantified at regular intervals till 24 hours**
4.2.1.1 Cell Culture, Neonatal Rat Cardiomyocytes

Neonatal cardiomyocytes from 2-3 days-old Fisher 344 rats (Charles River Laboratories International Inc.) were isolated using Worthington Neonatal Cardiomyocyte Isolation system (Worthington Biochemical Corporation, catalogue# LK003303), according to manufacturer's protocol. In brief, 2-3 days-old rat pups were sacrificed by decapitation. After sterilization with an antiseptic solution (surgical alcohol), the beating hearts were surgically removed, and immediately placed in centrifuge tubes containing 30-40 ml of sterile calcium-magnesium-free Hank's Balanced Salt Solution (CMF HBSS, pH 7.4, Reagent #1) to chill and rinse.

Tissue was minced with small scissors to less than 1 mm³ pieces keeping tissue at 0°C and incubated overnight (16-20 hours) at 2-8°C in 10 ml of CMF HBSS containing purified trypsin (Reagent #2) at a final concentration of 50 µg/ml. After the incubation time, tissue and buffer were mixed with trypsin inhibitor (Reagent #3) and oxygenated for 30 seconds by passing oxygen over the surface of the liquid. Tissue containing buffer was warmed to 30-37°C in a water bath. Then collagenase (Reagent #4) was added to the tubes and they were incubated for 30 to 45 minutes at 37°C on a slowly rotating instrument. The tubes were removed from the incubator, cells were released by triturating 10 times using standard 10 ml plastic serological pipettes, and then filtered through the cell strainer into a fresh 50 ml centrifuge tube.
Cells were sedimented at 180 x g for 5 minutes and the final cell pellet was suspended in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM / F-12) (Life Technologies, catalogue# 11320-033) growth media containing 7.5% horse serum (Life Technologies, Catalogue #16050-130), 7.5% fetal bovine serum (FBS) (Life Technologies, Catalogue# 26140-079) and 100 U/mL penicillin with 100 µg/mL streptomycin (Life Technologies, Catalogue # 15140122). The cells were plated for 1.5–2h to allow the differential attachment of non-myocardial cells (mostly fibroblasts). The non-adhesive cells (cardiomyocytes) were transferred to a centrifugation tube, washed and centrifuged again at 180× g for 5 minutes. After counting, the myocyte-enriched suspension was transferred to collagen I-coated culture dishes at density of 5×10⁴ cells per cm². The viability of cells (85–90%) was determined by exclusion of Trypan blue dye. Cells were incubated in 95% air and 5% CO2 at 37 °C and left untouched for at least 48 hours.

4.2.1.2 Cell Culture, Human Umbilical Vein Endothelial Cell (HUVECs)

HUVECs were kindly provided by Dr. Marsden, St. Michael’s Hospital, University of Toronto.

4.2.1.3 Adenoviral Transduction

Cultured neonatal rat cardiomyocytes (5×10⁴/cm² cells grown for 48 hours after seeding), were inoculated with Ad-CMV-Null (2.25×10¹⁰ PFU/ml, MOI: 100), Ad-CMV-emGFP-
hS100A6 (2.8×10^9 PFU/ml, MOI:100) and Ad-GFP-U6-rS100A6-shRNA (4.0×10^10 PFU/ml, MOI:100) adenoviral vectors in 250 μL/cm^2 of serum containing (7% Horse serum+7% FBS) DMEM/F12 growth media (Life Technologies, Catalogue#11330-032) for 24 hours. The virus containing growth media was replaced with fresh media after 24 hours but cells were incubated for another 24 hours to have maximal expression of overexpressed gene (human S100A6) and down regulation of knocked-down gene (rat S100A6) at the time of hypoxia/re-oxygenation (H/R) induction or calcium transient studies.

HUVECs (1×10^4/cm^2 cells grown for 48 hours after seeding) were inoculated with Ad-CMV-Null (2.25×10^10 PFU/ml, MOI: 500), Ad-CMV-emGFP-hS100A6 (2.8×10^9 PFU/ml, MOI: 500) and Ad-GFP-U6-rS100A6-shRNA (4.0×10^10 PFU/ml, MOI: 500) adenoviral vectors in 250 μL/cm^2 of serum containing (7% Horse serum+7% FBS) CS-C Medium (Sigma, Cat# C1556-100ML) for 24 hours. All adenoviral vectors were constructed in Vector BioLabs The Gene Delivery Company.

Ad-CMV-emGFP-hS100A6

Plasmid name: GFP-human S100A6 (rat Calcyclin tagged to GFP)

Vector backbone name: pc DNA6.2N-EmGFP-DEST

Insert DNA name: GFP tagged human S100A6 gene (calcyclin)

Gene bank accession# NM-053485
Enzymes used for releasing insert: They are made by GATEWAY technique

**Ad-CMV-Null**

Plasmid name: GFP-GUS

Vector backbone name: pc DNA6.2N-EmGFP-DEST

Insert DNA name: GFP tagged Glucuronidase

Enzymes used for releasing insert: They are made by GATEWAY technique

**shRNA sequence (TRCN0000087874):**

CCGG-GCTTTGATCTACAATGAAGCT-CTCGAG-AGCTTCATTGTAGATCAAAGC-
TTTTTGT

**Target sequence:** GCTTTGATCTACAATGAAGCT

**Hairpin sequence:** CTCGAG

### 4.2.1.4 Hypoxia/Re-Oxygenation (H/R) Induction

Hypoxic conditions were created by incubating culture dishes in an airtight Plexiglas chamber with an atmosphere of 1% O2, 20% CO2 and 79% N2 at 37°C for 24 hours. Serum free low glucose growth media, DMEM×1 (Life Technologies, Catalogue#11885-084) was pre-equilibrated in the hypoxic chamber overnight before use. Reperfusion/re-oxygenation was simulated by returning the cultured cells to the incubator with an atmosphere of 95% air and 5% CO2 at 37 °C for 6 hours in serum containing DMEM/F12 media.
4.2.1.5 Calcium Transients and Calcineurin Measurement Activity

Neonatal cardiomyocytes were incubated in 0.5 µM Fura-2AM in serum free media (DMEM/F12 media + 50 units penicillin/50 µg) for 1 hour and washed with serum free media. A fluorescence microscope (Olympus IX81, X-cite 120Q light source from EXFO, Fura 2B filterset from Semrock, using single band excitation [387 nm] and single band emission [510 nm], image acquisition with a Rolera MGi plus EM-CCD from Q-Imaging, via Metamorph v7.6.3. software) was used to acquire calcium transients.

A colorimetric assay kit was used to measure cellular Calcineurin (PP2B) phosphatase activity (Enzo Life Science, Catalogue#BML-AK816-0001) in cellular extracts. Human recombinant Calcineurin was included as a positive control.

4.2.1.6 Matrigel Tubule Formation and Migration Assay

Matrigel tube formation assay was performed to observe the effects of S100A6 on capillary-like network formation. 60 µl of matrigel (Becton Dickinson, Mississauga, ON, CA) was added to 96-well tissue culture plates (Corning, Tewksbury, USA) and allowed to polymerize at 37°C, 5% CO₂ for 45 minutes before plating the cells. HUVECs transduced with adenoviral vectors were plated in 100 µl HUVEC special medium onto the surface of the matrigel and stimulated with VEGF (50 ng/ml). Control group was also included which did not
undergo any transduction. The capillary-like network formation was observed at regular intervals till 24 hours and photomicrographs were recorded at each time interval for quantification.

For the migration assay, VEGF (50 ng/ml) and stromal cell-derived factor (SDF)-1 (100 ng/mL) were placed in each well of the Boyden companion plate. An 8µm (pore size) insert was placed in each well containing 500µL of HUVEC suspension. After incubation, each Boyden chamber insert was gently washed, and non-adherent cells were removed. Cells were fixed and stained using DiffQuik (Sigma), then allowed to dry overnight. The membrane was removed and mounted on a slide for quantification using light microscopy with a 20× objective.

4.2.1.7 Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL)

Neonatal cardiomyocytes were grown directly onto glass slides and transduced with adenoviral vectors (as described above) for overexpression or knockdown of S100A6. Hypoxia/re-oxygenation cell injury was created by incubation in the hypoxic chamber followed by re-oxygenation (as described above). Cells were washed twice with 1× PBS and fixed in 2% paraformaldehyde. Apoptotic cell labeling was performed using Apoptag Red in situ Apoptosis Detection kit as described in chapter 3.
4.2.1.8 Extracellular S100A6 Protein Uptake and Subcellular Localization

Neonatal cardiomyocytes cultured overnight were treated with pure recombinant S100A6 protein tagged to His (Life Technologies, MD) at concentration 100nM each for 1 hour or with vehicle (PBS). Cells were then fixed with 4% paraformaldehyde, permeabilized and incubated with sheep anti-S100A6 (1:400, R&D, Cat# AF4584) and rabbit Anti-6X His tag antibody (1:800, Abcam, Cat# ab9108) followed by fluorochrome-conjugated secondary antibodies, donkey anti-sheep Alexa 488 (1:400, Life Technologies, Cat# A-21206) or chicken anti-rabbit Alexa 543 (1:400, Molecular Probes), respectively. Extracellular protein uptake and subcellular localization was visualized by confocal microscopy. Images were generated with Zeiss LSM 700 confocal laser scanning microscope using excitation wavelength of 488nm and 543nm for Alexa green and red dyes respectively.

