JUNCTIONAL ADHESION MOLECULE-A PREVENTS CARDIAC

RUPTURE FOLLOWING MYOCARDIAL INFARCTION

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Summer 2011

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ACKNOWLEDGMENTS

all his advice as a member of my committee. Our discussions helped me understand my project, the previous research that was done, and the next step that we needed to take. Your expertise was a great benefit to my development and this project.

A special thank you goes to the fellow graduate students in my office who were always there to keep me sane. Thank you to Kristen Howell, Sasha Moseychuk, Michelle Pusey, and Bre Martin. I don't know how I could have got through grad school without your support.

I also recognize my family for always letting me pursue my dreams. You are extremely supportive of everything I do and have molded me into the person I am today. Thank you for giving me every opportunity to succeed and tons of great memories. Finally, I would like to thank my girlfriend Jacque Healey. You are there for me when I'm celebrating a positive result or depressed over a failed experiment. Our five years together have been amazing and I am always appreciative of everything you do for me.

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LIST OF ABBREVIATIONS

%FS   Percent Fractional Shortening

4E-BP1   4E Binding Protein 1

α-SMA   Alpha Smooth Muscle Actin

Apaf-1   Apoptotic Protease Activating Factor-1

ATP   Adenosine Triphosphate

Bax   Bcl-2-Associated X Protein

Bak   Bcl-2-Antagonist/Killer

Bcl-2   B-Cell Lymphoma-2

CRP   C-Reactive Protein

CVD   Cardiovascular Disease

DCM   Dilated Cardiomyopathy

DISC   Death Induced Signaling Complex

DNA   Deoxyribonucleic acid

ECM   Extracellular Matrix

EMT   Endothelial Mesenchymal Transition

eNOS   Endothelial Nitric Oxide Synthase

ER   Endoplasmic Reticulum

ERDF   Endothelial Derived Hyperpolarizing Factor

ERK1/2   Extracellular Signal-Regulated Kinase 1/2

FGF-2   Fibroblast Growth Factor-2

FOXO	Forkhead Box O
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GLUT4	Glucose Transporter 4
GSK-3β	Glycogen Synthase Kinase-3β
H&E	Hematoxylin and Eosin
HBSS	Hank's Balanced Salt Solution
HIF-1α	Hypoxia Inducible Factor-1α
IGF-1	Insulin-like Growth Factor-1
IgG	Immunoglobulin G
Il	Interleukin
JAM-A	Junctional Adhesion Molecule A
LV	Left Ventricle
LVIDD	Left Ventricular Internal Diameter-Diastole
LVPWD	Left Ventricular Posterior Wall Dimension
MAPK	Mitogen Activated Protein Kinase
MI	Myocardial Infarction
MMP	Matrix Metalloproteinase
mTOR	Mammalian Target of Rapamycin
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NO	Nitric Oxide
PCR	Polymerase Chain Reaction
PDZ	Postsynaptic Density 95/Disc-Large/Zonula Occludins-1

PECAM-1	Platelet/endothelial Cell Adhesion Molecule 1
PI3Kγ	Phosphatidylinositol 3-Kinase γ
PKC	Protein Kinase C
PMN	Polymorphonuclear Cell
PRR	Pattern Recognition Receptors
ROS	Reactive Oxygen Species
S6K1	70-kDa S6 kinase
SAPK/JNK	Stress Activated Protein Kinase/c-Jun N-terminal Kinase
SMC	Smooth Muscle Cell
SPARC	Secreted Protein, Acidic, Rich in Cysteine
SPF1	Supernatant Protein Factor 1
STAT3	Signal Transducer and Activator of Transcription
TGF-β1	Transforming Growth Factor-β1
TIMP	Tissue Inhibitor of Metalloproteinases
TTC	2,3,5 Triphenyl Tetrazolium Chloride
TNF-α	Tumor Necrosis Factor-α
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type

ABSTRACT

Cardiovascular diseases, including myocardial infarction, are the leading cause of death in the western world. While great strides have been made in understanding the pathophysiology and treatment of myocardial infarction, further elucidation will undoubtedly lead to better treatment options in the future. Myocardial infarction is the death of cardiac tissue due to a loss of oxygen and nutrients. Myocardial infarction may be followed by cardiogenic shock, ventricular arrhythmia, or cardiac rupture which can be responsible for sudden death. The cardiac repair process involves inflammation and the removal of dead cells which are replaced by deposited granular tissue providing tensile strength to the damaged area. This study details the role of Junctional Adhesion Molecule-A in the pathogenesis of myocardial infarction and in the cardiac repair process.

In order to determine the role of JAM-A in myocardial infarction, a surgical technique was utilized in which the left anterior descending coronary artery of wild type and *Jam-A* $^{gt/gt}$ mice was ligated, leading to a permanent ischemic injury. Following this procedure, *Jam-A* $^{gt/gt}$ mice exhibited an increased mortality rate attributed to cardiac rupture. Infarct size assessment revealed similar levels of initial injury between WT and *JAM-A* $^{gt/gt}$ mice. Contraction of the infarct occurred in both strains by 72 hours, but infarct size in *Jam-A* $^{gt/gt}$ mice was significantly greater at this timpoint. This indicated that JAM-A may play a role in the cardiac remodeling process. *Jam-A* $^{gt/gt}$ mice also were shown to develop heart failure 21 days following MI surgery.

While *Jam-A* $^{gt/gt}$ mice exhibited a distinct cardiac rupture phenotype following MI, no changes in the transcript levels of inflammatory cytokines, matricellular proteins, and extracellular matrix proteins involved in the cardiac remodeling process were found. There was also no change in signaling associated with the VEGF angiogenic pathway or growth factor signaling through Akt. Finally, JAM-A was not found to be expressed in fibroblasts and could, therefore, not cause an alteration in their matrix deposition. These data suggest that JAM-A plays a cardioprotective role in the heart, protecting it from the adverse condition of cardiac rupture following MI. However, it is still unclear how JAM-A confers this protection.

Chapter 1

INTRODUCTION

Cardiovascular disease (CVD), including heart failure, stroke, and coronary heart disease, is the leading cause of death in the western world. Approximately 1.3 million new coronary events develop each year resulting in 425,000 fatalities[1]. Thus, CVD accounts for one in every six deaths in the United States. Of particular interest is myocardial infarction (MI). 8.5 million individuals living in the United States today have had at least one MI[1]. The past decade has seen a distinct decrease in the incidence of both CVD and MI. In 2000, there were 280 new cases of MI per 100,000 individuals. This number decreased to 225 new cases per 100,000 individuals in 2008. This reduction results from increased awareness for the benefits of aerobic exercise and healthy blood pressure as well as the declining rate of cigarette smoking [2]. Research over the past three decades has led to numerous breakthroughs in the treatment of MI patients. These include various antithrombotic and fibrinolytic medications that disintegrate blood clots, angioplasty, coronary reperfusion, and coronary bypass surgery[3]. These strategies can increase lifespan and health following MI and some, if administered quickly following the ischemic event, can prevent the full extent of damage from occurring to the heart. Still, more research into the cellular mechanisms associated with ischemia in the heart and the intricacies of cardiac remodeling following MI will undoubtedly lead to more effective treatment and preventative options in the future.

1.1 Pathogenesis of Myocardial Infarction

MI, commonly referred to as a heart attack, is the death of cardiac tissue caused by a decreased supply of oxygen and other nutrients. In 2000, the American College of Cardiology set distinct parameters as to what constitutes an MI. These include myocardial cell death, the loss of electrically functioning cardiac tissue diagnosed using electrocardiography as a lengthened ST segment and/or loss of the Q wave, abnormalities in ventricular wall contractility assessed using echocardiography, and the presence of biological markers of myocyte death in the blood stream such as T- and I-troponin and creatinine kinase[4]. The resulting myocardial injury decreases the contractility of the heart and lessens cardiac output. If the damage is too severe, MI can lead to cardiac rupture, shock, ventricular arrhythmia, pulmonary edema, or death.

While MI is a prevalent and often deadly disease, many of its risk factors are largely preventable. The INTERHEART study, which analyzed nearly 30,000 male and female individuals worldwide, identified nine distinct risk factors. Since diet and exercise are the main determinant of cardiovascular health and the rate of atherosclerotic plaque buildup, it was not surprising that many of the risk factors for MI are related to obesity. Patients with hypertension, type 1 or type 2 diabetes, abdominal obesity, a fatty diet devoid of fruits and vegetables, an elevated apolipoprotein B/apolipoprotein A-1 ratio indicative of altered plasma lipid levels, and a lack of physical activity all greatly increase the risk of MI. Also, being a current or former smoker increases the risk. Psychosocial factors such as self-reported stress and depression also increase the risk of MI. Due to differences in the prevalence of these risk factors between the sexes, the first incidence of MI occurs on average, 9 years

later in women than men. Taken together, these findings indicate that 94% of all MI cases are derived from these preventable risks[5].

Lack of physical activity and unhealthy eating habits often lead to atherosclerotic plaque buildup in the vasculature (Figure 1.1). This plaque can grow large enough to completely occlude a coronary artery, leading to MI. Atherosclerotic occlusion of a coronary artery accounts for 80% of all incidences of MI. Less common causes of MI include the rupture of an unstable atherosclerotic plaque anywhere in the vasculature of the body. Part of the platelet thrombus may break away, travel throughout the vasculature until it encounters a narrow coronary artery, and become lodged stopping the flow of blood. Furthermore, platelet aggregates developed in a deep vein thrombosis may also break away and lodge itself in a nonatherosclerotic coronary artery preventing blood flow. Additionally, MI is seen after prolonged ischemia due to cardiac arrest and resuscitation[6].
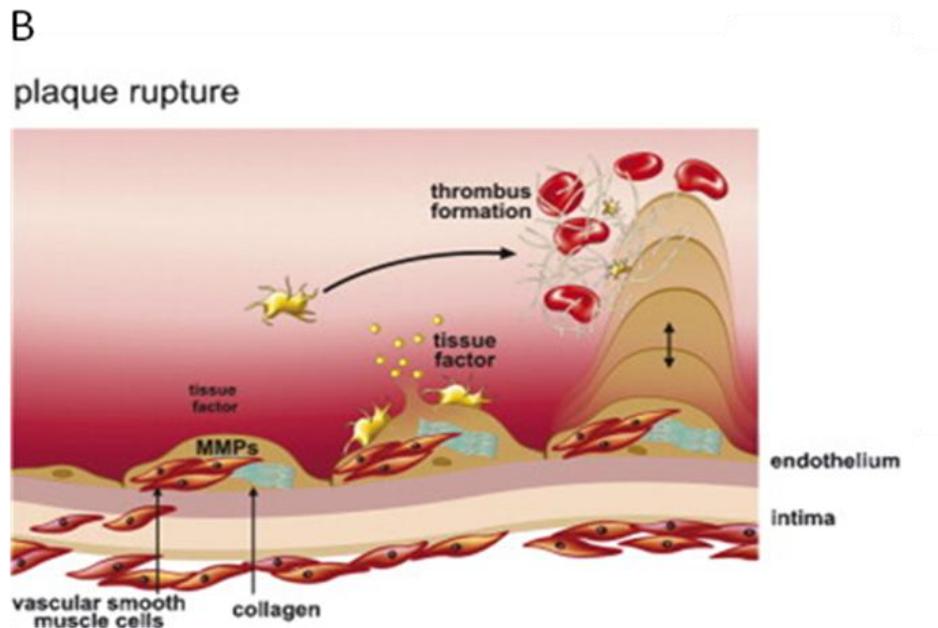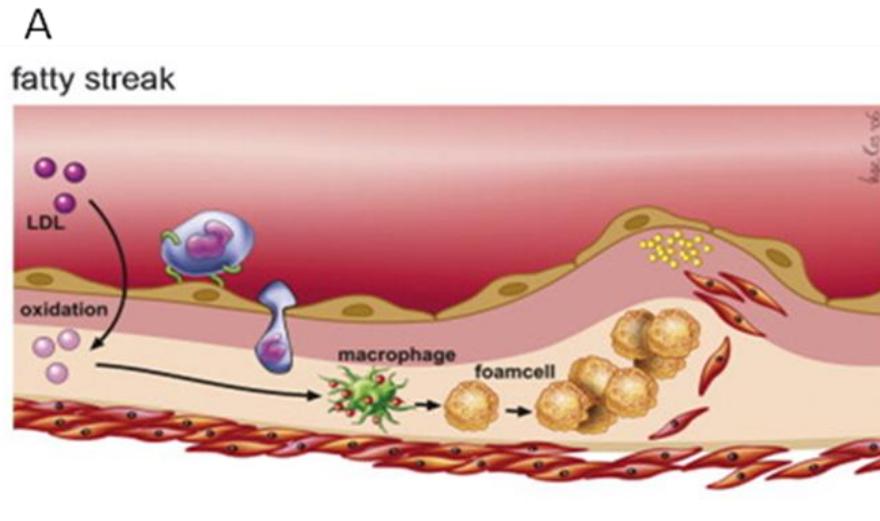
Figure 1.1: *Atherosclerotic Plaque Buildup and Rupture*
(A) Oxidized low density lipoproteins (LDL) are transported across the endothelium by scavenger receptors. Macrophages phagocytose the lipid particles, but are unable to degrade them. The lipids are stored within the macrophage which is now called a foam cell. (B) The activation of macrophages leads to cytokine release which causes dysfunction of the SMCs and fibroblast deposition of matrix proteins. The plaque expands and exposes ECM proteins of the endothelial basement membrane causing platelet aggregation and thrombus formation[7].

1.2 Cellular Response to Ischemia

Following an ischemic attack, not all cells distal to the occlusion die from hypoxia. The dense capillary network of the heart provides some nutrient supply to the tissue near the occlusion, preventing cell death. In this area, the little blood supply available causes the cells to undergo either autophagy or apoptosis[8]. This area of viable tissue is called the border zone and will be host to an intense inflammatory response to salvage the area. More distant from the site of occlusion, simple diffusion is the only mechanism of nutrient delivery to the tissue. Cells in this area with access to an acutely diminished oxygen supply undergo apoptosis. In the most remote area from site of occlusion, usually the apex of the heart, cells quickly and irreversibly become hypoxic causing cell necrosis[9].

Cellular changes in both the vasculature and cardiac muscle tissue itself occur quickly in response to ischemic stress. Cardiac myocytes, the muscle cells responsible for maintaining heart contractions, are highly metabolic with a large demand for oxygen and adenosine triphosphate (ATP). For this reason, myocytes have evolved several mechanisms to store oxygen and high energy phosphates as a reserve energy source during times of need. One such mechanism is the storage of oxygen in myoglobin. This protein is structurally similar to the blood protein hemoglobin, but becomes saturated at a lower oxygen concentration. This allows myoglobin to sequester oxygen inside the cell[10]. Myoglobin is able to support the metabolic activity of a hypoxic myocyte for approximately 8 seconds. Following this, the cell switches from aerobic to anaerobic respiration. High energy phosphates contained in ATP and the myocyte specific storage protein creatinine kinase decrease substantially after ischemia. After 30 seconds, 80% of the stored phosphates are depleted[11]. The switch

to anaerobic respiration forms new ATP through the breakdown of glycogen, but the acidic byproduct lactate is produced. Within ten minutes of ischemia, intracellular pH decreases to 6.0 or lower. With too few ATP molecules to properly regulate sodium potassium ATPases in the membrane, the electrochemical gradient of the myocyte becomes disregulated causing depolarization. This depolarization opens voltage gated calcium channels leading to the activation of calcium sensitive proteases and phospholipases inside the cell. Cell swelling also occurs as water diffuses into the cell by osmosis due to the increased intracellular ion concentration. Taken together, cellular edema and the destructive activity of the proteases and phospholipases lead to the necrosis of acutely hypoxic cells[12, 13]. Necrotic cells will burst, spewing their intracellular contents out haphazardly into the interstitial fluid. Necrosis occurs throughout the ischemic area, but is becomes predominant at points farther from the occlusion.

For ischemic cells closer to the occlusion that are supported mainly through passive diffusion and a diminished blood supply delivered by anastomoses, only the most basic cellular functions can be maintained. These cells undergo the energy dependant, orderly process of cell death known as apoptosis. While necrosis peaks approximately 24 hours following MI, apoptosis can continue for one week or longer and extend into the chronic period of remodeling[14]. This process is highly conserved and exhibits hallmark characteristics including the condensation and cleavage of deoxyribonucleic acid (DNA) inside the nucleus. The organelles and proteins inside apoptotic cells are randomly degraded by activated caspases, proteases, and other destructive proteins. The cell itself shrinks, invaginates, and blebs off forming tiny vesicles of cellular components that can be removed by inflammatory

phagocytes. This process allows the dead cell to be easily phagocytosed by leukocytes without damaging other viable cells in the area. Apoptotic signaling is tightly regulated and is initiated by both internal and external signals. Of these, the intrinsic pathway is highly active following MI. Various stressful signals including hypoxia, loss of growth factor and survival signal receptor binding, and ER or mitochondrial stress, all channel into the regulation of the B-cell lymphoma-2 (Bcl-2) family of proteins. Of these, Bcl-2–associated X protein (Bax) and Bcl-2–antagonist/killer (Bak) activation are required for apoptosis through the intrinsic pathway. It is believed that these and other accessory proteins create a pore forming complex that permeabilizes the mitochondria, allowing the release of cytochrome c. Cytochrome c then binds apoptotic protease activating factor-1 (Apaf-1) and complexes with procaspase 9 forming an apoptosome. The apoptosome subsequently activates the caspase cascade. Caspase proteins are responsible for the indiscriminate proteolytic cleavage of intracellular contents[15].

Once inflammation begins following MI, macrophages and other leukocytes will release Fas ligand, the driving force for the external apoptotic pathway. Nevertheless, this pathway is less dominant in the infracted myocardium than internally regulated apoptosis. Fas ligand binding to the Fas receptor triggers the formation of the Death Induced Signaling Complex (DISC). This complex directly activates procaspase 3 and the subsequent caspase cascade[12].

