Tissue Salvage in the Non-reperfused Myocardium Mediated by (the absence of the circadian rhythm gene) mPer2 and (the receptor tyrosine kinase) EphrinA1

by

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Major Department: Physiology

Alterations in circadian rhythm have been associated with numerous cardiovascular pathologies. In project 1, ***we tested the hypothesis that functional mutation of the Per2 circadian clock gene would provide cardioprotection to mice that had undergone permanent coronary ligation to induce myocardial infarction (MI)***. mPer2-M mice had a 43% smaller infarct size compared to wild type (WT), along with reduced leukocyte infiltration, increased capillary density, increased myocyte hypertrophy, and reduced myocyte apoptosis. This suggests that mutation of mPer2 is cardioprotective.

The heart lacks a sufficient capacity for endogenous repair after injury. ***We tested the hypothesis that intramyocardial administration of ephrinA1-Fc at the time of MI would promote cardiomyocyte survival, subsequently reducing infarct size and inflammatory cell infiltrate.*** The ephrinA1 ligand has been predominantly characterized as a pro-angiogenic factor in development and tumor progression, but is also involved in apoptosis and inflammation. The ephrinA1 ligand has not been studied in the adult myocardium or in the context of acute MI.

Intramyocardial injection of EphrinA1-Fc reduced infarct size, necrosis, chamber dilation, and left ventricular free wall thinning four days after MI. Inflammation was also

substantially reduced, with reductions in neutrophil and leukocyte density. We measured reductions in serum cTnI, and cleaved PARP, and increased bag-1 protein expression, suggesting reduced cell death. Phosphorylated AKT/total AKT protein was increased, indicating improved cellular survival. Our analysis of gene expression revealed that Eph receptors A1-A4, A6, and A7 were expressed in the uninjured adult myocardium. Expression of EphA1-A3 and EphA7 was significantly increased following MI while EphA6 expression was decreased. Treatment with ephrinA1-Fc further increased EphA1 and EphA2 gene expression, and also increased EphA4 expression.

To date, only reperfusion has been shown to reduce injury and improve long-term remodeling. We have discovered two new mechanisms by which this can be effected: 1) we have observed a dramatic reduction in cardiac injury in mice lacking a functional circadian gene product mPer2; and 2) we are the first to identify a role for ephrinA1/EphA signaling in the repair process following MI, and have identified a novel, protective role for ephrinA1-Fc administration at the time of MI.

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by

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DEDICATION

I dedicate this dissertation to my parents, who have instilled confidence, encouraged creativity, and demonstrated the importance of working hard in everything that I do. They have provided opportunities for me to pursue my dreams in any and every way possible. I am lucky and blessed beyond belief, and I thank them for that.

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The Department of Physiology embodies the “it takes a village….” philosophy, and I am grateful to so many of the faculty, staff, and students, who opened up their laboratories to share equipment, troubleshoot techniques, and provide feedback and advice. In addition, I wish to thank the department for taking a chance on an undergrad with no research experience, and giving me the opportunity to attend graduate school here. The administrative assistants, Laura Frye, Marsha Hall, and Crystal Stallings, do a remarkable job of keeping us all on our toes, finding solutions to anything that comes up, and being a constant source of encouragement.

In the Virag lab, Susan Kent has been a great friend and colleague, who is meticulous in her attention to detail, and an extremely fun personality with which to work. Jon DeAntonio

helped out with running qRT-PCR and western blots in the lab, and was such a quick learner. He'll make a great physician in a few more years.

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LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
Ang-2	Angiopoietin 2
BAG-1	Bcl-2 associated athanogene
Bcl-2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
Bmal1	brain and muscle aryl-hydrocarbon receptor nuclear translocator-like 1
CCM	Circadian Clock Mutant
CK	Creatine Kinase
CK-MB	Skeletal-Brain Hybrid Type Creatine Kinase
CLCs	Cardiomyocyte Like Cells
CO	Cardiac Output
Cry1	cryptochrome 1
cTnI	Cardiac Troponin I
EF	Ejection Fraction
Eph	Erythropoietin Producing Hepatocellular Carcinoma
Ephrin	Eph-Receptor Interacting Proteins
EPCs	Endothelial Progenitor Cells
ESPVR	End systolic pressure volume relationship
FABP	Fatty Acid Binding Protein
FGF	Fibroblast Growth Factor

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HIF-1α	Hypoxia Inducible Factor Alpha
HSCs	Hematopoietic Stem Cells
IFN-λ	Interferon gamma
I/R	Ischemia/Reperfusion
LAD	Left anterior descending coronary artery
LV	Left ventricle
LVEDV	Left ventricular end diastolic volume
LVPsys:	Left ventricular systolic pressure
MACS	Miltenyi Activated Cell Sorting
MAGIC	Myoblast Autologous Grafting in Ischemic Cardiomyopathy
MAPK	Mitogen-activated protein kinase
MI	Myocardial Infarction
MMP-9	Matrix Metallopeptidase 9
mPer2-M	Mammalian Per2 mutant
MSC	Mesenchymal Stem Cells
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
PARP	Poly-ADP-ribose-Polymerase
Per1, 2	Period 1, 2
PI3K	Phsphoinositide 3-kinase

PRSW	Preload recruitable stroke work
Rev-erb-α	reverse erythroblastosis virus alpha
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
SCN	Suprachiasmatic Nucleus
SGOT	Serum glutamic-oxaloacetic trasaminase
STEMI	ST elevation MI
SV	Stroke volume
TGF-β	Transforming Growth Factor Beta
TNFα	Tumor necrosis factor alpha
TLR	Toll-Like Receptor
VEGF	Vascular endothelial growth factor
WT	Wild Type

CHAPTER 1: INTRODUCTION

Acute Myocardial Infarction: Characterization and Significance

Despite considerable advances in recent years, cardiovascular disease continues to be a significant public health problem, affecting 81 million Americans and resulting in over 800,000 deaths each year. Almost 1 million new or recurrent heart attacks, or myocardial infarctions (MI), occur each year (Lloyd-Jones, Adams et al.). The damage resulting from MI is largely irreversible and often leads to heart failure. Currently, efforts are focused on reducing tissue damage following this ischemic event, or even generating replacement tissue.

Atherosclerosis often results in coronary artery thrombosis, leading to coronary occlusion and downstream ischemia. Prolonged ischemia results in the death of cardiomyocytes. This leads to the release of a number of different factors which may be used as plasma biomarkers for MI diagnosis. Factors released from apoptotic and necrotic tissue include fatty acid binding protein (FABP), creatine kinase, skeletal-brain hybrid type (CK-MB), serum glutamic-oxaloacetic trasaminase (SGOT), and cardiac troponins I and T (Masson, Latini et al. ; Cleutjens, Blankesteijn et al. 1999). In the days and weeks following MI, the infarct area undergoes a number of structural changes leading to the formation of non-contractile scar tissue, which over time will adversely affect function.

Cell Death and Myocardial Infarction

Death of myocytes following MI occurs through three different, but interdependent, pathways: necrosis, apoptosis, and autophagy (Whelan, Kaplinskiy et al. ; Kunapuli, Rosanio et al. 2006). Myocyte necrosis is a passive, unregulated process in which the cells swell and burst. Triggers include hypoxia and ischemia (Kunapuli, Rosanio et al. 2006). During this process, the plasma membrane becomes dysfunctional, causing the cells and their organelles to swell. As the contents leak out of these swollen cells, an intense inflammatory response is initiated (Whelan, Kaplinskiy et al.). Necrosis differs from apoptosis in that it is much less regulated, and does not require energy.

While initial reports suggested that necrosis was the characteristic mode of cell death that occurred in the acute stages after MI, there is also literature to suggest that apoptosis occurs acutely, as well (Itoh, Tamura et al. 1995; Bardales, Hailey et al. 1996; Olivetti, Abbi et al. 1997; Saraste, Pulkki et al. 1997; Buja and Entman 1998; Haunstetter and Izumo 1998; Kang and Izumo 2003). Some reports have suggested that, since apoptosis requires energy, it is more prominent following reperfusion, whereas necrosis is associated with prolonged ischemia (Gottlieb, Burleson et al. 1994; Kang and Izumo 2000). Apoptotic cell death is induced by cytokines including TNFα and Fas, which bind cell surface receptors and recruit intracellular adaptor proteins. This leads to activation of caspases, which mediate proteolytic cell death. In the case of Fas binding, pro-apoptotic proteins of the Bcl-2 family are activated. In contrast to necrosis, apoptosis consists of programmed death characterized by DNA fragmentation and shrinkage of the cells. Apoptosis is stimulated by external triggers such as a lack of growth

factors or changes in hormonal levels including increased norepinephrine and angiotensin II (Cigola, Kajstura et al. 1997; Communal, Singh et al. 1999; Kunapuli, Rosanio et al. 2006).

Cellular autophagy is the third, lesser known process which has only recently been proposed as a mechanism in myocyte survival and death. Autophagy is a form of programmed cell death in which a cell may re-direct nutrients to the most essential processes, through lysosomal degradation of less important components. Thus, it may be viewed as a survival mechanism. This process is typically observed in hibernating myocardium, or in the transition from hypertrophy to heart failure, and is a mechanism for cells to resist starvation in ischemic tissue. A protein often associated with cardiomyoycte autophagy is the Bcl-2 associated athanogene (BAG)-1 protein, which links heat shock proteins to the proteasome, leading to protein degradation (Townsend, Cutress et al. 2004; Gurusamy, Lekli et al. 2009). Under normal conditions, when a cell has a sufficient supply of nutrients, Tor kinase remains inactive. However, when the nutrient supply is insufficient for cellular survival, Tor kinase becomes inactivated, which initiates autophagy. A vesicle, termed the autophagosome, is formed from the endoplasmic reticulum, and becomes the repository for cell components slated to die. The contents of the autophagosome are then transferred to a lysosome via fusion, and the autophagosome is degraded by proteases (Kunapuli, Rosanio et al. 2006).

In the acute phase after MI, the heart undergoes a number of pathological and structural changes, often described as infarct repair. Many groups have described this infarct healing process extensively (Dobaczewski, Gonzalez-Quesada et al. ; Cleutjens and Creemers 2002; Frangogiannis, Smith et al. 2002; Holmes, Borg et al. 2005; Frangogiannis 2006). Perhaps, modulating part(s) of this process may help to preserve myocardial tissue and attenuate cardiovascular dysfunction. Three overlapping phases of infarct healing have been described

(Virag and Murry 2003; Frangogiannis 2006; Frantz, Bauersachs et al. 2009; Nah and Rhee 2009), which will be detailed below.

The Inflammatory Phase

In mice, the inflammatory phase of infarct healing typically lasts on the order of hours to days (approximately 3-72 hours) and is characterized by the induction of chemokines and cytokines in the infarcted heart. The onset of ischemia leads to activation of hypoxia-inducible factor (HIF)-1α. Growth factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are released following activation of HIF-1α, and stimulate growth of new vessels. Complement activation occurs, which may aid in inflammatory cell recruitment to the infarct (Frangogiannis, Smith et al. 2002; Frangogiannis 2006). Some of the infiltrating inflammatory cells may, in combination with cardiomyocytes and endothelial cells, generate reactive oxygen species (ROS) which may further contribute to leukocyte recruitment. Neutrophils infiltrate the tissue by adhering to endothelial cells in the vasculature and migrating into the ischemic tissue (Nah and Rhee 2009). In some animals that underwent ischemia/reperfusion, reduced levels of neutrophils was associated with significant decreases in infarct size (Romson, Hook et al. 1983; Jordan, Zhao et al. 1999). Infiltrating neutrophils can potentially be cytotoxic, but a rapid inflammatory response may also improve infarct healing (Ren, Dewald et al. 2003). Both neutrophils and macrophages participate in clearing the infarct zone from necrotic cellular debris. In addition, macrophages release growth factors and cytokines that aid in the forming the granulation tissue (Frangogiannis 2008). Toll-Like Receptor (TLR) Mediated Pathways are also activated during the inflammatory phase, leading to

activation of Nuclear factor (NF)-κB. NF-κB is located in the cytoplasm, associated with inhibitors of κB, or IκB. When IκB is phosphorylated, it ultimately becomes degraded by proteasomes, releases NF-κB, leading to NF-κB activation. NF-κB activation results in regulation of genes involved in inflammation, including chemokines and cytokines, as well as cellular adhesion (Frangogiannis 2008).

A provisional matrix, composed of fibrin, allows the infiltration of inflammatory and vascular cells to the healing infarct. This provisional matrix is formed when vascular permeability is increased through the release of inflammatory cytokines (Frangogiannis 2006).

The Proliferative Phase

The proliferative phase occurs 3 to 7 days following MI, and is characterized by proliferation of fibroblasts and endothelial cells to form a rich, cellularized granulation tissue. This granulation tissue must be vascularized, to provide nutrients and oxygen. Endothelial cells proliferate and contribute to formation of the vasculature. In addition, endothelial progenitor cells (EPCs) may be involved. These bone marrow-derived progenitors are produced in the bone marrow, and mobilized to the peripheral blood in response to ischemia (Asahara, Masuda et al. 1999; Takahashi, Kalka et al. 1999; Masuda, Kalka et al. 2007). The cells are then recruited to sites of vessel growth, where they may differentiate into vascular cells or contribute to vasculogenesis by secreting cytokines, chemokines, and growth factors that promote cell survival and growth (Kamihata, Matsubara et al. 2001; Rehman, Li et al. 2003). The Tie2 receptor tyrosine kinase is present on endothelial cells and EPCs. When the Angiopoietin (Ang)-2 ligand is activated during MI, it binds to, and activates, Tie2-expressing cells, which facilitates the onset

of angiogenesis. Under normal conditions, the Ang1 ligand is expressed and bound to Tie2-expressing cells. The expression of the Ang2 ligand seems to disrupt this signaling system (Frangogiannis 2006). Once vessels are formed, demarcation of vascular boundaries is necessary. The Eph Receptor Tyrosine Kinase (RTK) family and its ephrin ligands are involved in this process, with the EphB4 receptor expressed on venous endothelial and smooth muscle cells, while the ephrinB2 ligand is expressed on arterial vessels (Carmeliet 2003). Some of the newly formed vessels will be stabilized by pericytes. Vessels that are not coated with pericytes will eventually regress. The fibrin matrix is proteolytically cleaved, by the conversion of plasminogen to plasmin, and replaced with a more organized provisional matrix composed of fibronectin and hyaluronan (Frangogiannis 2006).

The Maturation Phase

The maturation phase typically occurs 7-14 days after injury in mice (Frangogiannis 2006). Ultimately, the proliferating fibroblasts and endothelial cells will undergo apoptosis, leaving a thin, collagenous scar in the infarct zone. The only vessels that remain are those that are coated with smooth muscle cells and have little to no angiogenic capacity. Fibroblasts, located predominantly at the infarct border zone differentiate into myofibroblasts and lay down extracellular matrix proteins, particularly collagen. Fibroblasts synthesize transforming growth factor-beta 1(TGF-β1), which further enhances collagen formation. Type I and III collagens are the principal components of the scar. The mature collagen matrix works to stabilize the scar (Sun and Weber 2000). If the resulting scar is large enough, heart failure may ensue due to decompensation of the remote regions of the myocardium. Cardiac fibrosis, characterized by the

accumulation of type I and type III collagen, replaces necrotic cardiomyocytes, and adversely affects myocardial tissue stiffness and diastolic function. Eventually, systolic function is also affected (Weber 1997). Structural changes of the heart occur due to several variables. Myocyte hypertrophy in remote regions of the myocardium may alter the shape of the heart and put additional strain on the infarcted region. Myocyte necrosis in the infarct zone may lead to thinning and dilation of the left ventricle. Cardiomyocytes lengthen and thin in response to infarction, and strain on the tissue, which may further result in wall thinning. Perhaps most importantly, groups of myocytes slip past one another during infarct repair, altering cell shape (Weisman, Bush et al. 1988). When left ventricular thinning and dilation occur, leading to infarct expansion, there is an increased incidence of clinical mortality (Weiss, Marino et al. 1991).

Current Therapeutic Strategies

Presently, there is great energy being devoted to exploring therapeutic strategies which could reduce infarct damage and/or promote regeneration of necrotic tissue. Early reperfusion and antithrombotic therapy have helped to reduce mortality by preventing myocyte necrosis, but therapeutic success is only accomplished if reperfusion is initiated immediately after MI. In fact, some suggest that the benefit to the patient begins to decline if therapy is initiated after a delay of 60 to 114 minutes (Segers and Lee ; Nallamothu and Bates 2003; Pinto, Kirtane et al. 2006). Pharmacological strategies already in place, such as the use of beta-blockers or ACE inhibitors following MI improve overall survival, but do nothing to improve actual cardiac function. Once

infarction has occurred, damage is irreversible so these strategies only work to delay the onset of heart failure.

The most recent interest has been in the fields of stem cell therapy and tissue engineering, but results thus far have been disappointing. Despite some remarkable advances in many animal models, improvements in the clinical setting have been modest at best. For example, a recent phase 1 clinical trial using intracoronary bone marrow mononuclear cell transplantation in patients who had suffered ST-elevation MI (STEMI) revealed no difference in the functional improvement observed between the cell treatment group and the placebo group. There was, however, a modest decrease in left ventricular end diastolic volume (LVEDV) following cell transplantation, suggesting a favorable effect on LV remodeling (Traverse, McKenna et al.). In rodents, transplantation of either mesenchymal stem cells (MSCs) or cardiomyocyte-like cells (CLCs) resulted in improved myocardial contractility, in a dose dependent manor. The CLCs were more effective, and exerted their effects mainly through engraftment in and mechanical support of the matrix, while MSCs promoted angiogenesis in the infarcted tissue (Shim, Tan et al.).

Much of the disparity in data reported from cell therapy experiments thus far is directly due to the variation in cell type and delivery method used, as well as concerns over safety. For example, autologous transplantation of skeletal myoblasts into rabbits that had undergone cryoinfarction resulted in improved myocardial function and incorporation of the skeletal muscle cells into the myocardium (Taylor, Atkins et al. 1998). However, results of the MAGIC clinical trial (Myoblast Autologous Grafting in Ischemic Cardiomyopathy) were disappointing, showing no substantial difference in function between myoblast- and placebo-treated patients. More importantly, there was a higher incidence of arrhythmias in myoblast recipients, suggesting that

safety of this therapy needs further evaluation (Menasche, Alfieri et al. 2008). Embryonic stem cells have also been used for cell therapy, with conflicting results. For example, transplantation of embryonic stem cells into infarcted mice resulted in the formation of teratomas, and little to no differentiation into cardiomyocytes (Nussbaum, Minami et al. 2007). Bone marrow-derived cells are less ethically controversial when compared to embryonic stem cells, but their efficacy is also questionable, ranging from no effect at all, to improved contractility and ejection fraction (for review see (Wei, Wong et al. 2009)). Questions also exist regarding the mechanism of cell therapy-mediated benefits. Cellular differentiation, engraftment, or paracrine actions are all possibilities, and may be specific to the cell type used.

An alternative to cell therapy that has been investigated in both the laboratory and clinical settings is protein therapy, which is attractive because it involves proteins that are already expressed *in vivo*, and are evolutionarily conserved. More importantly, therapies involving naturally occurring proteins avoid ethical debates that surround the use of stem cell therapy (ie, embryonic stem cells), and eliminate the possibility of rejection that may occur with cell therapy or organ transplantation. Availability of these compounds is relatively abundant and many are already commercially available, providing an attractive alternative to cultivating and expanding cell populations for autologous transplant. Proteins used for therapy after MI can be loosely categorized into four groups: (i) those used to promote angiogenesis, (ii) proteins to promote mobilization of stem and progenitor cells to the heart, (iii) proteins that may induce mitosis of native cardiomyocytes, and (iv) growth factors which induce proliferation and growth of stem and progenitor cells (Hwang and Kloner ; Segers and Lee).

Pro-angiogenic proteins, including fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) are of great interest due to the possibility of increasing cardiac

perfusion, which will ultimately promote cell survival and improve function. It has also been reported that following acute MI, there is a decrease in angiogenic factors including VEGF, associated with decreased myocardial contraction (Siddiqui, Fischer et al.). Thus, if various angiogenic proteins can be used to "supplement" the endogenous expression of these proteins in the heart, cardiac repair may be accelerated.

Both VEGF and FGF have been widely used in pre-clinical and clinical studies, but the promising results seen in laboratory animals have not been translated to human patients. An early study used VEGF infusion via osmotic minipumps in pigs that had undergone permanent coronary occlusion. This six week infusion of VEGF resulted in a reduced infarct volume and improved ejection fraction and myocardial wall thickening (Pearlman, Hibberd et al. 1995). In a rat model of MI, intracardiac injection of VEGF resulted in increased survival of cardiomyocytes, presumably by increasing myocyte mitotic activity as well as cytoprotection. Additionally, there was a greater intensity of neutrophil and macrophage infiltration with VEGF treatment, along with increased collagen formation (Dremina, Shurygina et al. 2009). In humans, plasmid DNA encoding VEGF has been delivered intramyocardially and endocardially, but with little to no functional improvement for those patients (Losordo, Vale et al. 1998; Vale, Losordo et al. 2001; Losordo, Vale et al. 2002; Rajagopalan, Mohler et al. 2003; Stewart, Kutryk et al. 2009).

FGF treatment has also produced promising pre-clinical data. In a dog model of coronary occlusion, FGF was infused into the left circumflex coronary (LCX), resulting in reduced infarct size, reduced necrosis, increased vascular density, and improved ejection fraction (Yanagisawa-Miwa, Uchida et al. 1992). A clinical study using recombinant FGF-2 intracoronary administration in patients with coronary artery disease demonstrated improved exercise tolerance

and reduced angina symptoms 90 days after treatment. However, the placebo group continued to improve, and by 180 days post-treatment, the differences between treatment groups were abolished (Simons, Annex et al. 2002). A more promising result came from a clinical trial that implanted microcapsules containing FGF into ischemic myocardium. The higher dose of FGF (100ug) had a favorable effect on infarct size and angina symptoms, while the lower dose (10ug) did not (Laham, Sellke et al. 1999). Clearly, protein or cell therapy (or some combination of the two) holds great promise for myocardial repair, but results are still conflicting. A system in which therapies could be targeted to specific cell types would be ideal, so that specific phases of infarct repair could be modulated independently. For example, promoting vasculogenesis in the ischemic myocardium is of interest to restore the flow of nutrients and oxygen to the infarct zone, so some would argue that therapies should be endothelial cell-specific. However, targeting the cardiomyocytes directly is also useful, in order to promote cellular survival and prevent death. To date, many of the therapies proposed have *not* been cell-specific.