4.2.1.9 Caspase 3/7 Activity Assay

Neonatal cardiomyocytes were grown directly in 96-well white plates, transduced with adenoviral vectors (as described above) for overexpression or knockdown of S100A6. Hypoxia/re-oxgenation cell injury was induced by incubation in the hypoxic chamber followed by re-oxgenation (as described above). Non-transfected neonatal cardiomyocytes and normoxic cells were grown in separate wells as control groups. Caspase 3/7-activity was measured by
adding 100µl of Caspase-Glo® 3/7 Reagent to each well of a white-walled 96-well as described in chapter 3.

4.2.1.10 Real Time-PCR

Total RNA extract from the cultured neonatal cardiomyocytes underwent RT-PCR using the method and primers described in chapter 3.

4.2.1.11 Western Blotting

Western blots were performed on protein extract from S100A6-overexpressing, S100A6-knockdown and non-transduced neonatal cardiomyocytes. Membranes were incubated with rabbit monoclonal anti-S100A6 antibody (Novus biologicals, Catalogue#NBP1-95284) or rabbit polyclonal anti-GFP antibody (Abcam, Catalogue#ab6556) and anti-rabbit IgG horseradish peroxidase (HRP) conjugated antibody (Promega, Catalogue#W401B). Blots of proteins were probed with rabbit polyclonal anti-GAPDH primary antibody (Santa Cruz Biotechnologies, Catalogue#sc-25778) for internal reference. Secondary antibodies were detected using Immobilon Western Chemiluminescence HRP substrate (Millipore, Catalogue#WBKLS0050). Band intensity was quantified by scanning densitometry using VersaDoc Imaging System.

4.2.2 In vivo Protocol

UTMD delivery of S100A6-plasmid or empty-plasmid was conducted 2 days prior to the myocardial I/R induction surgery, to ensure the peak of gene expression at the time of I/R
injury. Animals were followed by serial echocardiography to assess the LV systolic function and the circumferential extent of LV akinesia (Figure 21). The exogenous gene expression was measured at various time points post UTMD gene delivery and I/R induction surgery. Non-viable myocardium and collagen rich necrotic tissue were measured using TTC and MTC staining at days 1 and 28, respectively. Myocardial perfusion was measured by contrast-enhanced ultrasound perfusion imaging techniques. Hypertrophic response was investigated macroscopically through measurement of heart weight to tibia length or body weight ratio and microscopically in H&E stained sections.

* Echocardiography and sacrificing a subgroup of animals to collect tissue

**Figure 21 In vivo Experimental Protocol**

**4.2.2.1 Animal Preparation**

The study protocol (ACC Protocol # 392-2R) was approved by the Animal Care and Use Committee at the Keenan Research Centre for Biomedical Science, Li Ka Shing Knowledge
Institute, St Michael's Hospital, University of Toronto, in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

4.2.2.2 Plasmid-Gene Delivery via UTMD

An expression vector driven by a cytomegalovirus (CMV) promoter was constructed for transfection of human S100A6-N-emGFP, in which the human S100A6 sequence was fused with emGFP at the N-terminus to create a fusion protein. GUS-N-eGFP was used as the empty plasmid for control group. The plasmid vectors were acquired through SIDNET (Toronto, Ontario), and were sequence-verified prior to use in vivo.

Cationic lipid microbubbles (1x10⁹) were charge-coupled to 500 µg of plasmid DNA (hS100A6 or empty plasmid) and administered intravenously. UTMD was performed as previously described (Smith et al. 2012; Lee et al. 2014). High power ultrasound at a pulsing interval of 10 cardiac cycles at end-systole was transmitted over the left ventricle (LV) via an S12 transducer (Sonos 5500, Philips Healthcare, Andover, Massachusetts, USA) at a frequency of 5MHz, 2cm depth, and power 120V during intravenous infusion of plasmid-cationic microbubble complexes via the jugular vein over 5 minutes (Model AS50-Baxter)(Lee et al. 2014).

The probe was positioned to cut the LV in a transverse plane (short-axis) at the mid-papillary muscle level, and the transducer was slowly moved from the base to the apex to allow
maximal myocardial gene delivery. Ultrasound transmission was continued for an additional 15 minutes after plasmid-microbubble infusion to ensure maximal delivery of remaining circulating DNA-microbubble complexes. Animals were subsequently given Anafen 5mg/kg and placed on a warming pad at 37°C and allowed to recover.

4.2.2.3 Animal Experimental Protocol, I/R Induction Surgery

Myocardial I/R injury was induced via LAD coronary artery ligation for 30 minutes followed by reperfusion, 2 days post UTMD of Human S100A6-N-emGFP or GUS-N-eGFP. Control animals did not receive UTMD prior to I/R. The technique for I/R induction surgery is described in chapter 3.

4.2.2.4 Echocardiography and Cardiac Catheterization

Echocardiography was performed at day -1 (after UTMD gene delivery and before I/R induction surgery) and 1 day, 3 days, 7 days, 14 days and 28 days post I/R induction surgery as explained before in chapter 3.

4.2.2.5 Myocardial Contrast Echocardiography (MCE)

MCE was performed at day 28 to measure the blood flow in the LV myocardium at the level of papillary muscles, using gated pulse inversion imaging (HDI 5000, Philips Ultrasound; linear transducer (L7-4), mechanical index 1.0, transmit frequency 3.3 MHz). Gain settings were optimized and held constant for the remainder of the study. Background images were acquired
before microbubble infusion for subtraction of tissue signal. During continuous intravenous infusion of neutral lipid microbubbles (Definity, Lantheus Medical Imaging; 1x $10^7$ min$^{-1}$), triggered imaging was performed at increasing pulsing intervals (PI), specifically 1, 2, 3, 5, 7, 10 and 20s. Data was recorded on magnetic optical disks then transferred to a computer for analysis using HDI lab, DigiLink 1.7.2 and MCE2.9.4. Software programs. Five average background frames were digitally subtracted from average contrast-enhanced frames at each PI. Regions of interest were drawn over the risk area and the remote normal zones, and PI versus signal intensity (SI) were fit to the function $y = A (1 - e^{-\beta t})$, where $y$ is signal intensity at pulsing interval $t$. $A$ is plateau signal intensity representing micro-vascular blood volume. $\beta$ is the rate constant reflecting time to plateau signal intensity, representing blood flow velocity. Myocardial blood flow (MBF) was calculated as the product of $A$ and $\beta$. MBF in the risk area was normalized to the remote non-infarcted myocardium.

4.2.2.6 Post-mortem Analysis

Hematoxylin and Eosin Staining (H&E):

The extent of cardiomyocyte hypertrophy was determined from hemotoxylin-eosin stained sections. In brief, spindle-shaped cardiomyocytes with elliptical nuclei in non-infarct region of transverse myocardial section were selected. The area of each cell was measured at the level of the nucleus using Imaging Software NIS-Elements-Nikon, and the values were averaged.
Approximately 3 cells per field at x40 magnification were found with 2 fields randomly selected per sample slide. Each treatment group included 10 animals and 10 slides. The average diameter of 50-60 myocytes was then calculated for 10 animals in each treatment group.

**Triphenyltetrazolium chloride (TTC) Staining:**

We used 2,3,5-Triphenyltetrazolium chloride staining (Sigma-Aldrich, Catalogue# T8877-10G) to define the non-viable/infarcted LV myocardium at 24 hours post I/R. In brief, 24 hours post I/R induction surgery, a subgroup of animals from non-treated controls, empty-plasmid treated controls, and human S100A6-plasmid treated cases, were sacrificed. Hearts were collected right after sacrificing and 350-400 µm thick sections were cut under the ligation site. We prepared 1% fresh TTC solution and placed it at 37 °C. Then the heart sections were incubated in TTC solution and kept at 37 °C for 15 minutes. We flipped the slices at 7 minutes to ensure even staining. At the end of the incubation time, we carefully aspirated the TTC and added fresh 10% Formalin solution. Since the tetrazolium salts react with the dehydrogenases in the viable cells, viable tissue is stained as “brick-red” and the infarcted tissue, which lacks the enzymes, remains pale-white. Slices were photographed with a digital camera after 1 hour of fixation. We quantified the percentage of viable (red) and nonviable myocardium using ImageJ software (Liu Z1 2002).
Masson’s Trichrome (MTC) Staining:

We used Masson’s Trichrome Stain Kit (Polysciences Inc, Catalogue# 25088-100) to detect collagen fibers and necrotic tissue in the infarcted area of the LV myocardium. In brief, formalin fixed tissue sections were re-fixed in Bouin's solution overnight at room temperature. We removed the yellow color by rinsing in running tap water for 5-10 minutes. Then we stained the tissue sections in Weigert's iron haematoxylin working solution for 7 minutes. After rinsing in running warm tap water again for 10 minutes and washing in distilled water, we stained the tissue sections in Biebrich scarlet-acid fuchsin solution for 7 minutes.

We washed the sections in distilled water then differentiated them in phosphomolybdic-phosphotungstic acid solution until collagen was not red anymore. Then we transferred sections directly to aniline blue solution and stained for 5 minutes. Again, we rinsed them briefly in distilled water and differentiated in 1% acetic acid solution for 2-5 minutes. Sections were washed again in distilled water, dehydrated quickly through 95% ethyl alcohol and 100% ethyl alcohol then cleared in xylene. At the end, we mounted the sections with resinous mounting medium. Slices were photographed under light microscope (magnification x1). We quantified the percentage of collagen (blue) to the LV section area (red) by using ImageJ. Nuclei were seen in black.

Immunohistochemistry (IHC) Staining for Vessel Density Measurement:
IHC staining using monoclonal antibody against the rat endothelial cell adhesion molecule CD31 (Anti-CD31 antibody, ab64543) was used for quantifying vessels density. Number of micro vessels was counted within each visual field (magnification 40×) and results were presented as mean of the three counts.

**Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL):**

Analysis of apoptosis was performed in one LV section per animal, obtained from the region that showed maximal infarct size. Protocol is described in chapter 3.

**Caspase 3/7 Activity Assay:**

Caspase 3/7 activity was measured in the tissue total protein isolate as described in chapter 3.

**Real Time-PCR:**

Total RNA extract from myocardial tissue sample underwent RT-PCR. The method and primers are described in chapter 3.

The new additional primers are as follows:

- **Rat beta myosin heavy chain (βMHC):**
  
  5'-GCCAACTATGCTGGAGCTGA-3' (forward)
  
  5'-GATTTTCCCTGTGCAGTGCG-3' (reverse)

- **Rat atrial natriuretic factor (ANF):**
  
  5'-CTGGACTGGGAAGTCAACC-3' (forward)
  
  5'-GATCTATCGAGGGGTCCCA-3' (reverse)
Rat Calcineurin A: 5'-TCCGACGCAACCTTAACTC-3' (forward)  
5'-GCTGCTATTACTGCGTTGC-3' (reverse)  
Rat Calcineurin B: 5'-CCGTTCTTTTCCCCCAACT -3' (forward)  
5'-AACCCTGGGTATCCCATCCA-3' (reverse)  

3.3.2.7 Statistical Method  
Statistical methods are described in chapter 3.  

4.3 Results  
4.3.1 In vitro Results  
4.3.1.1 Localization and Confirmation of In vitro Transduction  

First, we aimed to demonstrate the location of endogenous rat S100A6 and our exogenous GFP-tagged human S100A6 to ensure binding to GFP (larger molecular mass of 27 kDa) did not influence its localization and hence activity. While endogenous rat S100A6 showed cytoplasmic and nuclear localization (Figure 22A), confocal microscopy imaging of transduced neonatal rat cardiomyocytes showed peri-nuclear and cytoplasmic localization of exogenous GFP-tagged human S100A6 protein (Figure 22B). Then we intended to show whether S100A6 protein could enter cardiomyocytes via endocytosis. After incubation of neonatal cardiomyocytes with
exogenous S100A6-His fusion protein and staining for endogenous S100A6 and exogenous extracellular S100A6-His fusion protein we observed cytoplasmic uptake of exogenously added S100A6 in the presence of 2mM Ca^{2+} after 1 hour which co-localized with endogenous rat S100A6 (both cytoplasmic and nuclear) (Figure 22C).

PCR data showed a significant rise in the expression of human/rat S100A6 gene in S100A6 over-expressing cardiomyocytes and significantly decreased expression of endogenous rat S100A6 in S100A6 knockdown cardiomyocytes. Over expression or knockdown of S100A6 had no influence on the expression of S100A1 and S100B (Figure 22D). We confirmed transduction of cardiomyocytes by western blotting in S100A6 overexpressing and S100A6 knockdown cardiomyocytes, compared to empty plasmid transfected and non-transfected controls (Figure 22E).
Figure 22 Localization and confirmation of in vitro viral transduction.

Immunocytochemical staining of endogenous rat S100A6 protein (green) in neonatal cardiomyocytes showing cytoplasmic and nuclear localization (magnification 60×) (A), exogenous GFP-tagged human-S100A6 (green) in adenoviral transduced neonatal cardiomyocytes showing peri-nuclear and cytoplasmic localization (magnification 40×) (B), Staining of endogenous S100A6 protein (top panel, green) and S100A6-His fusion protein (middle panel, red) after cytoplasmic uptake of exogenously added S100A6 protein in the presence of 2mM Ca²⁺ after 1hour showing co-localization (both cytoplasmic and nuclear) of these two molecules (bottom panel) (magnification 20×) (C), expression of rat-S100A6, rat-S100B, rat-S100A1 and exogenous human/rat-S100A6 mRNA in S100A6 over-expressing and S100A6 knockdown neonatal cardiomyocytes by qRT-PCR (Mean+/SEM, P<0.05 vs. non-transduced controls, N=7) (D), representative Western Blot membrane for S100A6 protein in S100A6-overexpressing and S100A6-knockdown cardiomyocytes compared to empty plasmid and non-transduced controls (E).
4.3.1.2 Calcium Transients and Calcineurin Activity

Calcium transients of S100A6 overexpressing and S100A6 knockdown neonatal cardiomyocytes were quantified using the fluorophore Fura-2AM 2 days after viral transduction. S100A6 overexpressing neonatal cardiomyocytes showed significantly increased calcium release amplitude. Moreover, S100A6 knockdown cardiomyocytes showed transients that had significantly lower amplitude and the overall Ca\(^{2+}\) release rate was significantly decreased in the S100A6 knockdown compared to control cardiomyocytes (Figure 23B, C and D).

We measured the phosphatase activity of Calcineurin in S100A6 overexpressing and S100A6 knockdown neonatal cardiomyocytes after hypoxia/reoxygenation (24h/6h) and normoxia compared to controls both as an indicator of free cytosolic calcium levels and as a key anti-apoptotic molecule. Calcineurin phosphatase activity was significantly higher in S100A6 overexpressing neonatal cardiomyocytes, compared to empty plasmid transduced and non-transduced controls both after normoxia and hypoxia/reoxygenation (H/R, 24h/6h). In comparison, S100A6 knockdown neonatal cardiomyocytes had significantly lower Calcineurin phosphatase activity compared to empty plasmid transduced and non-transduced controls both during normoxia and after hypoxia/reoxygenation. (Figure 23E). S100A6-over-expressing cardiomyocytes had significantly increased expression of Calcineurin A (CaN A) after H/R compared to empty plasmid transduced and non-transduced controls, while S100A6
overexpression had no significant effect on Calcineurin B (CaN B), neither during hypoxia nor after H/R in vitro (Figure 23F and G).
Figure 23 Calcium Transients and Calcineurin Activity in vitro.

Representative calcium transient imaging for non-transfected control, S100A6 over-expressing and S100A6 knockdown cardiomyocytes (A), the calcium release amplitude (Mean +/- SEM, P<0.05 vs. non-transduced controls, N=7) (B), the rate of calcium release (Mean +/- SEM, P<0.05 vs. non-transduced controls, N=7) (C) and uptake (Mean +/- SEM, P<0.05 vs. non-transduced controls, N=7) (D) in S100A6-overexpressing and S100A6-knockdown neonatal cardiomyocytes during spontaneous action potential. Calcineurin (CaN) phosphatase activity during normoxia and after hypoxia/re-oxygenation (24H/6H) in S100A6 over-expressing and S100A6 knockdown neonatal cardiomyocytes compared to empty plasmid transduced and non-transduced controls (Mean +/- SEM, P<0.05 vs. empty plasmid transduced and non-transduced controls, N=7) (E) and relative expression of CaN A and CaN B in S100A6 over-expressing, S100A6 knockdown, empty plasmid transduced and non-transduced cardiomyocytes during normoxia and hypoxia/re-oxygenation (Mean +/- SEM, P<0.05 vs. empty plasmid transduced and non-transduced controls, N=7) (F and G).
4.3.1.3 Effect of S100A6 on Apoptosis after Hypoxia/Re-oxygenation

S100A6 over-expressing cardiomyocytes showed significantly smaller number of apoptotic nuclei by TUNEL staining compared to empty plasmid transduced and non-transduced controls after H/R, while S100A6 knockdown cardiomyocytes showed a greater number of apoptotic nuclei both under normoxic conditions and after H/R (Figure 24A and B). TUNEL staining results were consistent with significantly lower caspase 3/7 activity in S100A6 over-expressing cardiomyocytes and higher caspase 3/7 activity in S100A6 knockdown cells compared to controls after H/R (Figure 24C).
**Figure 24** Effect of S100A6 on Apoptosis after Hypoxia/Re-oxygenation.

Representative images for TUNEL stained non-transduced (i), S100A6 overexpressing (ii) and S100A6 knockdown (iii) neonatal cardiomyocytes post hypoxia/reoxygenation. All Nuclei are shown in blue (DAPI+) while apoptotic nuclei are distinguished by red fluorescence (Rhodamine+) (60×) (A). Quantification of apoptotic nuclei detected by TUNEL staining in S100A6 over-expressing and S100A6 knockdown neonatal cardiomyocytes compared to empty plasmid transduced and non-transduced cells (Mean+/−SEM, P<0.05 vs. empty plasmid transduced controls, N=10) (B). Caspase 3/7 activity in S100A6 over-expressing and S100A6 knockdown neonatal cardiomyocytes compared to empty plasmid transduced and non-transduced control cells after hypoxia/reoxygenation (24hours/6 hours) (Mean+/−SEM, P<0.05 vs. empty plasmid transduced controls, N=10) (C).
4.3.1.4 Matrigel Tubule Formation

Using matrigel angiogenesis assay, S100A6 over-expressing HUVECs formed significantly greater number of nodes and tubes compared to empty plasmid transduced and non-transduced controls after 3 hours. In contrast, S100A6 knockdown HUVECs formed significantly less nodes and tubes compared to empty plasmid transduced and non-transduced controls (Figure 25A, B and C). Migration assay showed attenuated migratory responses from S100A6 knockdown HUVECs, while S100A6 over-expressing HUVECs showed enhanced migratory potential in response to VEGF and SDF-1 (Figure 25D).
A