The pathways of autophagy following myocardial infarction are just beginning to be understood in the heart. Autophagy is a cellular process involving the destruction of entire organelles inside an autophagosome. Under normal conditions, this process is initiated to remove old or damaged organelles from the cell. Under pathological conditions or a chronic loss of nutrients, autophagy of the mitochondria

and other organelles can distinctly lower the energy requirements of the cell permitting the cell to survive in stressful environments. It is known that hypoxic conditions somehow lead to BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3) activation. Bnip3 is known to downregulate mammalian target of rapamycin (mTOR) which initiates the autophagy pathway. The mitochondrion, and a few other organelles of the hypoxic myocyte, becomes surrounded by a lipid bilayer. This bilayer will then dock and fuse with a lysosome, allowing the organelle to be broken down[16, 17]. By decreasing the total number of organelles, especially mitochondria, the overall energy consumption of the cell decreases. With the decrease in nutrient supply present at the border zone, autophagy is a method for the myocytes to remain viable. However, it is unknown if this process causes a decrease in the contractile ability of the muscle tissue.

Cardiac muscle tissue is not the only structure to undergo physiological changes caused by ischemia. In the vasculature, the coronary arteries undergo vasodilation in order to recruit oxygen rich blood to the area of need. This process is mediated by both the endothelial and SMCs of the vasculature. Contraction and relaxation of the SMCs controls vascular tone and the flow of blood through the artery. In the SMCs, decreased ATP levels induced by hypoxia prevent the activity of sodium potassium ATPases leading to depolarization. The subsequent drop in intracellular potassium causes relaxation of the SMCs and vasodilation[18]. Hypoxic endothelial cells also release factors that regulate vascular tone by inducing vasodilation including prostacyclin, nitric oxide (NO), and a group of compounds called endothelial derived hyperpolarizing factors (EDHF). Of particular importance is the gaseous signaling molecule, nitric oxide. NO is synthesized inside endothelial cells from L-arginine by

endothelial nitric oxide synthase (eNOS). This enzyme is triggered by growth factor signaling, acetylcholine release, shear stress, and platelet aggregation. However, the exact mechanism of NO upregulation in pathologic hypoxia is still unknown[19]. Being nonpolar and gaseous, NO diffuses freely across cellular membranes. In SMCs, NO activates soluble guanylyl cyclase leading to cyclic guanosine monophosphate (cGMP) production and vasodilation[20]. While vasodilation of the coronary arteries occurs, this response is rendered useless as no increase in blood flow is possible through the occlusion. However, NO signaling to hypoxic cells, particularly myocytes, does have an important consequence. NO binds to the active site of cytochrome c oxidase, the last enzyme in the mitochrondrial respiratory transport chain. Cytochrome c oxidase transfers free electrons produced during aerobic respiration to an oxygen ion, allowing it to form a water molecule. By preventing the activity of this enzyme, NO slows down the metabolic activity of all cells in the hypoxic area to decrease the demand for oxygen[21].

1.3 The Cardiac Remodeling Process

1.3.1 Introduction

It was traditionally accepted that cardiac myocytes are terminally differentiated and are unable to proliferate. Therefore, there is no way to increase myocyte number when challenged with high blood pressure or pathological conditions such as aging or MI. This belief has recently been challenged with the identification of cKit+/CD45- cells that have been shown to produce new myocytes both *in vitro* and *in vivo*[22]. While these cells may provide therapeutic opportunities in the future, there is currently no way to repopulate dead myocytes in the ischemic myocardium with viable

cells. Instead, the heart undergoes an intricate repair process involving inflammation, angiogenesis, and fibrosis to provide the damaged area with tensile strength (Figure 1.2). The ischemic area of the heart becomes thin as leukocytes remove the damaged tissue, but local scar formation prevents myocardial rupture. Viable ventricular myocytes surrounding the infracted area undergo elongation and loosening of the extracellular matrix to increase end diastolic volume, counteracting the loss of contractility. The increase in diastolic volume exerts stress upon the ventricular wall. To compensate, eccentric hypertrophy occurs to increase wall thickness to withstand the stress. Unfortunately, the loss of myocytes has permanently prevented this area from contracting.
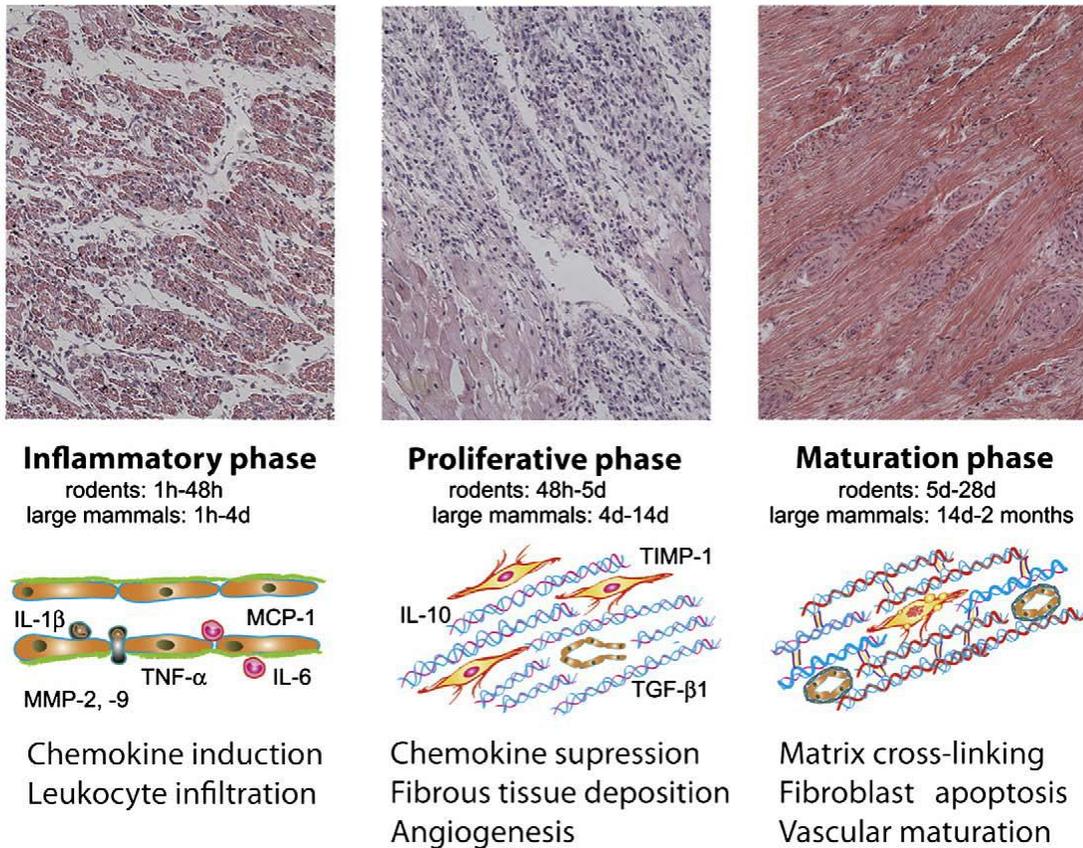
Figure 1.2: *The Phases of the Cardiac Remodeling Process*
The inflammatory phase begins immediately following MI as leukocytes infiltrate into the ischemic area and release inflammatory cytokines. The dead tissue is degraded. As the cytokine microenvironment changes with the migration of CD4+ helper T-cells, phagocytosis comes to an end. Fibroblasts migrate into the area and begin to lay down ECM proteins necessary for scar formation. Growth factor upregulation leads to angiogenesis and cell survival. In the maturation phase, myofibroblasts crosslink the ECM creating a complex scar. The new blood vessels mature and the heart is given tensile strength to resist cardiac rupture[23].

1.3.2 The Inflammatory Phase

Inflammatory cells, especially macrophages, dendritic cells, and plasma cells, can be found in all tissues of the body. These, and other innate immune cells, quickly activate when microbial invasion or local cellular damage occurs. Their activation depends on the binding of antigenic peptides to a group of membrane receptors known as pattern recognition receptors (PRRs). These receptors bind short protein sequences and lipopolysaccharides expressed both from external microbial sources and internally in normally functioning somatic cells. When necrosis occurs 20-40 minutes following ischemia, the breakdown of the phospholipid membrane allows the cellular contents to be expelled. This triggers the inflammatory phase of the cardiac remodeling process which begins immediately following MI and may last up to 4 days in humans and 48 hours in rodents[23]. The release of intracellular components such as heat shock proteins and fragments of hyaluronin and fibronectin from the damaged cells activates one class of PRRs called the toll-like receptors (TLR). Binding of these ligands to the TLR induces resident macrophages to release inflammatory cytokines, reactive oxygen species (ROS), pentraxins such as C-reactive protein (CRP), and the activation of intracellular signaling molecule nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[24]. NF-κB signaling in the macrophage is essential for the immune response. Over 50 genes for key inflammatory mediators such as interleukin-1β (Il-1β), tumor necrosis factor-α (TNF-α), matrix metalloproteinase 9 (MMP 9), and various cell adhesion molecules are activated by this transcription factor[25]. These cytokines affect all cells in the ischemic area and create a proinflammatory microenvironment necessary for cardiac remodeling.

Activated macrophages also begin to phagocytose necrotic cells and the blebbed membrane fragments of apoptotic cells.

One important effect of the mediators released by macrophages is the modulation of signaling in the vascular endothelium. The release of cytokines, particularly Il-1β and TNF-α, and reactive oxygen species during the innate response to tissue damage activates the endothelial layer. This activation has two major consequences. First, endothelial cells secrete many inflammatory mediators of their own. These include various interleukins, growth factors, and chemotactic compounds. The release of the CXC, CC, and CX3C families of chemotactic proteins creates a concentration gradient surrounding the infracted area. This attracts circulating inflammatory cells in the blood to the area of damage. Secondly, transcription for cell adhesion molecules such as various selectins, CD-44, inter-cellular adhesion molecule-1 (ICAM-1), P-selectin, and junctional adhesion molecule-A (JAM-A) is upregulated. Surface expression of these adhesion molecules increases and circulating inflammatory cells become clustered on the apical surface of the endothelial cell, priming the endothelium for leukocyte transmigration. Thus, the initial inflammatory burst by resident macrophages activates the endothelium, attracting other leukocytes into the area and allowing their transmigration into the cardiac tissue[26] (Figure 1.3).

As leukocytes flow through the blood stream, they are triggered by the chemokine gradient to increase their own surface expression of adhesion molecules. When the leukocyte encounters a primed endothelium, selectin molecules on both the leukocyte and the endothelial cell will bind. This forces the leukocyte to roll along the
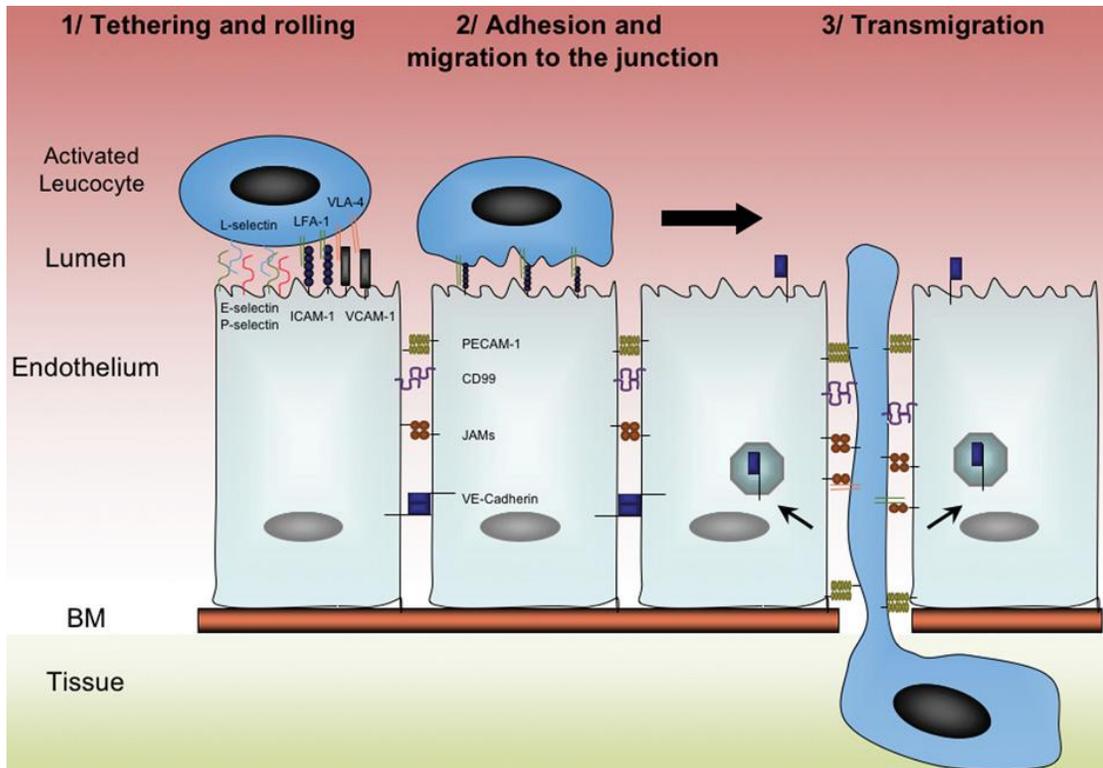
Figure 1.3: *Leukocyte Transmigration through the Vascular Endothelium*
Initial contact between the leukocyte and endothelium is made between various
selectin molecules. This allows the leukocyte to roll, slowing its travel through the
blood stream. Once the speed of the leukocyte has been reduced, stable interactions
between cell adhesion molecules and integrins on the leukocyte surface tether the cell
to the endothelium. Integrin binding allows lamellipodia formation permitting the
leukocyte to squeeze between the endothelial layer. The dissolution of the endothelial
tight junction allows the junctional proteins to bind the leukocyte and usher it
through[27].

endothelium as it breaks and forms numerous selectin interactions. Leukocyte rolling slows the cell down and allows binding of adhesion molecules such as ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1) to integrins, specifically CD11a/CD18 or CD49d/CD29, firmly anchoring the leukocyte on the endothelial surface. ICAM-1 and VCAM-1 binding induces intracellular calcium release and Rac1 activation in the leukocyte. Activation of Rac1 causes the leukocyte to rearrange its cytoskeleton, allowing the leukocyte to form lamellipodia and search for the junction between two endothelial cells. Once found, binding of the leukocyte to platelet/endothelial cell adhesion molecule 1 (PECAM-1) and tight junction proteins such as JAM-A allows the leukocyte to move through the cell junction[28]. The leukocyte must also break through the basement membrane of the endothelium. This is accomplished by the release of MMP9. MMPs are a family of zinc dependant proteases that degrade specific components of the extracellular matrix. MMP9 is a gelatinase, able to degrade fibrillar collagen, fibronectin, laminin, and proteoglycans. By releasing this compound, the leukocyte is able to degrade the basement membrane of the endothelial cell and finally break through to the tissue layer. Studies have shown that MMPs, especially MMP9 and the leukocyte/endothelial derived MMP2, are upregulated in the infracted area following MI. There, MMPs play an essential role in breaking down the ECM surrounding dead and damaged myocytes so they can be easily phagocytosed. The activity of the MMPs is opposed by the tissue inhibitor of metalloproteinases (TIMPs). All four members of this enzyme family bind to the catalytic domain of the MMP and prevent its function. TIMP activity is crucial in the healing infarct to progress from the initial stages of tissue degradation to the synthesis of the matrix scar[29].

The increase in leukocyte number in the infracted area leads to the release of numerous inflammatory cytokines. One important cytokine is interleukin-6 (Il-6), which is released by both macrophages and endothelial cells upon activation. Il-6 activates immune cells, heightening their response. It also travels through the blood stream and can activate the brain to increase body temperature. Most importantly, Il-6 induces signal transducer and activator of transcription 3 (STAT3) signaling in liver hepatocytes leading to CRP production. CRP acts similarly to immunoglobulin G (IgG) in that it can bind antigen and activate the complement system. CRP binds a number of intracellular compounds released following necrosis including phosphatidylcholine residues, histones, and monophosphate esters found in amino acids. CRP then binds to the C1 complement protein, leading to the activation of the complement cascade. The purpose of this cascade is to surround the antigen with complement proteins and antibody to allow for heightened phagocytosis by macrophages. This process is called opsonization. Without CRP, the clearance of the dead tissue in the infracted region would take much longer and cause dysfunction in the heart[30].

Other inflammatory cytokines that play a key role in the early inflammatory phase of cardiac remodeling include Il-1β and TNF-α. While TNF-α is a key acute inflammatory mediator, Il-1β plays a role throughout the entire course of cardiac remodeling. They both activate survival pathways and gene transcription in all cell types in the area. TNF-α induces ROS and NO formation, pro-inflammatory and pro-coagulant production in the endothelium, angiogenesis, and reduces calcium loading and contractile function of myocytes protecting them from excessive energy consumption[31]. In the inflammatory phase, Il-1β signals through the mitogen activated

protein kinase (MAPK) cascade leading to multiple changes in endothelial cells. These include the production of adhesion molecules needed for leukocyte transmigration, growth factors, chemokines, NO, and prostaglandins. Later, Il-1β will modify the activity of fibroblast cells allowing them to produce a collagenous scar[32].

1.3.3 The Proliferative Phase

The inflammatory phase of cardiac remodeling is both destructive curative. ECM proteins are degraded and necrotic tissue is removed by macrophages but numerous enzymes and processes are activated that enhance wound healing. Towards the end of this first phase, Th2 CD4+ helper T-cells migrate into the area of infarction driven by the cytokine and chemokine gradient. The appearance of these cells begins the proliferative phase of cardiac remodeling. This phase takes place approximately 4-14 days post-MI in humans[23]. Th2 helper T-cells become activated by the cytokine microenvironment of the healing infarct and release numerous cytokines of their own. These substances modify the activity of all cells in the area, in particular macrophages and fibroblasts. Th2 cells synthesize and secrete Il-10 and transforming growth factor-β1 (TGF-β1). Il-10 is a potent inhibitory cytokine affecting nearly all cell types. It's role is to downregulate the secretion of the acute phase inflammatory cytokines including TNF-α, Il-1β, -6,-8, and -12. Importantly, Il-10 has drastic effects on activated macrophages. It causes these cells to completely stop phagocytosis and the release of MMPs. Instead, Il-10 triggers these cells to produce TIMPs to inhibit matrix degradation[33].