Two Approaches Resulting in Infarct Salvage

An early interest in our laboratory was investigating the role of individual circadian clock genes on the response to acute MI. An early observation was made that mice lacking functional mammalian Period 2 protein (mPer2-M mice) had a reduced infarct size following 30 minutes of ischemia and 2 hours of reperfusion. This initiated a new set of experiments using a non-reperfused infarct model, to analyze various endpoints in response to permanent coronary occlusion. Data from these studies are represented in chapters 2.1 through 2.4. In short, we demonstrated that the absence of the circadian clock gene, mPer2, results in robust

cardioprotection following MI. One element of the response that was intriguing was a substantial improvement in capillary density and an increased number of bone marrow-derived progenitor cells in the mPer2-M mice. These experiments contribute a new understanding of circadian clock gene biology in the setting of cardiovascular disease, and our laboratory is currently investigating the cellular mechanisms of mPer2-M cardioprotection.

The direction of the laboratory then moved towards identifying therapeutic strategies that might promote revascularization of damaged myocardium, as well as provide an alternative to the convoluted and controversial field of cell therapy. Because of the challenges that have come from protein therapy involving VEGF and FGF, we were interested in identifying a new therapeutic family to use for post-MI repair.

Eph Receptor Tyrosine Kinase Family/Ephrin Ligands

The Eph Receptor Tyrosine Kinase (RTK) family and its respective ephrin ligands are widely under-explored in the cardiovascular arena. Many of these proteins are reported to be pro-angiogenic in other tissue settings, but have not been investigated in the injured adult heart. We were intrigued by studies showing that members of the Eph/Ephrin B family are involved in endothelial progenitor cell (EPC) adhesion (Foubert, Silvestre et al. 2007) and post-MI angiogenesis (Mansson-Broberg, Siddiqui et al. 2008). We constructed the hypothesis that members of the EphrinA family might also trigger therapeutic angiogenesis and attenuate the inflammatory response, resulting in reduced infarct size, as we saw in our mPer2-M mice. The EphrinA1 ligand, an angiogenic protein, was chosen for post-MI intramyocardial injection, and these results are discussed in chapters 3.1 through 3.4.

We have proposed two key projects, to elucidate the role of a specific peripheral clock gene, Per2, and hypothesize that its mutation will be protective to the heart. In contrast, we have chosen an angiogenic protein, ephrinA1, and propose that its administration to the infarcted heart will promote cell survival.

CHAPTER 2: mPER2 AND MYOCARDIAL INFARCT REPAIR

2.1: Background

Epidemiological Data

Incidence of cardiovascular disease appears to be correlated with the time of day, and has sparked a great deal of interest in basic and clinical research with regards to circadian clock gene regulation. Multiple studies have explored the time-of-day phenomena in the setting of myocardial infarction, and it is frequently reported that myocardial infarction is more likely to occur in the early morning hours (Durgan, Pulinilkunnil et al. ; 1992; Willich, Jimenez et al. 1992; Mitrovic, Stefanovic et al. 2008). By examining the blood levels of creatine kinase, it is evident that there is a greater incidence of myocardial infarction in the three hours after awakening (Willich 1999). Circadian fluctuations in blood pressure (BP) and heart rate (HR) have also been observed, often referred to as the "morning surge" due to the spike in both measurements upon waking, and a decrease in BP and HR while one sleeps (Neutel and Smith 1997; White 2001; Patel, Wong et al. 2008). Additional evidence of circadian variation in cardiovascular disease is demonstrated by increased risk of metabolic syndrome, myocardial infarction and atherosclerosis in shift workers (Karlsson, Knutsson et al. 2001; Ha and Park 2005; Copertaro, Bracci et al. 2008; Haupt, Alte et al. 2008). Interestingly, Esquirol and colleagues recently published the results of a clinical study that demonstrated differences in metabolic syndrome between shift workers and day workers, which took into account behavioral and lifestyle practices that may influence their risk for cardiovascular disease such as diet, alcohol consumption, smoking, and exercise. Even when these behaviors were accounted for,

there were still differences in triglycerides and HDL levels between shift and day workers (Esquirol, Bongard et al. 2009). Clearly, then, the differences observed in risk for developing cardiovascular disease must be regulated at a cellular or molecular level, rather than behavior at specific times of day. We are just beginning to elucidate regulation of clock gene expression in different cell types, and how alterations may influence the progression of disease processes.

Circadian Biology

Before characterizing clock gene expression in peripheral tissues, it is essential to understand the basic aspects of circadian biology. The central circadian clock is located in the suprachiasmatic nucleus (SCN) of mammals. Light cues from the environment are transmitted to the SCN by glutamate, ultimately leading to phase shift of the circadian clock. Light stimulation leads to induction of 'input genes' including the early transcription factor c-fos, and PER1 and PER2 genes. Output genes such as avp, dbp, and CREM transmit information from the SCN to downstream targets affecting physiological functions. Both input and output genes may overlap with pacemaker genes, which regulate circadian rhythms by using information from input genes to affect system function through output genes (Cermakian and Sassone-Corsi 2000).

Within most cell types in the body there are transcriptionally controlled circadian clocks which regulate many biological functions. It has been previously revealed that these peripheral clocks exist within many cells of the heart including cardiomyoyctes (Durgan, Hotze et al. 2005), as well as endothelial cells (Takeda, Maemura et al. 2007) and smooth muscle cells (McNamara, Seo et al. 2001; Nonaka, Emoto et al. 2001) of the vasculature. In circulation, clock genes have been identified on peripheral blood mononuclear cells (Teboul, Barrat-Petit et al. 2005),

hematopoietic stem cells (Tsinkalovsky, Rosenlund et al. 2005; Garrett and Gasiewicz 2006; Tsinkalovsky, Filipski et al. 2006), and immune cells (Nebzydoski, Pozzo et al. ; Wang, Reece et al. ; Du, Fan et al. 2005; Fukuya, Emoto et al. 2007; Hayashi, Shimba et al. 2007). The mPer1 and mPer2 genes were also identified in whole murine bone marrow, and exhibited a diurnal expression pattern that was differentiation- and lineage-stage specific, suggesting the presence of a circadian clock system in the bone marrow (Chen, Mantalaris et al. 2000). To assess the effect of myocyte-specific circadian clocks on cardiac function, Young and colleagues have developed a mutant mouse whose circadian clock in the cardiomyocytes is disrupted so that the morning to night transition is abolished. The authors reported that this cardiomyocyte-specific circadian clock mutant (CCM) did not exhibit the normal diurnal heart rate variations as seen in wild type (WT) mice. They also consumed more energy but with less efficient cardiac function (Bray, Shaw et al. 2008). Clearly, then, this cell-specific circadian clock can have a substantial effect on myocardial function and metabolism. One inherent weakness of the study is that it did not specifically address the contribution of individual clock genes to the peripheral clock function of the cardiomyocyte. To specifically investigate the effect of the cardiomyocyte-specific circadian clock on the response to myocardial ischemia, the same group performed ischemia/reperfusion (I/R) surgeries on WT and CCM mice and found that WT mice exhibited variation in infarct volume, remodeling, and function, depending on what time of day the I/R occurred. In contrast, CCM mice did not show this same time-of-day variation (Durgan, Pulinilkunnil et al.). This may provide an explanation at the cellular level as to some of the epidemiological findings described previously in this chapter, indicating that time of day is often related to severity of MI.

To fully understand the complexity of circadian variation and cardiovascular disease, a greater appreciation for the involvement of specific circadian genes is needed. In humans, PER1, PER2, BMAL1, and CRY1 are expressed in the heart, with all but CRY1 exhibiting circadian rhythm in the heart (Leibetseder, Humpeler et al. 2009). In mice, BMAL1, PER2, CRY1, and REV-erb-α were all expressed three and five days postnatally, but did not become synchronized to the expression of clock genes in the SCN until day five (Huang, Lu et al.).

The effect(s) of individual, specific clock genes on cardiovascular pathological conditions has been relatively unstudied. A recent study used mice with a mutated Per2 gene (mPer2-M), and evaluated a number of hemodynamic measurements in 12:12-hour light-dark cycle, as well as in constant darkness. mPer2-M mice had increased heart rate and reduced diastolic blood pressure. Interestingly, they did not undergo "nighttime dipping" when placed in constant darkness (Vukolic, Antic et al.). Dipping, or the fall in blood pressure at night when the patient is sleeping, is considered normal and healthy. When dipping does not occur, nocturnal hypertension may be diagnosed and treated with antihypertensive medication before bedtime (Vij and Peixoto 2009). In a rat model of ischemia/reperfusion, there was an attenuation of clock gene oscillations in the ischemic zone, but not in the non-ischemic zone. On the other hand, hypoxia induced through the use of a hypobaric chamber did not attenuate clock gene oscillations (Kung, Egbejimi et al. 2007). Interestingly, a 2003 report by Koyanagi and colleagues indicated that cotransfection of cultured sarcoma 180 cells with the *VEGF*-Luc reporter and HIF-1α led to a ~15-fold increase in VEGF promoter activity, which was dose-dependently repressed by cotransfection with PER2. Further, PER2 protein precipitated with HIF-1α protein, and there was an anti-phase accumulation of VEGF mRNA and PER2 protein, suggesting that PER2 protein may regulate the circadian expression pattern of VEGF in tumor

cells during periods of hypoxia (Koyanagi, Kuramoto et al. 2003). A potential role for the Per2 clock gene in the regulation of angiogenic proteins may be of interest in cardiovascular repair of ischemic tissue. Combined, these data suggests that circadian clock genes, including Per2, may be involved in regulating myocardial function, but their activity becomes impaired following myocardial ischemia. To our knowledge, the effect of a single circadian clock gene on the subsequent *response* to myocardial ischemia has not been investigated. Specifically, we are interested in the role of the Per2 gene in myocardial infarct repair. All facets of infarct repair (ie, cell death, proliferation, inflammation, vasculogenesis, and remodeling) will be evaluated in a Per2 functionally mutated mouse (mPer2-M). In addition, since circadian rhythms have been reported to influence bone marrow cell production and mobilization (Lucas, Battista et al. 2008; Mendez-Ferrer, Lucas et al. 2008), and the Per2 gene has been implicated in endothelial progenitor cell function (Wang, Wen et al. 2008), we will analyze bone marrow-derived progenitor cell counts in mPer2-M mice.

2.2: Attenuation of Myocardial Injury in Mice with Functional Deletion of the Circadian Rhythm Gene mPer2

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INTRODUCTION

Circadian rhythms are daily variations of physiological processes that are found in living organisms. In mammals, the circadian rhythms are regulated by the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN synchronizes the circadian rhythms of peripheral organs to each other and to the environmental light-dark cycle via integrated oscillatory expression of multiple circadian clock genes (Harmer, Panda et al. 2001; Dvornyk, Vinogradova et al. 2003; Dardente and Cermakian 2005; Merrow, Spoelstra et al. 2005). So far, eight core circadian clock genes have been identified in mammals, including a Clock gene, a gene encoding brain-muscle Arnt-like protein 1 (Bmal1) (Honma, Ikeda et al. 1998), three period genes (Per1, Per2, and Per3) (Sun, Albrecht et al. 1997; Zheng, Larkin et al. 1999; Shearman, Sriram et al. 2000), and two cryptochrome genes (Cry1 and Cry2) (Thresher, Vitaterna et al. 1998; Hardin and Glossop 1999; Sancar 2000).

Recently, it has been shown that clock genes are found in all peripheral tissues, including the heart. Epidemiologic studies demonstrate the existence of circadian patterns in the incidence

of cardiovascular disease. For example, the onset of non-Q-wave angina, unstable angina, myocardial infarctions, and sudden cardiac death all show marked elevations in occurrence between the hours of 6am and noon, compared to any other time of day (Muller 1999; Willich, Kulig et al. 2004). A better understanding of the function of circadian genes in the heart and in response to injury may lead to innovative therapies for cardiovascular disease (Fujino, Iso et al. 2006; Young 2006; Portaluppi and Lemmer 2007).

Cardiac tissue expresses all known isoforms of Cry and Per genes, with Cry2, Per1 and Per2 expressed to the greatest degree (Young 2006). However, all of these genes function essentially as reciprocally controlling transcription factors, and in many cases the expression of these genes is monitored by the modulation of many "non-circadian" proteins as readouts. Enzymes regulating cardiac metabolism (57), reactivity of vascular endothelial cells (22, 50, 54), modulation of inflammatory responses (3, 27, 33, 34, 44), bone marrow progenitor cell release (Bourin, Ledain et al. 2002), and apoptosis (25) all have circadian gene components of control, and all are associated with the myocardial response to coronary artery occlusion. However, the specific relationship between a circadian gene and the inflammatory response and injury associated with early myocardial infarction has not been determined. Given that circadian rhythms control the cell cycle and mutations in clock genes have been associated with tumor growth and altered regulation of apoptosis (Lamont, James et al. 2007), and altered contractile function, metabolism, and gene expression in clock gene mutant cardiomyocytes (Bray, Shaw et al. 2008) it would appear reasonable to suggest that Per2 may be capable of altering the response to ischemic injury. Therefore, these studies were designed to test the hypothesis that functional Per2 mutation would attenuate early post-MI injury.

METHODS

Animals. Male wild type C57BL/6J mice (aged 8-10 weeks) and homozygous mutant mPer2-M mice bred on a C57BL/6J background were obtained from Jackson Laboratories (mPer2-M Brdm1, stock #003819; Bar Harbor, Maine) (Zheng, Larkin et al. 1999). (Zheng, Larkin et al. 1999). Two segments of the PAS domain of the mPer2 gene were deleted, rendering a functional null mutant. RT-PCR indicated a mutant transcript, if translated, would generate an 87 amino acid protein (Zheng, Larkin et al. 1999). Mice possessing this ubiquitous mutation are morphologically indistinguishable, have a shorter circadian period, and lose rhythmicity in constant darkness compared to their WT counterparts (Zheng, Larkin et al. 1999). All animals were individually housed in a light-proof chamber and entrained in a 12 hour light and 12 hour dark (12:12 LD) cycle for at least 10 days before surgery. All procedures were approved by the East Carolina University Institutional Animal Care and Use Committee and are in compliance with NIH guidelines.

Surgical Procedure. Mice were anesthetized (20 μl/g Avertin i.p.), intubated and ventilated using a Kent Scientific TOPO ventilator. Briefly, the LAD coronary artery was permanently ligated or sham ligated in controls. The chest cavity was sutured closed and the animals were permitted to recover in a warming chamber before being returned to the vivarium. No analgesia was used and all experiments were performed during the light phase of the circadian cycle between ZT3 and ZT9. The surgical procedure is described in more detail elsewhere (Ismail, Poppa et al. 2003; Reinecke and Murry 2003).

At 4 days post-MI, mice were given a 0.5 ml i.p. injection of 5-bromodeoxyuridine (BrdU, 5mg/ml) to label proliferating cells and sacrificed 1 hour later with an i.p. injection of pentobarbital. The perfused heart and a segment of small intestine (used as a positive control for

BrdU+ proliferating cells) were immersed in zinc fixative and 4 transversely sectioned slices of equal thickness were processed and embedded in paraffin. Routine histological (hemtaoxylin and eosin, picrosirius red/fast green, toluidine blue, and congo red) procedures and immunostaining were performed using 5 μm sections.

Morphometry and Histology. For non-reperfused infarct studies, photographs of four hematoxylin and eosin stained sections per heart (uninjured control and 4 days post-MI, both WT and mPer2-M hearts) were taken at 20x using a DP70 digital camera. Scion imaging software (Scion Corporation, Frederick, MD) was used to trace the infarct zone (granulation tissue and necrosis), necrosis (no myocyte nuclei), and granulation tissue (inflammatory cells, fibroblasts, smooth muscle cells, and endothelial cells).

To assess myocyte cross-sectional area, 3 images were taken at 600x from both the epicardial and endocardial surface at the infarct border in 2 sections containing infarct (12 images total). In each image, 3-7 perpendicularly sectioned myocytes with centrally located nuclei were measured and the mean cross-sectional area was calculated.

Immunostaining. Tissue sections were deparaffinized and endogenous peroxidases quenched. After rinsing in PBS, slides were incubated with anti-smooth muscle α-actin (SMα-A; peroxidase-conjugated, DAKO, U7033) for myofibroblasts, anti-CD31 (PharMingen, #553371; 1:2000) for endothelial cells in infarcted hearts, isolectin B4 (Vector, #B-1205) for endothelial cells in control hearts (Ismail, Poppa et al. 2003), anti-CD45 (PharMingen, #550539; 1:2000) for leukocytes, and anti-Ly6G (BD PharMingen, #550291, 1:100) for neutrophils. Biotinylated Anti-MMP-9 (R&D Systems, BAF909, 1:3) was used to visualize the expression pattern of MMP-9 in infarcted tissues. The reaction product was visualized with DAB (Vector, SK-4100). For BrdU double-labeling of fibroblasts, slides previously stained with anti- SMα-A

were rinsed and incubated with peroxidase-conjugated anti-BrdU (Roche, #1585860; 1:25). The reaction product was visualized with Vector VIP (Vector, SK-4600).

Myofibroblast, capillary, macrophage, and neutrophil density was measured in the infarct zone in 5 fields/section of 2 sections of infarcted heart per specimen at 400x and numbers are expressed as #/0.1mm^2. Proliferating cells (DAB+/BrdU+) were counted in random fields throughout the infarct until a total of 500 DAB- positive cells had been counted in each of 2 sections containing infarct regions. Vascular smooth muscle cells that are clearly part of vessels can easily be seen at this magnification and were thus omitted from these counts. Measurements were expressed as the percentage of double-labeled cells in 1000 DAB- positive cells ± SEM.

For detection of DNA strand breaks in cell nuclei, we used TUNEL staining, a common means of detecting *in situ* cell death in tissue sections (Salloum, Abbate et al. 2008; Yoo, Lemaire et al. 2009) (Roche, #11684817910). All TUNEL positive (DAB+) cells were counted by inspecting random fields throughout the infarct until a total of 500 nuclei had been counted in each of 2 sections containing infarct regions and the data were expressed as a percentage of 1000 total nuclei. A double stain for TUNEL and anti-α-sarcomeric actin (Sigma, A2172, 1:4000; visualized with Vector Red, SK-5100) was done to determine the number of apoptotic cardiomyocytes in the border regions of 2 sections of the heart with infarct regions. The number of double positive cells was expressed as a percentage of 500 total cardiomyocyte nuclei.

Immunoblotting. Left ventricles of mouse hearts were snap frozen in liquid nitrogen at the time of harvest and subsequently homogenized in HEPES buffer containing protease inhibitors. Proteins from control WT and mPer2-M and 4 days post-MI WT and mPer2-M left ventricles (40ug) were resolved by SDS polyacrylamide gel electrophoresis (7-15%) and transferred to PVDF membranes. Chemiluminescence was used for immunodetection. Images of the western

blots were captured using the Typhoon 9410 Imager. Densitometry was performed using ImageQuant TL 1D and array image analysis software. All membranes were subsequently stained with Ponceau S (0.1% w/v in 5% acetic acid, Sigma P7170) to confirm equal loading and transfer (Moore and Viselli 2000).

RT-PCR. RNA was isolated from the whole LV according to routine Trizol method and purified using the RNeasy mini kit (Qiagen; #74104). RT was performed using the High Capacity cDNA RT kit (ABI; 4368814) and 100ng RNA was amplified with TaqMan Universal Master Mix (ABI; 4364338) using the Applied Biosystems 7900HT Fast Real-Time PCR machine. The following TaqMan primer/probes were purchased from ABI: clock (Mm00455950_m1), bmal1 (Mm00500226_m1), Npas2 (Mm00500848_m1), cry1 (Mm00514392_m1), cry 2 (Mm00546062_m1), mPer1 (Mm00501813_m1), mPer2 (Mm00478113_m1) and GAPDH (Mm99999915_g1) as an internal reference. For each gene, mRNA expression was analyzed in triplicate in three animals.

Hemodynamic Determinations. Uninfarcted mice and mice 4 days after infarction, were anesthetized (90 mg Ketamine :10mg Xylazine/100 g body weight i.p.). Echocardiography (Toshiba Nemio 30, Duluth, GA) using a 14-MHz linear array transducer (PLM 1204AT) derived left ventricular (LV) volume was used to calibrate the LV volume signal obtained by the conductance catheter (Joho, Ishizaka et al. 2007). Two dimensional images of the LV were obtained in the parasternal long axis and short axis views, and M-mode images were obtained at the midventricular level in both views, from which internal dimensions of the left ventricle were obtained at end diastole (LVIDd) and end systole (LVIDs). Left ventricular end-diastolic (LVEDV) and end-systolic volumes (LVESV) were determined using the area-length method as validated previously (Joho, Ishizaka et al. 2007).

Left ventricular pressure volume measurements were obtained using a 1.2 F pressure volume conductance catheter (Scisense Inc., London, Ontario) inserted into the carotid artery and advanced into the left ventricle as described previously (Nemoto, DeFreitas et al. 2002; MacGowan, Rager et al. 2005). Pressure volume data were recorded (Polyview, Grass Technologies, Warwick, RI) under baseline conditions and after transient occlusion of the inferior vena cava. Pressure volume loops were subsequently generated and analyzed offline, using CardioSoft (Sonometrics, London, Ontario) data analysis software. Hemodynamic measurements included peak systolic LV pressure (LVP), stroke volume, heart rate, cardiac output, stroke work, maximum rate of LV pressure development (LV dP/dt_{max}), end-systolic elastance (Ees) (Suga, Yamada et al. 1984), and preload recruitable stroke work (PRSW, linear regression of stroke work vs. end-diastolic volume) (Glower, Spratt et al. 1985), and are expressed as mean ± SD (Table 1).

Statistics. Data are expressed as mean ± SEM. Statistical significance between groups was determined by ANOVA and significance levels were $p< 0.05$. Statistical analysis of hemodynamic data was performed using two-factor ANOVA, comparing WT and mPer2-M mice at baseline, and at 4 days after infarction, and individual subgroup comparisons were made using Tukey's multiple range test ($p < 0.05$). Mortality between 4d WT and mPer2-mutants and RT-PCR data was analyzed with a Student's t-test ($p < 0.05$).

RESULTS

Morphometry and Histology. No significant differences were observed in mortality between the WT and mPer-M mice (survival rates - mPer2-M: 83% (n= 15 of 18); WT: 85% (n= 17 of

20). All mice were included in these analyses and the investigator was blinded while making measurements.