Non-Transduced HUVECs

S100A6 Over-Expressing HUVEC

S100A6-Knockdown HUVECs

Empty-Plasmid Transduced HUVECs

S100A6 Over-Expressing HUVECs

S100A6-Knockdown HUVECs
**Figure 25** S100A6 and Angiogenesis *in vitro.*

Representative images of matrigel angiogenesis assay showing capillary-like networks formed by HUVECs after 3 hours. Transfection with GFP tagged hS100A6 plasmid for S100A6 overexpression and S100A6-shRNA for S100A6 knockdown resulted in green fluorescence emitting tubes and nodes (A), number of nodes in S100A6 over-expressing and S100A6 knockdown HUVECs compared to empty plasmid transduced and non-transduced controls (Mean+/−SEM, P<0.05 vs. non-transduced and empty plasmid transduced controls, N=5) (B), tube formation in S100A6 over-expressing and S100A6 knockdown HUVECs compared to empty plasmid transduced and non-transduced controls (Mean+/−SEM, P<0.05 vs. non-transduced and empty plasmid transduced controls, N=5) (C), Migration assay data expressed as number of cells migrating to the opposite side of the chamber in S100A6 over-expressing and S100A6 knockdown HUVECs compared to empty plasmid transduced and non-transduced controls) (Mean+/−SEM, P<0.05 vs. non-transduced and empty plasmid transduced controls, N=7) (D).
4.3.2 Results, *In vivo*

4.3.2.1 UTMD Gene Delivery Efficiency

We delivered human S100A6-N-emGFP plasmid DNA by ultrasound targeted microbubble destruction (UTMD) 2 days prior to induction of ischemia-reperfusion. The regional expression of human/rat S100A6 in the LV myocardium (anterior and posterior) on days 1, 7 and 28 post cardiac I/R by qRT-PCR is shown in Figure 26. Our data shows increased expression of exogenous S100A6 in the anterior LV of S100A6-treated rats at all time points post I/R, with expression decreasing over time. Exogenous S100A6 expression was lower in the posterior LV (Figure 26A, B and C).
Figure 26 Expression of the exogenous transgene post UTMD of S100A6. Human/rat S100A6 gene expression in various regions of myocardium (anterior LV, posterior LV and RV) at day 1 (A), day 7 (B), and day 28 (C) post myocardial I/R normalized to normal control myocardium (Mean+/SEM, P<0.05 vs. all other groups, N=7).
4.3.2.2 LV Function and Akinetic Segment Size Post Myocardial I/R Injury

The mortality rate after I/R induction surgery (post ligation) was significantly lower in the S100A6-pretreated animals, compared to empty plasmid treated and non-treated controls (24.8% vs. 55.2% and 41.6%, respectively) (Figure 27A). LV ejection fraction (EF) and fractional area change (FAC) progressively improved in S100A6-treated group compared to empty plasmid treated and non-treated controls up to day 28. The circumferential extent of the akinetic segment of the LV progressively decreased in S100A6-treated rats from ay 3 out to day 28, and became significantly smaller compared to empty plasmid treated and non-treated controls (Figure 27B, C, D, E).
Figure 26 LV function and mortality in rats following myocardial I/R injury. Mortality rate during/after myocardial I/R in UTMD human-S100A6 plasmid pre-treated and UTMD empty plasmid pre-treated rats compared to non-treated controls (Mean+/−SEM, P<0.05, N=29) (A), Representative M-mode echo image of non-treated control (i), empty plasmid treated control (ii) and UTMD S100A6-plasmid treated rats (iii) (B), LV ejection fraction following myocardial I/R in human-S100A6 plasmid treated and empty plasmid treated rats compared to non-treated controls (Mean+/−SEM, P<0.05 vs. non-treated and empty plasmid treated controls, N=14-20) (C), LV fractional area change following myocardial I/R in human-S100A6 plasmid treated and empty plasmid treated rats compared to non-treated controls (Mean+/−SEM, P<0.05 vs. non-treated and empty plasmid treated controls, N=14-20) (D), circumferential extent of LV akinesia following myocardial I/R in human-S100A6 plasmid treated and empty plasmid treated rats compared to non-treated control rats (Mean+/−SEM, P<0.05 vs. non-treated and empty plasmid treated controls, N=14-20) (E).
4.3.2.3 Cardiac Hypertrophy Post Myocardial I/R Injury

Body weight changes over 28 days post I/R induction surgery did not show any significant difference between groups (data not shown). At day 28, S100A6 treated animals had a significantly lower heart weight to tibia length (HW/TL) and heart weight to body weight (HW/BW) ratios compared to empty plasmid treated and non-treated controls (Figure 28A and B). In keeping with less cardiac hypertrophy in S100A6 treated hearts, the size of cardiomyocytes was significantly smaller in S100A6-treated rats in comparison to empty plasmid treated and non-treated controls at day 28 following myocardial I/R (Figure 28 D). S100A6-treated hearts had significantly lower expression of βMHC and ANF, markers of myocardial hypertrophic phenotype, in the infarct and non-infarct regions of myocardium 28 days after I/R compared to empty plasmid treated and non-treated controls (Figure 28 E and F).
A  
Heart Weight/Tibia Length (Mean±SEM, mg/mm)  
- Non-Treated Controls  
- Empty Plasmid Treated Controls  
- S100A6 Plasmid Treated Rats

B  
Heart Weight/Body Weight (Mean±SEM)  
- Non-Treated Controls  
- Empty Plasmid Treated Controls  
- S100A6 Plasmid Treated Rats

C  
Non-Treated Control | Empty-Plasmid Treated | S100A6-Plasmid Treated

D  
Cardiomyocyte Size (Mean Area±SEM, µm²)  
- Non-Treated Controls  
- Empty Plasmid Treated Controls  
- S100A6-Plasmid Treated Rats
**Figure 27** Cardiac Hypertrophy Post Myocardial Ischemia-Reperfusion.

Ratio of heart weight to tibia length at day 28 post myocardial I/R (mean +/-SEM, P<0.05 vs. non-treated and empty plasmid treated controls, N=14-20) (A). Ratio of heart weight to body weight at day 28 post myocardial I/R (mean +/-SEM, P<0.05 vs. non-treated and empty plasmid treated controls, N=14-20) (B). Representative H&E stained images of non-infarct sections of LV myocardium at day 28 post I/R in Control (i), empty plasmid (ii) and S100A6 plasmid (iii) delivered rats (C). Cardiomyocyte size on day 28 post I/R (magnification 40X) (mean +/-SEM, P<0.05 vs. non-treated and empty plasmid treated controls, N=50-60 cells in each group) (D). Relative expression of βMHC in the infarct and non-infarct regions of myocardium 28 days post myocardial I/R (mean +/-SEM, P<0.05 vs. non-treated and empty plasmid treated controls, N=7) (E). Relative expression of ANF in the infarct and non-infarct regions of myocardium 28 days post myocardial I/R (F)(mean +/-SEM, P<0.05 vs. non-treated and empty plasmid treated controls, N=7).
4.3.2.4 Non-Viable Myocardium 24 Hours Post Myocardial I/R Injury

We used TTC staining to mark the non-viable infarcted area in LV myocardial sections 24 hours post I/R, demonstrating that S100A6-treated rats had significantly smaller infarct size/non-viable myocardium at 24 hours post myocardial I/R (Control: 24.8%, empty plasmid treated: 25.6% and S100A6-treated: 14.7%) (Figure 29A and B).
Figure 28 Non-viable LV myocardium 24 hours post myocardial I/R injury.
Representative TTC stained myocardial cryosections 1-day post I/R in non-treated controls, empty plasmid treated controls and human-S100A6 plasmid treated rats. The non-viable/infarcted myocardium is white and viable myocardium is red/pink (A), Quantified segment of non-viable myocardium/infarct area by TTC staining (Mean+/−SEM, P<0.05 vs. non-treated and empty plasmid treated controls, N=7) (B).
4.3.2.5 Apoptotic Index in the LV Myocardium 24 Hours Post Myocardial I/R Injury

We examined the anti-apoptotic effects of S100A6 pre-treatment on day 1 post myocardial I/R injury by TUNEL staining and caspase 3/7-activity assay. S100A6 pre-treated animals showed significantly lower numbers of apoptotic nuclei in the TUNEL stained infarct and non-infarct regions of myocardium compared to empty plasmid treated and non-treated controls (Figure 30A and B). Caspase 3/7 activity data at day 1 post myocardial I/R was lower in the infarcted myocardium of hS100A6-plasmid pre-treated animals compared to empty plasmid treated and non-treated controls (Figure 30C).
Figure 29 Apoptosis post myocardial I/R injury.