The other key mediator released by Th2 helper T-cells is TGF-β1. In fact, the changing cytokine microenvironment of the proliferative phase causes many cell

types to produce this cytokine including endothelial cells, macrophages, and fibroblasts. This cytokine is considered the key molecular switch to trigger the deposition of ECM proteins to form a fibrous scar. The three isoforms of TGF-β1, by signaling through MAPKs, RhoA, and the SMADs, exhibit a variety of cellular responses in multiple cell types. TGF-β1 signaling leads to myocyte hypertrophy and decreased cytokine, chemokine, and ROS production in macrophages. It causes endothelial cells to suppress adhesion molecule expression and chemokine synthesis, and aids in the initiation of angiogenesis. It also causes the activation of fibroblasts and is required for their differentiation to myofibroblasts[34]. All these changes are designed to allow the heart to grow new blood vessels, deposit scar tissue to give the infracted area tensile strength, and induce hypertrophy of surrounding myocytes to allow continued pumping activity of the heart.

 Following myocardial injury, cardiac myofibroblasts are the key cell type responsible for the deposition of new matrix proteins and the formation of proper matrix crosslinking. In the healthy myocardium, fibroblasts anchor themselves to both the ECM and cardiac myocytes where they maintain proper levels of ECM degradation and deposition. Normally, no myofibroblasts are present. Following myocardial injury, fibroblasts become activated by growth factors including TGF-β1 and fibroblast growth factor-2 (FGF-2) as well as shear stress to become proto-myofibroblasts. They then differentiate further into mature myofibroblasts which contain a higher amount of actin and myosin, allowing these cells to adopt SMC-like properties. The movement of the thinned ventricular wall during contraction exposes cells in this area to high levels of mechanical stress. By being able to quickly adapt their cytoskeleton to the changing

environment, the myofibroblasts can withstand these stresses in order to deposit new matrix materials[35].

While a number of fibroblasts are resident to the infracted area at all times, the number of differentiated myofibroblasts increases dramatically following injury. This increase is a result of a number of pathways. First, the cytokine gradient existing in the infracted area draws in neighboring fibroblasts from the surrounding heart tissue. Also, differentiation of new fibroblasts from stem cells within the bone marrow occurs and is responsible for approximately 50% of all fibroblasts in the healing infarct. Finally, proto-myofibroblasts can be derived from other cell types already existing in the infracted area including monocytes and endothelial cells[36].

Whatever their origin, fibroblasts differentiate into myofibroblasts and begin scar formation by secreting ECM proteins. The scar material is made up of nearly 80% collagen 1, 10% collagen III, and a number of other ECM proteins including laminin, elastin, and collagens IV, V, and VI[35]. Myofibroblasts also secrete a number of matricellular proteins into the ECM to regulate their own matrix production and help to properly package the secreted ECM proteins into a functioning scar. These include periostin, secreted protein acidic rich in cysteine (SPARC), osteopontin, tenascin-C, and thrombospondin. Tenascin-C is largely an autocrine factor that increases the secretion of ECM components. Tenascin-C has also been shown to cause detachment of myocytes from their basement membrane leading to anoikis. Periostin contains four fasciclin domains and is able to bind multiple types of ECM components simultaneously. It helps myofibroblasts bind to and migrate around the newly deposited ECM and mediates the tight association between collagen and other ECM proteins. SPARC directly binds collagen and fibronectin fibers regulating the assembly

of their tertiary structure. In addition to the formation of a fibrous scar, myofibroblasts help maintain a proper balance between TIMPs and MMPs to properly remodel the matrix[37].

Inflammatory cytokines and growth factors released during the late inflammatory and proliferative phases include vascular endothelial growth factor (VEGF), FGF-2, TGF-β1, the angiopoietins, TNF-α, Il-6, and Il-8. All these factors signal endothelial cells to produce new blood vessels in a process called angiogenesis. Since blood supply to the infracted area was halted, new vasculature must be constructed in this area to restore blood flow. The most important growth factors in this process, VEGF, FGF-2, and the angiopoietins, are upregulated within hours after occlusion in response to hypoxia[38]. The mechanism of VEGF upregulation is well documented. Hypoxia inducible factor-1α (HIF-1α) becomes active in the presence during low intracellular oxygen and binds to hypoxic response elements in the nucleus. This leads to gene transcription for a number of targets including VEGF[39].

Endothelial cells surrounding the area of infarction become activated by these growth factors and secrete MMPs to break down their own basement membrane. The junctions between neighboring endothelial cells will break, allowing them to migrate and sprout off to form a new vessel branch. The direction of migration is determined by the growth factor gradient. As the endothelial cells separate, the dissolution of the tight junction causes a momentary increase in vascular permeability. Once in position, the endothelial cells proliferate and the tight junctions are reformed[40].

1.3.4 The Maturation Phase

The maturation phase is the final step in the cardiac remodeling process and begins around 14 days post-MI in humans[23]. The maturation process may last up to two months or longer, depending on the infarct size. During this phase, endothelial and myofibroblast cells release even more matricellular proteins that cause the formation of a complex crosslinked scar. These proteins, such as lysyl oxidase and prolyl hydroxylase, catalyze reactions that bind neighboring collagen strands with each other as well as with other matrix proteins[41]. The hallmark of this phase is the decrease in myofibroblast number. The exact trigger and mechanism for this decrease is still debated; however, most agree that the myofibroblasts eventually undergo apoptosis. Since myofibroblasts are activated by shear stress, it is possible that the maturation of the scar creates a more rigid and stable environment that deactivates the stress induced survival pathways in these cells. Still, some myofibroblasts remain permanently within the scar indicating that constant matrix remodeling takes place indefinitely[23]. Also during this phase, the final maturation of newly formed vasculature occurs. The endothelial cells of the blood vessels lay down basement membranes and become stably linked to each other. Also, mural cells such as pericytes and SMCs surround the endothelial layer to form a fully functioning vessel[42].

1.4 Hypertrophy and Heart Failure

In order to compensate for the loss of myocytes in the infracted tissue, the ventricle must increase its end diastolic volume as per Starlings Law. This process is caused by an increase in myocyte size and a loosening of the extracellular matrix

allowing the heart to preserve stroke volume. The ventricular wall must undergo hypertrophy and thicken to withstand the increased intraventricular pressures. This type of hypertrophy, called eccentric hypertrophy, is triggered when mechanoreceptors in the myocytes are stretched, activating the renin-angiotensin pathway and causing the release of local growth factors such as endothelin-1. One effect of these factors is to induce fibrosis, which reinforces the tissue in response to the high pressure. Also, myocyte signaling pathways become activated by growth factor release leading to activation of such pathways as phosphatidylinositol 3-kinase γ (PI3Kγ) and Akt. These proteins depress contractility, prevent apoptosis, and allow the transcription of hypertrophic genes. The activation of these genes revert the myocyte back to an embryonic state, allowing the production of contractile machinery such as α-actin, β-myosin heavy chain, and the natriuretic peptides[43]. As contractile proteins are transcribed in eccentric hypertrophy, new sarcomeres are added in series, but not in parallel. This causes the myocyte cell to increase in length but not in overall thickness. As the myocytes lengthen, the overall length of the ventricle increases as well, causing heart to enlarge. If these changes are adequate to sustain the oxygen demand of the body, the heart will continue to pump and survive. However, if the heart is still unable to supply the body with an adequate cardiac output, this hypertrophic process continues. Eventually, myocytes lengthen too much to generate adequate contractile force[44]. This progressive increase in heart size with no beneficial increase in cardiac output is known as heart failure. As heart failure progresses blood pressure will drop, increasing capillary blood pressure. Fluid will move out of the blood causing both interstitial and pulmonary edema. If not corrected, heart failure will progress rapidly until death.

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1.5 Cardiac Rupture

An often fatal complication of MI is cardiac rupture. This describes the physical tearing of the heart muscle tissue, allowing blood to escape from the ventricular chamber. Since the heart is surrounded by a thin membrane called the pericardium, blood released from the heart through the ruptured wall is contained. Cardiac tamponade ensues as the blood filling this cavity exerts pressure on the heart, preventing it from filling properly[45]. The VALIANT study of 2010 estimated that 1% of all individuals with MI will experience rupture. While rupture is a rare complication following MI, it is almost always fatal. The same study indicated that cardiac rupture was responsible for nearly one fourth of all deaths following MI[46]. The most common locations of rupture occur in the interventricular septum, papillary muscles, and the LV[47].

The pathophysiology of cardiac rupture following MI is related to insufficient cardiac repair or an inappropriate response to the ischemic insult resulting in an substantial increase in ventricular wall stress. One possible cause of cardiac rupture lies in the extent of myocardial damage. If damage is too extensive, the remodeling process will thin the ventricular wall to the point where it cannot withstand the hemodynamic load. Also, if the damaged ventricular wall is not properly reinforced by scar tissue, the heart can rupture as well. Numerous mouse models have shown that defects in the cardiac remodeling process leads to cardiac rupture. These include improper regulation of inflammatory cytokines such as TNF-α and Il-10, poor induction of matricellular proteins such as SPARC, periostin, and biglycan, and the

excessive activity of MMPs. Also, defects in collagen production and improper myocyte tight junction formation all lead to cardiac rupture[45].

1.6 Junctional Adhesion Molecule-A

The focus of my research involves Junctional Adhesion Molecule-A (JAM-A), previously known as JAM-1 and F11R. Originally identified as the platelet receptor for the F11 antibody, JAM-A is found on the cell membrane of platelets, endothelial and epithelial cells, select leukocytes such as macrophages and neutrophils, and sperm cells[48, 49]. It is a member of the immunoglobulin superfamily and has two external variable Ig loops. Binding through the first Ig loop allows for homodimerization. In the extracellular domain, two putative sites of glycosylation exist at asparagine 185 and 191[49]. This 34 kDa, 299 amino acid, protein has multiple phosphorylation sites for PKC and other downstream effectors[50]. These sites are known to support the activation of PKC, cGMP or cAMP dependant protein kinase, and casein kinase II[51]. A type II postsynaptic density 95/disc-large/zonula occludins-1 (PDZ) domain binding motif is also present at the very end of the cytoplasmic tail[49]. In endothelial and epithelial cells, JAM-A is present at the tight junction. There, it homotypically binds to other JAM-A proteins allowing neighboring cells to adhere to each other[49]. At the tight junction, JAM-A interacts with the scaffold protein ZO-1through its PDZ domain. This scaffolding anchors multiple tight junction proteins such as occludin[52].

During endothelial activation by growth factors and cytokines, JAM-A dissociates from ZO-1 and the tight junction complex, redistributing itself along the

apical side of the cell[53]. This process is essential for endothelial migration as well as angiogenesis. Our laboratory has shown that JAM-A is essential for FGF-2 induced angiogenesis (Figure 1.5). FGF-2 signaling leads to a dissociation of JAM-A from integrin $\alpha_v\beta_3$ leading to proliferation and migration[54]. In fact, the FGF-2 induced angiogenic pathway in *Jam-A$^{gt/gt}$* mice is completely ablated. Dr. Cooke performed a matrigel plug assay in which WT and *Jam-A$^{gt/gt}$* mice were injected with 200µl of growth factor reduced matrigel with and without FGF-2. After fourteen days, the plug was removed and stained with H&E to detect the presence of vascularization. While blood vessels had formed within the matrigel of WT mice, none were present in the *Jam-A$^{gt/gt}$* FGF-2 treated plug. Unpublished data from our laboratory indicates that VEGF levels in the plasma are increased to compensate for this loss of FGF-2 signaling (Figure 1.6). The redundancy of growth factor signaling in angiogenesis allows *Jam-A$^{gt/gt}$* mice to exhibit normal embryonic and retinal vasculature[55].

While the function of JAM-A on leukocytes is still unknown, it has been shown to aid in leukocyte transmigration through the endothelium. During transmigration, adhesion molecules on the endothelial surface bind the β2 integrin, leukocyte function-associated antigen-1(LFA-1), on the surface of leukocytes. This binding induces endothelial signaling which separates JAM-A/JAM-A homodimers at the tight junction[56]. This helps the tight junction to loosen. JAM-A is then free to bind LFA-1, helping the leukocyte move through the vasculature[57]. JAM-A also plays a role in platelet physiology. In platelets, JAM-A binds to the Fc domain of the membrane receptor FcγRII. Upon stimulation of FcγRII receptor by its ligand F11, the subsequent activation of JAM-A leads to protein kinase C (PKC) phosphorylation. JAM-A activation in the platelet induces aggregation and granule secretion[50].
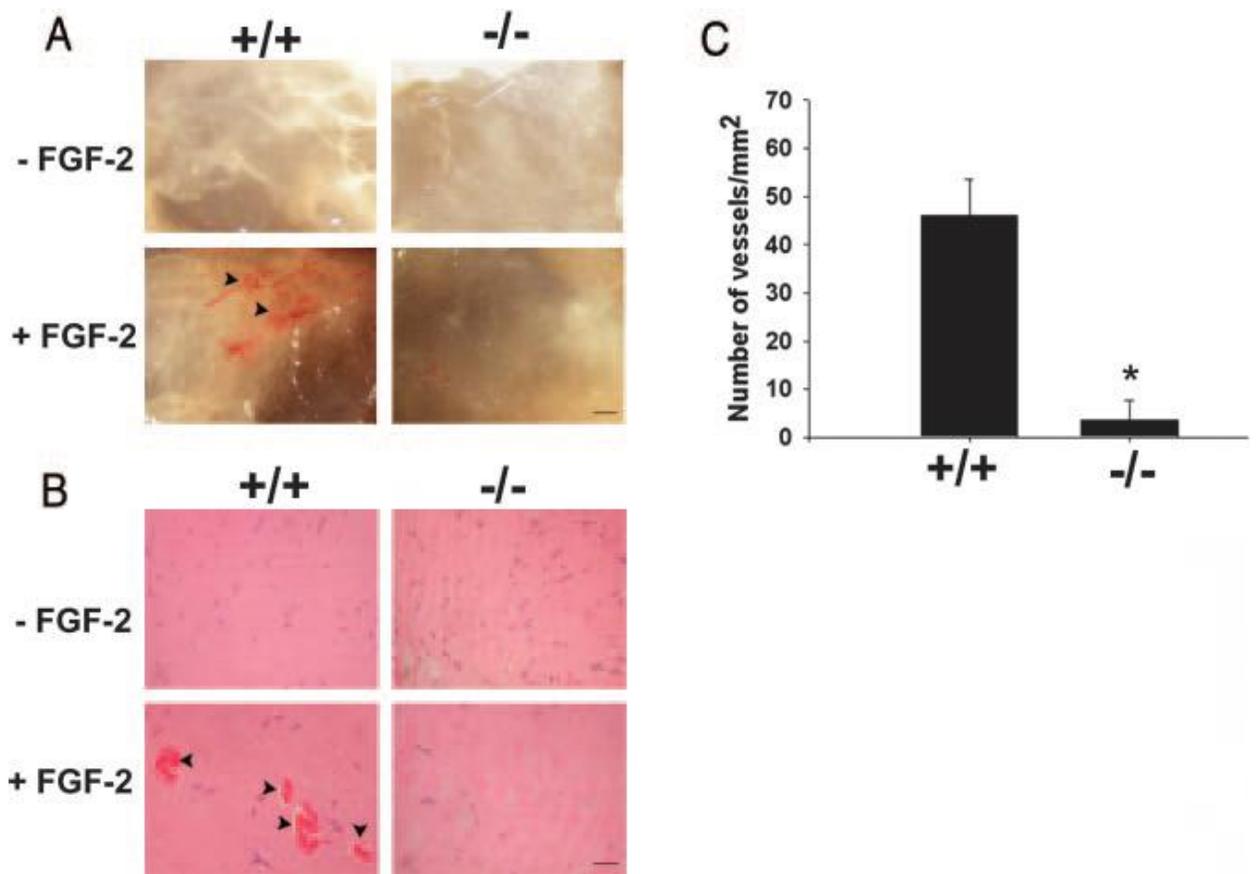
Figure 1.4: *FGF-2 Induced Angiogenesis is Perturbed in Jam-A$^{gt/gt}$ Mice*
(A) Light micrographs of matrigel plugs from WT and *Jam-A$^{gt/gt}$* mice with and
without FGF-2 treatment. (B) H&E staining of representative sections of the isolated
matrigel plugs. (C) Quantification of angiogenesis in the matrigel plug as determined
by number of cells per square millimeter. WT: n=6. *Jam-A$^{gt/gt}$*: n=9. p<.05[55.]
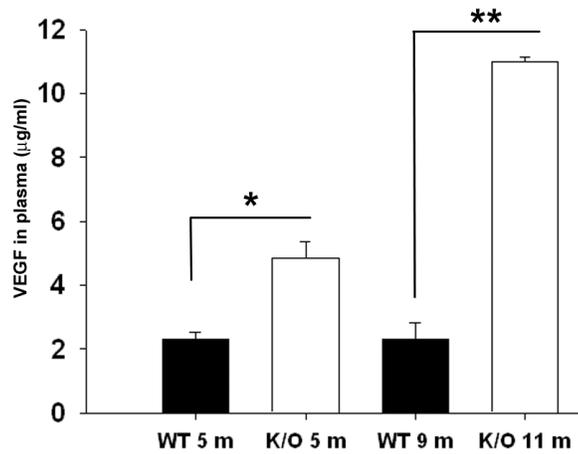
Figure 1.5: *Age Dependant Plasma VEGF Upregulation in Jam-A$^{gt/gt}$ Mice*
This unpublished work from our laboratory by Meghna Naik indicates that the VEGF
level in the plasma detected by ELISA of *Jam-A$^{gt/gt}$* mice is increased compared to the
WT at early and late adulthood. This phenomenon is likely a compensatory mechanism
to alleviate the ablation of the FGF-2 induced angiogenic pathway in *Jam-A$^{gt/gt}$* mice.

In order to study the role of JAM-A throughout the body, our laboratory has developed a transgenic mouse colony in which the activity of JAM-A is ablated. *Jam-A*$^{gt/gt}$ mice were developed using the gene trap method[58],[59] (Figure 1.6). Briefly, a trap vector containing a transmembrane domain, the β-galactosidase gene, an internal ribosomal entry site, and the gene for placental alkaline phosphatase was inserted between exon 4 and 5 of the JAM-A gene. This modification resulted in the production of a chimeric protein containing the external Ig loops of JAM-A while the cytoplasmic domain was replaced with β-galactosidase. All mice have been backcrossed to the tenth generation.