The infarct area was 43% smaller in the mPer2-M mouse hearts (Figure 2.1; WT: 5.4 ± 0.3 mm^2 versus mPer2-M: 3.1 ± 0.2 mm^2; $p<0.05$) and, as such, there was 48% less residual necrosis (infarct area minus granulation tissue area) in the mPer2-M (WT: 2.1 ± 0.2 mm^2 versus mPer2-M: 1.1 ± 0.2 mm^2; $p<0.05$) and 35% less granulation tissue in the mPer2-M (WT: 5.1 ± 0.4 mm^2 versus mPer2-M 3.3 ± 0.5 mm^2).

There were 40% less TUNEL positive apoptotic nuclei (as a percentage of all nuclei) in the mPer2-M infarcts as compared with WT mouse hearts (WT: 45 ± 3% versus mPer2-M: 27 ± 3%; $p<0.05$). Specifically, cardiomyocyte apoptosis in 4 day infarcts of mPer2-M mice versus WT 4 day hearts was significantly less (Figure 2.2A; 26 ± 1.9% and 41 ± 1.6% respectively). The average myocyte cross-sectional area (MCSA) was not different between uninjured control hearts of WT and mPer2-M mice (WT: 203 ± 22 μm versus mPer2-M: 225 ± 23 μm). There was a non-significant trend for MCSA to be higher in the epicardium (220 ± 23 μm) than endocardium (186 ± 22 μm) in WT hearts in contrast to mPer2-M hearts which tended to have larger myocytes in the endocardium (247 ± 17 μm) versus the epicardium (208 ± 33 μm). At 4 days post-MI, the average MCSA (both endocardial and epicardial) was increased in mPer2-M hearts whereas it decreased in WT mouse hearts (Figure 2.2B; WT: 174 ± 6 μm versus mPer2-M: 262 ± 9 μm). There was no difference between epicardial MCSA versus endocardial MCSA in either WT or mPer2-M hearts at 4 days post-MI.

Representative micrographs of immunohistochemical staining for the pan-leukocyte marker CD45 in WT and mPer2-M mouse hearts are shown in Figure 2.3 (A and B, respectively). Macrophage density was 25% lower in the infarct zone of mPer2-M hearts at 4

days post-MI as compared with WT mouse hearts (Figure 2.3C; WT: 84 ± 8 versus mPer2-M 63 ± 5; $p<0.05$). There was no significant difference between neutrophils (WT 4d 13 ± 2 versus mPer2-M 4d 12 ± 3). Similarly, eosinophils counts using congo red staining and toluidine blue staining for mast cells were present in very low numbers and thus yielded no significant differences between the groups (data not shown).

There was no difference in the vessel density per $0.1mm^2$ in uninfarcted control hearts (WT: 103 ± 8 versus mPer2-M: 112 ± 4). Representative images of $CD31^+$ endothelial cells in the infarct zone at 4 days post-MI are shown in WT (Figure 2.3D) and mPer2-M (Figure 2.3E) mouse hearts. We observed a 43% increase in vessel density per $0.1mm^2$ in the infarct zone of mPer2-M mice compared to WT (Figure 2.3F; WT: 49 ± 10 versus mPer2-M: 87 ± 8; $p<0.01$). Although there was no difference in the average area/vessel in the WT versus mPer2-M control mice or in the vessels in the infarct region, there was a significant difference in the area/vessel in the uninjured tissue regions of the infarcted heart (WT: 6.3 ± 0.8 μm^2 versus mPer2-M: 9.7 ± 0.4 μm^2; $p<0.01$).

Immunohistochemistry for activated fibroblasts was performed using an anti- SMα-A antibody and representative images of infarct zone of WT and mPer2-M hearts at 4 days post-MI are shown in Figure 2.3 (G and H, respectively). Fibroblast density in the infarct zone was 44% higher in mPer2-M hearts at 4 days post-MI than in WT mouse hearts (Figure 2.3I; WT: 60 ± 6 versus mPer2-M: 108 ± 17, $p<0.05$). There was no difference in fibroblast proliferation rate (SMA^+ + $BrdU^+/SMA^+$) between the 2 groups at 4 days post-MI or interstitial fibrosis (data not shown). It is possible that proliferation occurs earlier since there was less injury to the mPer2-M hearts but this was not determined.

Figure 2.4 shows representative micrographs for MMP-9 immunohistochemistry in 4 day WT and mPer2-M hearts and a western blot to demonstrate changes in the expression level. The images demonstrate the presence of this protein in inflammatory cells. One representative sample per group most closely approximating the average of the 3 per group measured was used for the western blot. Densitometry of the bands shows there is no difference in the expression of MMP-9 between control WT or mPer2-M hearts, however, in contrast to the 40% increase in expression in 4 day WT infarcted hearts compared to control, the expression level does not change in mPer2-M hearts at 4 days post-MI.

RT-PCR. The level of transcripts as measured by the C_t (detection threshold) of each gene in each sample was normalized to the constitutive housekeeping gene GAPDH to control for sample to sample differences. The groups (n=3)were compared as follows: control mPer2-M versus control WT, 4d WT versus 4d control, 4d mPer2-M versus control mPer2-M, and 4d mPer2-M versus 4 d WT. There were no differences in the expression levels of any of the 7 genes (mPer2 was not measured in mPer2 mutant mice), except for bmal, with a measured 1.54 fold increase in 4D mPer2-M from 4d WT; $p<0.05$.

Cardiac Function. Pressure-volume loops for WT and mPer2-M mice are shown in Figure 2.5 (n=6 per group). There were no differences in ventricular performance between the groups before infarction. After infarction, as expected, indices of ventricular performance decreased in both WT and mPer2-M 4 days post-MI (Table 2.1). However, the loss of ventricular contractile function was significantly attenuated in the mPer2-M animals compared to infarcted WT animals. Peak LVP (WT, 67 ± 3; mPer2-M, 79 ± 4, $p < 0.05$), ESPVR (WT, 2.23 ± 0.23mmHg/µL; mPer2-M 2.98 ± 0.20 mmHg/µl, $p<0.05$), and PRSW (49.1 ± 2.8 mmHg/µl; mPer2-M, 61.4 ± 2.4 mmHg, $p <0.05$) all were significantly better in the mPer2 animals, while

heart rate, cardiac output, ejection fraction, and stroke volume were not different between the groups. Since bradycardia can decrease cardiac output by limiting stroke volume(Hart, Burnett et al. 2001), it is possible that anesthesia induced bradycardia created some degree of ventricular dilatation that partially masked differences between the groups in measurements of output and stroke volume. However, the ESPVR and PRSW are widely held to be more sensitive measures of performance. Clearly, at four days, significant dysfunction compared to control values exists in both groups, but the improved performance in the mPer2-M animals is consistent with the decreased inflammation and reduced apoptosis also described in these animals.

Figure 2.1. mPer2-M hearts have reduced infarct size at 4 days post-MI.

Representative histology of WT (top left) and mPer2-M (top right) control mouse hearts and WT (bottom left) and mPer2-M (bottom right) hearts 4 days after chronic MI. Infarct size was 46% smaller in mPer2-M hearts 4 days post-M.I ($p<0.05$). Arrows point to granulation tissue, asterisks indicate necrosis, RV= right ventricle, LV = left ventricle.

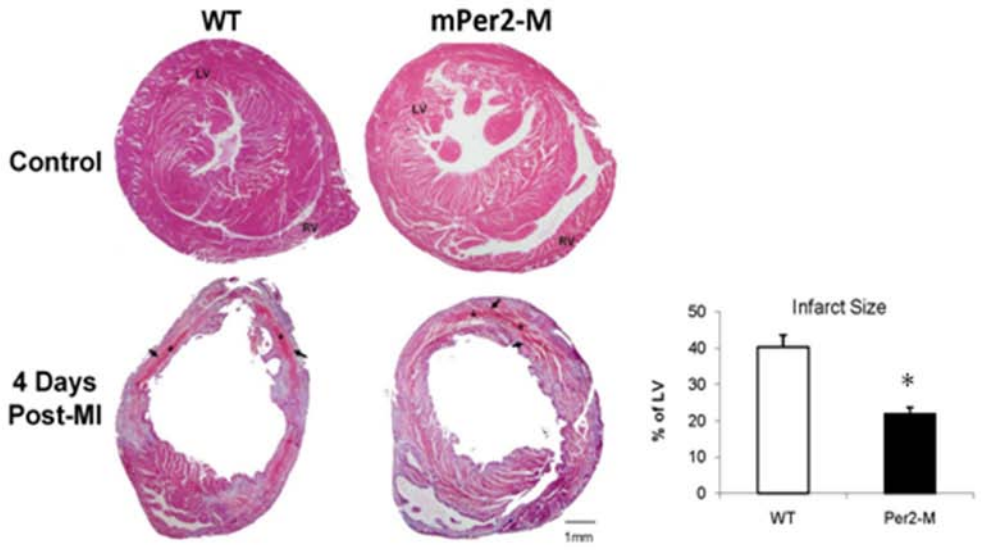

WT
mPer2-M
Control
4 Days
Post-MI
LV
RV
1mm
Infarct Size
% of LV
50
40
30
20
10
0
*
WT
Per2-M

Figure 2.2. A) Reduced cardiomyocyte apoptosis and B) increased myocyte cross-sectional area in mPer2-M mouse hearts 4 days after chronic infarction.

There were 15% less TUNEL-positive apoptotic cardiomyocytes observed in mPer2-M mice (top right) as compared with WT (top left; * $p < 0.05$). The average myocyte cross-sectional area (μm^2) was increased in mPer2-M mice (bottom right) versus WT (bottom left).

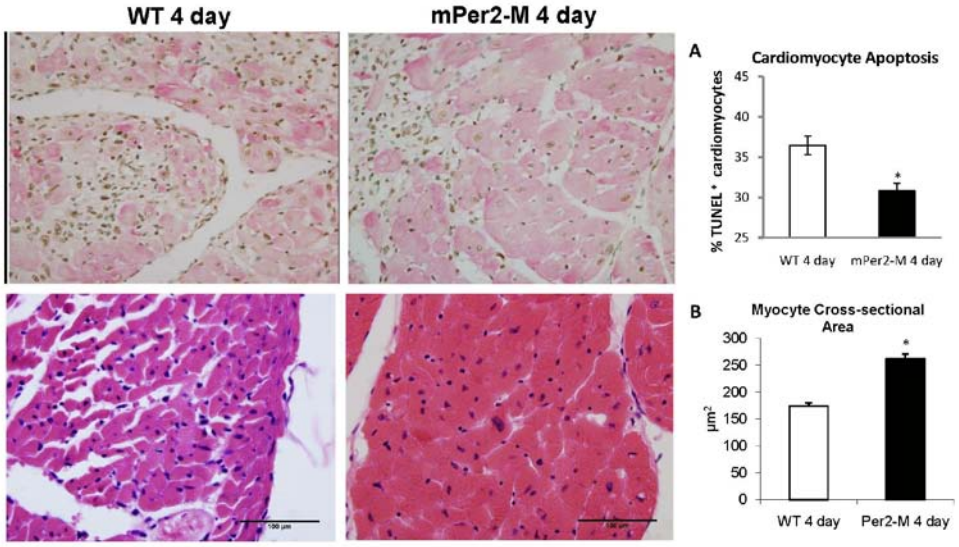

WT 4 day
mPer2-M 4 day
A
Cardiomyocyte Apoptosis
% TUNEL + cardiomyocytes
45
40
35
30
25
*
WT 4 day
mPer2-M 4 day
B
Myocyte Cross-sectional Area
μm²
300
250
200
150
100
50
0
*
WT 4 day
Per2-M 4 day

Figure 2.3. Pan-leukocyte marker CD45 staining showed reduced CD45-positive inflammatory cells in mPer2-M mice (*B*) compared with WT mice (*A*) at 4 days after infarction (*C*; **P* <0.05). KO, knockout. There was a significant increase in vessel density in mPer2-M (*E*) hearts compared with WT hearts (*D*) at 4 days post-MI (*F*; †*P* < 0.01). Fibroblast density was also increased in mPer2-M (*H*) compared with WT (*G*) mouse hearts at 4 days post-MI (*I*; **P* < 0.05).

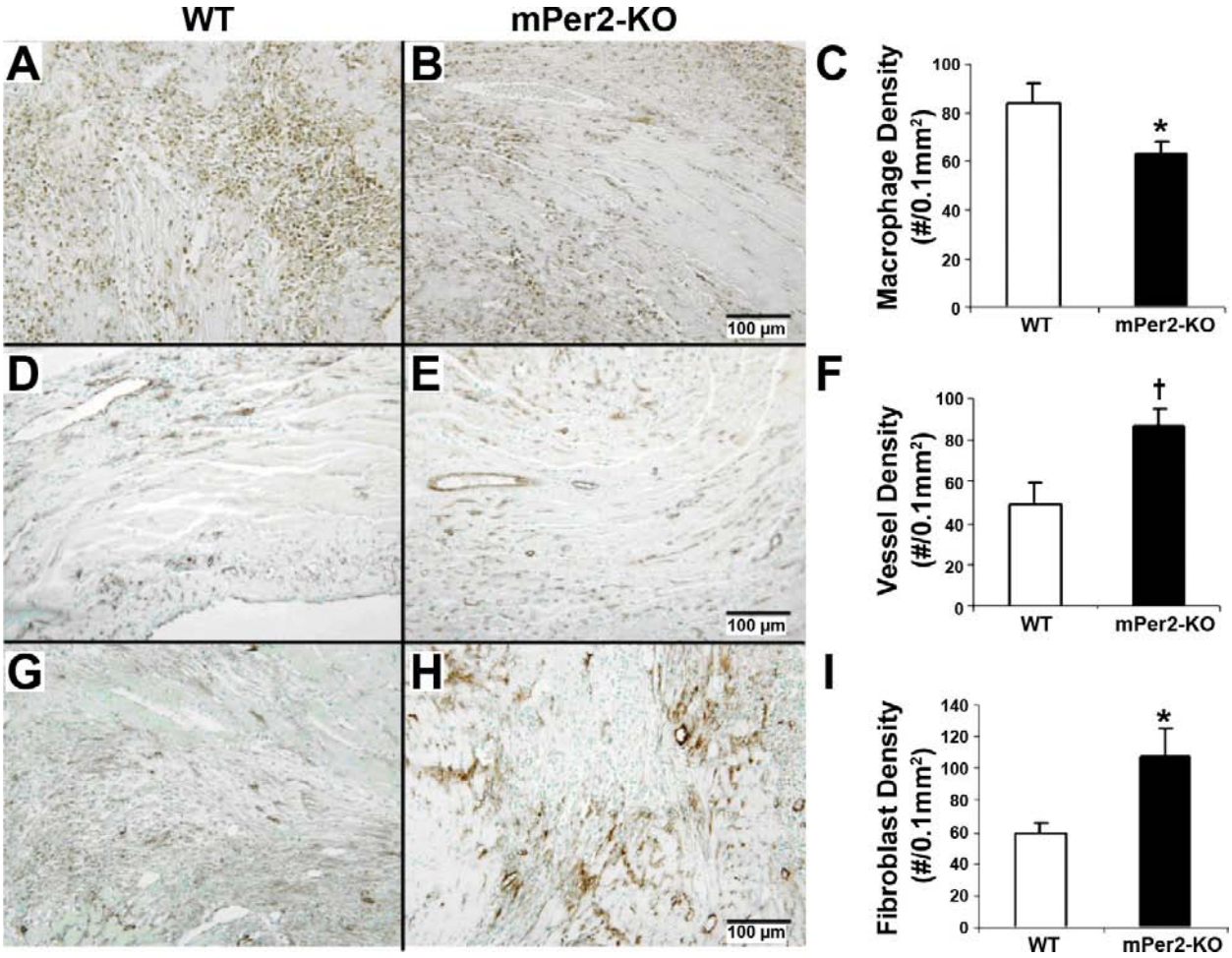
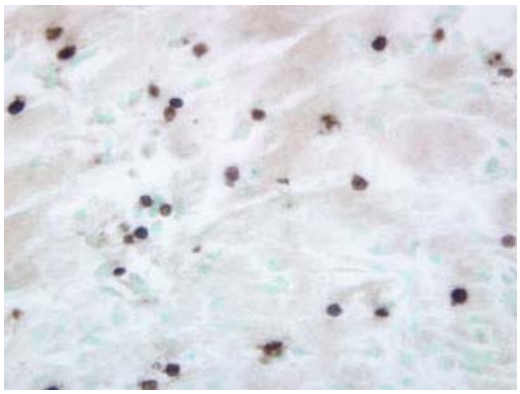
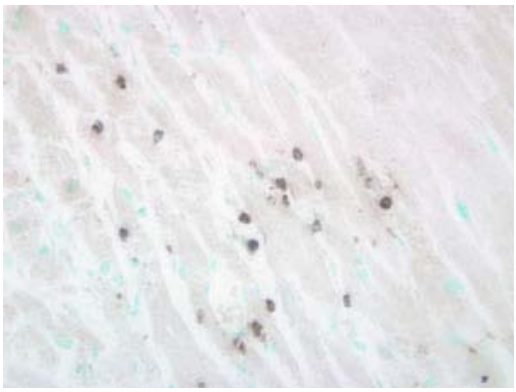

WT
mPer2-KO
A
B
C
D
E
F
G
H
I
100 μm
100 μm
100 μm
Macrophage Density (#/0.1mm²)
Vessel Density (#/0.1mm²)
Fibroblast Density (#/0.1mm²)
WT
mPer2-KO
*
†
*

Figure 2.4. MMP-9 expression in inflammatory cells is reduced in mPer2-M hearts 4 days after chronic infarction.

MMP-9 is expressed in inflammatory cells in mPer2-M hearts (right) compared to WT (left) at 4 days after infarction. A representative western blot for MMP-9 shows that there is no difference between uninjured WT and mPer2-M hearts. In contrast to the increased expression observed in WT hearts at 4 days post-MI, there is no change in MMP-9 expression in mPer2-M hearts at 4 days post-MI.

WT 4 Day mPer2-M 4 Day





WT mPer2-M WT 4Day mPer2-M 4 Day

Figure 2.5. mPer2-M mice showed improved LVP, ESPVR, and PRSW 4 days after infarction.

Pressure-volume loops, showing pressure generated (y-axis) with volume ejected (x-axis). ESPVR: slope of the best fit line through the end-systolic pressure volume points in the series of PV loops. With linear fit, all slopes have regressions $r>0.94$.

PRSW: $r > 0.95$ for each of the Stroke work/End diastolic volume slopes

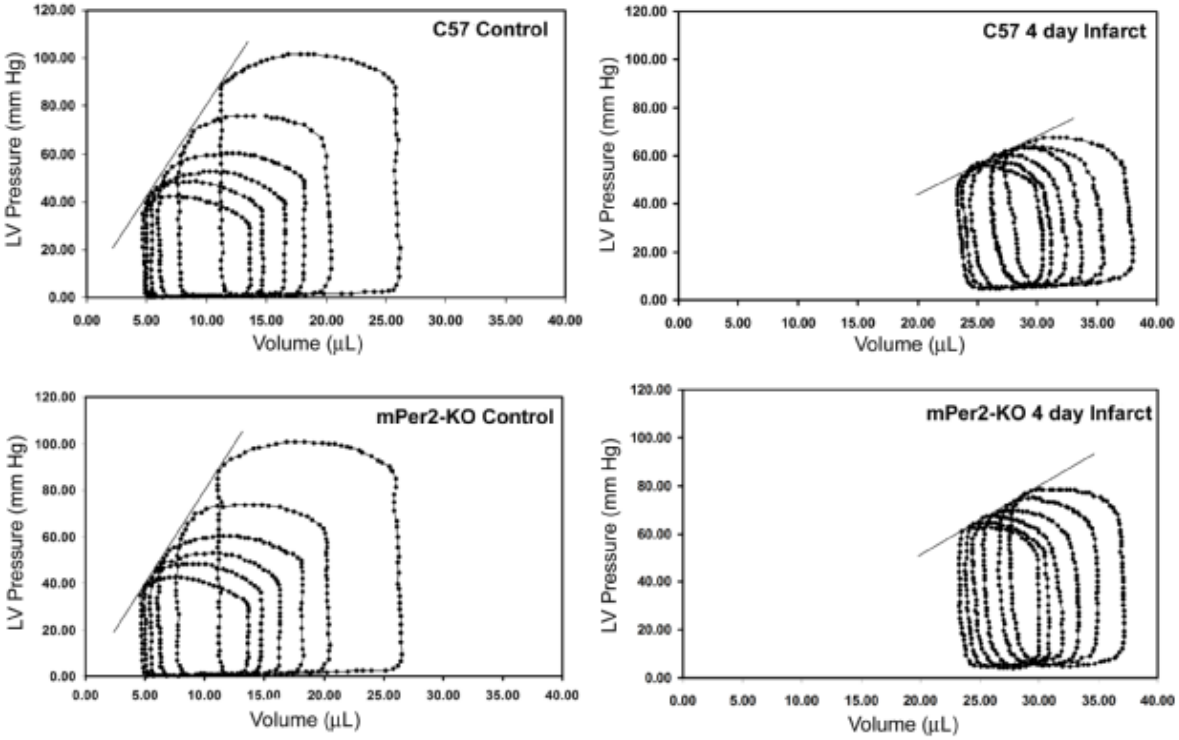

C57 Control
C57 4 day Infarct
mPer2-KO Control
mPer2-KO 4 day Infarct
LV Pressure (mm Hg)
Volume (µL)
0.00
20.00
40.00
60.00
80.00
100.00
120.00
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00

Table 2.1. Pressure-Volume (PV) Loop data. Hemodynamics in C57 WT and mPer2-M mouse hearts without injury (control) and with chronic infarction 4 days post-MI. HR: heart rate; SV: stroke volume; EF: ejection fraction; CO: cardiac output; EDV: end diastolic volume; LVPsys: left ventricular systolic pressure; ESPVR: end systolic pressure volume relationship; PRSW: preload recruitable stroke work (values are mean ± SEM; * significantly different from respective control, ŧ = significantly different 4 day WT vs. 4 day mPer2-M).