Representative images of IHC stained infarct regions of myocardium 1-day post myocardial I/R [Sarcomeric α-actinin (red), nuclei (blue) and FITC positive apoptotic nuclei (green)] (A), Apoptotic index in the infarct and non-infarct regions of myocardial cryosections 1-day post myocardial I/R in hS100A6-plasmid treated and empty plasmid treated rats compared to non-treated controls (Mean +/-SEM, *P<0.05 vs. non-treated and empty plasmid treated controls, N=7-9) (B), Caspase 3/7 activity in the infarct and non-infarct regions of myocardium 1-day post I/R in hS100A6-plasmid treated and empty plasmid treated rats compared to non-treated controls (Mean +/-SEM, *P<0.05 vs. non-treated and empty plasmid treated controls, N=6) (C).
4.3.2.6 Collagen Rich Necrotic Segment of LV Post Myocardial I/R Injury

Using Masson’s Trichrome staining on LV sections at day 28 post myocardial I/R, we showed significantly smaller collagen rich scar tissue in the LV of hS100A6-plasmid treated rats compared to non-treated and empty plasmid treated controls (Control: 24.0%, empty plasmid treated: 25.6% and S100A6-treated: 11.6%) (Figure 31A and B, N=7).
**Figure 30** Collagen rich necrotic tissue post myocardial I/R injury.
Representative Masson’s trichrome (MTC) stained myocardial cryosections 28 days post I/R in non-treated and empty plasmid treated controls versus hS100A6-plasmid treated rats. The collagen debris in the central compact necrotic region of the infarcted area is shown in blue, myocardial fibers in pink and nuclei in purple (A), quantified infarct area/scar size by MTC staining in non-treated and empty plasmid treated controls versus hS100A6-plasmid treated rats (% Mean+/−SEM, P<0.05 vs. non-treated and empty plasmid treated controls, N=7)(B).
4.3.2.7 Perfusion and Microvascular Density Post Myocardial I/R Injury

Myocardial contrast echocardiography at day 28 post myocardial I/R demonstrated significantly higher myocardial blood flow in the risk area of the LV (infarct and peri-infarct regions) in the hS100A6-plasmid treated group compared to non-treated and empty plasmid treated controls. Data was normalized to the remote non-infarct regions of the LV (Figure 32A and B). IHC staining for CD31 vascular marker showed increased vessel density in the peri-infarct regions of hS100A6-plasmid treated hearts, compared to empty plasmid and non-treated controls (Figure 33A and B).
Figure 31 Myocardial Perfusion Post Myocardial Ischemia-Reperfusion.
Representative images of myocardial contrast echocardiography (MCE) on day 28-post myocardial I/R injury in the hS100A6-plasmid treated group, non-treated and empty plasmid treated controls. White arrows show the LV myocardium with reduced perfusion. Perfusion is improved in the infarct region of the LV in hS100A6-plasmid treated group compared to non-treated and empty plasmid treated groups (A), quantitative MCE-derived myocardial perfusion in the infarct/peri-infarct area normalized to non-infarct segments at 28 days post I/R (Mean+/-SEM, p<0.05, N=12-20) (B)
Figure 32  Microvascular Density Post Myocardial Ischemia-Reperfusion.
Representative images of IHC staining for CD31 marker in the peri-infarct region of myocardium in the hS100A6-plasmid treated group, non-treated and empty plasmid treated controls, 28 days post myocardial I/R [CD31 (red), nuclei (blue)] (A), quantitative IHC-derived vessel density in the peri-infarct region of myocardium in the hS100A6-plasmid treated group, non-treated and empty plasmid treated controls, 28 days post myocardial I/R (B) (Mean+/-SEM, P<0.05 vs. non-treated and empty plasmid treated controls, N=8).
4.4 Discussion

Myocardial I/R injury involves a wide range of pathological processes at the cellular and molecular levels. While many therapeutic strategies addressing myocardial I/R injury have shown benefits in experimental levels, their impact on current clinical practice has been modest (Hausenloy and Yellon 2013; Kloner 2013). Among all the potential therapies, expressional modulation of genes involved in the post myocardial I/R remodeling process has attracted the most recent attention. For instance, the S100 family of EF-hand Ca\(^{2+}\) binding proteins including S100A1, S100B and S100A6 has become a focus of cardiovascular research because of their proposed roles in the regulation of cardiomyocyte contractility, hypertrophy and apoptosis. S100A1 and S100B are extensively studied and suggested as potential therapeutic targets to prevent ventricular remodeling and HF. S100A6, a least known member of this family, regulates a wide array of cellular and molecular functions, including cell proliferation, differentiation and survival, as well as Ca\(^{2+}\) dynamics, cardiomyocyte contractility, hypertrophy and apoptosis (Nowotny et al. 2000; Brinks et al. 2011).

Comparisons between S100A6 and S100A1, the most widely studied member of the S100 family of EF-hand calcium binding proteins, show key similarities but also demonstrate important differences. S100A1 is highly expressed under basal conditions and is down regulated post MI (Tsoporis et al. 2003) and in the setting of heart failure (Remppis et al. 1996). On the
contrary, S100A6 exhibits low basal cardiac expression and gets upregulated after acute MI (Tsoporis, Marks, Haddad, O'Hanlon, et al. 2005) and in failing hearts due to ischemia and idiopathic cardiomyopathy. Extracellular S100A1 inhibits apoptosis through both RAGE dependent and RAGE independent signaling pathways (Leclerc et al. 2009; Donato 2001). Intracellular S100A1 can also inhibit apoptosis but through interacting with P53 (Fernandez-Fernandez, Rutherford, and Fersht 2008; Pleger et al. 2011). Similarly, forced expression of S100A6 in cardiomyocytes prevents apoptosis by interfering with p53 phosphorylation (Tsoporis, Izhar, and Parker 2008a) and extracellular S100A6 released from damaged cells following MI has been shown to prevent apoptosis by interacting with RAGE receptors (Leclerc et al. 2007).

S100A1 was shown to be involved in maintenance of the program of normal adult gene expression. It has been speculated that S100A1 down-regulation following infarction is permissive for induction of fetal genes, including skeletal α-actin (skACT) and β-myosin heavy chain (β-MHC) leading to more hypertrophic response (Tsoporis et al. 2003). Correspondingly, S100A6 overexpression has been shown to inhibit the induction of the cardiac fetal gene promoters, β myosin heavy chain and skeletal alpha actin (Tsoporis, Marks, Haddad, O'Hanlon, et al. 2005). Intracellular S100A1 overexpression enhanced the Ca\(^{2+}\)-transient amplitude in cardiomyocytes via a stimulatory action on sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase.
(SERCA2a) and ryanodine receptor 2 (RyR2). It is hypothesized that improved Ca\textsuperscript{2+} handling resulting from S100A1 treatment in failing heart might beneficially effect myocardial apoptosis, hypertrophy, or gene expression by modulating various Ca\textsuperscript{2+}-dependent signaling pathways involving calcineurin, calmodulin kinase, or protein kinase C isoforms (α, β, γ) (Tsoporis et al. 2003). The effect of S100A6 gene expression on the intracellular calcium dynamics was investigated in only one study, which showed no significant effect on stimulated action potentials in adult cardiomyocytes. They did not test the influence of S100A6 gene expression on spontaneous action potentials (Wang et al. 2014).

Another functional role proposed for S100A1 has been its pro-angiogenic properties. Most et al demonstrated that S100A1 knockout mice have a defective angiogenic response to hindlimb ischemia due to increased VEGF receptor-2 degradation and blunted signaling through the phosphoinositide-3-kinase/Akt/eNOS pathways (Most et al. 2013). In fact, nitric oxide supplementation rescued the defective angiogenic response in S100A1 knockout mice after femoral artery ligation. Human endothelium has significant expression of S100A6, being present in both the nucleus and the cytoplasm. Depletion of S100A6 protein levels by RNA interference has resulted in increased cell-cycle arrest and a reduction in cyclin-dependent kinase 1 (CDK1) and phospho-CDK1 levels with effects on cell-cycle progression, suggesting an anti-angiogenic effect of S100A6 depletion (Bao et al. 2012).
Tsoporis et al conducted the only in vivo study on the therapeutic function of S100A6 gene overexpression on myocardial ischemia. He demonstrated that cardiomyocyte-specific transgenic mice overexpressing S100A6 subjected to permanent LAD coronary ligation exhibited less myocyte hypertrophy, interstitial fibrosis and cardiomyocyte apoptosis, leading to greater preservation of LV systolic function compared to control animals.

Our study was the first in vivo experiment applying UTMD for localized gene delivery to the myocardium in a cardiac ischemia/reperfusion model. The results of our study adds to the existing data on S100A6, showing that locally delivered S100A6 gene 2 days prior to LAD ligation and reperfusion helped ameliorate cardiac I/R injury. We performed LAD ligation and reperfusion blinded to which pre-treatment animals had received, allowing us to use mortality as one of the endpoints. While S100A6 gene therapy provided a survival advantage after I/R, the challenge for translating this to clinical practice will be optimizing gene delivery for rapid transfection prior to or immediately after reperfusion.

The exact mechanism behind this protective effect is unclear, however we can speculate that enhanced Ca\(^{2+}\) cycling during spontaneous action potential would regulate calcium induced calcium release from sarcoplasmic reticulum especially in pacemaker cells, thus suppressing arrhythmogenesis and leading to a reduction in reperfusion arrhythmias and early mortality (Wang, Chen, and Valderrabano 2012; del Monte et al. 2004). All mortality occurred during
occlusion or immediately upon reperfusion, supporting an arrhythmic death. We examined the role of intracellular S100A6 on Ca\textsuperscript{2+}-homeostasis in neonatal ventricular cardiomyocytes *in vitro*. While overexpression of S100A6 leads to increased amplitude of Ca\textsuperscript{2+} transients with enhanced release and uptake rates between the SR and the cytosol, knockdown of S100A6 had the opposite results. This effect on intracellular calcium transients is similar to S100A1 (Most et al. 2005; Kettlewell et al. 2005).

Our data on enhanced Ca\textsuperscript{2+} transients is the likely link to increased Calcineurin activity, which is driven by higher availability of free Ca\textsuperscript{2+}. Given the pro-survival and anti-apoptotic role of calcineurin-NFAT signaling in the heart (Obasanjo-Blackshire et al. 2006), the enhanced Calcineurin activity likely contributed to the existing anti-apoptotic effects of S100A6 mediated through p53 phosphorylation (Tsoporis, Izhar, and Parker 2008a). Interestingly, we have shown that up-regulation of S100A6 following MI or overexpression of 100A6 by gene delivery will inhibit β-MHC and ANF expression and prevent myocardial hypertrophy post I/R that is comparable to S100A1 anti-hypertrophic effect.