1.7 Dilated Cardiomyopathy

Previously, our laboratory noted that some *Jam-A*$^{gt/gt}$ mice greater than six months of age have a tendency to develop dilated cardiomyopathy (DCM) (Figure 1.7). DCM is a clinical phenotype in which thinning of the ventricular wall and enlargement of the ventricular chamber lead to a decrease in contractility. In DCM, myocyte death triggers the formation of fibrotic tissue. Impairment of myocyte contractility or cell death prevents the heart from providing normal stroke volume. DCM hearts also exhibit cardiomegaly, as the organ potentially doubles or triples in size. The decrease in cardiac output usually causes increased intravascular volume, leading to congestive heart failure. Both genetic defects and acquired factors have been indicated in the pathogenesis of this disease. Genetic mutations in over 40 genes that generate diverse proteins involved in myocyte contraction, nuclear envelope function, calcium homeostasis, transcription, and splicing machinery are known to
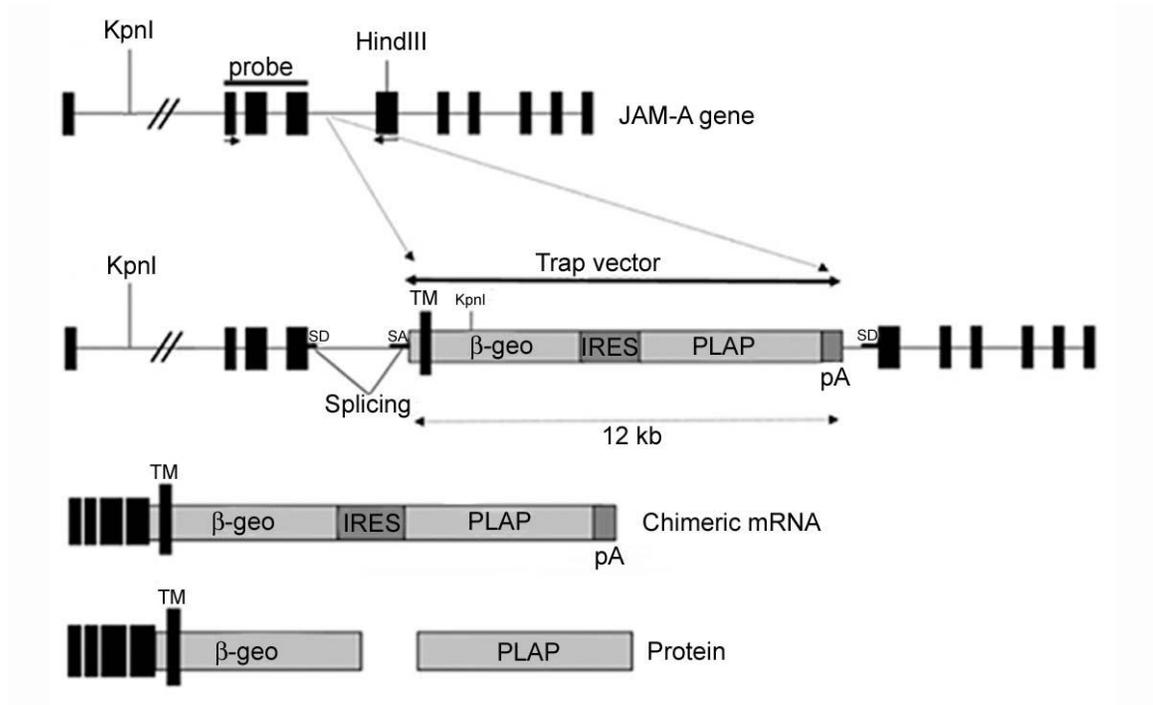
Figure 1.6 *Gene Trapping of the Jam-A Gene*
The trap vector containing a transmembrane domain (TM), the β-galactosidase (β-geo)
gene, an internal ribosomal entry site, and the placental alkaline phosphatase (PLAP)
gene was inserted between exon 4 and 5 of the *Jam-A* gene. Splicing and translation of
the chimeric mRNA produces a nonessential PLAP protein as well as the chimeric
JAM-A protein. Chimeric JAM-A contains the normal extracellular domain of JAM-
A, while the cytoplasmic tail is replaced by β-geo. This ablates the intracellular
signaling and activity of JAM-A[62].
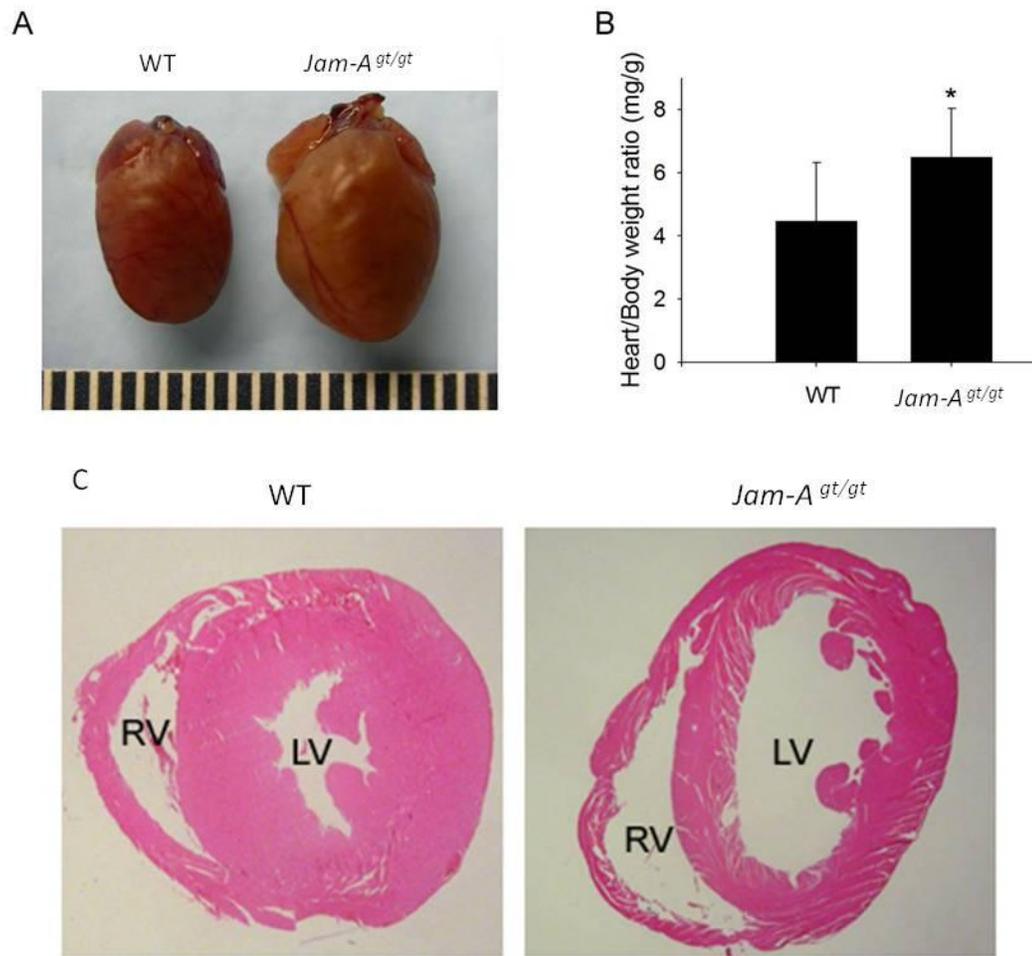
Figure 1.7 *Tendency of Jam-A$^{gt/gt}$ Mice to Develop Dilated Cardiomyopathy*
(A) Some *Jam-A$^{gt/gt}$* mice greater than 8-18 months of age develop an enlarged heart.
This phenotype is quantified in (B) as a ratio between the heart weight to body weight.
P=.006. (C) H&E staining of heart sections from WT and *Jam-A$^{gt/gt}$* mice show
enlarged ventricular chambers and thinner ventricular walls indicative of DCM. RV,
right ventricle. LV, left ventricle [62].

cause DCM. Also, secondary DCM is often caused by viral myocarditis and the drug anthracycline[60, 61].

Previous work in our laboratory by Dr. Vesselina Cooke found that histological sections of *Jam-A$^{gt/gt}$* mice showed a distinctly enlarged heart compared to wild type (WT) samples. The interior diameter of the LV was increased while the ventricular wall itself showed significant thinning[62]. Hematoxylin and Eosin (H&E) staining of left ventricular myocytes showed the myocytes were enlarged and interstitial edema was present. However, Masson's trichrome staining for fibrotic tissue showed no difference between WT and *Jam-A$^{gt/gt}$* mice. Finally, echocardiography of live WT and *Jam-A$^{gt/gt}$* mice showed both increased ventricular diameter and thinner ventricular walls. However, these results were not found to be significant[62]. These data indicate that some *Jam-A$^{gt/gt}$* mice greater than six months of age have a tendency to develop DCM.

1.8 JAM-A in MI

Since DCM only arises in some older *JAM-A$^{gt/gt}$* mice, it is a difficult and costly condition to study. Mice must be allowed to grow undisturbed for six months or longer in order for the phenotype to develop. Therefore, in order to elucidate the role of JAM-A in the observed cardiomegaly, ventricular thinning, and fibrotic deposition prevalent in DCM, we decided to use a model of acute myocardial infarction. The loss of viable myocardial tissue by the permanent ischemic injury following MI leads to geometric changes in the LV induced by the cardiac remodeling process. This results in similar changes to the myocyte apoptosis, hypertrophy, and fibrosis seen in clinical

DCM. Also, MI can be performed on younger mice allowing for a more efficient and cost effective study.

In addition to its role in pathologic conditions resulting in tissue injury such as MI, the cardiac remodeling process may take place at all times in the heart. The repair process can be triggered by minor injury and myocyte turnover. Whatever the cause of remodeling, the cardiac repair processes would not progress without the release of cytokines and growth factors by myocytes, endothelial cells, and inflammatory cells. As mentioned before, JAM-A has been indicated to play a role in leukocyte transmigration through the vascular endothelium[56]. Additionally, JAM-A has been shown to reduce leukocyte movement through the endothelium in pathologic conditions. The transmigration of polymorphonuclear cells (PMN), namely neutrophils, basophils, and eosinophils identified by their lobed nuclear membrane, is impaired in *Jam-A*$^{-/-}$ mouse models of inflammatory peritonitis[63] and hepatic ischemia/reperfusion injury[64]. Therefore, it is possible that the chronic DCM phenotype seen in older *Jam-A*$^{gt/gt}$ mice is the result of improper leukocyte recruitment to the cardiac tissue. With less leukocytes entering into the tissue level, the release of cytokines such as TNF-α and TGF-β1 may be insufficient to support the proper remodeling of an otherwise normally functioning heart. Over time, this disturbance could lead to the development of the DCM phenotype we observe.

The following studies were begun by a former member of the Naik laboratory, Dr. Vesselina Cooke. The materials and methods used for her experiments can be found in her PhD dissertation. Experimental induction of MI was performed surgically by Dr. Erhe Gao through the ligation of the left anterior descending coronary artery. Following this procedure to both WT and *Jam-A*$^{gt/gt}$ mice , Dr. Cooke observed

the mice for 14 days. She noted a significant decrease in survival in *Jam-A$^{gt/gt}$* mice

compared to the WT. I continued these experiments and can be found in Chapter 3 of

this thesis. Upon necropsy of the deceased mice, Dr. Cooke found that all *Jam-A$^{gt/gt}$*

mice died of cardiac rupture whereas none of the WT mice experienced this

phenotype.

Having observed a decrease in survival following MI and the high

incidence of cardiac rupture, Dr. Cooke went on to analyze whether this phenotype

could be a result of decreased leukocyte transmigration in the gene trapped strain. The

cardiac remodeling process would not be possible without the influx of

polymorphonuclear cells and macrophages into the area of infarction within hours of

the injury. Resident macrophages in the injured myocardium detect tissue damage and

release cytokines such as Il-1β and ROS. These and other cytokines induce the

production of chemotactic compounds and the endothelial expression of adhesion

molecules. This attracts leukocytes in the blood to the area of damage and allows their

transmigration into the tissue. Leukocyte transmigration has been shown to be

impaired in *Jam-A $^{-/-}$* mice[63, 64]. If leukocytes are prevented from entering the tissue

layer following infarction, this could potentially interfere with the cardiac remodeling

process and lead to cardiac rupture. To determine if there was a defect in leukocyte

transmigration following MI, Dr. Cooke performed immunohistochemistry on isolated

cardiac tissue sections three days post-MI. Staining was able to detect colocalization

between PMN cells and MMP9, released by leukocytes as they break through the

vascular endothelium, with PECAM-1, an endothelial adhesion molecule and a marker

for endothelial cells (Figure 1.8). She determined that a significant amount of
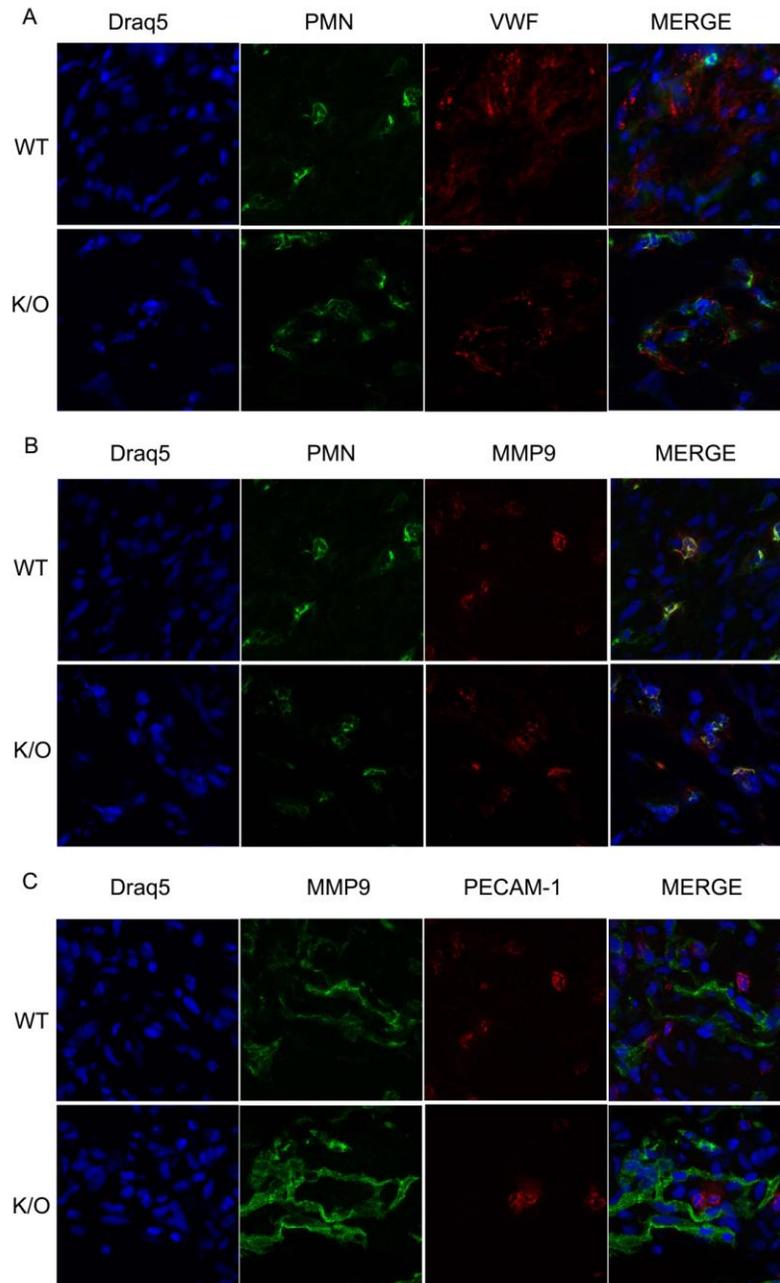
Figure 1.8: *Immunohistochemical Analysis of PMN and MMP9 Localization*
(A) PMN cell colocalization with von Willebrand Factor (vWF), a marker of
endothelial cells, is increased in *Jam-A$^{gt/gt}$* mice compared to the WT. (B) PMN and
MMP9 colocalize. (C) *Jam-A$^{gt/gt}$* mice also exhibit increased colocalization of MMP9
with the endothelial marker PECAM-1[60].

leukocytes were trapped in the vascular endothelium of *Jam-A$^{gt/gt}$* mice compared to the WT. This decrease in the number of leukocytes able to reach the tissue layer may prevent the proper release of inflammatory cytokines and decrease the removal of apoptotic and necrotic cells via phagocytosis. All would have drastic effects on the cardiac remodeling process and could potentially lead to cardiac rupture.

Since DCM was observed in some adult *Jam-A$^{gt/gt}$* mice, we wanted to determine if a similar phenotype of ventricular thinning, decreased cardiac output, and eventual heart failure could be found in *Jam-A$^{gt/gt}$* mice following MI. Dr. Cooke isolated hearts from WT and *Jam-A$^{gt/gt}$* mice 72 hours following MI surgery and weighed them. A ratio of heart weight to each mouse's body weight was determined. Both WT and *Jam-A$^{gt/gt}$* mice had increased heart/body weight ratios following MI, and *Jam-A$^{gt/gt}$* mice exhibited significantly larger hearts than the WT (Figure 1.7). Cardiomegaly is a characteristic of DCM, in which the heart can increase two to three times in size. Increased preload and hypertrophy following MI is also known to increase heart size[44].