TABLE 1

	C57 Control; n=6	*mPer2-M Control; n=6*	*C57 4 Days; n=6*	*mPer2-M 4 Days; n=6*
HR (bpm)	389 ± 29	397 ± 37	432 ± 56	443 ± 48
SV (uL)	14.9 ± 2.1	15.4 ± 2.0	10.8 ± 1.4 *	9.7 ± 2.3 *
EF (%)	57 ± 6	58 ± 7	28 ± 3 *	26 ± 3 *
CO (mL/min)	5.80 ± 1.2	6.13 ± 1.3	4.67 ± 1.2	4.31 ± 1.3
EDV (uL)	26 ± 1.2	26.5 ± 1.1	38 ± 1.6 *	37.2 ± 2.0 *
LVPsys (mm Hg)	101 ± 6	100 ± 7	67 ± 3 *	79 ± 4 * ŧ
ESPVR (mm Hg/uL)	7.77 ± 0.65	7.86 ± 0.83	2.23 ± 0.19 *	2.98 ± 0.20 * ŧ
PRSW (mm Hg)	114 ± 7.0	119.2 ± 8.2	49.1 ± 2.8 *	61.4 ± 2.4 * ŧ

DISCUSSION

Our data represent novel findings regarding the interaction between the circadian rhythm gene mPer2 and cardiac injury. In summary, we observed significant myocardial protection in mPer2-M mouse hearts as evidenced by a 43% reduction of infarct area, a 43% increase in vascular density, 25% less leukocyte infiltration, 17% more hypertrophy, and 15% less cardiomyocyte apoptosis in the infarct zone of mPer2-M mouse hearts compared to WT mouse hearts 4 days post-MI. Our hemodynamic data confirm less dysfunction, as exhibited by preservation of contractility and indices of cardiac work such as in ESPVR, PRSW, and LVP in mPer2-M, 4 days post-MI. There was better functional preservation in the mPer2-M animals, although the large difference in infarction was accompanied by modest improvements in indices of contractility. In part, some of the differences may have been masked by the bradycardia induced by the anesthetic, but more likely, the severity of the infarction, and the early time point of these data both contribute to limiting differences that might be observed in functional performance. However, the improvement in performance, combined with the decreased inflammation and volume of necrosis would be consistent with an expectation of better longer term recovery once the infarction completely resolves.

In the absence of reperfusion, at 4 days post-MI, granulation tissue comprised of macrophages, endothelial cells, and fibroblasts are at the peak of proliferation and migration to initiate scar formation (Virag and Murry 2003). Mice lacking functional mPer2 protein contained less macrophages, more myofibroblasts, better preservation of capillary density, and displayed decreased total and cardiomyocyte apoptosis compared to matched C57 controls at 4 days post-MI. Previous studies showing that mPer2-M mutant mice failed to show a daily rhythm in levels of IFN-γ a potent pro-inflammatory cytokine and activator of macrophages that

is secreted by NK cells (Arjona and Sarkar 2006; Liu, Mankani et al. 2006). The decreased responsiveness of the inflammatory cascade may be responsible in part for the decreased apoptosis due to reduced oxidative stress and cytokine elaboration (Sun 2007; Frangogiannis 2008; Hori and Nishida 2009) . The lack of functional Per2 protein in the heart may be directly responsible for the reduced apoptosis as well since a previous study showing mPer2 overexpression in mouse Lewis lung carcinoma cells and mammary carcinoma cells (EMT6) resulted in rapid apoptosis by downregulation of c-Myc, Bcl-X(L) and Bcl-2 and upregulation of p53 and bax (Hua, Wang et al. 2006). Also, it has previously been shown that the inflammatory response following an LPS challenge in mPer2 mutant mice, the inflammatory response is blunted due to deficient NK cell function (Liu, Mankani et al. 2006). Further, when the circadian system is uncoupled centrally, rats kept in total darkness during the first 48 hours following brain injury exhibited improved recovery (Corwin and Vargo 1993; Vargo, Lai et al. 1998; Vargo, Grachek et al. 1999). These data coordinately suggest that reduced injury in the non-reperfused model occurred by decreased immune cell infiltration and function as well as reduced cardiomyocyte apoptosis, leading to expeditious resolution of infarct repair. Increased myofibroblast density in the infarct zone is also suggestive of faster healing. This notion is further supported by the decreased expression of MMP-9 in mPer2-M hearts at 4 days post-MI. MMP-9 is known to be upregulated early in response to injury and is often found to be expressed by leukocytes (Heymans, Luttun et al. 1999). Further, MMP-9 null mice exhibit reduced infarct area in response to acute ischemia/reperfusion and this was attributed to less neutrophil infiltration (Romanic, Harrison et al. 2002) and in the absence of reperfusion, there was less deleterious remodeling, dilation, and fewer macrophages (Ducharme, Frantz et al. 2000). Indeed, we observed decreased MMP-9 expression in inflammatory cells and this, combined

with the reduced density of inflammatory cells and reduced cardiomyocyte apoptosis indicates that less injury is part of the mechanism by which infarct area is reduced in mPer2-M hearts. Furthermore, the observed decrease in cardiomyocyte apoptosis, increased cardiomyocyte hypertrophy, and reduced infarct size imply that less death and more robust compensation act coordinately to preserve cardiac function.

Mechanistically, *Per1, Per2, Cry1, and Cry2* interfere with *Clock-Bmal1* activity to repress transcription targets (Gauger and Sancar 2005; Shimba and Watabe 2009). Since Per2 protein is non-functional in these mPer2-M mice and bmal1 (arntl) gene expression is increased in response to ischemia in the absence of reperfusion in mPer2-M versus WT, this would suggest that the repressor activity normally effected by mPer2 is alleviated and so targets could be upregulated. These targets include ET-1, VEGF, and metabolic enzymes known to play a role in hypertrophy and angiogenesis (Thackaberry, Gabaldon et al. 2002). Also, the work by Kotanagi, S. et al (2003) showing that transfection of tumor cells with Per2 dose-dependently inhibits VEGF induced by hypoxia via inhibition of HIF-1α/ARNT-induced VEGF promoter activity, further lends support to the idea that mPer2 mutants probably have increased VEGF levels and hypertrophic mediators in response to hypoxia/ischemia (Thackaberry, Gabaldon et al. 2002; Koyanagi, Kuramoto et al. 2003). Further investigation into vascular changes, as well as potential mediators of this and the observed increase in cardiomyocyte hypertrophy is currently underway.

We postulate that synchronization between the SCN and peripheral clocks is a continuous process and that the pressure to maintain this synchronization involves signaling mechanisms that are energetically demanding for the peripheral target tissues. When coupled with an underlying pathophysiology that generates a vulnerable substrate, such as coronary artery disease

or pressure overload, the pressure to normalize desynchronized rhythms may increase the progression of injury. Additional mechanistic studies are being conducted to understand the signaling pathways between clock genes and cardiac genes that afford this protection. Long-term studies are also in progress to determine if the observed enhancements in the repair process result in reduced scar formation and consequent ventricular remodeling as well as amelioration of cardiac dysfunction.

2.3: mPer2 Mice Have Increased Frequency of Bone Marrow-Derived Progenitors

Introduction:

We observed 43% increased vessel density in mPer2-M mice at four days post-MI, and there was no significant difference in endothelial cell proliferation (chapter 2.2). Interestingly, preliminary data from our laboratory indicates increased ephrinA1 protein in mPer2-M mice (Figure 2.6). EphrinA1 is an angiogenic protein, discussed in more detail in chapter 3. The increased expression of an angiogenic protein, coupled with increased capillary density, is suggestive of an increased angiogenic response. Further, ephrinA1 has been implicated in hematopoietic stem cell adhesion (Ting, Day et al.), so it is plausible that upregulation of this protein is contributing to progenitor cell recruitment to the infarcted heart. Thus, we postulated that this observation may be vasculogenesis due to mobilization of circulating endothelial progenitor cells (EPCs). These cells are thought to originate in the bone marrow, and mobilize into circulation in response to ischemia, cytokines, growth factors, and even exercise. They are also present to participate in normal endogenous maintenance of the endothelium, and have been used as biomarkers to predict the severity of cardiovascular events (Seguin, Braun et al. 2007; Povsic and Goldschmidt-Clermont 2008; Mobius-Winkler, Hollriegel et al. 2009). In 1997, Asahara and colleagues were the first to identify EPCs, using a combination of the hematopoietic cell surface marker CD34 and the vascular endothelial growth factor (VEGF) receptor Flk-1 (Asahara, Murohara et al. 1997). When CD34+ mononuclear blood cells were plated on fibronectin, a small population (approximately 80 cells per mm^2) attached and formed spindle shapes, suggesting differentiation to an endothelial phenotype. It was later reported that

producing a regional ischemia increased circulating EPC frequency in both mice and rabbits. Additionally, treating rabbits with GM-CSF prior to inducing hindlimb ischemia led to further increases in circulating EPCs and improved hindlimb vascularization (Takahashi, Kalka et al. 1999).

EPCs are a relatively small population in the bone marrow and peripheral blood. Recent reports indicate that these cells represent anywhere from 70-210 cells per mL of blood to 3000-5000 cells per mL of blood (Dome, Dobos et al. 2008), to approximately 2% of the peripheral blood population (Chang, Leu et al.), depending on which markers are used. In the bone marrow, these cells represent less than 0.003% of circulating bone marrow-derived progenitors (Taylor, Rossler et al. 2009).

To assess the total number of circulating bone marrow-derived progenitor cells in C57 and mPer2-M mice, Miltenyi Biotec's Magnetic Activated Cell Separation was used to enrich the bone marrow population by depleting mature hematopoietic cells through the use of magnetically labeled microbeads labeled against mature hematopoietic cells. Markers included in the linage cell depletion kit (Miltenyi # 130-090-858) include CD5, CD45R/B220, CD11b, Gr-1/Ly6G/C, 7-4, and Ter-119. We chose to isolate a bone marrow-derived subpopulation of progenitor cells, which are lineage cell-negative (antigens listed above), and CD34, sca-1 double-positive. While there is not uniform agreement on the specific definition for an EPC, it is generally accepted that EPCs are derived from a CD34+ bone marrow subset, and typically express CD133 and KDR. There is still disagreement on including CD133 as a marker, however, because mobilized EPCs typically lose CD133 expression early in the mobilization process (Leone, Valgimigli et al. 2009). CD34 was one of the original markers used to identify EPCs (Morrison, Hemmati et al. 1995; Asahara, Murohara et al. 1997; Kawamoto, Ohmura et al. 1997). In our studies, the CD34

and sca-1 antigens were chosen due to their documented expression on bone marrow multipotent hematopoietic stem cells in mice. Both markers represent primitive populations of bone marrow cells that have not yet committed to a particular lineage.

Methods:

Bone marrow isolation: Isolation of bone marrow was performed under sterile conditions. Mice were euthanized with an i.p. injection of 0.1mL pentobarbital (390mg/mL). Each hind leg was wiped with betadine, followed by 70% ethanol. Sterile scissors and forceps were used to isolate the femur and tibia of each leg. These bones were placed in a sterile petri dish, and immersed in 70% ethanol. The ends of each bone were cut off with sterile scissors, and 10mL of sterile MACS buffer (Miltenyi # 130-091-221) was flushed through each bone using a 27.5 gauge needle and syringe. The suspension, containing bone marrow cells, was collected in a 50mL conical tube. The sample was centrifuged at 1500rpm for 10 minutes at 4°C to pellet the cells. Cells were washed once with MACS buffer, then re-centrifuged. The pellet was then resuspended in 1mL MACS buffer, a 10ul aliquot was taken for cell counting (using a hemocytometer), and the suspension was transferred to a 1.7mL eppendorf tube. The tube was then centrifuged at 1500rpm for 10 minutes at 4°C. Bone marrow from 2-3 mice per experimental group was pooled to obtain an adequate number of cells to analyze.

MACS Purification: The supernatant was vacuumed off, and the pellet was resuspended in 40ul of MACS buffer per 10^7 cells. Ten microliters of Lineage biotin-antibody cocktail (Miltenyi # 120-001-547) was added to the cell suspension, the tube was briefly vortexed, and incubated on ice for 10 minutes. Next, 30ul of MACS buffer per 10^7 cells and 20ul of anti-biotin microbeads (Miltenyi #120-000-900) per 10^7 cells were added to the suspension. The suspension was

vortexed and incubated on ice for 15 minutes. Cells were washed with 1mL MACS buffer per 10^7 cells and centrifuged at 300xg for 10 minutes. The supernatant was aspirated with vacuum filtration, and the cell pellet was resuspended with 500ul MACS buffer, per 10^8 cells. One MS magnetic column (Miltenyi # 120-000-472) per pooled sample was used. Each column was rinsed with 500ul of MACS buffer, followed by the addition of the 500ul sample suspension. The column was washed three additional times with 500ul MACS buffer. The entire effluent was collected in a 15mL conical tube. This is the enriched cell population, depleted of lineage positive cells.

Antibody Staining and Flow Cytometry: The collected cells were centrifuged for 10 minutes at 300xg and 4°C. The supernatant was aspirated, and the cell pellet was resuspended in 100ul MACS buffer, transferred to a 1.7mL eppendorf tube, and chilled on ice. Two microliters of FITC rat anti-mouse Ly6A/E (anti-sca-1) antibody (BD Pharmingen # 557405) and 5ul of PE anti-mouse CD34 antibody (Biolegend # 119308) were added to the 100ul cell suspension, and incubated on ice for 10 minutes. After incubation, 1mL MACS buffer was added to the tube, which was then centrifuged for 15 minutes at 1500rpm and 4˚C. The supernatant was removed; the pellet was resuspended in 500ul MACS buffer, and transferred to a polystyrene flow cytometry tube. Cells were analyzed on a Becton Dickinson FACScan flow analyzer. Results are expressed as the percent double-positive for sca-1 and CD34. Due to the need to pool samples from multiple mice to attain an adequate number of cells for flow cytometric analysis, the results represent pooled populations of 2-3 mice each. Thus, standard error of the mean cannot be calculated.

Results:

mPer2-M exhibit greater numbers of BM-derived progenitor cells before and after MI compared to C57 WT mice.

Our results indicate that the frequency of CD34+/sca-1+ progenitors in the bone marrow tended to be higher in mPer2-M mice than in C57 WT mice at control, basal levels (0.62% vs. 0.45%) (Figure 2.7). Bone marrow was collected from both strains of mice 24 hours after surgery. The mPer2-M mice again had relatively higher total levels of CD34+/sca-1+ progenitors compared to C57 WT mice (1.01% vs. 0.73%). Although there was a trend for increased levels of the mPer2-M-derived bone marrow EPCs, we could not verify this statistically, so we are unable to conclude that there was increased production of BM-derived progenitors in mPer2-M mice.

Figure 2.6: Western blot showing increased ephrinA1 protein expression (MW: 28kDa) in mPer2-M mice compared to WT mice, at control, 2 days, and 4 days post-MI.

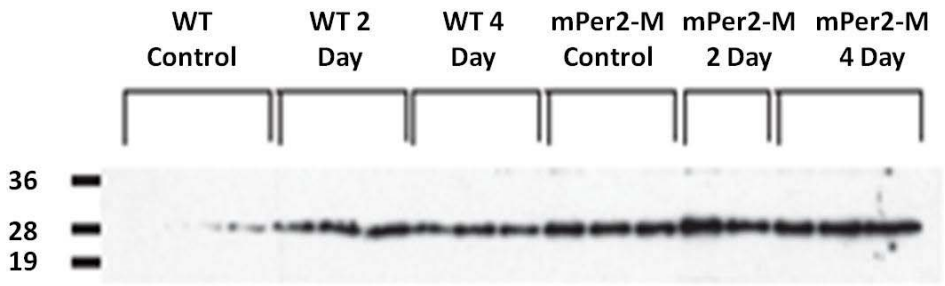
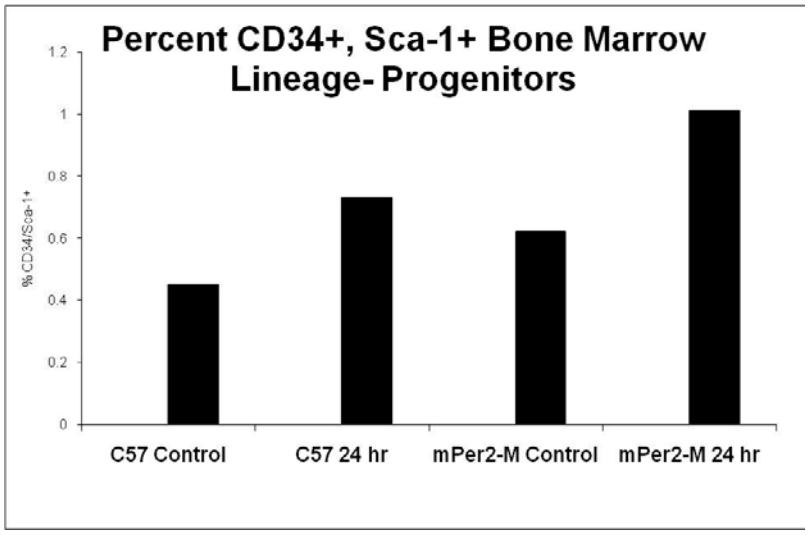
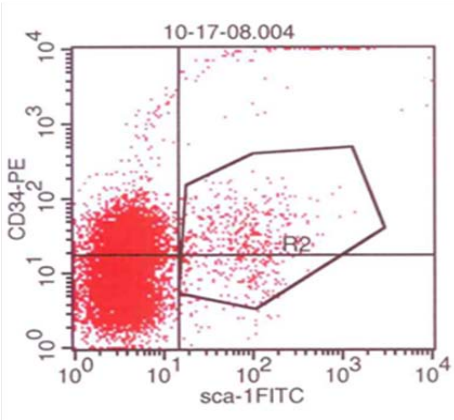

WT Control
WT 2 Day
WT 4 Day
mPer2-M Control
mPer2-M 2 Day
mPer2-M 4 Day
36
28
19

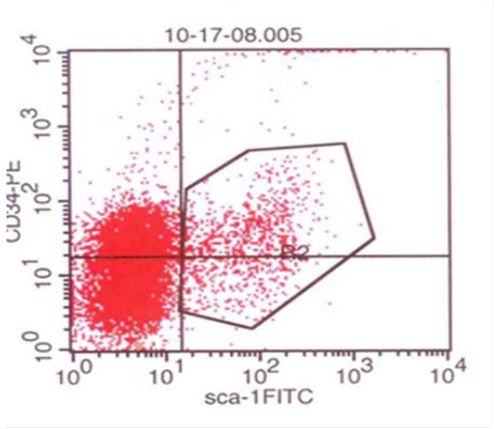
Figure 2.7: mPer2-M mice exhibit higher levels of CD34+, sca-1+ progenitor cells before and 24 hours after acute myocardial infarction, compared to wild type controls. Top panel: CD34+, sca-1+ frequencies. Bottom panel: representative dot plots of C57 and mPer2-M bone marrow progenitors at 24 hours post-MI.



C57 24 hours



mPer2-M 24 hours



Discussion:

This data suggests that mPer2-M mice tended to have elevated levels of CD34+/sca-1+ progenitors in their bone marrow, which may be contributing to the cardioprotection observed in these mice. There was a trend toward increased EPC production in mPer2-M mice, compared to WT, but we were unable to perform statistics on this data due to a small sample number. Increased bone-marrow derived progenitors may be involved in the improved vascularity we observed in mPer2-M mice following MI.

Our findings contradict that of a 2008 report from Wang *et al.* which investigated the angiogenic response in mPer2-M mice using a model of hind limb ischemia. The authors found that cultured endothelial cells showed earlier growth arrests after multiple passages than their C57 WT counterparts. This was coincident with increased Akt signaling in mPer2-M which may be responsible for the increased vascular senescence. Additionally, matrigel was implanted with and without VEGF, and hemoglobin content of the matrigel was measured as an index of vessel formation. mPer2-M mice had substantially less hemoglobin in the presence and absence of VEGF, indicating reduced angiogenesis in these mice. Hind limb ischemia in mPer2-M led to autoamputation, but did not in WT mice, which may suggest impaired blood flow in response to ischemia. To elucidate a mechanism for this, the authors measured circulating EPC levels and found that baseline levels did not differ between WT and mPer2-M mice, but the number of EPCs 5 days post-ischemia was significantly less in mPer2-M (Wang, Wen et al. 2008). These findings indicate that mPer2-M mice actually have an impaired angiogenic response to ischemia, which is in contrast to our findings. However, differences in the experimental paradigm between the two studies make direct comparisons in the data difficult.

While we cannot directly explain the differing results between our study and that of Wang and colleagues, there are several notable differences. First, it is possible that the mPer2 gene simply plays differing roles in different tissue beds. Since the endothelial cell senescence study was performed *in vitro,* and involved cultured cells, and the *in vivo* work was carried out in a model of hind limb ischemia, these results may not be directly comparable to our studies, which were designed to evaluate the response to myocardial ischemia *in vivo*. Cells in the Wang *et al.* study were cultured, while ours were analyzed upon collection. *Further*, the markers used to characterize EPCs were different. In our study, we chose to characterize cells expressing CD34 and sca-1, which represents a larger pool of bone marrow derived cells with which to work, and from which we believe EPCs are derived. Wang and colleagues used a different set of markers- acetylated low-density lipoprotein (Ac-LDL), lectin, and endothelial nitric oxide synthase (eNOS). As discussed earlier, the dissimilar set(s) of cell surface markers used to identify EPCs is still a topic of debate in this young field, and makes it difficult to make direct comparisons between different studies, especially when dealing with such a rare population of cells. Additionally, different markers are present at different lineage stages of development.

There have been several reports in the last decade that have characterized the expression of clock genes in bone marrow-derived cells, as well as the circadian rhythm variation in cultured bone marrow cells. Tsinkalovsky and colleagues (2005) analyzed bone marrow side populations by staining mouse bone marrow with Hoechst 33342 fluorescent dye and enriching the cell population through FACS. Approximately 80% of the BM side population cells they isolated expressed sca-1 and c-kit, which are markers for early hematopoietic progenitors. The authors reported that circadian genes mPer1, mPer2, mBmal1, mCry1, mClock, and mRev-erbα were all expressed in whole bone marrow and BM side populations. There were different

expression patterns for several circadian genes- mPer1 and mCry1 mRNA expression was higher in bone marrow side populations than in whole bone marrow (Tsinkalovsky, Rosenlund et al. 2005).

The same group reported in 2006 that mPer2 displays a circadian rhythm in bone marrow progenitors in the absence of light-dark synchronization. Other clock genes that were investigated did not appear to exhibit circadian variation when examined in side population cells. However, in whole bone marrow and liver cells, mPer1 and mRev-erbα displayed circadian variation, indicating that expression of circadian clock genes may be regulated during development (Tsinkalovsky, Filipski et al. 2006).

A 2008 report in Nature elucidated some of the questions surrounding hematopoietic stem cell (HSC) release with regards to circadian rhythms. The authors measured levels of circulating HSCs following exposure to photic stimulation. Additionally, expression of the Cxcl12 chemokine was measured, as it is the only chemokine known to be involved in HSC migration. When CXCL12 expression was at its lowest, HSC levels in the blood were at their highest, while high levels of CXCL12 corresponded to low levels of circulating HSCs. When mice were exposed to 12 hours of light and 12 hours of darkness, there was a regular oscillation of HSCs in circulation. However, when the light-dark cycle was advanced by 12 hours, to simulate "jet lag," circulating HSCs did not oscillate with any kind of regularity with regard to time (Mendez-Ferrer, Lucas et al. 2008). Based on these findings, it was crucial that blood in our study was collected at approximately the same time each day. In this study, it was collected in the mornings, between 9 and 11am. Likewise, all mice in our studies are housed in 12 hours of light/12 hours of dark conditions, to ensure consistency during sample collection and analysis.