One novel finding of our study was the pro-angiogenic effect of S100A6. We showed a pro-angiogenic effect for S100A6, both *in vitro* and *in vivo*. S100A6 transduced HUVECs had increased tube formation by matrigel angiogenesis assay, and increased migratory capacity under stimulation by chemoattractants SDF-1 and VEGF, while knockdown of S100A6 had the
opposite effects. The pro-angiogenic effect of S100A6 is similar to that proposed recently with S100A1. While our study is the first to show the angiogenic potential of S100A6 gene delivery using UTMD, the exact mechanism behind this novel finding still needs to be elucidated. We believe this pro-angiogenic effect of S100A6 contributes to its beneficial influence after cardiac ischemia-reperfusion injury, given the role of angiogenic growth factors in I/R injury (Hausenloy and Yellon 2009).

4.5 Conclusion

In summary, the present study is the first to demonstrate the therapeutic benefit of S100A6 gene therapy, with improved survival and increased LV systolic function in the setting of acute MI and reperfusion injury. These positive effects were not only attributable to the known anti-apoptotic and anti-hypertrophic effects of S100A6, but also enhanced Ca$^{2+}$ cycling and Calcineurin activity, and to its pro-angiogenic property. These results warrant further studies exploring the therapeutic potential of S100A6 in cardiac diseases.
Chapter 5

UTMD Mediated S100A6-Minicircle Delivery Immediately After Myocardial I/R, Attenuates Cardiomyocytes Apoptosis, Prevents Necrosis and Improves Myocardial Perfusion and LV Systolic Function
Abstract

**Background:** We have previously shown that pretreatment with hS100A6-plasmid using UTMD, two days prior to the myocardial I/R, attenuates myocardial reperfusion injury and improves LV function by inhibition of cardiomyocytes apoptosis, decreasing necrotic scar expansion and hypertrophic response, improving calcium handling and enhancing angiogenesis. While S100A6 gene therapy prior to myocardial I/R injury provided a survival advantage, the challenge for translating this to clinical practice motivated us to optimize our gene delivery techniques for a more rapid transfection immediately after reperfusion through application of minicircle DNA instead of conventional plasmid vectors.

**Methods:** Neonatal rat cardiomyocytes were transfected with minicircle-GFP and plasmid-GFP using electroporation to compare transfection efficiency of minicircle vectors versus conventional plasmids. Myocardial I/R was induced by ligation of the LAD for 30 minutes, followed by reperfusion in rats. UTMD of S100A6-Minicircle (S100A6-MC) (1x10^9 cationic microbubbles charge-coupled to 200 µg of S100A6-MC) was performed immediately after reperfusion, with controls receiving empty-Minicircle (1x10^9 cationic microbubbles charge-coupled to 200 µg empty-MC) or no treatment post myocardial I/R injury. 2D-echo was performed at day 1, day 3, day 7, day 14 and day 28 post myocardial I/R. TTC staining was used to assess the non-viable LV segment as well as TUNEL staining for measurement of apoptosis.
both 1 day post myocardial I/R. Masson’s trichrome staining was used to detect collagen deposition and necrotic scar formation in addition to myocardial contrast echocardiography (MCE) for measurement of myocardial perfusion both 28 days post myocardial I/R.

**Results:** Transfection of MC vectors resulted in more rapid (at day 1) and longer transfection compared to parental plasmid *in vitro*. UTMD of S100A6-MC resulted in significantly increased expression of S100A6 in the infarct, peri-infarct and non-infarct regions at days 1 and day 3-post myocardial I/R. In the S100A6-MC treated group, LV ejection fraction and fractional shortening was significantly improved and the extent of the akinetic segment was smaller at all time points post I/R injury compared to the empty-MC treated and non-treated controls. S100A6-MC UTMD treated rats showed less apoptosis and smaller non-viable LV segment at day 1. S100A6-MC treated rats showed improved myocardial perfusion in the infarct region as well as less collagen deposition and scar formation at day 28-post myocardial I/R.

**Conclusion:** Treatment with hS100A6-Minicircle (S100A6-MC) using UTMD technique immediately posts myocardial I/R is an effective therapy for myocardial reperfusion injury, which is applicable in the clinical setting.
5.1 Introduction

Among all members of S100 family of proteins, S100A1, S100B and recently S100A6 have attracted much attention in cardiovascular research because of their proposed role in the regulation of cardiomyocyte contractility, hypertrophy and apoptosis, and are considered as potential therapeutic targets to prevent ventricular remodeling and heart failure. S100A6 has been the focus of our studies. We have used a safe and efficient gene delivery technique that has been well established in our lab, Ultrasound Targeted Microbubble Destruction (UTMD). UTMD is non-invasive, target-tissue specific and easily applicable method of gene delivery (Kobulnik et al. 2009).

We have previously determined the pattern of LV dimensional/functional alterations, apoptotic changes and S100A6 expression/secretion in various regions of rat myocardium post I/R over a 28-day period. We have also performed Pre-I/R delivery of S100A6-plasmid using UTMD. Our results showed that UTMD mediated delivery of hS100A6-plasmid prior to I/R can ameliorate myocardial I/R injury, resulting in lower mortality and improved LV systolic function, via attenuation of apoptosis, enhanced angiogenesis, and reduction in cardiac hypertrophy and infarct size. Although UTMD delivery of hS100A6-plasmid yielded significant therapeutic benefits, its application prior to myocardial I/R is not clinically translatable. In order
to overcome this drawback, we have now studied the application of minicircle vectors instead of conventional plasmids, with the goal of maximizing gene delivery immediately after reperfusion.

Minicircle (MC) DNA is similar to plasmid DNA. They both have a transgene cassette, an results in rapid expression after gene delivery (Darquet et al. 1997). MC lacks some prokaryotic sequences in the backbone such as replication region and antibiotic resistance segment. These sequences are removed before episomal DNA isolation by site-specific recombination in Escherichia coli (Chen, He, and Kay 2005; Kay, He, and Chen 2010). The small size of MC compared to plasmid DNA results in higher transfection efficiency and more sustained expression of the transgene (Molnar et al. 2004). In this phase of my study, we tested Post-I/R delivery of S100A6-minicircle (S100A6-MC) using UTMD as a clinically applicable gene therapy to prevent cardiac I/R injury.
5.2 Methods and Experimental Protocol

5.2.1 *In vitro* Protocol

5.2.1.1 Cell Culture, Neonatal Cardio-Myocytes

Rat neonatal ventricular cardiomyocytes were isolated as described in chapter 4 and transfected with minicircle-green fluorescent protein (GFP) or plasmid-GFP using electroporation. Fluorescence imaging and RT-PCR were used to assess transfection efficiency out to day 21.

5.2.1.2 Electroporation Gene Transfer

Electroporation or electro-permeabilization applies an electrical field to cells. The electrical field increases the permeability of the cell membrane, which allows DNA to be introduced into the cells. In brief, cells were suspended in an appropriate electroporation buffer and placed into an electroporation cuvette. Then DNA was added and the cuvette was connected to a power supply. This subjected the cells to a high-voltage electrical pulse of defined magnitude and length (2.1 kV, 100 Ω, and 25 µF, time constant ~2.6 milliseconds). The cells were then allowed to recover briefly and finally placed in DMEM/F12 medium containing 7.5% FBS, 7.5% horse serum and 1% Penicillin/Streptomycin.
5.2.2 *In vivo* Protocol

5.2.2.1 Animal Preparation

The method is described in chapter 4.

5.2.2.2 Animal Experimental Protocol, I/R Induction Surgery

Myocardial I/R injury was induced via LAD ligation for 30 minutes followed by reperfusion as described in chapter 3. UTMD of human S100A6-MC or Empty-MC was performed within 15 minutes following reperfusion and chest closure. A control group received no therapy after myocardial I/R induction surgery. Animals were followed out to day 28, with a subgroup sacrificed at day 1 and day 3 post I/R to study gene expression at different time points. After sacrificing animals, tissue was harvested for post-mortem analyses (Figure 34).

* Echocardiography and sacrificing a subgroup of animals to collect tissue
**Figure 33** *In vivo* Experimental Protocol.

### 5.2.2.3 Minicircle (MC) Preparation

Parental minicircle plasmids (pMC), MN501A1-S100A6 and MN501A1-GFP, were purchased from System Biosciences (CA, USA). Parental MC-plasmids were transfected into ZYCY10P3S2T *E.coli*, (System Biosciences) according to manufacturer’s protocol. Plasmid containing bacteria growth and MC induction was carried out as previously reported (Mark A Kay 2010). Briefly, parental plasmid containing *E.coli* were grown in 400mL supplemented Terrific Broth (Teknova, CA, USA) for 16 hours and subsequently incubated with 0.01% L- (+)-Arabinose in Luria Bertani (LB) broth (Sigma, ON, Canada) for 5 hours.

Bacteria were then harvested and plasmid was isolated using Macherey Nagel (PA, USA) DNA purification kit protocol, modifying manufacturer’s protocol. DNA impurities consisting of remnant parental plasmid and bacterial genomic DNA were removed using DNAse digestion. Briefly, the sample was digested with NdeI restriction enzyme (Thermo Scientific, MA, USA), to linearize parental plasmid and break down genomic DNA, and further treated with Plasmid-Safe DNAse (Epicentre, WI, USA). The sample was then filtered through a 30kDa centrifugal filter (Millipore, MA, USA) to remove the digested impurities and enzymes. This purified MC was further analyzed by spectrophotometer NanoDrop2000 (Thermo Scientific) and run on a 1% agarose gel to validate its purity.
5.2.2.4 Minicircle-Gene Delivery via UTMD

For gene delivery, cationic lipid microbubbles (1x10^9) charge-coupled to 200 µg of human S100A6-MC or empty-MC were administered intravenously. UTMD was performed as previously described (Lee et al. 2014). High power ultrasound at a pulsing interval of 10 cardiac cycles at end-systole was transmitted over the left ventricle (LV) via an S12 transducer (Sonos 5500, Philips Healthcare, Andover, Massachusetts, USA). The detailed methods are described in chapter 4.