Dr. Cooke also performed echocardiographic analysis on mice surviving two weeks following MI surgery (Figure 1.9). Echocardiography allows for detailed assessment of cardiac function during systole and to provide measurements of ventricular wall thickness. She first measured the left ventricular internal dimension-diastole (LVIDD), the diameter of the LV at the end of diastole. At this time, the heart is filled with its maximum amount of blood. The study found that LVIDD increased in both WT and *Jam-A$^{gt/gt}$* mice after MI with *Jam-A$^{gt/gt}$* mice showing a significantly greater diameter. Dr. Cooke also analyzed the left ventricular posterior wall dimension (LVPWD). This measure of ventricular wall thickness decreased in both strains

following MI. However, *Jam-A*$^{gt/gt}$ mice exhibited a distinctly thinner ventricle than the WT. Finally, the percent of fractional shortening (%FS) was found. This parameter is a comparison of the thickness of the LV when it is widest at the peak of diastole to when it is thinnest following contraction at the end of systole. %FS indicates the overall contractility of the heart and can predict cardiac output. Following MI, %FS decreased in both WT and *Jam-A*$^{gt/gt}$ mice. *Jam-A*$^{gt/gt}$ mice showed a further decrease in contractility compared to the WT (Figure 1.9). Decreased contractility and LV wall thickness are characteristics of the cardiac remodeling process following MI. These parameters, however, are significantly intensified in *Jam-A*$^{gt/gt}$ mice, suggesting that ventricular walls tress is also increased in these mice. These data also correlate nicely with the tendency of *Jam-A*$^{gt/gt}$ mice to develop DCM.

The DCM like phenotype seen in *Jam-A*$^{gt/gt}$ mice following MI often leads to heart failure as cardiac output progressively decreases. In order to determine if *Jam-A*$^{gt/gt}$ mice are undergoing heart failure, Dr. Cooke performed an analysis of BNP activity and collagen 1 immunostaining 21 days after MI (Figure 1.10). High pressure in the left ventricle triggers ventricular myocytes to synthesize and release BNP. BNP production was similar in the WT after MI, but significantly increased in *Jam-A*$^{gt/gt}$ mice. Also, an increase in cardiac fibrosis accompanies both DCM and heart failure[65]. Qualitative analysis of collagen 1 expression identified by immunohistochemistry showed that very little collagen was present in either WT and *Jam-A*$^{gt/gt}$ sham operated mice. Staining for collagen 1 was vivid in *Jam-A*$^{gt/gt}$ mice compared to the WT after MI. Taken together, these results indicate that *Jam-A*$^{gt/gt}$ mice show greater dysfunction after MI and are likely undergoing heart failure.
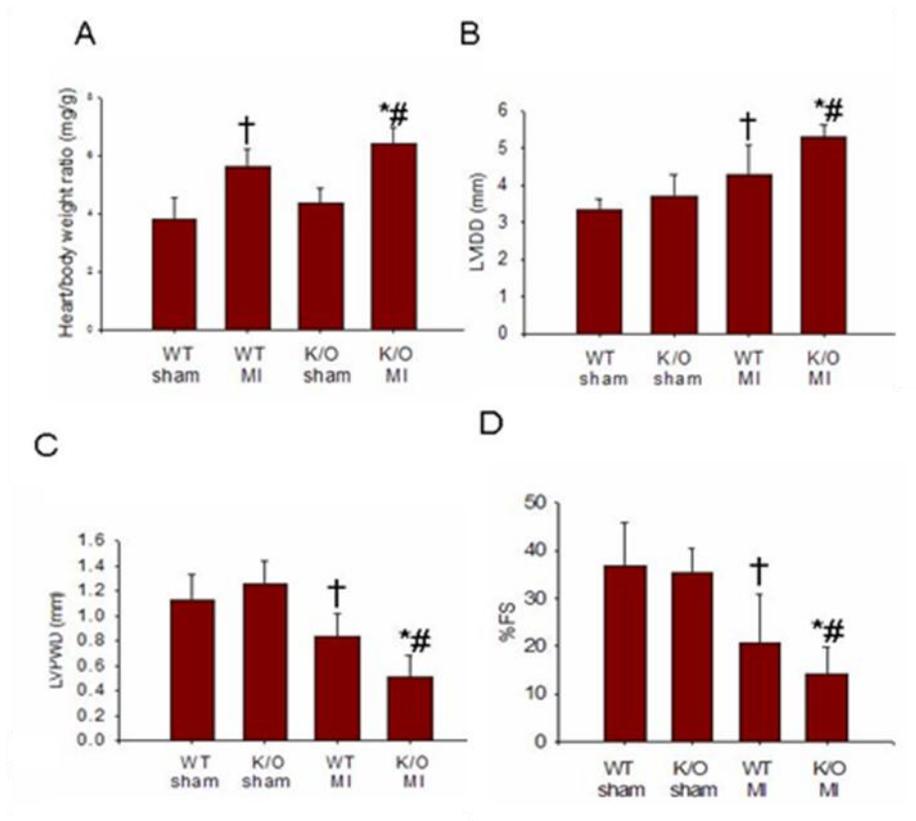
Figure 1.9: *Echocardiographic Analysis of Cardiac Function*
(A) Heart/body weight ratio 72 hours post-MI. [†]P=0.0005 WT MI vs. WT sham,
*P=0.016 *Jam-A*[gt/gt] MI vs. *Jam-A*[gt/gt] sham, [#]p=0.016 *Jam-A*[gt/gt] MI vs. WT MI. (B)
LVIDD 14 days post-MI. [†]P=0.009 WT MI vs. WT sham, *P=0.011 K/O MI vs. *Jam-A*[gt/gt] sham, [#]P=0.04 *Jam-A*[gt/gt] MI vs. WT MI. (C) LVPWD 14 days post-MI. [†]P=0.01
WT MI vs. WT sham, *P=0.001 *Jam-A*[gt/gt] MI vs. *Jam-A*[gt/gt] sham, [#]P=0.006 *Jam-A*[gt/gt]
MI vs. WT MI. (D) %FS 14 days post-MI. [†]P=0.008 WT MI vs. WT sham, *P=0.0001
Jam-A[gt/gt] MI vs. *Jam-A*[gt/gt] sham, [#]P=0.05 *Jam-A*[gt/gt] MI vs. WT MI[62].
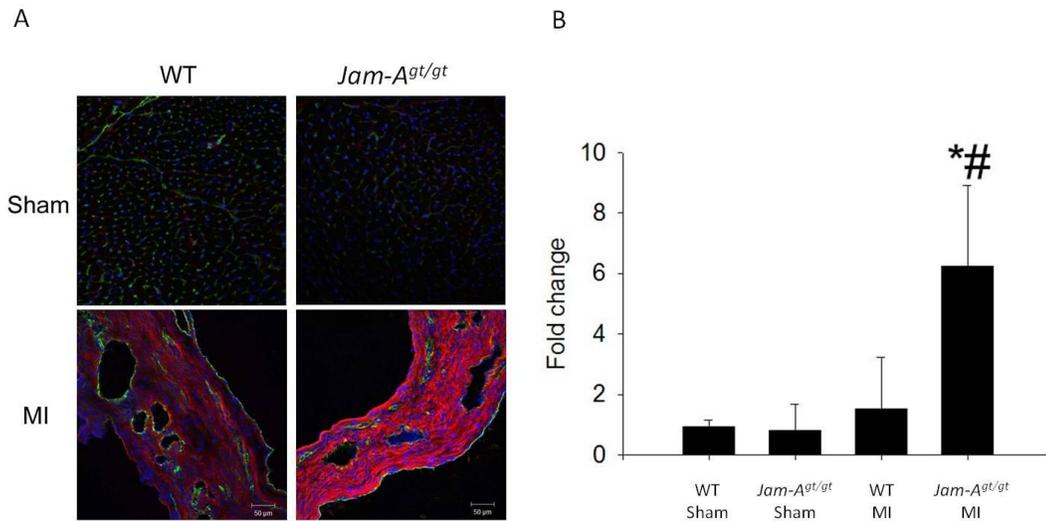
Figure 1.10: *Indicators of Heart Failure*
(A) Little fibrosis is seen in sham operated mice 21 days post-MI. Collagen 1 staining following MI is vivid in *Jam-A*[gt/gt] mice compared to the WT. Blue: draq5, nuclear stain. Green: PECAM-1, marker of endothelial cells. Pink: Collagen 1. (B) BNP production assessed by real time PCR analysis is greatly increased in *Jam-A*[gt/gt] mice 21 days post-MI compared to the WT. *P=0.014 *Jam-A*[gt/gt] MI vs WT MI; #P=0.0004 *Jam-A*[gt/gt] MI vs *Jam-A*[gt/gt] sham[62].

Chapter 2

MATERIALS AND METHODS

2.1 Animal Use

All experiments were approved by the University of Delaware's Institutional Animal Care and Use Committee.

2.2 Experimental MI Surgery

Surgical induction of MI was performed by Dr. Erhe Gao, as previously described[66]. Male mice aged 12-20 weeks were anesthetized with 2% isofluorane ventilation. After a skin incision in the chest, a purse suture was made in preparation to close the wound using 4-0 silk thread. The pectoral muscle was then retracted exposing the fourth intercostal space. Through this space, a clamp was inserted to open the rib cage, exposing the pleural cavity. Manual pressure on the chest caused the heart to extrude through the opening made at the fourth intercostals space. A ligation was made on the left anterior descending coronary artery approximately 3mm from its origin using a 6-0 silk suture. The heart was placed back inside the chest and air was manually evacuated from the surgical site. The purse suture was tightened closing the surgical wound. A sham surgery was performed using the same procedure. However, the left anterior descending coronary artery was not ligated. Mice were then allowed to recover while being monitored for any signs of distress. Following the surgery, the

mice were administered the analgesic buprenorphine (.05mg/kg) immediately following surgery and every six hours for 24 hours.

2.3 2,3,5 Triphenyl Tetrazolium Chloride Staining

Staining with 2,3,5 triphenyl tetrazolium chloride (TTC) was performed as previously described[67, 68]. Hearts were isolated from the mouse and snap frozen in liquid nitrogen. The hearts were stored at -80° for no more than six hours prior to staining in order to prevent freezer burn. A buffer solution of 77.4ml of .1M Na_2HPO_4 with 22.6ml of .1M NaH_2PO_4 was prepared followed by adjusting the pH to 7.4 by adding either buffer. 1% weight/volume TTC was added to this solution. Hearts were then removed from the freezer and sliced in approximately 3mm sections. Sections were placed in bromosilicate tubes containing the 1% TTC solution and incubated at 37°C for 20 minutes. The tubes were agitated periodically. After incubation, the sections were removed from the 1% TTC solution and placed in 10% buffered formalin. To quantify, the heart sections were individually weighed. The sections were then placed between two glass panes and secured with metal binder clips. Images were taken on a Nikon SMZ 1500 dissecting microscope. Using ImageJ software, both sides of each section were measured for the total area of the section as well as the area of infarction. The percent infarction was found for each side and averaged to find the percent infarction for each slice. This percent was multiplied by the weight of the slice to find the weight of the infarction for each slice. The total weight of the slices was divided by the total weight of the infracted area for a particular heart and expressed as a percent.

2.4 Real Time Reverse Transcriptase Polymerase Chain Reaction Analysis

Total RNA from mouse hearts was isolated using the RNeasy® Fibrous Tissue Mini Kit (QIAGEN®, Valencia, CA) according to the manufacturer's instructions. One modification of the QIAGEN® protocol was performed. To remove all DNA from the isolated genetic material, the Ambion® Turbo DNA-*free*™ Kit (Applied Biosystems™ -by Life Technologies Corporation™, Carlsbad, CA) was used according to the manufacturer's instructions. The concentration of the total RNA was found using a nanodrop. cDNA was created from the isolated RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad™, Hercules, CA) according to the manufacturer's instructions. qRT-PCR was performed using the iQ™ SYBR® Green Supermix (Bio-Rad™, Hercules, CA) according to the manufacturer's instructions on the iCycler iQ™ Real Time PCR Detection System (Bio-Rad™, Hercules, CA). qRT-PCR for the following genes of interest were performed: Il-1β, Il-10, TNF-α, MMP2, MMP9, TIMP1, TIMP2, TIMP3, TGF-β1, Periostin, SPARC, Tenascin-C, Collagen 1, Collagen 2, and JAM-A. Expression levels were compared to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences for all target genes can be found in the following table. (Table 2.1)

Table 2.1: *Primer Sequences for Real Time PCR*

Name	Symbol	Primer Sequence (5' to 3')
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	For: GATTGTCAGCAATGCATC
		Rev: GCATGGACTGTGGTCATGAG
Periostin	POSTN	For: GAACGAATCATTACAGGTCC
		Rev: GGAGACCTCTTTTGGCAAGA
Secreted Protein, Acidic, Rich in Cysteine	SPARC	For: ATGAGGGCCTGGATCTTCTTTC
		Rev: GGAAGAGTCGAAGGTCTTGTTGTC
Tenascin-C	TSC	For: GCAAGGGGTAACTTCCAATG
		Rev: ACAGGGGACATGGTCATTCT
Interleukin 1β	IL1B	For: AAAATACCTGTGGCCTTGGGCCTCAA
		Rev: CAGGGTGGTTGTGCCGTCTTTCATT
Interleukin 10	IL10	For: CTGGCTCAGCACTGCTATGC
		Rev: CTGGCCCCTGCTGATCCT
Tissue Inhibitor of Metalloproteinases 1	TIMP1	For: CATGGAAAGCCTCTGTGGATATG
		Rev: AAGCTGCAGGCACTGATGTG
Tissue Inhibitor of Metalloproteinases 2	TIMP2	For: CCAGAAGAAGAGCCTGAACCA
		Rev: GTCCATCCAGAGGCACTCATC
Tissue Inhibitor of Metalloproteinases 3	TIMP3	For: GGCCTCAATTACCGCTACCA
		Rev: CTGATAGCCAGGGTACCCAAAA
Transforming Growth Factor-β1	TGFB1	For: TGAGTGGCTGTCTTTTGACG
		Rev: AGCCCTGTATTCCGTCTCCT
Collagen 1	COL1A1	For: GCGAGTGCTGTGCTTTCTG
		Rev: TCCCTCGACTCCTACATCTTC
Collagen 3	COL3A1	For: CCCAAGCCAGAGATCCCATT
		Rev: GAAGCACAGGAGCAGGTGTAGA
Matrix Metalloproteinase 2	MMP2	For: GATAACCTGGATGCTGTCG
		Rev: CCAAACTTCACGCTCTTG
Matrix Metalloproteinse 9	MMP9	For: TGAATCAGCTGGCTTTTGTG
		Rev: ACCTTCCAGTAGGGGCAACT
Tumor Necrosis Factor-α	TNF	For: TAGCCAGGAGGGAGAACAGA
		Rev: TTTTCTGGAGGGAGATGTGG
Junctional Adhesion Molecule-A	JAM-A	For: GGTCAGCATCCACCTCACTGT
		Rev: AGGTCAGCACTGCCCTGTTC

2.5 Preparation of Protein Lysate

Preparation of protein lysates from heart tissue was performed as previously described with minor modifications[69]. Hearts were isolated from mice, snap frozen in liquid nitrogen, and stored at -80°C. Hearts were thawed on ice and homogenized by hand using a glass mortar and plastic pestle. The heart was placed in the mortar tube along with 500µl digestion buffer (250mM sucrose, 10mM Tris pH 7.5, 1mM ethylenediaminetetraacetic acid pH 7.5, 10µg/ml aprotinin, 10µg/ml leupeptin, 1mM NaF, 1mM PMSF, and 2mM sodium orthovanadate). Hearts were ground by hand. The solution was removed and placed in a 1.5ml eppendorf tube. To this, 500µl lysis buffer (2% NP40, 250mM sucrose, 10mM Tris pH 7.5, 1mM ethylenediaminetetraacetic acid pH 7.5, 10µg/ml aprotinin, 10µg/ml leupeptin, 1mM NaF, 1mM PMSF, and 2mM sodium orthovanadate) was added. The lysate was incubated on ice for 30 minutes, then centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was removed and stored at -80°C.

2.6 Bicinchoninic Acid Assay

To assess the concentration of protein in the lysate from each heart sample, the BCA Protein Assay Kit was used (Pierce®, Thermo Scientific, Rockford, Il). 50 parts BCA Reagent A was mixed with 1 part BCA Reagent B. 500µl of this solution was added to cuvette. There was one tube for each bovine serum albumin standard. Heart lysates were done in duplicate. .Bovine serum albumin was diluted following the manufacturer's instructions to create a standard curve. 25µl of each standard was placed in the corresponding cuvette. All protein lysates were diluted in a 1:50 ratio. 25µl of this dilution was added to the corresponding cuvette. Tubes were

incubated for 30 minutes at 37°C. Following this incubation, optical density was measured using a spectrophotometer at 562nm wavelength. The final heart lysate concentrations were multiplied by 50 to account for the initial dilution.

2.7 Western Blotting

Western blot was performed as previously described[70]. 40µg of protein was combined with 2X Laemmli Buffer (.5M Tris HCl pH 8, 10% glycerol, 2% SDS, .0025% bromphenol blue, 5% β-mercaptoethanol) and boiled for five minutes. Samples were run on an SDS-PAGE gel for 1.5 hours at 100 volts. Protein was then transferred to PVDF membrane at 30 volts for 1 hour. The membrane was blocked with either 3% bovine serum albumen for phosphorylated antibodies or 5% milk for non-phosphorylated antibodies. The following primary antibodies were used: phosphorylated SAPK/JNK Thr183/Tyr185 rabbit IgG (Cell Signaling Technology, Boston, MA), SAPK/JNK rabbit IgG (Cell Signaling Technology, Boston, MA), phosphorylated p38 MAPK Thr180/Tyr182 rabbit IgG (Cell Signaling Technology, Boston, MA), p38 MAPK rabbit IgG (Cell Signaling Technology, Boston, MA), phosphorylated p42/p44 MAPK (Erk1/2) Thr202/Tyr204 rabbit IgG (Cell Signaling Technology, Boston, MA), p42/p44 MAPK (Erk1/2) rabbit IgG (Cell Signaling Technology, Boston, MA), phosphorylated Src family Tyr 416 rabbit IgG (Cell Signaling Technology, Boston, MA), Src rabbit IgG (Cell Signaling Technology, Boston, MA), phosphorylated Akt serine 473 rabbit IgG (Cell Signaling Technology, Boston, MA), Akt (pan) rabbit IgG (Cell Signaling Technology, Boston, MA), phosphorylated GSK-3β serine 9 rabbit IgG (Cell Signaling Technology, Boston, MA), GSK-3β rabbit IgG (Cell Signaling Technology, Boston, MA), JAM-A rabbit IgG

(Zymed-by Life Technologies Corporation™, Carlsbad, CA) and HSC 70 (Santa Cruz Biotechnology®, Inc., Santa Cruz, CA). Secondary antibodies used were HRP-linked anti-Rabbit IgG (Cell Signaling Technology, Boston, MA) and HRP-linked anti-mouse IgG (Cell Signaling Technology, Boston, MA). Blots were developed using 20X LumiGLO® Reagent and 20X Peroxide (Cell Signaling Technology, Boston, MA) and HyBlot CL® Autoradiography Film (Denville Scientific, Inc., Metuchen, NJ). Blots were stripped of primary antibody using Restore™ Western blot Stripping Buffer (Thermo Scientific, Rockford, Il).