Our data in chapter 2.2 indicated a robust cardioprotection in mPer2-M mice following acute myocardial infarction that was, in part, characterized by elevated capillary density. Investigating the specific mechanism for this apparent revascularization is of great interest to our laboratory. One initial hypothesis was that mPer2 mice may have higher levels of EPCs, or may have improved mobilization of their bone marrow-derived EPC pool. Clearly, more work must be done to definitively answer this intriguing question, but these initial data suggest that EPCs may in fact play a role. In addition to measuring EPC levels in the blood (to contrast with levels from the bone marrow), it will also be imperative to measure CXCL12 and CXCR4 expression, to explore whether or not that mobilization signaling cascade may be involved, as well as enhanced myocardial expression of homing signals.

2.4: Conclusions

mPer2-M mice subjected to 4 days non-reperfused myocardial ischemia are protected more than their wild type (WT) counterparts in the days following MI. This was characterized by a 43% reduction in infarct size, 25% less macrophage infiltrate, 43% increased capillary density, 17% increased myocyte hypertrophy, as well as 15% less myocyte apoptosis. In addition, we observed higher levels of bone marrow-derived progenitors in mPer2-M mice compared to WT, in control mice and 24 hours post-MI. Together, this data suggests that functional deletion of the mPer2 clock gene provides cardioprotection from ischemic events in mice.

Interestingly, our results are in conflict with several recent reports also looking at the Per2 gene. For example, hemodynamic data from Vukolic *et al.* indicated that Per2 mutant mice abolishes the "dipping" phenomena, which may indicate nocturnal hypertension (Vukolic, Antic et al.). Our data suggests an *improved* functional response in mPer2-M to myocardial ischemia, with no notable differences between mPer2-M and WT mice under control conditions. However, we did not investigate functional parameters in animals housed under constant darkness, which may elicit different results.

As discussed in chapter 2.3, our preliminary EPC data conflicts with that of Wang and colleagues (Wang, Wen et al. 2008), who found that mPer2-M mice have significantly less EPCs following an ischemic event than WT mice. However, differences between our study and theirs may simply be due to different experimental models (LAD ligation vs. hind limb ischemia) or the use of different cell surface markers to characterize EPCs.

Another study that looked at gene expression in bone marrow from mPer2-M mice was published in 2009. Luo and colleagues demonstrated that circadian oscillations of several clock genes (Per1, Bmal1, Clock, and Rev-erbα) were disrupted in mPer2-M mice, but present in wild type mice (Luo, Tian et al. 2009). This suggests that mPer2 plays a critical role in regulating circadian oscillations in the bone marrow. In addition, the authors revealed that mPer2-M mice had downregulated Ly49C and Nkg2d mRNA. Both of these genes are involved in cytotoxicity regulation, which may support our findings if the functional absence of Per2 promotes cellular survival.

A recent report by Kakan and colleagues demonstrated that mPer2-M mice had less severe acetaminophen- (APAP) induced liver damage than wild type mice when APAP was administered in the evening. There was no difference in liver injury between the two strains when APAP was administered in the morning. It was hypothesized that the mPer2-M protection from injury was mediated by reduction of an important cytochrome P450 isoform, Cyp1a2 (Kakan, Chen et al.). P450 enzymes are known to be expressed in the heart, but it is not yet clear what their role is in the progression of MI (Sunder-Plassmann 2007; Zordoky, Aboutabl et al. 2008; Chaudhary, Batchu et al. 2009). This would be a unique direction to expand on our current studies in the mPer2-M mice.

Because of the epidemiological interest in circadian rhythm and its involvement in cardiovascular disease, our study may provide new insight into the molecular involvement of a specific clock gene in the infarct repair process. More studies are certainly needed, to ascertain specific mechanisms which may be involved in this cardioprotection. A basic understanding of the interplay between circadian clock genes and cardiovascular disease may lead to novel insights in this field.

CHAPTER 3: EPHRINA1/EPHA RTK SIGNALING AND MYOCARDIAL INFARCT REPAIR

3.1: Introduction

Discovered in 1987, Eph receptors represent the largest family of receptor tyrosine kinase (RTK) receptors. Ephrins, or "Eph receptor-interacting ligands," bind these receptors. Since their discovery, there has been a rapidly growing body of literature representing interest in the field, with approximately 1200 PubMed listings in 2008 (Lackmann and Boyd 2008). The vast majority of these publications are focused on development and cancer. In fact, only a handful of publications exist that document the expression or role of Eph or Ephrin proteins in the heart- either developmentally (Li, McTiernan et al. 1998; Gerety, Wang et al. 1999; Li, Mi et al. 2001; Gerety and Anderson 2002; Cowan, Yokoyama et al. 2004; Wang, Cohen et al. 2004; Grego-Bessa, Luna-Zurita et al. 2007; Stephen, Fawkes et al. 2007) or in adult (Mansson-Broberg, Siddiqui et al. 2008).

There are two subclasses of Eph receptors and ephrin ligands, based on their structure, binding affinity and sequences. The A sub-class consists of 10 EphA receptors, which bind the six ephrinA ligands. Likewise, there are six EphB receptors which may bind the three ephrinB ligands. For the most part, binding is said to be "promiscuous," with only ligands and receptors of the same class interacting. However, there are several exceptions and cross-talk between subclasses can occur. The ephrinA5 ligand will bind the EphB2 receptor, and the ephrinB2

ligand will bind the EphA4 receptor (Mosch, Reissenweber et al. ; Kullander and Klein 2002; Himanen, Saha et al. 2007). We did measure expression of other ephrin ligands (data not shown) and did not see changes in any of them with MI or with ephrinA1 treatment, so the effects reported here are most likely due to direct modulation of EphA receptors by ephrinA1.

Both ligands and receptors are membrane-bound, meaning that cell to cell contact is required for interactions. As stated previously, a major difference between the two sub-classes is structure of the proteins- specifically, how they are anchored to the membrane. EphrinA ligands are bound to the membrane by a glycosylphosphatidylinositol (GPI) linkage, while EphrinB ligands consist of a transmembrane-spanning domain. Receptors consist of both an extracellular and intracellular domain. On the intracellular side is the tyrosine kinase domain, as well as a sterile alpha motif and a Postsynaptic density 95-Discs large-Zonula occludentes-1 (PDZ) binding motif (Mosch, Reissenweber et al. ; Brantley-Sieders, Schmidt et al. 2004).

Eph/Ephrin Signaling

When an ephrin ligand binds an Eph receptor, forward signaling occurs in the direction of the receptor-expressing cell. This signaling cascade is dependent on the tyrosine kinase domain as well as associations with other effector proteins. Simultaneously, signals to the ligand-expressing cell initiate reverse signaling (Holland, Gale et al. 1996; Himanen, Saha et al. 2007; Pasquale 2008). Recently, it has been proposed that ephs and ephrins on the same cell surface may interact to silence bidirectional signaling, but the mechanism has yet to be elucidated (Egea and Klein 2007). As is the case for most RTKs, dimerization of the receptors is necessary for phosphorylation and activation to occur. However, multimerization of the ligands is also

required, and in some cases, the degree of activation can be amplified by the extent of ligand aggregation (Gale and Yancopoulos 1997; Surawska, Ma et al. 2004).

Many of the initial discoveries made regarding Ephs and ephrins involved the processes of cellular repulsion and adhesion. Forward signaling is typically associated with cellular repulsion, while reverse signaling is associated with cellular adhesion. Following cell-to-cell contact of the receptor- and ligand-expressing cells, cell separation must occur if repulsion is to happen. This is typically accomplished by either cleavage of the cell surface proteins or trans-endocytosis of the entire Eph/ephrin complex (Himanen, Saha et al. 2007; Kuijper, Turner et al. 2007). Adhesion and repulsion are important processes in vascular development, axon guidance, tumor metastasis, and cellular invasion.

Involvement of EphA/ephrinA in Disease

As previously mentioned, much of the interest in Eph/ephrin signaling has been in the cancer literature. Briefly, EphA2 has been identified on the endothelium of tumor blood vessels, and its ligand, ephrinA1, is expressed on tumor cells (Brantley, Cheng et al. 2002). Additionally, EphrinA1 has been identified as a downstream target of VEGF, TNFα, and HIF-2α (Pandey, Shao et al. 1995; Cheng, Brantley et al. 2002; Yamashita, Ohneda et al. 2008). Upregulation of EphA2, particularly in breast and prostate cancer, is often correlated with a poor prognosis in cancer patients (McCarron, Stringer et al. ; Ireton and Chen 2005; Landen, Kinch et al. 2005; Wykosky and Debinski 2008). However, EphA1 downregulation has been observed in skin and colorectal cancers (Hafner, Becker et al. 2006; Herath, Doecke et al. 2009).

While there is minimal literature documenting the expression patterns of Eph receptors or ephrin ligands in response to ischemic cardiovascular events, more is known about their response to central nervous system (CNS) injury, where there have been reports of upregulated Eph receptors and ephrin ligands (Du, Fu et al. 2007; Pasquale 2008). In 1999, Moreno-Flores and colleagues induced neuronal death by kainite injection, and found EphA4, EphB2, and EphA5 expression in the hippocampal neurons three days after injury. The authors suggest that these receptors may be involved in axonal migration in response to injury, or in post-injury glial activation (Moreno-Flores and Wandosell 1999). A more recent report by Liu and colleagues showed that EphB3-expressing macrophages were recruited to injured axons expressing the ephrinB3 ligand following injury of the adult retinal ganglion (RGC), suggesting a role for EphB3 in axon plasticity (Liu, Hawkes et al. 2006). EphA4 was found to be accumulated in injured axons following spinal cord injury in rats, while its ephrinB2 ligand was upregulated in the astrocytes surrounding the injury (Fabes, Anderson et al. 2006). In a non-human primate model, EphA4 was upregulated on astrocytes around a lesion site in the primary visual cortex, and could be activated by ephrinA5-Fc to stimulate astrocyte proliferation. It was reported that activation led to downstream signaling of both the mitogen-activated protein kinase (MAPK) and Rho pathways (Goldshmit and Bourne).

In the heart, the EphA3 receptor is critical for development of the atrioventricular valves and atrial septum. Both the A3 receptor and ephrinA1 ligand were identified in adjacent endocardial cells. EphA3 mutant mice had atrioventricular canal and septal defects, were bradycardic, and approximately 75% died of cardiac defects (Stephen, Fawkes et al. 2007). The ephrinA5 ligand has also been identified in isolated neonatal cardiomyocytes, and when it was overexpressed in these cells, the EphA3 receptor was downregulated, and there was reduced

bromodeoxyuridine incorporation (Li, Mi et al. 2001). To our knowledge, only one study has evaluated the expression pattern of Eph receptors and ephrin ligands in the heart, which will be discussed in more detail in chapter 3.2 (Mansson-Broberg, Siddiqui et al. 2008).

Eph RTKs and ephrin ligands are also involved in the inflammatory process, by enhancing epithelial/endothelial barrier permeability, and promoting leukocyte extravasation. Both ligand and receptor are expressed in the vascular endothelium as well as the epithelium, and also on leukocytes (Ivanov and Romanovsky 2006). One study demonstrated that ephrinA1 stimulation of T cells and thymocytes inhibited their migration in transwell plates (Sharfe, Freywald et al. 2002). However, another study showed the opposite- stimulation of CD4+ T cells with ephrinA1 promoted chemotaxis. Further, the EphA1 and EphA4 receptors were identified on CD4+ T cells (Aasheim, Delabie et al. 2005). Clearly, there is a role for Ephs and ephrins in the inflammatory process. However, the role of these proteins in modulating the inflammatory response following MI remains un-explored.

Eph/Ephrin signaling also appears to be involved in cell survival and death. In one study, T cells which expressed the ephrinA4 ligand were stimulated with EphA2-Fc. Activation of ephrinA4 by EphA2 partially abolished cell death, as evaluated by thymidine incorporation. By using propidium iodide staining and a TUNEL assay, the authors were able to conclude that EphA2-stimulated reverse signaling, through ephrinA4, inhibited T cell apoptosis. In addition, it was concluded that EphA2/ephrinA4 reverse signaling induced Akt phosphorylation (Holen, Shadidi et al. 2008).

The goal of the studies in chapter 3 is to elucidate the role(s) of Eph/ephrin signaling in the context of non-reperfused myocardial infarction. Specifically, we hypothesize that the ephrinA1 ligand and its receptors will be expressed in the heart, and expression of the angiogenic

ephrinA1 ligand will decrease in response to injury. Supplementation with chimeric ephrinA1-Fc will ameliorate the infarct response and promote tissue salvage. Based on its purported roles in angiogenesis, inflammation, and cell survival, this signaling cascade would be an attractive therapeutic target in reducing cellular damage and death following MI.

3.2: Intramyocardial Administration of EphrinA1-Fc Promotes Tissue Salvage Following Myocardial Infarction in Mice

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Introduction

The heart lacks an endogenous regenerative capacity sufficient for repair after injury. Consequential left ventricular remodeling after myocardial infarction (MI) leads to LV dilatation, ultimately leading to heart failure (Pfeffer and Braunwald 1991; Gaudron, Eilles et al. 1993; Goldstein, Ali et al. 1998; Holmes, Borg et al. 2005). To reduce this epidemiologic and fiscal burden, it is imperative that strategies be developed to preserve cardiomyocyte survival, subsequently reducing myocardial infarct size, and reducing overall LV remodeling.

Immediately after coronary occlusion, ischemic myocytes downstream from the occlusion become necrotic and/or undergo apoptosis (Cheng, Kajstura et al. 1996; MacLellan and Schneider 1997; Freude, Masters et al. 1998) or autophagy (Nakai, Yamaguchi et al. 2007; Dorn and Diwan 2008; Porrello and Delbridge 2009) . Cardiac troponin I is released, which can be measured in plasma and correlates to the size of injury (Bodor, Porterfield et al. 1995; Chapelle 1999; Braunwald, Antman et al. 2002; Nageh, Sherwood et al. 2003; Oyama and Sisson 2004; Jaffe 2005). Neutrophils infiltrate the tissue immediately, while leukocytes, predominantly macrophages, arrive shortly thereafter and participate in digestion of necrotic cellular debris. Neutrophils in the ischemic tissue can be toxic to the surrounding myocytes, because they release reactive oxygen species and proteolytic enzymes which further injure the

surrounding myocytes (Lefer and Granger 2000; Frangogiannis, Smith et al. 2002; Frangogiannis 2008; Lambert, Lopez et al. 2008; Nah and Rhee 2009). Once damage occurs, a hypocellular scar forms that leads to contractile dysfunction and heart failure (Fishbein, Maclean et al. 1978; Frangogiannis, Smith et al. 2002; Virag and Murry 2003; Dorn 2009).

Since the discovery of the Eph (erythropoietin-producing hepatocellular carcinoma) receptor tyrosine kinase (RTKs) in 1987 (Hirai, Maru et al. 1987), a great deal of effort has been focused on elucidating Eph receptor tyrosine kinase (RTK) and ephrin ligand signaling in the context of numerous pathologies. A distinguishing characteristic of Eph-ephrin interactions is the ability to generate bidirectional signaling. "Forward" signaling occurs in the direction of the receptor-expressing cell, while "reverse" signaling occurs in the direction of the ligand expressing cell (Bruckner, Pasquale et al. 1997; Mellitzer, Xu et al. 1999; Klein 2001; Kullander and Klein 2002). Upon ligand binding and receptor activation, endocytic internalization of the complex occurs (Pasquale 2010), leading to downregulation of the protein. Intracellular cascades downstream of Eph/ephrin signaling are involved in cellular survival, growth, differentiation, and motility (Zhou 1998; Kullander and Klein 2002; Arvanitis and Davy 2008; Pasquale 2008; Pasquale 2010). The EphA1 receptor has been linked to angiogenesis through endothelial cell migration. Like the ephrinA1 ligand, EphA1 is induced by TNF-α, VEGF, and IL-1β, leading to cellular adhesion via integrins and vessel destabilization (Pandey, Shao et al. 1995; Cheng, Brantley et al. 2002; Cheng, Brantley et al. 2002; Moon, Lee et al. 2007). Similarly, the EphA2 receptor, expressed on endothelial cells, is widely reported as a key player in angiogenesis, particularly in development and cancer (Ogawa, Pasqualini et al. 2000; Brantley-Sieders, Schmidt et al. 2004; Brantley-Sieders, Fang et al. 2006; Wykosky, Palma et al. 2008).

Of the five ephrinA ligands, ephrinA1 is unique in that it is the only ligand which binds all eight EphA receptors known to be expressed in mice. Aside from its predominant characterization as a pro-angiogenic factor in adult mouse tumors, (Easty, Hill et al. 1999; Ogawa, Pasqualini et al. 2000; Iida, Honda et al. 2005), ephrinA1 appears to be involved in inflammation and apoptosis, two very important facets of infarct progression. It was reported in 2006 that Eph receptors are differentially expressed at early and late stages of inflammation (Ivanov and Romanovsky 2006). For example, at earlier stages of inflammation, EphA2 and EphrinB2 expression is predominantly localized to epithelial and endothelial cells, promoting disruption of the endothelial/epithelial barrier. However, as the inflammatory process progresses, expression of EphA1, EphA3, EphB3, and EphB4 on these cells decreases, allowing infiltrating leukocytes to adhere to endothelial cells by disrupting endothelial/epithelial barriers (Ivanov and Romanovsky 2006). EphrinA1/EphA receptor expression changes also appear to be involved in regulating pathways involved with apoptosis. In 2006, Munoz and colleagues reported that EphA4 deficient mice exhibited both defective T cell development and increased numbers of apoptotic cells (Munoz, Alfaro et al. 2006). These two reports suggest a role for the EphA4 receptor in mediating cell death, and it is reasonable to suspect that activation of this receptor is anti-apoptotic, while inhibition or removal of this receptor is pro-apoptotic.

The present study was designed to characterize the expression of ephrinA1/EphA RTKs in the uninjured adult myocardium, in response to ischemia in non-reperfused myocardium, and the role of exogenous ephrinA1 in limiting myocardial infarct injury. Specifically, we tested the hypothesis that intramyocardial administration of ephrinA1-Fc at the time of injury would promote myocyte survival and subsequently reduce infarct size and inflammatory cell infiltrate.

Our results indicate a novel and robust cardioprotective role for ephrinA1-Fc in limiting excessive infarct injury in the nonreperfused myocardium.

Methods

Ethical Approval. All procedures were approved by the East Carolina University Institutional Animal Care and Use Committee and the investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Animals. Six week old B6/129S breeder pairs were obtained from Jackson Laboratories (Bar Harbor, Maine) to establish an in-house colony (strain # 101045). Animals were housed in 12-12 light/dark cycle conditions and received food and water *ad libitum*.

Surgical Procedure. Male 8-10 week old mice (22-28g) were anesthetized (20 μl/g Avertin i.p.), intubated, and mechanically ventilated. The left anterior descending (LAD) coronary artery was permanently ligated using 8-0 suture. Sham controls in which the suture was pulled through the heart but not ligated, and either IgG-Fc or ephrinA1-Fc was injected, were done to ensure that there was no injury caused by the injection (data not shown). Infarction was confirmed by blanching of the myocardium distal to the site of ligation. Following coronary occlusion, using a Hamilton syringe with a sterile 30 gauge needle, animals received a single intramyocardial injection of either 6ug IgG-Fc (R&D #110-HG), or 6ug ephrinA1-Fc (Sigma #E9902) resuspended in 6ul sterile PBS at the peri-infarct zone. This dose was chosen based on prior studies showing effective doses of intramyocardial injections of Tβ4 (Bock-Marquette, Saxena et al. 2004) and intraperitoneal injection of ephrinB2-Fc (Mansson-Broberg, Siddiqui et al. 2008).

Additionally, this dose is within the therapeutic range (for humans) of the maximum recommended therapeutic dose (MRTD) 0.00001 to 1000mg/kg-bw/day, as defined by the FDA (Contrera, Matthews et al. 2004). Taking into account heart weight, potential for efflux of the protein from the heart via the injection site, and that an average mouse left ventricle weighs approximately 150mg, injecting 6µg of protein intramyocardially is within this range (approximately 40mg/kg). The investigator performing the surgery was blinded as to the treatment, which were randomized by another investigator. Once the animals recovered, they were returned to the vivarium. The surgical procedure is described in more detail elsewhere (Virag and Murry 2003; Virag, Dries et al. 2010).

Four days after surgery, mice were given a 0.5 ml i.p. injection of 5-bromodeoxyuridine (BrdU, 5mg/ml) to label proliferating endothelial cells and anesthetized 1 hour later with an i.p. injection of 0.1mL pentobarbital (390mg/mL) (Virag and Murry 2003). The heart was arrested in diastole using cold KCl (30mM), excised, rinsed in PBS, and immersed in zinc fixative with a segment of small intestine (used as a positive control for $BrdU^+$ proliferating cells). Hearts were sectioned transversely into 4 slices of equal thickness and were processed and embedded in paraffin. Routine histological (hematoxylin and eosin) procedures and immunostaining were performed using 5 µm sections, as described below (Virag and Murry 2003).

EphrinA1-Fc Distribution in the Myocardium: To determine the distribution pattern and duration of persistence of ephrinA1-Fc in the nonreperfused myocardium, an anti-human IgG-Fc was used to immunolocalize the ephrinA1 chimera in hearts at 30min, 4hr, and 24h post-injection (n=3 per group). A representative image (Figure 1) shows prominent epicardial and transmural staining at 30min. Light staining was observed in 2 of 3 hearts at 4hr but none was observed at

24hr or 4 days post injection, saline injected hearts, or in tissues incubated without the primary antibody (data not shown).

Histology and Morphometry. Images of four hematoxylin and eosin stained sections per heart were taken at 20x using a DP70 digital camera. Scion imaging software (Scion Corporation, Frederick, MD) was used to trace the cross sectional area of the left ventricular wall and chamber, as well as the infarct zone (necrosis + granulation tissue) and necrosis. Measurements from three to four complete, transverse profiles per heart were averaged. Septal and free wall thicknesses were also measured using the average of three radial measures in each of two sections containing infarct. The investigator was blinded as to the treatment while obtaining morphometric measurements.