5.2.2.5 Echocardiography and Cardiac Catheterization

Under inhalation anesthesia, echocardiography was performed using Vevo® 2100 system (Visual Sonics) at various time points (1, 3, 7, 14 and 28 days after myocardial I/R injury). LV fractional shortening (FS), LV ejection fraction (LVEF), fractional area change (FAC) and the circumferential extent of the akinetic segment of the LV were measured. The detailed method is described in chapter 3.

5.2.2.6 Myocardial Contrast Echocardiography (MCE)

Quantitative MCE was performed at day 28 to measure myocardial blood flow. The protocol is described in chapter 4.
5.2.2.7 Post-mortem Analysis

Triphenyl-tetrazolium chloride (TTC) Staining

2,3,5-Triphenyl-tetrazolium chloride staining defined the non-viable/infarcted LV myocardium at 24 hours post I/R. The protocol is described in chapter 4.

Masson’s Trichrome (MTC) Staining

Masson’s Trichrome staining was used to detect collagen content and scar tissue at day 28 post I/R. The protocol is described in chapter 4.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL):

Analysis of apoptosis at day 1 was performed from the region that showed maximal infarct size, using TUNEL staining. The protocol is described in chapter 3.

Caspase 3/7 Activity Assay

Caspase 3/7 Activity was measured using Caspase-Glo® 3/7 Assay Systems Promega as described in chapter 3.

Real Time-PCR

PCR amplification was performed to measure gene expression levels of Human 100A6 normalized to rat TBP (housekeeping gene), and data were analyzed with StepOne software v2.1. The protocol is described in chapter 3.
Western Blotting

Western blot was performed on protein extract from various regions of LV (infarct, peri-infarct, non-infarct) in S100A6-MC treated group and in non-treated controls to show the level of exogenous human S100A6. GAPDH was used as the housekeeping protein. The protocol is described in chapter 3.

5.2.2.8 Statistical Methods

The statistical method is described in chapter 3.
5.3 Results

5.3.1 *In vitro* Results

5.3.1.1 Transfection Efficacy of GFP-MC versus GFP-Parental Plasmid

`IHC staining of neonatal cardiomyocytes 24 hours post transfection with GFP tagged hS100A6-MC using electroporation technique showed nuclei and cytoplasm localization of GFP protein. The cardiomyocyte origin of cells was confirmed by IHC staining for anti-sarcomeric α-actinin as opposed to the fibroblastic origin. GFP-MC transfection of neonatal cardiomyocytes showed significantly higher GFP expression at various time points (day 1, 7, 14 and 21) when compared to parental plasmid-GFP (Figure 35A, B and C).`
**Figure 34** Transfection Efficacy of GFP-MC vs. GFP-Parental Plasmid.

Representative images of IHC stained neonatal cardiomyocytes 24 hours post transfection with GFP tagged S100A6-MC using electroporation technique (separate channels and merged image). GFP tagged hS100A6-MC is shown in green, anti-sarcomeric α-actinin in red and DAPI+ nuclei in blue (A). Representative images of neonatal cardiomyocytes taken by light and fluorescent microscope at various time points (6 hours, 1 day and 21 days) post transfection with GFP tagged hS100A6-MC and GFP tagged hS100A6-parental plasmid (B). Quantification of GFP mRNA by RT-PCR in neonatal rat cardiomyocytes at various time points (6 hours, 1 day and 21 days) post transfection with GFP tagged hS100A6-MC and GFP tagged hS100A6 parental plasmid using ionoporation technique (Mean+/−SEM, p<0.05 vs. parental plasmid treated group, N=5)(C).
5.3.2 In vivo Results

5.3.2.1 UTMD Mediated Gene Delivery Efficiency

UTMD mediated delivery of hS100A6-MC immediately after myocardial I/R resulted in significant expression of S100A6 in the infarct, peri-infarct and non-infarct regions of left ventricle measured by RT-PCR both at day 1 and day 3 post myocardial I/R. The results were confirmed by western blot analysis of protein extracts from the cells 3 days post I/R (Figure 36A, B and C).
Figure 35 UTMD Mediated Gene Delivery Efficiency.
Quantification of hS100A6 mRNA by RT-PCR in myocardium 1 day post UTMD mediated post-I/R delivery of S100A6-MC (Mean+/SEM, N=8-10)(A), Quantification of S100A6 mRNA by RT-PCR in myocardium 3 days following UTMD mediated post-I/R delivery of S100A6-MC (Mean+/SEM, N=8-10)(B), S100A6 protein levels in various regions of LV (infarct, peri-infarct and non-infarct regions) 3 day following UTMD mediated post-I/R delivery of S100A6-MC measured by Western Blotting (Mean+/SEM, P<0.05 vs. Healthy Control and Control MI, N=8-10) (C).
5.3.2.2 LV Function and Akinetic Segment Size Post Myocardial I/R Injury

UTMD mediated delivery of hS100A6-MC immediately post myocardial I/R resulted in improved LV ejection fraction (EF) and fractional shortening (FS) when compared to empty-MC treated and non-treated controls at all time points. Additionally, the size of the akinetic segment of the LV was significantly smaller in hS100A6-MC treated group than empty-MC treated and non-treated controls at all time points (Figure 37A, B, C, D and E).
**Figure 36** LV function and Akinetic Segment Size Post Myocardial I/R Injury.

Representative echocardiograms in parasternal long axis view (PSLAX) from a non-treated control (left) and S100A6-MC treated rat 28 days post myocardial I/R injury (A), quantification of EF (B), FS (C), fractional area change (FAC) (D) and akinetic segment of LV circumference (E) at various time points (days 1, 3, 7,14 and 28) post myocardial I/R injury in S100A6-MC post-I/R treated, empty-MC post-I/R treated and non-treated controls (Mean+/±SEM, P<0.05 vs. empty-MC post-I/R treated and non-treated controls, N=12).

5.3.2.3 Non-Viable Myocardium 24 Hours Post Myocardial I/R Injury

The extent of non-viable myocardium measured by Triphenyl-tetrazolium chloride (TTC) staining 24 hours post I/R induction surgery was significantly less in the hS100A6-MC post-I/R treated rats than empty-MC treated and non-treated controls (Figure 38, A and B).
**Figure 37** Non-Viable Myocardium 24 Hours Post Myocardial I/R Injury.

Representative images from the TTC stained sections of LV myocardium at the level of papillary muscles 24 hours post myocardial I/R induction in the hS100A6-MC post-I/R treated, empty-MC treated and non-treated controls (A), quantification of non-viable LV myocardium 24 hours post myocardial I/R in the hS100A6-MC post-I/R treated, empty-MC treated and non-treated controls (Mean+/-SEM, P<0.05 vs. empty-MC treated and non-treated controls, N=8-10) (B).
5.3.2.4 Apoptotic Index in LV Myocardium 24 Hours Post Myocardial I/R

The apoptotic index measured by TUNEL apoptosis detection kit 24 hours post myocardial I/R injury was significantly lower in the peri-infarct region of LV in the hS100A6-MC post-I/R treated group compared to empty-MC treated and non-treated controls (Figure 39A and B).

![Representative images from TUNEL stained sections 24 hours post myocardial I/R injury in S100A6-MC post-I/R treated, empty-MC treated and non-treated controls (A), quantification of apoptotic index in TUNEL stained sections 24 hours post myocardial I/R injury in S100A6-MC post-I/R treated, empty-MC treated and non-treated controls (B) (Mean+/-SEM, P<0.05 vs. empty-MC treated and non-treated controls, N=5-7)(B).]

**Figure 38** Apoptotic Index in peri-infarct region of LV Myocardium. Representative images from TUNEL stained sections 24 hours post myocardial I/R injury in S100A6-MC post-I/R treated, empty-MC treated and non-treated controls (A), quantification of apoptotic index in TUNEL stained sections 24 hours post myocardial I/R injury in S100A6-MC post-I/R treated, empty-MC treated and non-treated controls (B) (Mean+/-SEM, P<0.05 vs. empty-MC treated and non-treated controls, N=5-7)(B).
5.3.2.5 Collagen Rich Necrotic Segment of LV Post Myocardial I/R Injury

The size of collagen-rich necrotic myocardium measured by Masson’s Trichrome (MTC) staining 28 days post myocardial I/R induction surgery was significantly smaller in the hS100A6-MC post-I/R treated rats compared to empty-MC treated and non-treated controls (Figure 40A and B).

Figure 39 Collagen Rich Necrotic Segment of LV Post Myocardial I/R Injury.
Representative images from Masson’s Trichrome (MTC) stained LV sections 28 days post myocardial I/R induction surgery in hS100A6-MC post-I/R treated, empty-MC treated and non-treated controls (A), quantification of the collagen rich fibrotic tissue in LV myocardium 28 days post myocardial I/R induction surgery in hS100A6-MC post-I/R treated, empty-MC treated and non-treated controls (Mean+/−SEM, P<0.05 vs. empty-MC treated and non-treated controls, N=5-7) (B).
5.3.2.6 Perfusion and Microvascular Density Data Post Myocardial I/R

MCE at day 28 post myocardial I/R in hS100A6-MC post-I/R treated, empty-MC treated and non-treated controls showed significantly greater myocardial perfusion in the infarct and peri-infarct regions of the LV in UTMD hS100A6-MC post-I/R treated animals compared to empty-MC treated and non-treated controls (Figure 41A, B and C).