2.8 Primary Cardiac Fibroblast Isolation and Culture

Primary cardiac fibroblasts were isolated from WT mice in a protocol adapted from Dr. Takeshi Tsuda's laboratory. The heart was removed and placed in 10ml of ice cold Hank's Balanced Salt Solution (HBSS) (Invitrogen™ -by Life Technologies Corporation™, Carlsbad, CA). Under the hood, hearts were squeezed with forceps to remove excess blood. HBSS was discarded. Two hearts were pooled together and minced with a razor. Minced tissue was then placed in a polystyrene Petri dish and washed with 20ml cold HBSS. HBSS was removed by a pipette. Washing was repeated until the solution was clear. A solution was made containing 100ml HBSS, 0.6mg/ml collagenase A, and 4ml (0.01%) trypsin. 5ml of this solution was added to the minced tissue and incubated at 37°C for 10 minutes. After incubation, the tissue was broken up using a syringe with 22 gauge needle. The solution was then passed through 40μm nylon cell strainer into a 50ml conical tube. 15ml of the HBSS/collagenase A/trypsin solution was added and the incubation process was repeated 5 times. The HBSS solution containing the heart cells was then centrifuged at

2000 rpm for 10 minutes. The supernatant was discarded. The pellet was resuspended in Dulbecco's Modified Eagle Medium (Invitrogen™ -by Life Technologies Corporation™, Carlsbad, CA) with 15% fetal bovine serum and 1% penicillin/streptomycin. Cells were plated on 2% gelatin coated dishes for 1 hour to allow fibroblasts to adhere. The supernatant was removed and the plate was washed two times with HBSS. Growth media was then added and the fibroblasts were allowed to grow.

2.9 NIH-3T3 Fibroblast Cell Line Culture

NIH-3T3 fibroblast cells were maintained in Dulbecco's Modified Eagle Media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

2.10 Preparation of Protein Lysate from Cultured Cells

Media was removed from the cells. Cells were washed twice with phosphate buffered saline and incubated with ice cold lysis buffer (1% NP40, 150mM NaCl, 50mM Tris HCl pH 7.5 with 10μg/ml aprotinin, 10μg/ml leupeptin, 1mM NaF, 1mM PMSF, and 2mM sodium orthovanadate) for half an hour on ice. Lysate was then centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was removed and frozen at -80°C.

<h1 align="center">Chapter 3</h1>

<h1 align="center">RESULTS</h1>

3.1 JAM-A in MI and the Cardiac Remodeling Process

3.1.1 Introduction

While previous work by Dr. Cooke identified a distinct phenotype of decreased survival and cardiac rupture in the *Jam-A$^{gt/gt}$* mouse following MI, more work was needed to determine the role of JAM-A in this process. First, to further document the mortality seen in *Jam-A$^{gt/gt}$* mice, an expansion of the survival curve was needed. As stated previously, cardiac rupture is caused by either an excessive amount of infarct to the heart or a defect in the cardiac remodeling process. To rule out the possibility that infarct size is larger in *Jam-A$^{gt/gt}$* mice, I performed TTC staining to assess the area of damage. Also, Dr. Cooke noted that an increased number of PMN cells were trapped within the vasculature of *Jam-A$^{gt/gt}$* mice compared to the WT following MI. Since leukocytes are less able to traverse the endothelium in *Jam-A$^{gt/gt}$* mice, the release of inflammatory cytokines may be delayed or decreased leading to a defect in cardiac remodeling. To analyze if this is the case, I performed real time PCR analysis, along with Jennifer Joyce from Dr. Takeshi Tsuda's laboratory, of various key genes involved in remodeling.

3.1.2 Survival of *Jam-A$^{gt/gt}$* Mice and Cardiac Rupture Following MI

The survival of *Jam-A$^{gt/gt}$* and WT mice, given either the experimental MI or sham surgeries, was monitored for two weeks. Following this, all mice were

<div align="center">47</div>

sacrificed. The pooled data from both Dr. Cooke and myself were displayed in a

Kaplan-Meier plot (Figure 3.1). The results indicate that WT sham and *Jam-A*[gt/gt] sham

mice both had 100% survival two weeks post-MI. WT mice undergoing MI surgery

experienced 75% survival, similar to previously reported values for the C57BL/6

strain[71]. *Jam-A*[gt/gt] survival following MI fell to 50% following MI, a significant

reduction (p=0.0003). This result indicates that JAM-A plays a cardioprotective role

after MI. Also, necropsy of the mice that died during this two week observation period

indicated the cause of death of all *Jam-A*[gt/gt] was cardiac rupture compared to only two

such cases in the WT. (Figure 3.1) Cardiac rupture can be caused by a number of

genetic abnormalities resulting in improper myocyte junction formation and contractile

protein activity, poor regulation of inflammatory cytokines, and impaired fibroblast

matricellular and matrix protein production[45]. It can also be caused by ventricular

dilation after a large infarction due to ventricular thinning. These abnormalities all lead

to negative manifestations in the cardiac remodeling process. Taken together, these

data indicate that JAM-A either plays a role in the cardiac remodeling process or is

involved in cardioprotection from myocardial injury.

3.1.3 Infarct Size Assessment Using TTC Staining

To determine if the observed cardiac rupture was caused by increased

damage in the *Jam-A*[gt/gt] mice, I performed a TTC staining assay. TTC is a compound

that is able to pass freely through the cell membrane. Once inside the cell, TTC is

converted to a formazan pigment by various dehydrogenase enzymes. The formazan

pigment gives off a characteristic red color and can be used to identify living cells.

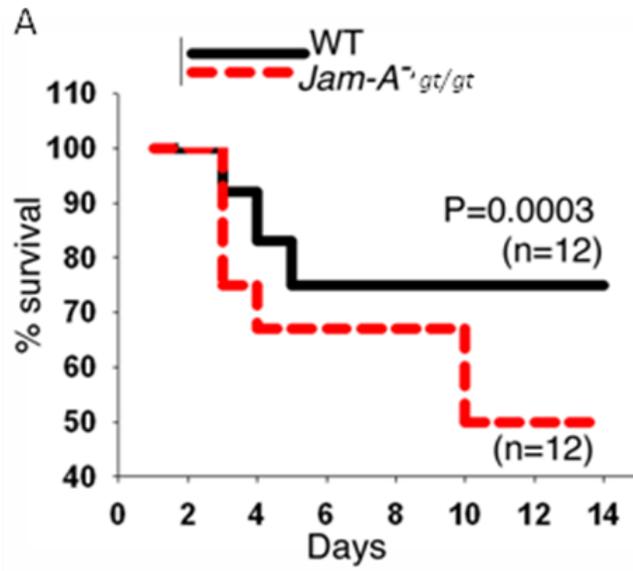Dead tissue is decolorized by a solution of buffered formalin, allowing the

Figure 3.1: *Myocardial Infarction Survival Curve*
WT mice experienced 75% survival two weeks following MI surgery. *Jam-A$^{gt/gt}$* two week survival of 50% was significantly less (p=0.0003). No mortality occurred in sham operated mice of either strain. Necropsy revealed the cause of death of all *Jam-A$^{gt/gt}$* mice was due to cardiac rupture.

differentiation between viable and dead tissue[68]. Using this technique, infarct size at 24 and 72 hours was determined for both strains. At 24 hours, infarct area in both WT (32.77%) and *Jam-A*$^{gt/gt}$ mice (37.26%) was similar indicating that initial damage was not different. However, at 72 hours a significant difference of p=.00374 was found in infarct area between WT (20.65%) and *Jam-A*$^{gt/gt}$ mice (33.38%) (Figure 3.2). Both strains show a decrease in infarct size from their 24 hour levels with the WT decrease being significantly greater than the *Jam-A*$^{gt/gt}$. This could indicate that WT mice are remodeling the ischemic area at a greater rate compared to the *Jam-A*$^{gt/gt}$.

3.1.4 Real Time PCR Analysis of the Cardiac Remodeling Process

Having observed a decrease in PMN transmigration in *Jam-A*$^{gt/gt}$ mice, it was necessary to see if any changes in the inflammatory and early proliferative phases of cardiac remodeling occurred. The decrease in transmigration should cause a disturbance in the synthesis of inflammatory cytokines. Since angiogenesis, fibroblast recruitment, and scar formation following MI are dependent on the cytokine release, this would impair these processes. To determine if cardiac remodeling was effected, I employed real time PCR analysis of inflammatory cytokines, matricellular proteins, MMPs, TIMPs, and matrix proteins to determine if gene transcription was occurring properly. Aiding in this work was Jennifer Joyce from Dr. Takeshi Tsuda's lab. Surprisingly, no significant difference in the transcription of any of our cardiac
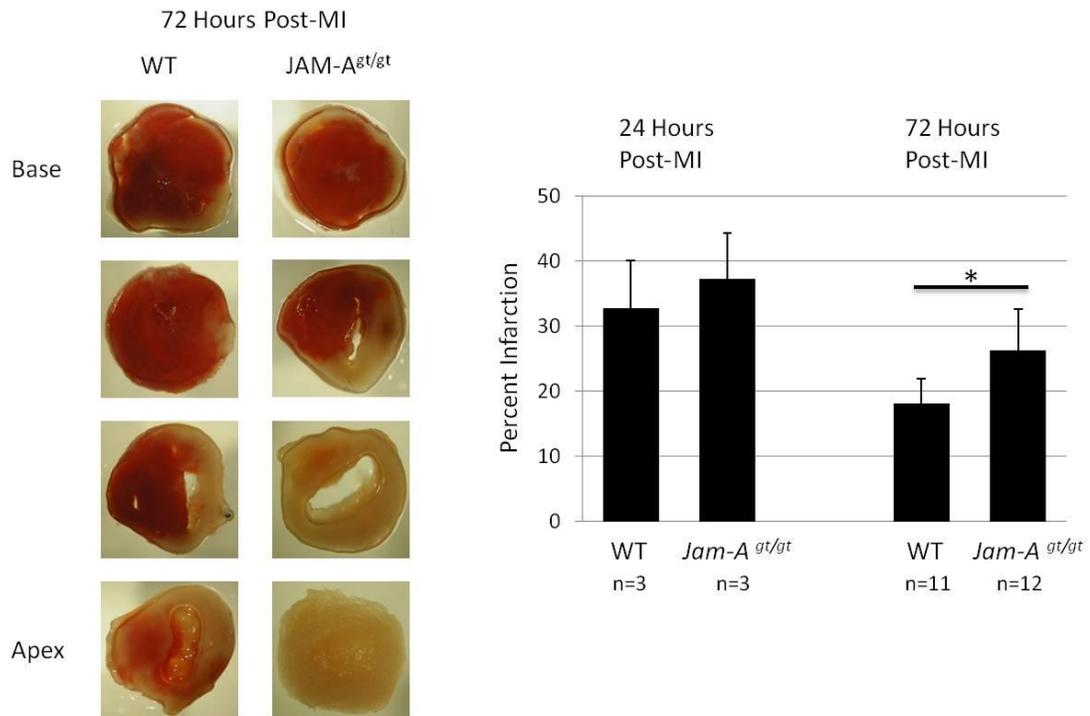
Figure 3.2: *Assessment of Infarct Size*
Infarct size was measured by 2,3,5 triphenyl tetrazolium chloride staining. At 24 hours post-MI, there was no difference in % infarction between WT and *Jam-A*[gt/gt] mice. 72 hours post-MI, WT mice show a significant decrease in infarct size. Infarct size in *Jam-A*[gt/gt] mice did not change between 24 and 72 hours post-MI (p=0.0037).

remodeling gene targets was found. These included Il-1β, Il-10, TNF-α, MMP2, MMP9, TIMP1, TIMP2, TIMP3, TGF-β1, Periostin, SPARC, Tenascin-C, Collagen 1 and Collagen 2 (Figure 3.3). While leukocytes are being trapped in the vasculature, they are still able to produce inflammatory cytokines that influence other cells involved in cardiac remodeling. At 72 hours post-MI, the increased expression growth factors such as FGF-2, VEGF, and the angiopoietins induce endothelial cells to proliferate. Our lab has shown that JAM-A overexpressing endothelial cells exhibit increased tube formation,[72] indicating that JAM-A aids in the progression of angiogenesis. Therefore, I performed real time PCR analysis at 72 hours post-MI to detect any change in JAM-A transcript levels. (Figure 3.4) While an increase in JAM-A expression following MI surgery in the WT does occur, it is not significant.

Figure 3.3: *Real Time PCR Analysis of Cardiac Remodeling Genes*
No significant difference was seen in any key mediator of the cardiac remodeling process 24 hours post-MI. WT sham n=4. WT MI n=7. *Jam-A$^{gt/gt}$* sham n=4. *Jam-A$^{gt/gt}$* MI n=6.

Figure 3.4: *JAM-A Transcript Levels 72 Hours Post-MI*
Following MI, JAM-A shows a slight but insignificant increase in transcript level
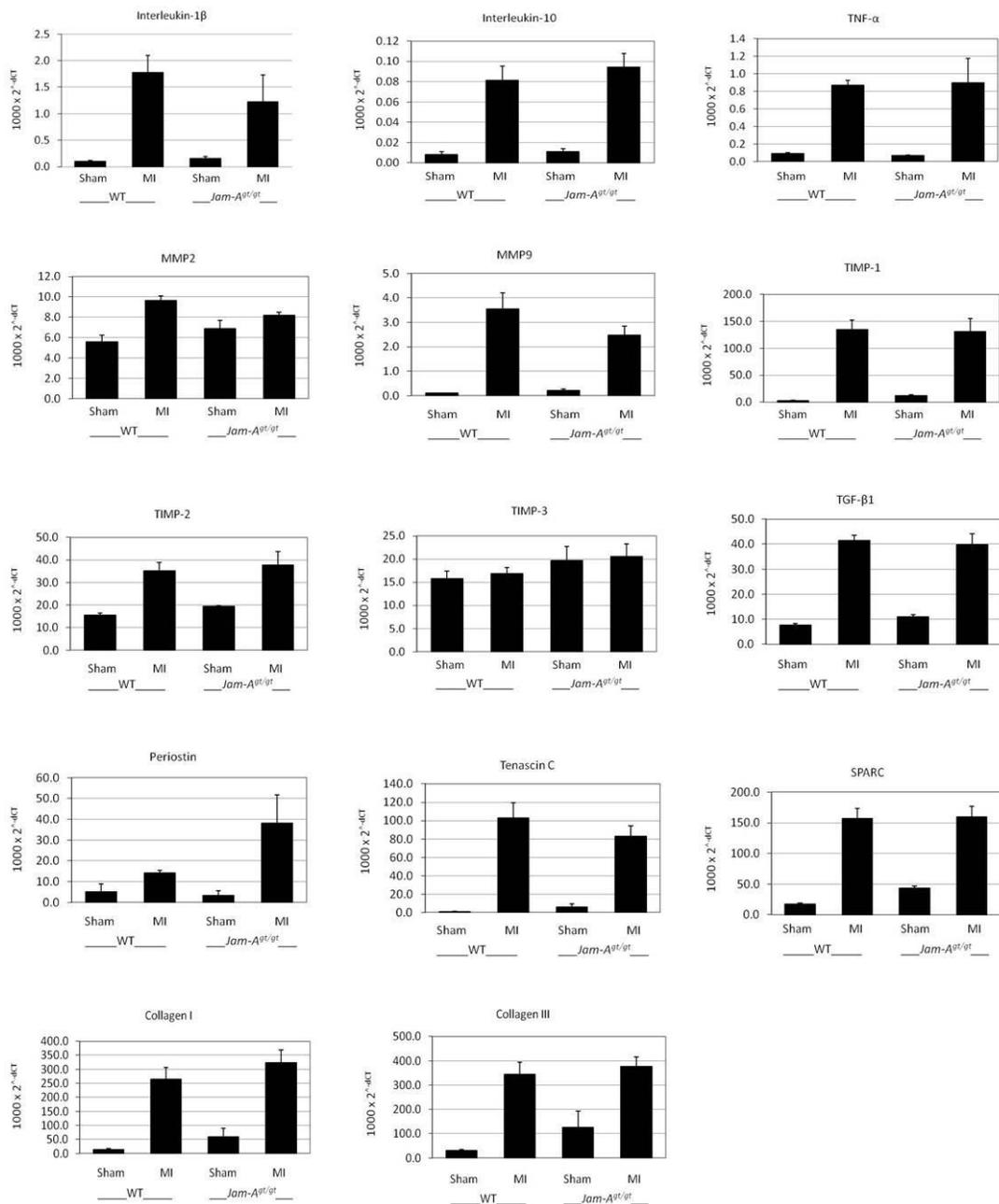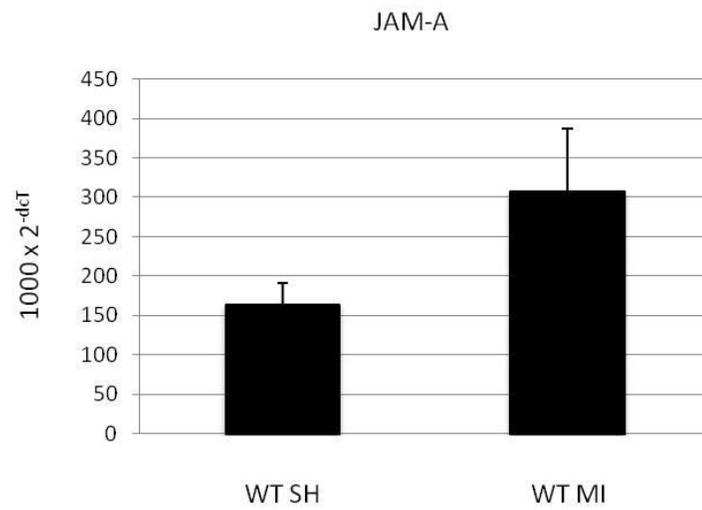following MI. p=0.1327. n=4 for each group.