Immunostaining. Tissue sections were deparaffinized in xylene and endogenous peroxidases quenched with 3% H_20_2 in methanol. Slides were rinsed in PBS and incubated with anti-ephrinA1 (Zymed # 34-3300), CD45 (PharMingen, #550539; 1:2000) for leukocytes, Ly6G (PharMingen #550291) for neutrophils, or CD31 (PharMingen #553371) and anti-BrdU (Roche #11585860001) for proliferating endothelial cells. Slides were incubated with appropriate biotinylated secondary antibodies and then with Avidin Biotin Complex (Vector Labs PK-6100). The reaction product was visualized with DAB (Vector, SK-4100), counterstained with methyl green, dehydrated in xylene, and slides were coverslipped. For the ephrinA1 staining, a second antibody, anti-ephrinA1 (Santa Cruz, # sc-911) was used to verify consistent staining pattern. Negative controls were performed in the same manner but without a primary antibody. For mast cell staining, slides were sent to Histo-Scientific Research Laboratories (Mount Jackson, Virginia) for pinacyanol erthrosinate staining to identify mast cells (Murray, Gardner et al. 2004). Leukocyte, neutrophil, and mast cell density was measured in 3 fields per section of 2

sections of infarcted heart at 400X. Results were expressed as the number of cells per 0.1mm^2. For proliferating endothelial cells ($BrdU^+$ + $CD31^+$), numbers are expressed as a percentage of 1000 endothelial cells ($CD31^+$ only).

Cardiac Troponin I (cTnI) Measurements. Approximately 50-100µl of whole blood was collected from mice pre-surgery and at the time of euthanasia by a submandibular bleed, stored in lithium heparin coated tubes on a rocker to prevent clotting, and analyzed within 30 minutes of collection on an i-STAT Handheld Clinical Analyzer with cTnI cartridges (Abbott labs #06F15-04). Values are expressed as ng/mL.

Protein Isolation: Whole left ventricles were snap frozen in liquid nitrogen at the time of collection, and stored at -80˚C until use. The whole LV was homogenized in a lysis buffer containing 50mM hepes, 10mM EDTA, 100mM NaF, 50mM Na pyrophosphate, and 1% each of protease and phosphatase inhibitors. Protein was quantified using the Bradford Assay.

Western blotting: Western blotting was performed on a 4-12% gradient Bis-Tris gel (BioRad) in 1X MOPS running buffer. 50ug of sample was loaded per well, and the gel was run for 1 hour at 155V, and transferred for 55 minutes (for ephrinA1, BAG-1 and GAPDH) or 1hr 30 min (for cleaved PARP, AKT, and pAKT) onto pure nitrocellulose membranes (BioRad). The membrane was incubated with one of the following antibodies: cleaved PARP (89kDa; Cell Signaling #9544; 1:1000), ephrinA1 (28kDa; Santa Cruz, sc-911; 1:100), AKT (Cell Signaling #4691, 1:1000), phospho-AKT (Cell Signaling #4060, 1:2000), and GAPDH (37kDa; Millipore #MAB374; 1:100), followed by appropriate secondary antibodies. EphrinA1 and cleaved PARP were run on the same membrane, which was cut horizontally at 50kDa, with the bottom half of the membrane used for the ephrinA1 blot, and the top half used for the cleaved PARP blot. The ephrinA1 blot was then stripped/reprobed for anti-GAPDH to confirm equal protein loading. All

blots were detected with Amersham ECL Advance (GE Healthcare #RPN2135) and imaged on a Typhoon Imager. Densitometry was performed using Image J software and the intensity of each protein was normalized to GAPDH. In the case of pAKT/AKT, the amount of phosphorylated AKT protein was normalized to total AKT.

RNA extraction and real-time RT-PCR. The Trizol method was used for RNA isolation, followed by the Quiagen RNeasy kit for additional purification. cDNA was synthesized using a high capacity cDNA kit. Real-time RT-PCR was conducted on an Applied Biosystems thermocycler. A reaction mixture of 10 µL containing 100ng RNA was amplified using recommended conditions for TaqMan primers provided by Applied Biosciences. TaqMan primers and probes were obtained from Applied Biosciences (EphrinA1: Mm00438660_m1), EphA1: Mm00445804_m1, EphA2: Mm00438726_m1, EphA3: Mm00580743_m1, EphA4: Mm00433056_m1, EphA5: Mm00433074_m1, EphA6: Mm00433094_m1, EphA7: Mm00833876_m1, GAPDH: Mm99999915_g1). In each experiment, fluorescence data were analyzed using the ΔΔCT method. Gene expression was normalized to the housekeeping gene GAPDH. No Template Controls (NTC) were included in each experiment, and all samples were run in triplicate.

Statistics. Student t-tests were used to test statistical significance between 4 day MI and ephrinA1-Fc-treated MI for RT-PCR, relative infarct size, and necrosis. ANOVAs and student Newman-Keuls post-hoc analyses were used to determine differences between control, 4 day MI, and ephrinA1-Fc-treated MI for cTnI, inflammatory cell density, chamber area, and left ventricular free wall thickness. The number of hearts analyzed for each endpoint and significance levels have been specified for each experiment in the Figure legends. Four animals

were excluded from all experiments: two from each group, based on suboptimal cTnI and/or overall health of the animals. P values less than 0.05 were considered significant.

Results

EphrinA1-Fc Reduces Infarct Size, Necrosis, Chamber Dilation, and Left Ventricular Free Wall Thinning.

EphrinA1-Fc or IgG-Fc was injected into the border zone of the infarct immediately after coronary ligation. Four days after surgery, tissue was collected and either fixed for histology and immunohistochemistry, or frozen for RNA and protein isolation. Overall survival for this study was 70%, and there was no difference in survival between experimental groups. Histological staining and morphometric analyses (Figure 2) show a 50% reduction in the size of the infarct (expressed as a percent of the left ventricle), 64% less necrotic area, a 35% reduction in chamber dilation, and 32% less thinning of the infarcted left ventricular free wall. Of note, there was no significant difference in chamber area between uninjured control hearts and those treated with ephrinA1-Fc at day 4 post-MI.

Cardiac Troponin I Levels Reduced with ephrinA1-Fc Administration

In the present study, serum cTnI levels were measured prior to surgery and at the time of euthanasia (four days post-MI) in the same animals. There was an 89% increase in cTnI levels following MI in vehicle treated hearts. However, cTnI levels in ephrinA1-Fc treated hearts were 54% lower than those from vehicle treated animals (Figure 3A), $p<0.05$. Interestingly, there was

no significant difference between pre-surgery levels and those of ephrinA1-Fc treated animals four days post-surgery.

EphrinA1-Fc Treated Hearts Show Diminished cleaved PARP Expression and Increased BAG-1 Expression.

In the present study, cleaved PARP, the main target of caspase-3 and an indicator of increased apoptosis (Nicholson, Ali et al. 1995; Tewari, Quan et al. 1995; Oliver, de la Rubia et al. 1998), increased by approximately 88% in response to MI, but diminished with ephrinA1-Fc treatment (Figure 3B) below control levels. Although we did not observe a change in the level of Bcl-2 protein expression with ephrinA1-Fc treatment (data not shown), we did observe a change in Bcl-2-associated athanogene-1 (BAG-1). BAG-1 is a protein that enhances the anti-apoptotic effects of Bcl-2 and has also been identified as a cardioprotective protein through interactions with heat shock proteins (Doong, Vrailas et al. 2002; Townsend, Cutress et al. 2004). We report here that ephrinA1-Fc administration upregulated the expression of the BAG-1 protein by approximately 54% (Figure 3C).

EphrinA1-Fc Treatment Reduces Inflammatory Cell Infiltration to Infarcted Myocardium

Our results indicate a 57% reduction in neutrophil density (Figure 4A) and a 21% reduction in leukocyte density in ephrinA1-Fc-treated versus IgG-Fc-treated hearts at 4 days post-MI (Figure 4B), indicating ephrinA1-Fc attenuates the inflammatory response. We

observed no statistical differences in the numbers of mast cells between ephrinA1-Fc and vehicle treated hearts, with only a few (1-6 per section of LV) mast cells per heart (data not shown).

EphrinA1-Fc Treatment Does not Influence the Angiogenic Response to MI.

No differences were seen in endothelial cell proliferation (5.0 ± 1% vs. 6.1 ± 1.3%; n=3 vehicle, n=5 ephrinA1-Fc) or capillary density (111 ± 26.4 vs. 111 ± 26.0 vessels per 40X high power field, n=4 per group) between vehicle- and ephrinA1-Fc-treated hearts, respectively.

EphrinA1 and EphA Receptor Gene Expression in Response to EphrinA1-Fc Treatment

EphrinA1 gene expression was quantified using qRT-PCR. mRNA levels decrease significantly by 35% following MI, and remain unchanged with ephrinA1-Fc treatment (Figure 5). Of the eight receptors, EphA1, A2, A3, and A7 were all significantly upregulated four days after MI (5-fold, 2-fold, 5-fold, and 28%, respectively); EphA1 andA2 were further upregulated with ephrinA1-Fc treatment (10-fold and 3-fold, respectively, from control). Despite not changing in response to MI, EphA4 was significantly upregulated 2-fold with ephrinA1-Fc treatment. EphA6 was detected in control hearts, but significantly decreased in response to MI, and expression in the ephrinA1-Fc- treated group was unchanged relative to the untreated MI group (Figure 5). Ligands ephrinA2-A5 and EphrinB3 (the only B ligand known to bind to an EphA receptor, specifically, EphA4) were also detected in the heart, but their expression did not change in response to MI or ephrinA1-Fc administration (data not shown).

Endogenous EphrinA1 Tissue Expression Pattern post-MI and in Response to EphrinA1-Fc Treatment

In uninjured control hearts, endogenous ephrinA1 protein expression appeared to be expressed at a low, basal level on cardiac myocytes throughout the myocardium. Four days after MI, ephrinA1 protein expression was expressed in cardiomyocytes throughout the uninjured regions of the hearts and was also localized to the spared cardiac myocytes on both the epicardial and endocardial surfaces of the myocardium, at the border zones of the infarct (Figure 6A and 6B). In the ephrinA1-Fc-treated hearts at 4 days post-MI, endogenous ephrinA1 protein expression appeared to be localized not only to the cardiomyocytes, but also to infiltrating granulation tissue cells throughout the infarct zone (Figure 6C).

EphrinA1 Protein Expression post-MI and in Response to EphrinA1-Fc Treatment

Western blotting was used to quantify endogenous ephrinA1 expression. Since anti-IgG-Fc immunostaining (Figure 1) shows that expression of the chimeric protein is greatly reduced by 4 hours post-injection, and completely abolished by 24 hours, ephrinA1 protein expression detected at 4 days is only the endogenous protein. In addition, the molecular weight for the chimera is 42kDa (not observed), vs. 28kDa for the native protein. Endogenous ephrinA1 protein expression decreased 50% with MI, but was only diminished by approximately 36% with ephrinA1-Fc treatment (Figure 6D).

EphrinA1-Fc Administration Increases pAKT/total AKT Ratio.

Total and phosphorylated AKT protein was measured using western blotting. While total AKT remained unchanged in the three groups (Control, 4 day MI, and EphrinA1-Fc treated), phosphorylated AKT levels increased with EphrinA1-Fc treatment following MI (Figure 7). The pAKT/AKT ratio in EphrinA1-Fc treated hearts was significantly different from control and MI.

There was a trend for increased pAKT/AKT from control to MI, but this was not statistically significant**.**

Figure 3.1: EphrinA1-Fc distribution in the infarcted myocardium. Anti-human IgG-Fc staining to detect exogenous ephrinA1-Fc in the myocardium 30 min after injection. This representative image shows an abundant concentration of ephrinA1-Fc on the epicardial surface, as well as transmural expression of the protein. To a lesser extent, ephrinA1-Fc was also detected 4 hours post-injection, but could not be detected 24 hours or 4 days post-injection.

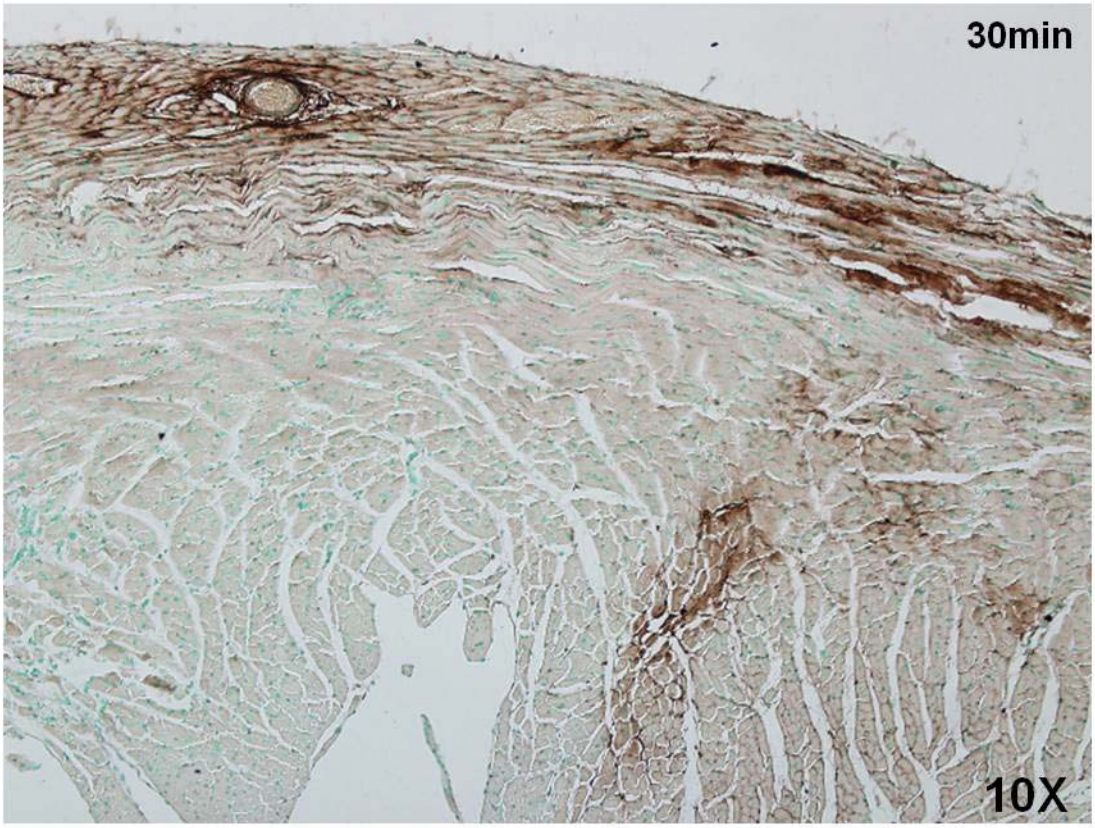

30min
10X

Figure 3.2: EphrinA1-Fc administration reduces infarct size, chamber dilation, necrosis, and thinning of the left ventricular free wall.

Representative histological images shown are of vehicle-treated (A) and ephrinA1-Fc-treated (B) hearts four days post-MI. There was a 50% reduction in infarct size (C), 64% less necrosis (D), 35% less chamber dilation (E), and 32% less thinning of the left ventricular free wall (F). n=7 control, 9 IgG-Fc, 9 ephrinA1-Fc, $p<0.05$. † different from control, * different from IgG-Fc. LV: Left Ventricle; RV: Right Ventricle; N: Necrosis; G: Granulation tissue

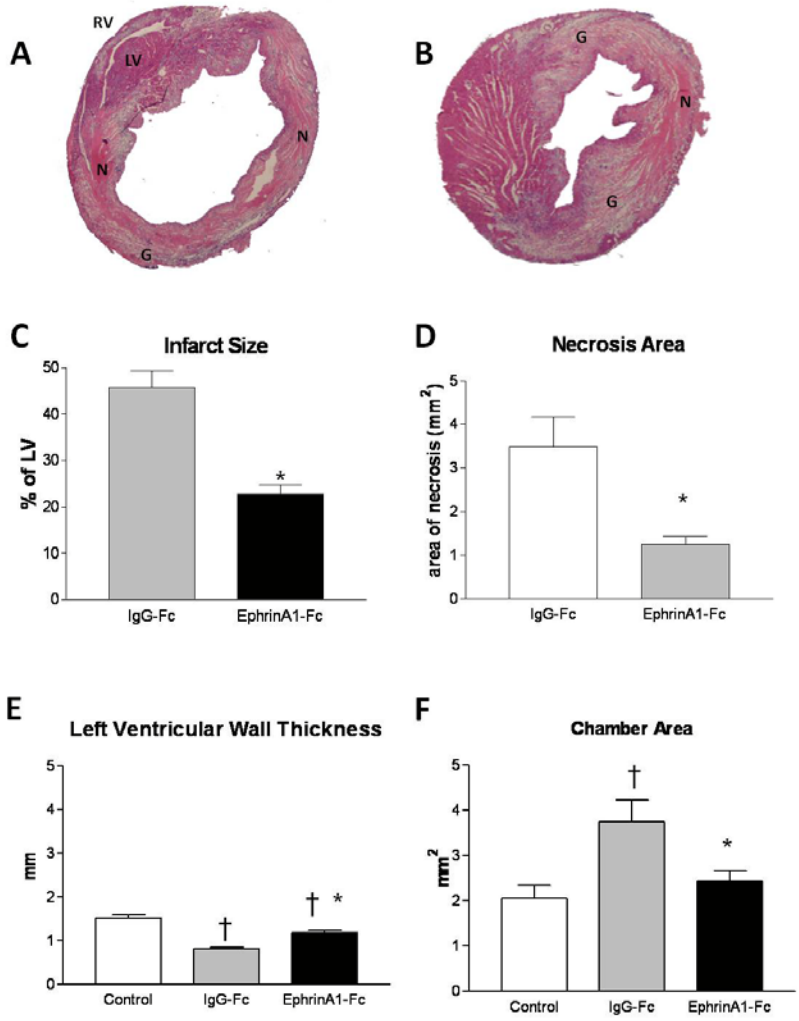

A
RV
LV
N
N
G
B
G
N
G
C
Infarct Size
% of LV
50
40
30
20
10
0
*
IgG-Fc
EphrinA1-Fc
D
Necrosis Area
area of necrosis (mm2)
5
4
3
2
1
0
*
IgG-Fc
EphrinA1-Fc
E
Left Ventricular Wall Thickness
mm
5
4
3
2
1
0
†
† *
Control
IgG-Fc
EphrinA1-Fc
F
Chamber Area
mm2
5
4
3
2
1
0
†
*
Control
IgG-Fc
EphrinA1-Fc

Figure 3.3: Intramyocardial EphrinA1-Fc administration reduces tissue injury.

Cardiac troponin I (cTnI) levels in serum decreased 54% four days post-MI (A). Cleaved PARP expression was reduced with ephrinA1-Fc administration (B). BAG-1 protein (C) increased with ephrinA1-Fc administration by 54% when normalized to GAPDH. n= 8 control, 13 vehicle, 11 ephrinA1-Fc. $p<0.05$, † different from control, * different from MI.

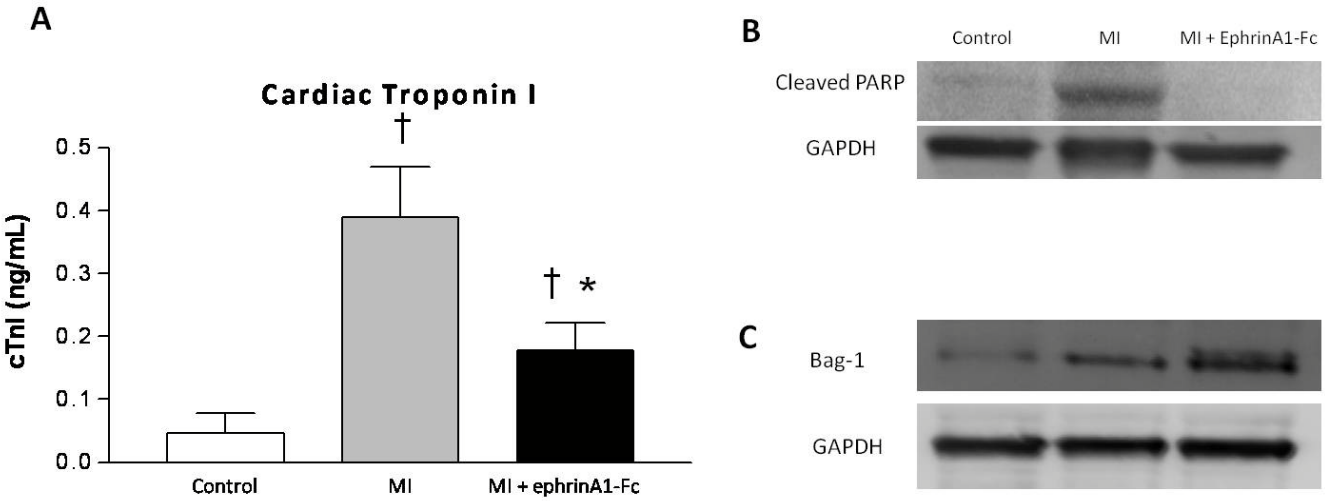
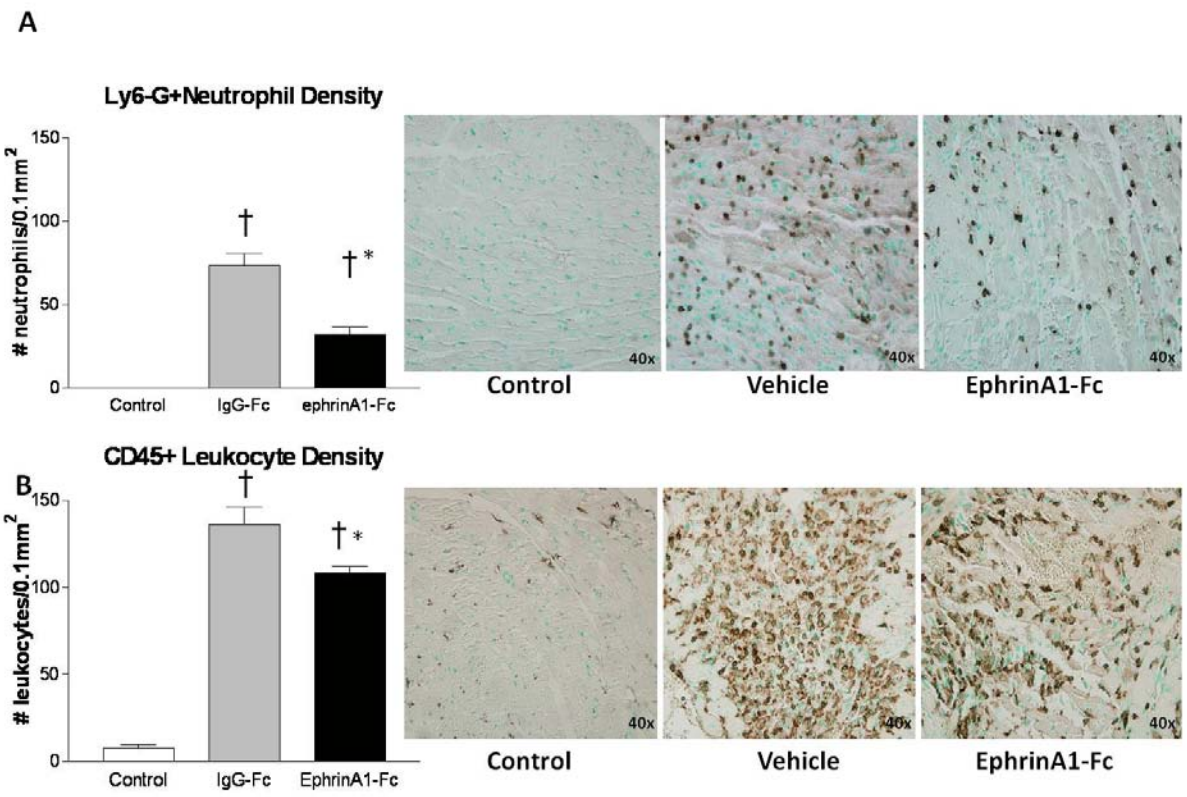

A
Cardiac Troponin I
cTnI (ng/mL)
0.5
0.4
0.3
0.2
0.1
0.0
†
† *
Control
MI
MI + ephrinA1-Fc
B
Control
MI
MI + EphrinA1-Fc
Cleaved PARP
GAPDH
C
Bag-1
GAPDH

Figure 3.4: EphrinA1-Fc reduces inflammatory cell infiltration.