**Figure 40** Myocardial Perfusion Post Ischemia-Reperfusion Injury.
Representative contrast enhanced ultrasound images at day 28 post myocardial I/R in hS100A6-MC post-I/R treated, empty-MC treated and non-treated controls. Arrows show the LV myocardial region with reduced perfusion (A), quantification of myocardial perfusion in the infarct region at day 28 post I/R in hS100A6-MC post-I/R treated, empty-MC treated and non-treated controls (B), (Mean+-SEM, P<0.05 vs. empty-MC treated and non-treated controls, N=8).
5.4 Discussion

S100A6 expression is increased in the heart following MI (Cai XY 2011). We have previously shown a progressive increase in S100A6 gene expression in the infarct and peri-infarct regions of myocardium post I/R injury that was after the peak of LV functional deterioration and simultaneous with or just after the peak of cardiomyocyte apoptosis. Existing data has demonstrated that overexpression of S100A6 gene in vitro prevents cardiomyocyte apoptosis induced by tumor necrosis factor-alpha through interference with p53 phosphorylation (Tsoporis, Izhar, and Parker 2008b) and inhibits the induction of the cardiac fetal gene promoters in cultured neonatal rat cardiomyocytes (Tsoporis, Marks, Haddad, O'Hanlon, et al. 2005) suggesting a role in modulating cardiac hypertrophy. Most recently, cardiomyocyte-specific transgenic mice overexpressing S100A6 gene showed attenuation of cardiac hypertrophy, decrease in interstitial fibrosis and reduction of cardiomyocytes apoptosis compared to control animals in a coronary ligation model of MI (Tsoporis et al. 2014).

Our previous data has shown that UTMD mediated delivery of hS100A6-plasmad prior to myocardial I/R injury prevented reperfusion injury and preserved cardiac function by prevention of apoptosis and reduction of necrotic scar formation, as well as improvement of calcium handling and angiogenesis. Our results are confirmatory of existing data on transgenic mice while additionally highlighting the application of the UTMD technique for localized gene
delivery for myocardial ischemia/reperfusion injury instead of a permanent LAD ligation/infarction. Since UTMD gene delivery prior to the myocardial I/R is not easily translatable to the clinical setting, we needed a vector with more rapid and efficient transfection ability to administer immediately after myocardial I/R and have a therapeutic effect.

Our *in vitro* data showed several advantages for minicircle vectors over conventional plasmids such as more efficient transfection, faster and more sustained expression over a period of weeks as compared to plasmids where expression wanes after 7 days. It is also known that avoidance of antibiotic resistance genes as selection markers for plasmid production eliminates the safety concerns of potential allergic reactions due to the residual antibiotic. The combination of improved transfection efficiency and improved safety profile make minicircle vectors a highly attractive alternative to conventional plasmid gene vectors for clinical applications.

### 5.5 Conclusion

Our current data demonstrate that UTMD mediated delivery of hS100A6-MC immediately after myocardial I/R instead of UTMD mediated delivery of hS100A6-plasmid 2 days prior to the myocardial I/R yielded similar therapeutic benefits. Immediate delivery of hS100A6-MC after myocardial I/R attenuated myocardial ischemia-reperfusion injury by reducing infarct size, improving LV systolic function and enhancing myocardial perfusion confirming hS100A6-MC as an efficient therapy for myocardial I/R injury.
Chapter 6

Summary
6.1 Conclusion

First, we showed that S100A6 gene expression gradually increases in the infarct and peri-infarct regions of myocardium following I/R, which may not be early enough to prevent cardiomyocyte apoptosis and LV functional deterioration. Then we hypothesized that forced overexpression of S100A6 gene at an earlier time point may prevent cardiomyocyte apoptosis and subsequent adverse remodeling of the LV and would improve LV systolic function, post I/R. We tested this hypothesis by UTMD delivery of hS100A6-plasmid 2 days prior to myocardial I/R injury. Our data demonstrated that the therapeutic benefit of S100A6 gene therapy, with improved survival and better LV systolic function post myocardial I/R injury. These positive effects were not only attributable to the known anti-apoptotic and anti-hypertrophic effects of S100A6, but also to enhanced Ca\(^{2+}\) cycling and Calcineurin activity, and to its pro-angiogenic properties.

To enhance the clinical relevance of our findings we used minicircle vectors instead of conventional plasmid because of their higher transfection efficacy and faster and more sustained expression, allowing us to pursue gene therapy immediately after I/R injury. UTMD delivery of hS100A6-MC immediately after myocardial I/R instead of UTMD mediated delivery of hS100A6-plasmid 2 days prior to the myocardial I/R yielded similar therapeutic benefits. Immediate delivery of hS100A6-MC after myocardial I/R attenuated myocardial ischemia-
reperfusion injury by reducing infarct size, improving LV systolic function and enhancing myocardial perfusion confirming the potential for hS100A6-MC gene delivery as an effective therapy to prevent myocardial I/R injury.

6.2 Limitations

My studies have several important limitations. Firstly, $\text{Ca}^{2+}$ transients were measured during the absence of stress (spontaneous action potential). Examining $\text{Ca}^{2+}$ transients under stimulation, and after hypoxia-re-oxygenation could provide us with additional valuable information. We did not include sham-operated animals or control groups receiving the microbubbles or DNA vectors alone. Including a control group undergoing sham operation in which the left coronary artery is loosely encircled by silk suture but not ligated is reasonable.

Although our results have been very promising in a rat model of ischemia/reperfusion injury, there may be many barriers to translation of this basic science work to the patients’ bedside and the clinical scenario given the differences between rodent and human anatomy and cardiovascular physiology. The therapeutic benefits of S100A6 gene therapy should be reassessed in the canine or pig myocardial I/R model before application to humans. Similar microbubbles to what were used for UTMD gene delivery have been safely used in patients for other purposes such as LV opacification and contrast perfusion imaging, with proven safety.
However, the power of ultrasound transmission may need to be adjusted in order to reach appropriate mechanical index to prevent undesired biological effects of high power ultrasound.

6.3 Future Direction

Previous studies have demonstrated transcriptional regulation of S100 genes expression (Lesniak and Kuznicki 1999), based on our data we can suggest the presence of additional translational regulation on S100A6 expression following hypoxic stress such as involvement of specific micro-RNAs, which can be an interesting avenue of research for future studies.

No significant interaction has been reported between S100A1 and S100A6 so far. In this study, the contradictory expression of S100A1 and S100A6 post myocardial I/R injury suggests a trans-repression effect for S100A6 and S100A1 genes, this should be explored in future studies.

Our results for the first time have shown pro-angiogenic function for S100A6. We proved it in vitro by the increased endothelial cells migration and tube formation. Our in vivo data not only confirmed improved perfusion in the myocardium of rats over-expressing S100A6 gene, but also showed increased vessel density in the peri-infarct region of myocardium in S100A6 overexpressing group. The mechanism underlying this effect is not yet determined. Previous studies had shown the pro-angiogenic function for S100A1, which was mainly through direct and indirect (through PKC) activation of eNOS (Most et al. 2013). On the other hand, it has been shown that S100A6 is a regulator of the cell cycle progression and sequence in the endothelial
cells through regulation of CDK1, cyclin A1 (CCNA1) and cyclin B (CCNB1) genes expression (Bao et al. 2012). Considering existing data and our finding it will be worth to measure expression level of HIF-1, VEGF-A, VEGF-R2, CDK1, Phospho-CDK1, CCNA1, CCNB1 and Beta-galactosidase genes as well as eNOS activity in S100A6-knock down and S100A6 overexpressing endothelial cells compared to controls under normoxic condition and post hypoxia/re-oxygenation.

Our calcium transient measurements have shown regulatory function for S100A6 for the first time. Calcium transient regulatory function had been shown for S100A1 gene before through directly interaction with ryanodine receptors (RYR) and by stimulating CICR pathway (Donato 2001; Pleger et al. 2011; Most et al. 2005). To explain the mechanism underlying calcium transient regulatory function of S100A6, it will be reasonable to test the co-localization of S100A6 protein with ryanodine receptors and SERCA as well as its influence on CICR pathway.

During my in vivo experiments most of the mortality occurred 15-30 minutes post LAD ligation, before reperfusion and was mostly due to arrhythmia (Ventricular Tachycardia/Fibrillation). I also showed that mortality rate was significantly lower in S100A6 pre-delivered rats. It has recently been shown that calcium dysregulation plays a significant role in atrial fibrillation pathogenesis (Heijman et al. 2014; Dobrev, Teos, and Lederer 2009).
Considering S100A6 regulatory function on calcium transient, it will be interesting to test the correlation between S100A6, S100A1 and S100B expressional level and paroxysmal or persistent atrial fibrillation post myocardial infarction. Biopsy sampling from atrial myocardium of patients undergoing CABG surgery post myocardial infarction and checking the mRNA and protein levels by PCR and western blotting may help us to test this hypothesis.

We have measured the size of infarcted myocardium 24 hours post myocardial I/R injury by TTC staining. We have also calculated the size of collagen rich necrotic tissue 28 days post I/R by MTC staining. We did not assess the size of the area at risk (AAR) post myocardial I/R injury in our groups, to ensure relatively homogenous AARs between groups. To assess AAR, one could inject Evans blue dye [(1.5%, 1.0 mL) in phosphate-buffered saline (PBS)] in the ventricle or through jugular vein catheter during coronary artery ligation. Subtracting the non-viable myocardial region (White on TTC stained sections) from the AAR (Pink in Evans blue stained sections) would show the extent of myocardial salvage with our gene therapies.
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