3.2 Vascular Permeability and MI

3.2.1 Introduction

Since the endothelium plays a unique role in regulating myocyte function in the heart, I wanted to determine if this interaction could be the cause of the dysfunction seen following MI. The most important role of the vasculature is to provide blood as a source of nutrients, growth factors, waste removal, etc. to a tissue. The growth of blood vessels beginning in development up through adulthood is controlled by the interplay of various growth factors including VEGF, FGF-2, angiopoietin, and others. Of these, the two most important growth factors to coronary development and maintenance are VEGF and FGF-2[73]. Previously research performed in our laboratory indicated that JAM-A plays a crucial role in regulating FGF-2 induced angiogenesis (Figure 1.4). In fact, the FGF-2 induced angiogenic pathway in *Jam-A*$^{gt/gt}$ mice is completely ablated. To compensate for this loss of FGF-2 signaling, unpublished data from our lab indicates that VEGF levels in the plasma are increased (Figure 1.5). The redundancy of growth factor signaling in angiogenesis allows *Jam-A*$^{gt/gt}$ mice to exhibit normal embryonic and retinal vasculature[55].

The defect in FGF-2 signaling or the upregulation of VEGF in *Jam-A*$^{gt/gt}$ mice may contribute to the dysfunction seen in following MI. In the heart, FGF-2 signals to cardiac myocytes, fibroblasts, and endothelial cells. In myocytes, FGF-2 signaling through the MAPK pathway induces a hypertrophic response[74]. Following ischemia, FGF-2 decreases myocyte contractility and energy consumption by uncoupling gap junctions between cells[75]. FGF-2 is also known to drive the recruitment and activation of CD4+ T-cells following myocardial injury[76]. FGF-2, along with TGF-β1, drives the differentiation of fibroblasts to myofibroblasts allowing

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fibrosis following MI[31]. Finally, FGF-2 is involved in endothelial proliferation and migration associated with angiogenesis[77]. Conversely, VEGF upregulation may have unwanted vascular effects leading to myocyte dysfunction.

Unlike the varied role of FGF-2 in the heart, VEGF is only involved in angiogenesis and the regulation of endothelial function. Hypoxic conditions activate HIF-1α which leads to transcription of the VEGF gene. Stress activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) works cooperatively with HIF-1α, enhancing this production[78]. VEGF is known to cause vasodilation and induce endothelial cell proliferation and migration to sprout new vessels[79]. To accomplish this, VEGF signaling induces extracellular signal-regulated kinase 1/2 (ERK1/2) leading to endothelial proliferation[80]. Also, VEGF activation of p38 MAPK pathway leads to actin reorganization necessary for endothelial cell migration[81]. Signaling by VEGF is also required for endothelial cells to break tight and adherans junctions with neighboring endothelial cells to allow for migration to occur. VEGF binding to the VEGF-R2 receptor leads to Src activation and the eventual phosphorylation of VE-cadherin. This phosphorylation allows VE-cadherin to be endocytosed, aiding in the dissolution of endothelial junctions[82]. The separation of neighboring endothelial cells causes a local increase in vascular permeability as the barrier function of vasculature is disrupted. It has been shown that, following MI, this increase in VEGF induced vascular permeability is associated with local edema. Also, microthrombi develop at the site of separation that can cause complete occlusion of the microvasculature. Extravazation of red blood cells into the tissue also occurs and causes dysfunction of neighboring cells[83]. Unpublished data from out laboratory by Meghna Naik indicates that plasma VEGF levels are upregulated in *Jam-A$^{gt/gt}$* mice compared to WT in both

early adulthood and in aged mice (Figure 1.5). This is likely a compensatory mechanism since the FGF-2 induced angiogenic pathway is completely ablated in the gene trapped strain. Therefore, I hypothesized that the increased vascular permeability induced by high serum VEGF levels in *Jam-A$^{gt/gt}$* mice led to myocardial dysfunction and damage to cardiac myocytes. To determine this, western blotting of signaling molecules associated with the VEGF-R2 receptor was conducted.

While previous data from out lab indicates that *Jam-A$^{gt/gt}$* mice exhibit greater serum VEGF levels and increases in vascular permeability, these experiments demonstrate that no differences in signaling downstream of the VEGF-R2 receptor occurs between WT and *Jam-A$^{gt/gt}$* mice. Specifically, phosphorylation of SAPK/JNK (Thr183/Tyr185), ERK1/2 (Thr202/Tyr204), p38 (Thr180/Tyr182), and Src (Tyr416) are unchanged between WT and *Jam-A$^{gt/gt}$* mice following MI (Figure 3.5).

3.2.2 Western Blot Analysis of Vascular Permeability

Phosphorylation of ERK1/2 and SAPK/JNK increased 24 and 72 hours following MI, but was not significantly different between *Jam-A$^{gt/gt}$* and WT mice (Figure 3.5). Interestingly, there was no significant upregulation after MI in phosphorylation of p38 and Src at either timepoint following MI.
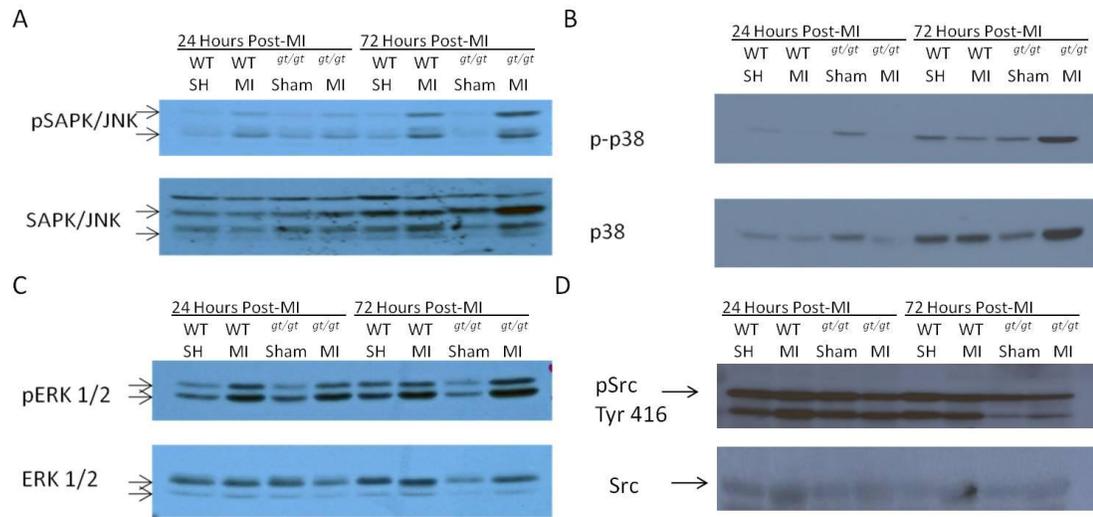
Figure 3.5: *Western Blot Analysis of VEGFR2 Associated Signaling*
(A) Phosphorylation of SAPK/JNK occurred 72 hours post-MI, but was not
significantly different between *Jam-A$^{gt/gt}$* and WT mice. (B) No upregulation of p38
phosphorylation occurred following MI. (C) ERK1/2 activation was present both 24
and 72 hours post-MI, but was not different between *Jam-A$^{gt/gt}$* and WT mice. (D)
There was no change in Src phosphorylation after MI.

3.3 JAM-A and Akt Signaling

3.3.1 Introduction

In cardiac myocytes, endothelial cells, and a number of other cell types, the activation of the serine/threonine protein kinase Akt is the most important signaling pathway involved in cell survival. The release of growth factors occurs regularly to maintain a healthy myocardium as well as following an acute injury such as MI. In all cases, the major growth factors signal through Akt in a variety of cell types leading to a diversity of cellular responses including hypertrophy, hyperplasia, and the prevention of apoptosis. In endothelial cells, this pathway can be initiated through the binding of angiogenic growth factors such as FGF-2 and VEGF, among others, to their membrane receptors[84]. Binding to the growth factor receptor leads to activation of PI3K which helps recruit inactive Akt to the membrane. Akt is then phosphorylated by PDK1 and PDK2, allowing it to change conformation to its active state. Once activated, Akt phosphorylates downstream targets leading to four major outcomes in endothelial cells. First, it applies an inhibitory phosphorylation on the apoptotic proteins Bad, Forkhead and others, preventing cell death. It also helps the endothelial cell proliferate by mediating the progression through the cell cycle, activating such regulators as E2F and p21. Akt enhances cellular energy consumption by phosphorylating glycogen synthase kinase-3β (GSK-3β) leading to the translocation of glucose transporter 4 (GLUT4) to the membrane. Finally, Akt induces protein translation by activating various transcription factors including eukaryotic initiation factor 4Ebinding protein 1 (4E-BP1) and 70-kDa S6 kinase (S6K1). Initiation of these pathways by VEGF and FGF-2 are required for endothelial proliferation, migration, and tube formation necessary for angiogenesis[85]. Therefore, the induction of Akt in

endothelial cells following MI leads to angiogenesis and the proper regulation of cardiac remodeling.

Akt signaling also plays a vital role in maintaining healthy myocytes and in their adaptation to stressors following MI. Growth factors that bind myocytes such as insulin-like growth factor-1 (IGF-1), TGF-β1, and endothelin-1 all lead to Akt activation in the same PI3K dependant manner as endothelial cells. Additionally, Akt activity in myocytes has many similar outcomes as that in endothelial cells. It causes similar increases in glucose metabolism through the addition of membrane GLUT4 receptors. It also prevents apoptosis by deactivating Bad and Forkhead. While many similarities exist between Akt signaling in these two cell types, the major difference lies in its regulation of cell growth. In endothelial cells, Akt has an effect on cell cycle regulation and proliferation. Since myocytes are considered to have very little proliferative capabilities, Akt induces cell growth not by an increase in cell number, but in hypertrophy. Both physiological and pathological hypertrophy requires the activity of this signaling pathway. Akt exerts this influence by activating mTOR, leading to subsequent upregulation of a number of transcription factors for hypertrophic genes. Proper Akt signaling is required to add sarcomeres in series and in parallel. This phenomenon seen in physiologic hypertrophy allows myocytes to grow both in length and thickness. This is in contrast to the excessive thinning seen during pathological eccentric hypertrophy following ventricular pressure overload. Akt regulates the type of hypertrophic response of the cell by activating both GSK-3β and the forkhead box O (FOXO) family of transcription factors. Both these targets downregulate the translation of particular genes involved in the hypertrophic response. While this downregulation seems counterintuitive, these genes would otherwise drive

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the myocyte toward eccentric hypertrophy and excessive thinning of the walls of the heart[85, 86, 87].

In a recent report by Nava et al, it was found that JAM-A regulates endothelial proliferation through the modulation of Akt signaling. Endothelial cells isolated from *JAM-A*^{-/-} mice showed increased Akt phosphorylation compared to the WT. Akt was then free to activate β-catenin, leading to its nuclear translocation and function as a transcription factor for proliferative genes. JAM-A homodimerization is believed to prevent the activation of PI3K, downregulating Akt activity[88]. It is also known β-catenin expression increases during endothelial migration[89]. Since both endothelial proliferation and migration are required for angiogenesis following MI, it is possible that the absence of JAM-A creates a defect in β-catenin function. Furthermore, human hearts exhibiting dilated cardiomyopathy removed from transplant patients show distinctly decreased β-catenin expression levels[90]. Therefore, I hypothesized that the absence of JAM-A leads to improper regulation of Akt and β-catenin signaling and possibly β-catenin expression leading to DCM and cardiac rupture following MI.

In these experiments, I performed Western blot analysis of Akt and GSK-3β phosphorylation 24 and 72 hours following experimental MI. I found no differences in activity levels of these proteins following MI (Figure 3.6).

3.3.2 Western Blot Analysis of Akt and GSK-3β

Western blot analysis of pAkt serine 473 and pGSK-3β serine 9 were performed, then quantified using densitometric analysis (Figure 3.6). A significant

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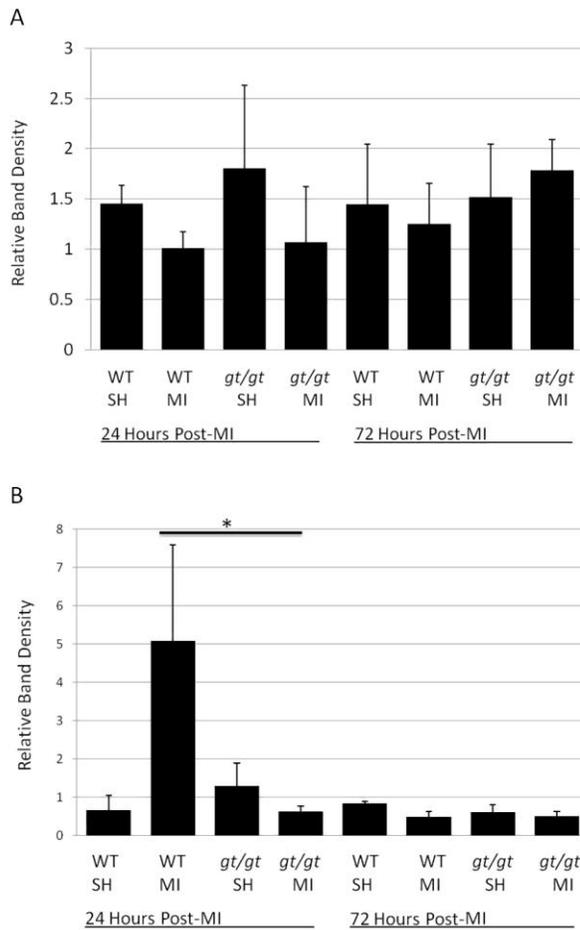
Figure 3.6: *Western Blot Analysis of Akt and GSK-3β*
(A) No significant differences are seen in Akt phosphorylation after MI. At 24 hours post-MI, both WT MI and *JAM-A* $^{gt/gt}$ MI mice show a slight reduction in activity. At 72 hours post-MI, there is a slight increase in *JAM-A* $^{gt/gt}$ MI Akt activity not seen in the WT MI samples. (B) Little change in GSK-3β occurs between mice given sham or MI surgery or between strains. The significant increase in GSK-3β signaling at 24 hours is likely due to an outlier sample with very high activity. 24 Hour WT MI vs. 24 Hour *JAM-A* $^{gt/gt}$ MI: p=0.043.

increase in GSK-3β is seen in the WT MI sample 24 hours following surgery. A substantial outlier in band intensity is likely the cause of this upregulation, rather than any physiological finding.

3.4 Absence of JAM-A in Cardiac Fibroblasts

3.4.1 Introduction

As mentioned before, cardiac fibroblasts play a crucial role in the cardiac remodeling process. In fact, fibrosis is associated with a majority of disease states of the heart. In the healthy heart, resident fibroblasts in the cardiac tissue maintain a strong and functioning ECM by constantly degrading old proteins and depositing new material. Following myocardial infarction, fibroblasts migrate from other locations in the heart to the area of injury. Once there, FGF-2, TGF-β, and other growth factors induce fibroblasts to adopt smooth muscle like characteristics and differentiate to myofibroblasts. This allows the cells to withstand the mechanical stress of the remodeling myocardium[91]. A handful of other cell types, especially endothelial cells, are able to differentiate into fibroblasts during tissue damage. Polarized endothelial cells undergoing this endothelial mesenchymal transition (EMT) lose the expression of proteins such as E-cadherin and transcribe fibroblast proteins such as α-smooth muscle actin (α-SMA) and supernatant protein factor (SPF1)[92]. This increases the pool of myofibroblasts available to deposit matrix material, allowing the heart to form a scar to prevent rupture.

Additionally, studies have indicated that fibroblasts can regulate the hypertrophy and function of cardiac myocytes. This can be seen following MI when decreased cardiac output reduces the flow of blood to the kidneys. The kidneys will

then release renin, initiating the renin-angiotensin system leading to vasoconstriction. Angiotensin II has been shown to trigger cardiac fibroblasts to release hypertrophic growth factors such as TGF-β1 and endothelin-1[93]. Also, fibroblasts are able to form direct gap junctions with myocytes through connexin-43 and connexin-45[94]. This indicates that fibroblasts may link distant myocytes electrochemically that are separated by the insulating ECM[95]. Thus it is possible that a defect in fibroblast activity can result in electrochemical dysfunction in myocytes, delayed fibrosis, or poor crosslinking of ECM components.

A report by Morris et al indicated that JAM family members are differentially expressed on human and mouse fibroblasts (Figure 3.7)[96]. RT-PCR analysis of primary human fibroblasts isolated from the lung, cornea, and dermis indicated that the JAM family member JAM-C, but not JAM-A or B was expressed by these cells. Analysis of mouse fibroblast cell lines, however, indicated that L-cells and NIH-3T3 cells expressed JAM-A, but not JAM-B or C. The 3T3 L1 cells showed very little JAM expression. These data indicate that JAM-A protein may exist on mouse fibroblast cells. Furthermore, fibroblast cells are very similar in cell types to endothelial cells. In fact, endothelial cells are able to differentiate to fibroblasts during times of tissue repair in the process of EMT. Previous research from our laboratory indicated that JAM-A plays a role in endothelial migration. Isolated endothelial cells in which JAM-A levels were knocked down using siRNA showed a significant decrease in wound induced migration[97]. It is possible that a delay in fibroblast migration or overall function due to the absence of JAM-A can cause the defect in myocyte function seen in our mice following MI. Therefore, I hypothesized that
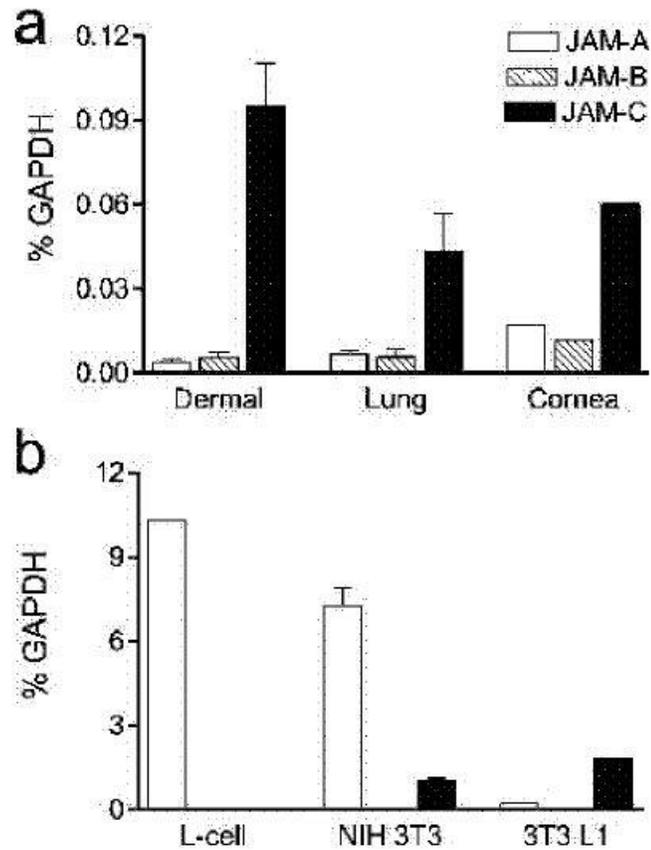
Figure 3.7: *JAM Family Member Transcript Levels in Human and Mouse Fibroblasts*
(A) Human primary fibroblasts isolated from the dermis, lung, and cornea indicate the
presence of JAM-C RNA. (B) The mouse fibroblast immortalized cell lines L-cell and
NIH-3T3 found JAM-A transcript to be elevated. 3T3 L1 cells showed now significant
expression of a JAM family member[96].

fibroblasts in *Jam-A$^{gt/gt}$* mice following MI may also have a defect in migration leading to their improper recruitment to the site of ischemia.

3.4.2 Cardiac Fibroblasts Do Not Express JAM-A

Western blot analysis of isolated primary cardiac fibroblasts and the NIH-3T3 fibroblast cell line do not express JAM-A protein (Figure 3.8). However, the lysate of the entire WT heart does show the presence of JAM-A as expected.
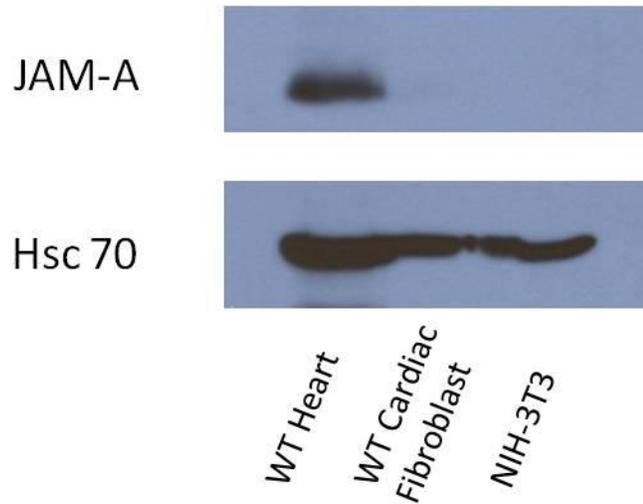
Figure 3.8: *Cardiac Fibroblasts Do Not Express JAM-A*
Protein lysates from total WT heart tissue, isolated WT cardiac fibroblasts, and the fibroblast cell line NIH-3T3 were blotted for the presence of JAM-A. Only the total heart lysate contains JAM-A.

Chapter 4

DISCUSSION

This report, together with previous experiments conducted by Dr. Vesselina Cooke, demonstrates that *Jam-A$^{gt/gt}$* mice show decreased survival after experimental MI. The mortality in the *Jam-A$^{gt/gt}$* mice all resulted from cardiac rupture. Immunohistochemical studies performed by Dr. Cooke demonstrated that the presence of JAM-A in the vasculature is required for PMN transmigration following MI. However, even though a significant amount of PMN cells were trapped, transcription of key cardiac remodeling genes occurred normally. Echocardiographic analysis revealed that *Jam-A$^{gt/gt}$* mice developed more ventricular dilatation, lower systolic function, and thinner LV wall than WT mice two weeks post-MI. Elevated BNP transcript levels and fibrosis suggest that *Jam-A$^{gt/gt}$* mice are undergoing heart failure at a greater rate compared to the WT. Even so, the absence of JAM-A had no effect on signaling associated with the VEGF Receptor 2 or on the growth factor induced Akt pathway. Finally, JAM-A was found to not be present on cardiac fibroblasts.

In cardiac tissue, JAM-A is only present in the endothelium and some resident leukocytes. Therefore, either of these two cell types must be regulating ventricular myocyte cells to induce the hypertrophy associated with this condition. The cardiac endothelium is known to regulate myocytes locally through the release of various compounds including TNF-α, NO, and endothelin[98]. An example of this interaction occurs when cardiac myocytes are cocultured with endothelial cells from the cardiac microvasculature, a distinct decrease in contractility was found upon

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activation with inflammatory cytokines[99]. Defects in endothelial function could disregulate myocyte contractility leading to the loss of function seen in DCM. Also, an association between myocyte blood flow and DCM has been found. Myocardial vascularization was diminished in some patients with DCM although an increase in circulating VEGF and endothelial progenitor cells was present[100]. Also, endothelial expression of β-catenin, necessary for endothelial proliferation during angiogenesis, is decreased in DCM[88]. Taken together, these studies implicate a role for endothelial vascularization and cytokine release in proper myocyte function. Improper regulation of the endothelium can lead to DCM and decreased heart function.

Heart failure is commonly seen in the advanced stages of DCM and post-MI remodeling. The studies performed previously by Dr. Cooke indicate that *Jam-A$^{gt/gt}$* mice are prone to develop heart failure following infarction. One indicator of this is the high level of BNP seen in the gene trapped mice, but not in the WT strain three weeks following MI. Serum BNP is an approved biomarker for congestive heart failure as outlined by the United States Food and Drug Administration[101]. BNP is synthesized, stored, and released by ventricular myocytes in response to high pressure within the ventricle. It acts against the renin-angiotensin system to decrease plasma sodium concentration and opposes vasoconstriction allowing fluid to be excreted in the urine. Thus, BNP release often parallels the rise in pulmonary edema seen in heart failure[102]. BNP release has also been shown to attenuate myocyte hypertrophy by inhibiting Gαq phosphorylation downstream of a growth factor receptor[103] as well as decrease the fibrotic deposition of fibroblasts[104]. Additionally, immunohistochemical staining for collagen 1 expression three weeks post-MI indicates that *Jam-A$^{gt/gt}$* mice are undergoing a greater level of fibrosis than the WT. Fibrosis following MI is

reparative, as the heart attempts to provide the ischemic area with tensile strength to prevent rupture. Ischemic fibrosis is often found in the subendocardium and may extend to viable areas surrounding the ischemic tissue[105]. Increased walls tress exacerbates this process during heart failure. Effector hormones of the renin-angiotensin system as well as TGF-β1 released during heart failure are known to increase fibroblast deposition of ECM proteins. Crosstalk between compounds released during heart failure by activated macrophages, myocytes undergoing hypertrophy, and fibroblasts are also indicated to progress fibrosis in heart failure[106]. Clearly, JAM-A plays a cardioprotective role in the heart. By preventing aberrant cardiac remodeling and fibrosis, JAM-A is able to prevent the cardiac rupture phenotype following an acute injury such as myocardial infarction and the chronic dysfunction of DCM.

Additionally, Dr. Cooke's results corroborate previous studies pertaining to the role of JAM-A in leukocyte transmigration through the endothelium in normal and pathological conditions. JAM-A binding of LFA-1 on the surface of leukocytes is known to help the immune cell pass through the endothelial barrier into the tissue[55]. The ablation of JAM-A in a mouse model of hepatic ischemia-reperfusion injury reduced neutrophil migration into the injured tissue[61]. Immunostaining in *Jam-A$^{gt/gt}$* mice showed that 15% of PMN cells were trapped in the vasculature compared to just 10% of the WT strain. However, this significant increase in PMNs sequestered in the endothelium was not associated with a disturbance in the transcription of key genes involved in the cardiac remodeling process. It is likely that a 5% difference in transmigration, while statistically significant, is not physiologically so. During the inflammatory phase, an abundance of activated macrophages and neutrophils are

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migrating into the ischemic area. Such a small change in the number of leukocytes within the tissue will not affect the remodeling process or lead to such a severe phenotype as cardiac rupture. While no change in gene transcript levels of the key cardiac remodeling genes occurred, it is possible that a change in mRNA translation occurred. If so, protein levels in the tissue for these targets would be different. By analyzing plasma levels of these cytokines and growth factors in the plasma by ELISA, it is possible that a difference could be found. Also, although the level of MMP2 and MMP9 gene transcription was similar, a difference in the activated form of the protein could still be present. MMPs are produced as a proprotein. In order to become active, the c-terminal prodomain must be cleaved by a protease. This leads to the binding of both a zinc and calcium ion inducing a conformational change that allows the MMP to undergo autolytic cleavage creating the active protein[107]. Since MMPs are being trapped in the vasculature of *Jam-A$^{gt/gt}$* mice, it is possible that a difference in MMP activation or activity could cause damage to the local vasculature. Assessing the activity of this enzyme through zymography can identify if this is the case.

While our lab has previously shown an upregulation of plasma VEGF levels in *Jam-A$^{gt/gt}$* mice as a compensatory mechanism for the ablation of the FGF-2 angiogenic pathway, I have found no significant difference in signaling associated with VEGF production or the VEGF-R2 receptor between WT and *Jam-A$^{gt/gt}$* mice following MI. It is possible that the upregulation of angiogenic growth factors following an acute injury such as MI reaches a level of saturation in both strains that masks the predisposed increase in *Jam-A$^{gt/gt}$* mice. More likely, though, is the fact that endothelial cells only make up a fraction of the cell types found in cardiac tissue. Since survival signaling such as the upregulation of ERK1/2 is common after the MI

injury[108], it could be that signaling in myocytes, fibroblasts, and leukocytes mask any changes taking place in the endothelial population. Whatever the reason, I found no difference in VEGF-R2 signaling either before or after MI, indicating that vascular permeability is not the cause of the dysfunction seen in *Jam-A*$^{gt/gt}$ mice.

In chapter 3.3, I demonstrate that there is no significant difference in Akt activation in either WT or *JAM-A* $^{gt/gt}$ mice following MI. At 24 hours post-MI, both strains show a slight decrease in Akt phosphorylation. This reduction likely correlates with the presence of apoptosis in the ischemic tissue. The loss of Akt activity allows apoptotic proteins such as BAD to upregulate, leading to the mitochondrial release of cytochrome C and cell death. This data also correlates with the results of my TTC staining. Since we see the initial size of infarction is the same in WT and *JAM-A* $^{gt/gt}$ mice, it is likely that a similar level of cell death is occurring in both strains. Therefore, the downregulation of Akt activity at 24 hours should be comparable. At 72 hours post-MI, levels of active Akt in the WT return to a similar level as sham while the *JAM-A* $^{gt/gt}$ level is slightly higher. At this time point, apoptosis is still occurring throughout the damaged tissue which will cause a decrease in Akt signaling in the cells undergoing this process. Additionally, 72 hours post-MI in mice marks the beginning of the proliferative phase of cardiac remodeling. Growth factor release such as TGF-β, VEGF, and FGF-2 are occurring. Viable cells in the ischemic tissue will be responding to these growth factors leading to an upregulation in Akt activity. This difference in signaling could muddle the results at this timepoint. The slight increase in Akt phosphorylation we see in *JAM-A* $^{gt/gt}$ mice could be the result of the loss of JAM-A in endothelial cells, leading to the increase in Akt activity described by Nava et al[88]. Nevertheless, the data gave no significance and this analysis is only conjecture.

Akt is known to phosphorylate GSK-3β leading to an increase in glucose utilization in all cell types and the induction of hypertrophic signaling in myocytes. Nevertheless, no change was seen in GSK-3β signaling after MI compared to the sham operated mice in either strain. In the WT MI sample taken 24 hours following surgery, a significant increase in GSK-3β signaling is seen. However, this large increase is likely due to a statistical outlier rather than any physiological response since there was no change in activity of Akt at 24 hours, its direct upstream regulator.

Since any effect of JAM-A on the signaling of both the VEGFR2 and Akt pathways would likely be found in endothelial cells, preparing a protein lysate from the entire heart tissue may have been imprudent. It is possible that a change is occurring but is being masked by signaling of these molecules in other cell types such as myocytes and leukocytes. To identify if this is the case, western blotting of isolated endothelial cell from the entire cardiac tissue at 24 and 72 hours following MI should be performed. Any effect JAM-A is having on this cell type would be clearly demonstrated without interference from other cell types.

Finally, while fibroblasts closely resemble endothelial cells in structure and protein expression patterns, cardiac fibroblasts and the fibroblast cell line NIH-3T3 do not express JAM-A. When EMT is initiated, the expression patterns of various cell adhesion molecules are modified. For example, the endothelial adhesion molecule E-cadherin is known to be downregulated while the integrin $\alpha_v\beta_6$, a receptor for fibronectin and tenascin, are upregulated[109]. Since JAM-A is present on endothelial cells but not fibroblasts, it is likely that expression of JAM-A is lost during EMT. It would be interesting to see if this is the case by inducing EMT in an endothelial population and identifying changes in surface expression of JAM-A using flow

cytometry. Since JAM-A is not ordinarily found on cardiac fibroblasts, it cannot play a role in the migration of fibroblasts or in their interaction with myocytes following MI.

The data presented in this thesis shows that the actions of JAM-A in the myocardium have still not been fully deciphered. Our lab has demonstrated a clear phenotype of reduced survival and cardiac rupture in *Jam-A*$^{gt/gt}$ mice following experimental induction of MI. However, the cause of this dysfunction is still unknown. Since JAM-A is only found on endothelial cells, leukocytes, and platelets the contribution of one of these cell types must be responsible for the decreased survival we see in *Jam-A*$^{gt/gt}$ mice. It is known that the vascular endothelium, by supplying proper blood flow and the release of local mediators, maintains a healthy myocyte population within the heart. It is possible that the loss of JAM-A from this cell type somehow prevents proper endothelial function leading to the disregulation of myocytes. In order to determine if this is the case, it would be useful to see if any changes in the expression of endothelial mediators has occurred with the ablation of JAM-A. Real time PCR and ELISA assays for factors released by the endothelium including endothelin-1, angiotensin II, and other factors that induce hypertrophy must be performed. Also, nitric oxide is known to effect both myocyte hypertrophy and heart chamber dilation. In the body, NO that is produced is unstable and quickly converts to NO_3^- and NO_2^- through the Griess reaction. Although NO itself is difficult to measure, the presence of its metabolites can be detected in a colorimetric assay and read on a spectrophotometer[110]. These experiments would indicate whether the endothelium is functioning normally to support a healthy myocyte population.

Another culprit in the phenotype we see in the *Jam-A*^{gt,gt} mice may be the structure of the microvasculature. Schafer et al found a distinct decrease in the protein

level of β-catenin and the endothelial junction protein VE-Cadherin in human hearts with DCM removed from transplant patients. Immunohistochemical analysis indicated that while the vasculature was intact, its structure appeared to lack integrity. Also, endothelial expression of VE-Cadherin and β-catenin were unaffected by VEGF treatment[90]. This group, however, was unable to pin down the mechanism behind this phenotype. It is possible that one cause of DCM is the improper structure of the microvasculature leading to poor delivery of nutrients and general dysfunction of the endothelial cells. We have seen a defect in FGF-2 induced angiogenesis in *Jam-A$^{gt.gt}$* mice that is compensated for by an increase in VEGF signaling[55]. While the number of blood vessels is similar in the gene trapped strain compared to the WT, perhaps the structure of the vessels are altered. Comparing immunohistochemical sections of the microvasculature of WT and *Jam-A$^{gt.gt}$* mice stained for JAM-A and other markers of endothelial cells such as PECAM-1 and vWF may determine if this is the case.

Recently, a report by Liu et al[111] demonstrated a novel role for platelets in the healing infarct. They found that the initial infiltration of leukocytes into the ischemic area is simultaneously accompanied by platelet aggregation. Antiplatelet therapy following MI reduced local inflammation, indicating that platelets play a unique role in the activation of inflammatory cells. This activation is likely through the direct interaction of P-selectin and its receptor on the leukocyte. Also, Liu et al found that clopidogrel therapy prior to MI reduced both the presence of urokinase plasminogen activator in the plasma and, importantly, the rate of cardiac rupture. Our laboratory has previous shown that *Jam-A$^{gt/gt}$* platelets exhibit hyperaggregation[50]. It is possible that the overactive platelets in *Jam-A$^{gt/gt}$* mice are somehow leading to the

cardiac rupture phenotype. Still, much more work needs to be done in this area to understand the exact role of platelets in the healing infarct.

   While I was unable to determine the mechanism behind the cardiac rupture and DCM phenotypes we see in *Jam-A*$^{gt/gt}$ mice, the experiments outlined in this report have been useful. Previously, the only report of an endothelial tight junction protein involved in either of these two conditions is the decrease in VE-cadherin seen in human patients with DCM. If the endothelium is truly responsible for these phenotypes, these results will shed light on a previously unknown facet of cardiac remodeling and endothelial/myocyte interaction. Also, the cardiac rupture we see may be caused by aberrant platelets in *Jam-A*$^{gt/gt}$ mice. The role of platelets in cardiac repair remains elusive. If JAM-A is involved in platelet induced cardiac rupture, it may prove useful to administer antiplatelet medications immediately following MI. Furthermore, there is no direct therapy that can prevent cardiac rupture. Once rupture has occurred, it is almost certain that death will follow. As for DCM, treatment involves medication which causes an increase cardiac output and a decrease blood volume. Due to the complexity of DCM, there is no direct intervention available to correct this condition. Understanding the role of JAM-A in the pathogenesis of these conditions may lead to novel medications and improved patient outcomes in the future.

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