EphrinA1-Fc administration significantly reduced infiltration of neutrophils (A) and leukocytes (B) at 4 days. n= 3 control, 9 IgG-Fc, 9 EphrinA1-Fc, $p<0.05$, † different from control, * different from MI. Representative images of $Ly6G^+$ neutrophil infiltration (top panels) and $CD45^+$ pan-leukocyte infiltration (bottom panels) are shown in control (left), vehicle-treated (middle), and ephrinA1-Fc treated (right) hearts.


A
Ly6-G+Neutrophil Density
neutrophils/0.1mm2
150
100
50
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Control
IgG-Fc
ephrinA1-Fc
40x
40x
40x
Control
Vehicle
EphrinA1-Fc
B
CD45+ Leukocyte Density
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150
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Control
IgG-Fc
EphrinA1-Fc
40x
40x
40x
Control
Vehicle
EphrinA1-Fc

Figure 3.5: Altered gene expression of EphrinA1 and EphA receptors in response to MI and MI + EphrinA1-Fc.

Following MI, ephrinA1 gene expression was significantly reduced (black bars), and remained relatively unchanged in response to ephrinA1-Fc administration (grey bars). Receptors A1, A2, A3, and A7 were significantly upregulated in response to MI, by 5-fold, 2-fold, 5-fold, and 1-fold, respectively, while EphA4 remained unchanged. EphA6 was detected in control hearts but dropped significantly following MI, and expression was not recovered with ephrinA1-Fc administration. In response to ephrinA1-Fc administration, receptors A1 and A2 were further upregulated, by approximately 2-fold each, and A4 was also upregulated by almost 2-fold. Values were calculated using the ct method, normalized to GAPDH, and presented here as fold changes relative to uninjured control (white bars). n=8 control, 8 MI, 8 ephrinA1-Fc, $p<0.05$, † different from control, * different from MI.

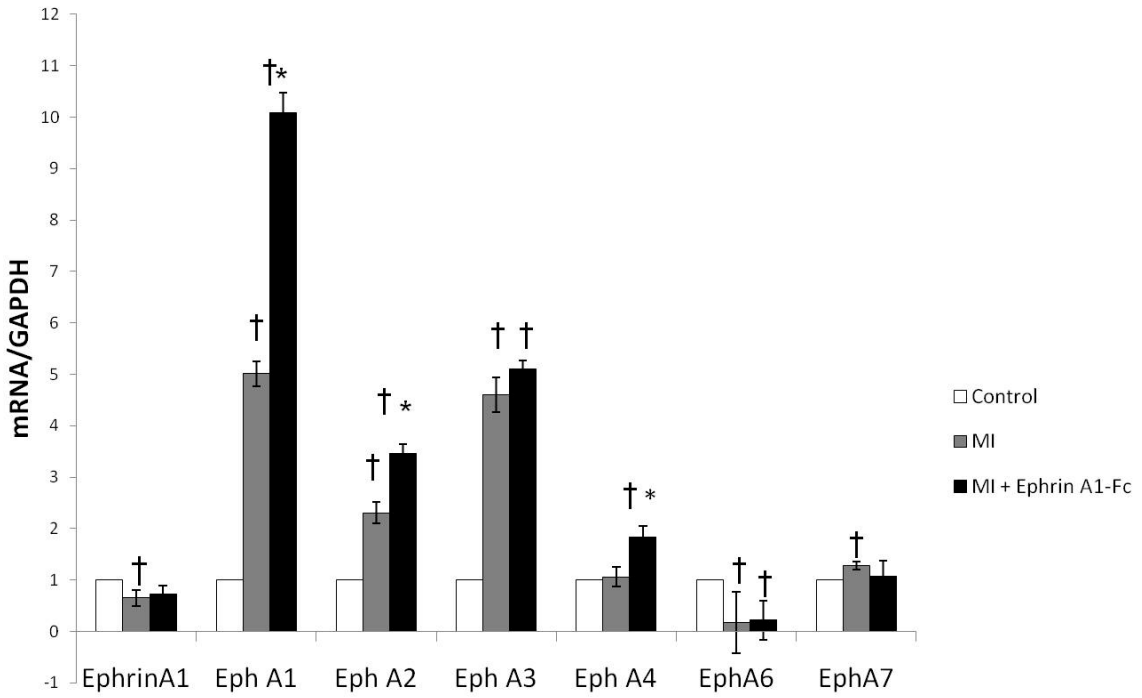
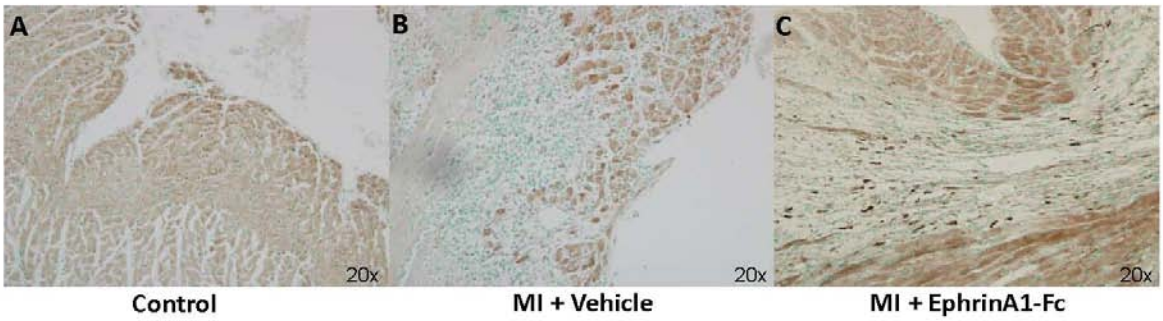

mRNA/GAPDH
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
EphrinA1
Eph A1
Eph A2
Eph A3
Eph A4
EphA6
EphA7
Control
MI
MI + Ephrin A1-Fc

Figure 3.6: EphrinA1 protein distribution in the myocardium.

Representative immunostaining for ephrinA1 protein showed a low basal expression of ephrin A1 in cardiomyocytes of control hearts (A), intense staining in endo- and epi-cardial myocytes following 4 days non-reperfused MI (B), and more intense staining in myocytes as well as numerous granulation tissue cells in the infarct zone following ephrinA1-Fc treatment at 4 days post-MI (C). EphrinA1 total protein expression (D) was reduced by 50% in response to MI, but only reduced 36% in response to ephrinA1-Fc administration (normalized to GAPDH).


A
20x
B
20x
C
20x
Control
MI + Vehicle
MI + EphrinA1-Fc

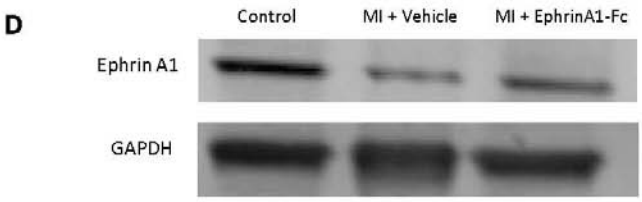
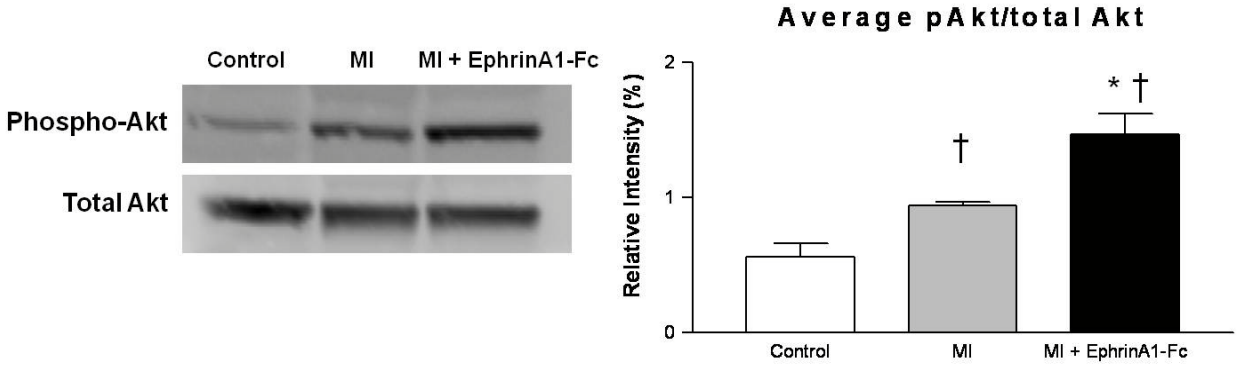

D
Control
MI + Vehicle
MI + EphrinA1-Fc
Ephrin A1
GAPDH

Figure 3.7: Akt Expression with ephrinA1-Fc Administration

Representative blot of phosphorylated and total Akt, with the average densitometric analysis of three repeated blots. n=3 control, 3 MI, 3 ephrinA1-Fc. $p<0.05$,† different vs. control, * different vs. MI.


Control
MI
MI + EphrinA1-Fc
Phospho-Akt
Total Akt
Average pAkt/total Akt
Relative Intensity (%)
2
1
0
*
†
†
Control
MI
MI + EphrinA1-Fc

Discussion

We report here that ephrinA1, and several of its receptors, are expressed in the adult myocardium, and their expression profile is altered in response to ischemia in the non-reperfused myocardium. In addition, we have identified a novel and protective role for intramyocardial administration of ephrinA1-Fc at the time of MI, leading to reduced infarct size, necrosis, chamber dilation, and wall thinning, as well as less inflammatory cell infiltration. A significant decrease in cardiac troponin I levels, coupled with reduced cleaved PARP and increased BAG-1 protein expression, indicates less overall cell death. This is further supported by the fact that ephrinA1-Fc administration leads to increased phosphorylated AKT protein, a known modulator of myocyte survival (Matsui, Nagoshi et al. 2003; Latronico, Costinean et al. 2004; Matsui and Rosenzweig 2005; Hausenloy and Yellon 2006; Miyamoto, Murphy et al. 2009).

The heart lacks significant regenerative capacity to overcome myocardial injury. Therefore, there has been much investigation into a number of cell-, gene-, and protein-based therapeutic strategies aimed at augmenting the cardiac regenerative potential and promoting tissue salvage (Urbich, Rossig et al. 2006; Laflamme, Zbinden et al. 2007; Dorn and Diwan 2008; van Rooij, Marshall et al. 2008; Abbate, Biondi-Zoccai et al. 2009; Bartunek, Vanderheyden et al. 2010; Hwang and Kloner 2010). To effectively reduce injury and limit the progression of remodeling and dysfunction, it is necessary to reduce inflammation and cell death, and promote revascularization. Optimization of the mode of delivery, timing, and bioavailability of proteins and growth factors has been an attractive target for infarct salvage and regeneration. In 2002, Edelberg *et al*. reported that pre-treatment of an infarcted, non-reperfused rat heart with Platelet Derived Growth Factor-AB (PDGF-AB) resulted in a ~50% reduction in

infarct size. However, treatment at the time of coronary occlusion did not alter infarct size (Edelberg, Lee et al. 2002). There has also been interest in using the thymosin β4 peptide for myocardial salvage. A 2004 report by Bock-Marquette *et al.* concluded that thymosin β4 was crucial for myocyte survival, migration, and repair (Bock-Marquette, Saxena et al. 2004). In that study, intramyocardial (400ng in 10µl collagen) and intraperitoneal (150µg in 300µl PBS) delivery of the thymosin β4 peptide immediately after permanent coronary ligation in mice reduced infarct volume by 50% and improved contractile performance. The authors identified AKT activation as a potential mechanism of Tβ4-mediated protection. More work is needed to identify the factor(s) and their mechanisms of action that will promote optimal therapeutic efficacy (Segers and Lee).

The literature is currently limited in the number of reports involving ephrin and/or Eph expression and signaling in the adult heart. In 2008, Mansson-Broberg and colleagues demonstrated a protective role for ephrinB2/EphB4 signaling in the repair process after MI (Mansson-Broberg, Siddiqui et al. 2008). An intraperitoneal injection of 100ug ephrin B2-Fc 1 week after ligation of the left anterior descending coronary artery in mice resulted in increased capillary density. This study suggests a role for Eph/ephrin signaling in the infarcted heart and its effect on capillary density, but did not investigate how these interactions influence infarct size or cell behavior, including that of cardiac myocytes, infiltrating inflammatory cells, and fibroblasts. Additionally, the authors reported that ephrinB2-Fc treatment of cultured human aortic endothelial cells induced proliferation and that ephrinB2-Fc also induced increased sprouting in murine aortic ring studies, demonstrating a pro-angiogenic role for the EphB4/ephrinB2 signaling cascade (Mansson-Broberg, Siddiqui et al. 2008). More recently, it has been proposed that downregulation of angiogenic factors in early injury impairs performance

and this can be remediated by administration of an angiogen (Siddiqui, Fischer et al.). In accordance with this notion, the results of our study demonstrate decreased protein expression of the angiogenic factor ephrinA1, yet implicate a *non*-angiogenic role for exogenous chimeric ephrinA1-Fc-induced signaling in the context of acute MI that has not been previously reported. Further, Mansson-Broberg and colleagues reported that Eph receptors A1- A4, A6, B1, and B4, as well as ephrin ligands A1, A2, A5, B2, and B3 were expressed in adult hearts. We have also detected the EphA receptors mentioned above, in addition to the EphA7 receptor. The EphA6 and EphA7 receptors have been implicated in angiogenesis, both are expressed on vascular endothelium (Shaut, Saneyoshi et al. 2007), and EphA7 is also expressed on mural cells (Stadler, Higgins et al. 2001), making it an attractive target to modulate vessel integrity via cell adhesion. Pathologically, increased expression of EphA7 correlates with increased severity of disease outcome in gliobastoma patients (Wang, Fokas et al. 2008). Although we did not observe an angiogenic effect in the present study, long-term studies are underway to examine later time points, since involvement of these receptors could potentially mediate vessel persistence and/or revascularization of the infarcted heart.

As in other RTK's, activation of Eph receptors by their ephrin ligands results in autophosphorylation of the receptors, and endocytic internalization and degradation of the ligand-receptor complex (Pasquale 2010), which would result in reduced protein expression. In our study, we identified increased mRNA expression of several receptors (A1, A2, and A4) following ephrinA1-Fc administration. This is likely due to a compensatory increase in mRNA following internalization and degradation of the receptors. Of particular interest was the significant upregulation of EphA4 receptor expression following ephrinA1-Fc administration, since expression of this receptor was unaffected by MI alone. EphA4 and EphA1 are both

expressed on T cells. EphrinA1 stimulation of EphA4-expressing T cells resulted in cell migration (Aasheim, Delabie et al. 2005; Holen, Nustad et al. 2010), so it is possible that activation of this receptor in our model muted the inflammatory response, reducing necrotic debris and tissue damage, with an overall reduced inflammatory cell population at 4 days post-MI. This is further supported by a recent report showing that a small subset of T-cells, which express the angiotensin AT2R, are noncytotoxic compared to other T cells, and their transplantation into the ischemic myocardium increased expression of the protective cytokine IL-10, thus reducing injury (Curato, Slavic et al. 2010). EphA4 is also involved in apoptosis. Furne and colleagues reported that removal of the ephrinB3 ligand from EphA4 resulted in caspase-dependent cell death (Furne, Ricard et al. 2009). We did not see changes in ephrinB3 mRNA expression, but it is plausible that ephrinA1-Fc stimulation of EphA4 in our model reduced, or inhibited, apoptotic cell death. A 2006 study by Muñoz and colleagues showed that EphA4-deficient mice had increased numbers of apoptotic cells, again suggesting a role for EphA4 forward signaling in the inhibition of cellular apoptosis (Munoz, Alfaro et al. 2006). More studies are needed to determine the cell-specific expression of EphA4 and its level of activation in response to ephrinA1-Fc.

There is evidence that ephrinA reverse signaling results in Akt phosphorylation and inhibition of apoptosis (Holen, Shadidi et al. 2008). Since ephrinA1-Fc administration increased the endogenous protein expression of ephrinA1 in the myocardium (Figure 6), this may also play a role in the observed protection. The EphA1 and EphA2 receptors have mainly been characterized in the setting of tumor angiogenesis (Giaginis, Tsourouflis et al. ; Wykosky, Gibo et al. 2005; Wykosky and Debinski 2008; Chen, Wang et al. 2010). Further investigation into the mechanism of salvage afforded by forward signaling by each receptor and reverse signaling

by the ligand in addition to the role that activation of these pathways may play in promoting vessel stability and/or angiogenesis in the infarcted heart will be determined in future investigations by in vitro studies and examination of later timepoints.

In the early stages following myocardial infarction, cardiomyocytes are lost through regulated cell death, or apoptosis, as well as unregulated death, or necrosis. Protein expression of cleaved PARP, a marker of cellular apoptosis, was substantially reduced with ephrinA1-Fc administration, as shown in Figure 3. Self digestion, or autophagy, can also occur in cardiomyocytes during MI, and may be involved in survival mechanisms, as well as cell death (Whelan, Kaplinskiy et al.). Autophagy is activated in hibernating myocardium, an adaptive feature of cardiomyocytes to survive limited flow and depleted oxygen supply (Slezak, Tribulova et al. 2009). In addition to enhancing the anti-apoptotic effects of the bcl-2 protein (Reed, Zha et al. 1996; Tang 2002), it has been previously reported that the BAG-1 protein can induce autophagy in a rat model of ischemia-reperfusion by linking heat shock proteins Hsc70/Hsp70 with the proteasome (Gurusamy, Lekli et al. 2009)., leading to cardioprotection. In accordance with these findings, in this study, we observed increased BAG-1 expression coupled with the significant reduction in myocardial injury following ephrinA1-Fc administration. Although we did not observe a change in Bcl-2 protein expression, we hypothesize that BAG-1 expression is leading to increased cellular survival through myocyte autophagy and studies in our laboratory are currently underway to specifically explore the cellular mechanism by which increased BAG-1 expression affords protection in ephrinA1-Fc treated hearts (Terman and Brunk 2005).

Cardiac Troponin I (cTnI) is a highly sensitive, specific, and reliable serum biomarker for cardiac injury in the clinical setting (Chapelle 1999; Nageh, Sherwood et al. 2003; Oyama and Sisson 2004), and there is a proportional relationship of the extent of myocardial injury with the

measured level of cTnI (Bodor, Porterfield et al. 1995; Braunwald, Antman et al. 2002; Jaffe 2005). In our study, cTnI levels were reduced by approximately 55% ($p<0.05$) with ephrinA1-Fc administration. Combined with the reduced cleaved PARP and increased BAG-1 protein expression, these data suggest reduced cardiomyocyte injury as the mechanism for the observed salvage.

Moreover, our data demonstrate that EphrinA1-Fc administration post-infarction leads to phosphorylation of AKT, a protein involved in cellular survival. Our findings are in agreement with similar studies, including a recent paper which showed that administration of Nerve Growth Factor induced neovascularization and improved cardiac function in a permanent coronary occlusion model, which was coincident with a significant increase in phosphorylated AKT three days post-MI (Meloni, Caporali et al.). In another study by the same group, inhibition of PI3K signaling led to reduced pAKT/AKT, increased cardiomyocyte apoptosis *in vitro* and reduced infarct size in mice 14 days following permanent coronary occlusion (Siragusa, Katare et al.). Treating swine for 7 days with subcutaneous injections of G-CSF beginning 24hrs after MI led to reduced infarct size, increased VEGF expression, and increased pAKT/AKT (Iwanaga, Takano et al. 2004). Clearly, AKT activation is involved in cellular survival and favors cardiac salvage. While several other groups have identified AKT activation as a survival mechanism in the setting of non-reperfused MI (Patten and Karas 2006; Haider, Jiang et al. 2008; Shujia, Haider et al. 2008), the downstream targets for AKT in this setting are not fully understood. It has been proposed that three main methods of AKT cardioprotection involve anti-apoptotic factors, promotion of cell growth, and promotion of survival and improved function of *dys*functional cardiomyocytes (Matsui and Rosenzweig 2005). Studies are currently underway to investigate this signaling process.

In summary, our data provide the first evidence that intramyocardial administration of recombinant ephrinA1-Fc promotes myocardial tissue salvage. Modulating ephrinA1/EphA signaling may play a significant role in governing the repair process following myocardial infarction, and so exogenous ephrinA1-Fc may prove to be an attractive therapeutic target. Although reperfusion has been the clinical standard for post-MI therapy, approximately 25% of these patients still have an infarct size greater than 75% of the ischemic zone, which is associated with an even greater incidence of mortality and poor outcome (Miura and Miki 2008). In addition, reperfusion should be initiated within 2 hours of the onset of MI for the greatest success in salvaging ischemic tissue (Milavetz, Giebel et al. 1998). Thus, reperfusion after MI may not always be feasible, so investigating the time-dependency of ephrinA1-Fc administration may provide new insight into a promising treatment option. Our laboratory is currently working to elucidate the mechanism for the considerable degree of salvage observed by examining the effects of ephrinA1-Fc on cell-specific receptor expression patterns, signaling cascades activated, and isolated cell behavior and metabolism. Additionally, we are examining the long-term impact that ephrinA1-Fc administration has on remodeling and cardiac function, as well as the time frame and frequency of administration required to elicit protection.

3.3: Metabolic Effects of EphrinA1 Administration

PI3K-Akt Signaling and Myocardial Infarction

We hypothesized that the cardioprotection observed following EphrinA1-Fc administration is due to promotion of cardiomyocyte survival in the ischemic setting, because revascularization was not observed (chapter 3.2). Further, we have ruled out regulation of apoptosis because changes in bcl-2 protein expression were not altered with EphrinA1-Fc administration (chapter 3.2), and the overall contribution of apoptosis in a non-reperfused infarct is relatively small when compared to necrosis.

The phosphatidylinositol 3-kinase (PI3-K) pathway is a survival pathway resulting in activation of protein kinase C (PKC) as well as many other downstream signaling pathways, through phosphorylation of Akt. Briefly, growth factors and hormones stimulate a cell and activate PI3K, leading to phosphorylation of the D-3 position phosphatidyl-inositol-4,5-bisphosphate on the cell membrane, resulting in formation of phosphatidyl-inositol-3,4,5-triphosphate. Ultimately, phosphatases can degrade $PI(3,4,5)P_3$ which will halt PI3K signaling (Cantley 2002; Hausenloy and Yellon 2006).

PI3K-Akt signaling is of interest in the present study because of promising pre-clinical and clinical data suggesting a role for this survival cascade in ameliorating reperfusion injury. For example, the reperfusion injury salvage kinase (RISK) pathway has been studied extensively in the settings of both ischemic pre- and post-conditioning to reduce myocardial infarct size. Repeated bursts of ischemia and reperfusion at the initiation of myocardial reperfusion (ischemic

post-conditioning) resulted in reduced infarct size, and eNOS and Akt phosphorylation. However, treatment with a PI3K inhibitor abolished this protection. A similar effect was seen with ischemic pre-conditioning, which is initiated before global ischemia, suggesting that the RISK pathway may be recruited either before or after ischemia while still being beneficial (Hausenloy, Lecour et al. ; Hausenloy, Tsang et al. 2005; Hausenloy, Tsang et al. 2005).

In 2000, Tong and colleagues demonstrated that performing IPC before 20 minutes of global ischemia led to protein kinase B phosphorylation and nitric oxide production, which was inhibited by treatment with the PI3K inhibitor wortmannin (Tong, Chen et al. 2000). This suggests that the protective effects often observed with IPC may be, at least in part, regulated by PI3K signaling. Interestingly, Tsang and colleagues reported in 2005 that the diabetic rat heart can be protected with IPC, indicated by Akt phosphorylation, but only when three cycles of IPC were used. In wild type rats, one, two, or three cycles of IPC were all beneficial. Thus, it appears that there is a threshold in diabetic hearts, and if that threshold is reached using IPC, protection may be afforded via the RISK pathways (Tsang, Hausenloy et al. 2005).

There are conflicting reports describing changes in total and phosphorylated Akt following MI and in response to therapeutics. A recent report by Siddiqui and colleagues revealed that both total and phosphorylated Akt are reduced 2 days after coronary ligation in C57/BL6 mice, compared to sham controls (Siddiqui, Fischer et al.). Sumi *et al* initiated 30 minutes of ischemia and 48 hours of reperfusion in rabbits, then immediately treated them with G-CSF or saline. G-CSF treatment reduced infarct size and increased phosphoryated Akt levels at 10 minutes and 48 hours post-MI (Sumi, Kobayashi et al.). In a rat model, Yamazaki *et al* pre-treated their animals with epicatechin, which significantly reduced infarct size and improved function up to 3 weeks after surgery. However, the authors did not observe any difference in

total or phosphorylated Akt two hours after coronary occlusion, which suggests that the cardioprotection from epicatechin is not Akt-dependent (Yamazaki, Taub et al.). Treatment of cultured H9c2 rat cardiomyocytes with M-CSF for 10 minutes induced phosphorylation of Akt, leading to upregulation of a downstream anti-apoptotic gene, Bcl-xL, suggesting a role for pAkt in myocyte survival (Okazaki, Ebihara et al. 2007). Our data in chapter 3.2 supported the hypothesis that ephrinA1-Fc administration would increase levels of phosphorylated ephrinA1-Fc. To assess mitochondrial content, we were next interested in evaluating citrate synthase activity in whole heart homogenates.

Citrate Synthase and Myocardial Mitochondria Function

Changes in the metabolic activity of the heart are common following MI (Heather, Carr et al. ; Moncada ; Neubauer, Horn et al. 1995; Lesnefsky and Hoppel 2003; Chen, Moghaddas et al. 2008). The mitochondria are critical for the regulation of cellular survival and death, through either apoptosis or necrosis, which have made them attractive therapeutic targets (Bouchier-Hayes, Lartigue et al. 2005). An analysis of mitochondrial activity in the infracted heart would potentially yield important information on the metabolic activity of the cells, providing mechanistic insight into the EphrinA1-Fc-mediated cardioprotection observed in our studies.

To assess mitochondrial content and function, citrate synthase activity is frequently measured. Citrate synthase is an enzyme located in the mitochondrial matrix, which catalyzes the reaction of acetyl CoA and oxaloacetate combining to form citrate, with CoASH released as a byproduct. When the yellow colormetric agent 5,5-dithiobis-2-nitrobenzoate (DTNB) is

combined with CoASH, a mercaptide ion is released and the change in absorbance can be measured at 412nm (Goncalves, Paupe et al. ; Reisch and Elpeleg 2007).

Citrate synthase activity is frequently used as a measure of the oxidative capacity of a tissue. It has been previously reported that citrate synthase activity is significantly reduced two days post-MI in mice (Siddiqui, Fischer et al.). Additionally, it has been reported that left ventricular dysfunction following MI resulted in reduced citrate synthase activity in type IIB muscle fibers (Delp, Duan et al. 1997).

Methods:

Surgical procedure: As previously described (Chapter 3.2), MI was induced in B6129s mice, followed by intramyocardial injection of EphrinA1-Fc . Animals were euthanized four days post-surgery, and tissue was collected. Left ventricles were snap-frozen in liquid nitrogen, and stored at -80˚C until used for citrate synthase activity assay.

Citrate synthase activity:

Homogenization: Whole left ventricles were isolated from saline- (n=5) and ephrinA1-Fc- (n=6) treated mice four days post-MI, as well as from naive control animals (n=6). The tissue was snap frozen in liquid nitrogen and stored at -80˚C. Frozen left ventricles were weighed, and diluted 1:10 in 100mM Tris buffer for homogenization. A small glass homogenizer and vial were used, with homogenization occurring on ice the entire time. The homogenate was transferred to an eppendorf tube, and centrifuged at 1500xg for 10 minutes to pellet any debris

from the homogenization procedure. The supernatant was aliquoted into fresh eppendorf tubes and stored at -80˚C until used for the citrate synthase assay.

Citrate Synthase Assay: A reaction cocktail was made consisting of the following, (volume per sample): 100mM Tris Buffer (40ul), 1mM DTNB (20ul), 3mM AcCoA (30ul), and 10mM oxaloacetate (10ul). One well of a 96 well plate was used for each sample, with three additional wells used as blanks. To each well, 90ul of 100mM Tris buffer was added, along with 10ul of homogenized protein. In the case of the blanks, 10ul of Tris buffer was substituted for the protein. Finally, 100ul of the reaction cocktail was added to each well to initiate the reaction, and the plate was immediately placed into the plate reader for subsequent analysis. The plate reader used was an Epoch (BioTek) with Gen5 software. The software was set up for a kinetic read, at 412nm, under the "sweep" mode, with absorbance readings made at 15 second intervals for a total of 5 minutes. The most linear portion of the plotted data points was selected (typically from 0 to 3 minutes) and the slope was calculated for each sample for the specified range of time (see figure 3.8 for a representative data plot). The specific activity was calculated using previously described methods (Brown, Jew et al. 2003; Brown, Chicco et al. 2005).

Statistics: The pAkt/Akt blots were repeated three times, with densitometry performed on each blot to normalize the pAkt levels to total Akt. The densitometric data reported is the average of the three, and the images shown are representative blots. InStat Software was used to perform One-way ANOVA with Tukey-Kramer Multiple Comparisons Test used to make comparisons between groups. For the citrate synthase data, results of the three experiments were averaged, and a one-way ANOVA was used to compare means across groups. $p<0.05$.

Results:

Citrate Synthase Activity is Not Altered by EphrinA1-Fc Administration.

Frozen hearts from control animals (n=6), saline-treated infarcted animals (n=5) and EphrinA1-Fc-treated infarcted animals (n=6) were analyzed for citrate synthase activity, and the assay was repeated three times. Results of all three assays were pooled and analyzed for statistical significance. There was no significant difference between any of the three groups in citrate synthase activity, as shown in Figure 3.9 (4.60 control vs. 4.42 saline vs. 4.54 ephrinA1-Fc). This finding was surprising, since it is generally assumed that citrate synthase activity will decrease following MI. Interestingly, though, in one of the three runs for this experiment, There *was* a significant decrease in citrate synthase activity from control to saline-treated animals ($p=0.03$), with a trend for a decrease from control to EphrinA1-Fc treated animals ($p=0.06$). However, there was no statistically significant difference between saline-treated and EphrinA1-Fc-treated mice, and when the results from each of the three experiments were compiled, there was no significant difference.

Figure 3.8: Representative plot of citrate synthase activity

Representative plot of citrate synthase activity, measured as absorbance (OD) at 412nm. The area of linearity is highlighted in a yellow box, from zero to two minutes. This is the region used to calculate specific activity. When the slope of the line begins to level off, it is presumed that the reaction is limited by one of the substrates, so accurate calculations cannot be made. In this case, the first two minutes of the reaction appear to be most linear. Each point represents a specific absorbance, for each sample (y axis) plotted over time (x axis).

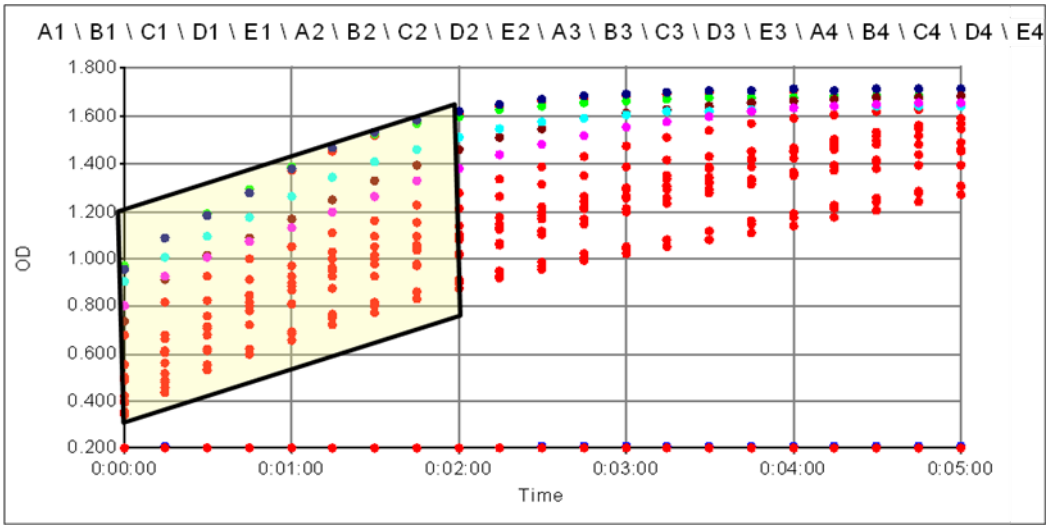
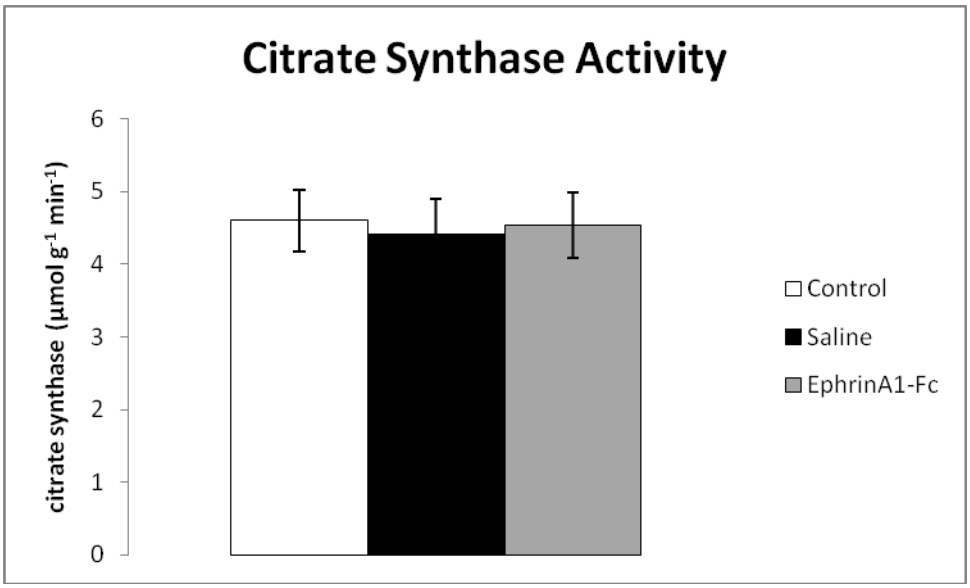

A1 \ B1 \ C1 \ D1 \ E1 \ A2 \ B2 \ C2 \ D2 \ E2 \ A3 \ B3 \ C3 \ D3 \ E3 \ A4 \ B4 \ C4 \ D4 \ E4
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
OD
0:00:00
0:01:00
0:02:00
0:03:00
0:04:00
0:05:00
Time

Figure 3.9: Administration of ephrinA1-Fc does not alter citrate synthase activity in the heart.

The calculations from each of the three assays were averaged, for control, saline-treated, and EphrinA1-Fc-treated, and represented in a bar graph. One-way-ANOVA did not detect statistical significance for any of the comparisons between groups. Results are expressed as mean +/- SEM.


Citrate Synthase Activity
citrate synthase (µmol g-1 min-1)
6
5
4
3
2
1
0
Control
Saline
EphrinA1-Fc

Discussion:

To our surprise, citrate synthase activity was not altered with EphrinA1-Fc treatment, suggesting that mitochondrial content and function is not influenced by this treatment. These findings were surprising, in that other studies have reported a decrease in citrate synthase activity with MI. It was anticipated that our infarcted hearts, treated only with saline, would demonstrate a similar effect, and that treatment with EphrinA1-Fc would abolish the reduced citrate synthase activity.

Several factors may have affected this outcome, including timing. While our study examined the effects of permanent coronary occlusion on citrate synthase activity four days after infarction, other studies have analyzed citrate synthase two days after permanent occlusion (Siddiqui, Fischer et al.) as well as after ischemia reperfusion (Brown, Jew et al. 2003; Brown, Chicco et al. 2005). In addition to timing, the entire left ventricle was homogenized and used for this assay, so it is possible that any measurable citrate synthase activity was too dilute to be measured.

Many studies have examined citrate synthase activity in skeletal muscle, but less is known about its activity in the myocardium. In one study, myocardial infarction was induced in rats, and then some rats were given propionyl-L-carnitine (PLC) 10 days after surgery, and exercise capacity of the rats was assessed. Interestingly, no differences in skeletal muscle citrate synthase activity were seen between sham and MI groups (Koh, Brenner et al. 2003).

Based on the results obtained, it can be concluded that ephrinA1 administration *does not* affect the oxidative capacity of the infarcted heart. However, earlier time points should be examined, and an ischemia/reperfusion model may also be beneficial.

3.4: Conclusions

The data reported in chapter three clearly suggest a protective role for EphrinA1 in the context of acute, permanent myocardial ischemia. Histological analysis revealed a significant reduction in infarct size, necrosis, chamber dilation, and left ventricular wall thinning. We have also observed reduced inflammatory cell infiltration. Cardiac Troponin I levels were significantly reduced with EphrinA1-Fc administration, suggesting reduced myocardial tissue damage. Further, there was a substantial reduction in cleaved PARP protein expression, along with upregulated BAG-1 expression. Combined, this suggests a reduction in cardiac myocyte death. Finally, we have demonstrated that administration of EphrinA1-Fc increases phosphorylated Akt levels.

To our knowledge, we are the first to demonstrate a role for EphA/EphrinA signaling in the context of acute myocardial infarction, and the first to use EphrinA1 therapeutically in this setting. In the context of cardiac repair, reperfusion has become the accepted standard in post-MI therapy, but alone is not sufficient to promote survival or repair of damaged tissue. Angiogenic protein therapies thus far have failed when translated from bench to bedside, due to the formation of a leaky and unstable vasculature. The potential to activate a complex and varied system such as the Eph RTKs is exciting and holds therapeutic promise, due to the potential to modulate specific cellular interactions through individual receptors. Future studies should certainly involve the use of EphrinA1-Fc administration in larger animal models of myocardial infarction, as well as in conjunction with reperfusion therapy. Additionally, the use of receptor-

specific knockout mice will be helpful in elucidating the contributions of each Eph receptor to EphrinA1-mediated cardioprotection.

CHAPTER 4: GENERAL CONCLUSIONS

The work presented in this dissertation is a compilation of studies which investigate two separate mechanisms for understanding tissue salvage following myocardial infarction. In the first study, we have identified a role for the Per2 protein in cardiac repair. Specifically, functional mutation of this protein was protective in the ischemic heart. In the second study, we are the first to identify a protective role for exogenous ephrinA1 administration at the time of MI, and this may open the door to new post-MI therapies involving the Ephs/ephrins. Future studies should aim to develop a detailed understanding of the mechanisms involved in myocardial infarct progression, as well as investigate ways to manipulate this process to enhance repair. Currently, most pre-clinical studies of infarct repair have failed when translated to human beings. Our work provides a new understanding of the contribution of a single clock gene, Per2, to cardiovascular repair. These results initiated a new set of experiments attempting to ameliorate damage caused by infarction through exogenous protein administration (ephrinA1). In the setting of non-reperfused MI, we have presented a novel mechanism for infarct salvage, which may prove to be a legitimate adjuvant, or even alternative, to reperfusion or the newly popular cell therapy.

4.1: mPer2 Mutation is Cardioprotective

In the first set of experiments, we connected a single circadian clock gene, Per2, with infarct repair processes, and concluded that functional mutation of this gene protected the heart

from ischemic damage. Our findings are significant, since they provide a basic understanding of the role of Per2 in cardiovascular repair, and may help explain some of the epidemiological data that has been collected over the last decade. In addition to its role in circadian clock biology, Per2 is a metabolic gene, and may be altering the metabolism of cardiomyocytes, promoting improved cell survival in an ischemic tissue. To further investigate this theory, assays should be performed that evaluate cellular metabolism, including citrate synthase assay to measure mitochondrial content and function. Isolated mitochondria studies may also be useful, to measure O_2 consumption and H_2O_2 emission. Generally, it is accepted that a reduction in infarct size below 20% of the left ventricular area is therapeutic and associated with improved outcome (Miura and Miki 2008). When expressed as a percent of the left ventricle, the mPer2-M mice had an infarct size of approximately 24%, which is certainly closer to this therapeutic window than the 37% infarct size of WT mice.

Of interest to this study was preliminary data from our laboratory (Figure 2.6) which showed that there was increased ephrinA1 protein expression in mPer2-M mice. Since this protein has been associated with pathological angiogenesis, and we observed increased capillary density in mPer2-M mice after infarction, we hypothesized that administration of ephrinA1 protein to infarcted mice would provide protection.

4.2: EphrinA1 Administration Promotes Infarct Salvage

The second set of experiments was initially an attempt to exploit a system with a known role in angiogenesis, in an attempt to promote revascularization of the infarcted heart. Our

findings were surprising, in that there was a robust reduction in infarct size, which was not paralleled with increased capillary density or endothelial cell proliferation. Clearly, at least in the acute stages after MI, some other mechanism was responsible for the beneficial effects observed. The mechanism is not fully clear yet, but there are several possibilities. For example, it is possible that the reduced infarct size was a direct modulation of inflammatory cell recruitment by ephrinA1, and that less inflammation resulted in less tissue damage. Equally plausible, though, is that less tissue damage, produced by a separate mechanism, simply resulted in reduced inflammation. Because ephrinA1 administration resulted in increased phosphorylated Akt, it is highly likely that survival pathways were modulated and less tissue damage was the end result. Clearly, studies must be done to clarify the means by which this signaling cascade is working in the heart.

As discussed earlier, it is generally accepted that reducing infarct size below 20% of the total left ventricle is therapeutic (Miura and Miki 2008). Clearly, the ephrinA1-Fc administration brings our infarct sizes close to this target (22.85%), and additional studies with higher doses may prove to be even more effective. An added benefit is that, unlike reperfusion therapy, ephrinA1-Fc administration attenuates the inflammatory response, which reduces the possibility of further tissue damage from therapy.

Certainly, these pre-clinical results are promising, and these studies should be scaled to larger animal models to assess their translational potential. In addition, administration of ephrinA1 in the setting of reperfusion, in animals with diseases such as diabetes, or coupled with other pharmacological therapies such as statins, would be essential. Functional measurements should also be made, to assess the ability of ephrinA1 to improve cardiac performance. Multiple injections, rather than one single bolus, may also prove to be beneficial in large animal models.

However, due to the small size of a mouse heart, this likely would have produced *more* damage. It would also be useful to assess the ability of ephrinA1 to improve stem cell retention and survival in an infarcted heart, since this has been an ongoing challenge in the regenerative medicine field. Many questions remain unanswered, but the studies described here provide much to be optimistic about. Ultimately, protein therapy, such as ephrinA1, may prove to be a successful alternative to cell therapy. Because of the potential to activate or repress multiple receptors on several different cell types, the ephrin family may provide a unique opportunity for cardiac repair that has not been available with previous methods.

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APPENDIX

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

March 11, 2009

Jitka Virag, Ph.D.
Department of Physiology
239 Ed Warren Life Sciences Bldg.
ECU Brody School of Medicine

Dear Dr. Virag:

Your Animal Use Protocol entitled, "Murine Model of Myocardial Infarct Repair," (AUP #Q228a) was reviewed by this institution's Animal Care and Use Committee on 3/11/09. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to biohazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

East Carolina University

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Animal Care and
Use Committee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

December 8, 2009

Jitka Virag, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Virag:

The Amendment to your Animal Use Protocol entitled, "Murine Model of Myocardial Infarct Repair", (AUP #Q228a) was reviewed by this institution's Animal Care and Use Committee on 12/8/09. The following action was taken by the Committee:

"Approved as amended"

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure