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Understanding Neonatal Cardiac Mitochondria Morphology and Structure and the Effect of Macrophage Phenotype on Cardiomyocyte Morphology

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Understanding Neonatal Cardiac Mitochondria Morphology and Structure and the Effect of Macrophage Phenotype on Cardiomyocyte Morphology

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Biomedical Engineering

by

Jeoung Hyun Kawk

Thesis Committee:
Assistant Professor Anna Grosberg, Chair
Assistant Professor Wendy F. Liu
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ABSTRACT OF THE THESIS

Understanding Neonatal Cardiac Mitochondria Morphology and Structure and the Effect of Macrophage Phenotype on Cardiomyocyte Morphology

By

Jeoung Hyun Kawk

Master of Science in Biomedical Engineering

University of California, Irvine, 2017

Assistant Professor Anna Grosberg, Chair

Neonatal cardiomyocytes are not the most mature form of cardiomyocytes and may contain different structures and morphology compared to those of adult cardiomyocytes. Especially, mitochondrial morphology and structures has not been extensively studied in neonatal cardiomyocytes, leaving the criteria for normal neonatal mitochondrial morphology and structure unclear. Due to this uncertainty, the source of variability, whether it is physiology or external factors, in neonatal mitochondrial morphology and structure could not be accurately assessed. In this study, fixation and/or staining protocols were explored to test their influence on mitochondrial morphology and structure in neonatal cardiomyocyte culture. It was concluded that alterations in PFA concentration and/or incubation time and stain and primary antibody concentrations did not affect mitochondrial morphology and network. In the second part of the study, influence of cytokines in cardiac hypertrophy was investigated by analyzing changes in cell area occurred post introduction of specific cytokines. There are different macrophage populations, and they are often classified into fully polarized states of M1 and M2. M1

macrophages have pro-inflammatory characteristics whereas M2 macrophages have anti-inflammatory characteristics. It was shown that conditioned media obtained from M2 polarized macrophages that contain anti-inflammatory cytokines reduced the cardiomyocyte area, suggesting that cytokines released by M2 can protect and rescue the heart from cardiac hypertrophy. This finding substantiates the capacity of cytokines released from M2 polarized macrophages as possible treatment option for cardiac hypertrophy and adverse remodeling. Although the results regarding the stimulating factors and M1 polarized, or inflammatory macrophages, conditioned media on cardiomyocyte area were unconvincing and not fully understood, this study was successful in validating promising effect of anti-inflammatory cytokines in resolving cardiac hypertrophy.

Chapter 1

Introduction

The heart is a four-chambered muscular organ that is essential for survival in vertebrates. It pumps blood to entire body through blood vessels to supply nutrients and oxygen to cells and remove metabolic wastes. Due to its critical role in circulation, abnormalities and dysfunctions of the heart can be critical in one's health. Unfortunately, a significant portion of the world's population is suffering from heart diseases varying in severity. This encourages breakthroughs in cardiovascular research striving to better understand the structures and function of the heart in the hopes of preventing deterioration of its function. The heart is a complex organ composed of cardiomyocytes, fibroblasts, endothelial cells and perivascular cells, and each component plays important roles in maintaining normal heart function (1). In order to achieve proper circulation, cardiomyocytes must contract both consistently and synchronously to produce enough force to supply the body with blood. The organization of myocardial sub-structures including actin fibers, sarcomeres, nuclei, microtubules and mitochondria is critical in cardiac force production as it can alter both mechanistic and energetic properties of the heart (2). Consistent contraction of heart muscle requires continual supply of energy in the form of ATP, which is needed to release actin from myosin in cardiomyocyte contraction and to sequester calcium back into the sarcoplasmic reticulum during diastole (3). Therefore, it is not surprising that heart contains the most number of mitochondria compared to other organs to accommodate higher energy demand and that its sub-constructs are organized to transfer energy efficiently.

Mitochondria are highly dynamic double membrane organelles that act as the powerhouse of cells due to the production of ATP, a high-energy molecule. ATP production occurs through oxidative respiration, more specifically oxidative phosphorylation. This process

involves coupling oxygen to the phosphorylation of ADP to produce ATP (4). Abnormalities within the mitochondria impair energy supply and cause damage and even cell death. In addition to their role in energy production, mitochondria also play important role in cell differentiation, cell death and cell cycle control (5, 6). Since the normal heart function heavily depends on proper mitochondrial function, mitochondrial dysfunction is one of the main causes of cardiovascular diseases. As highly dynamic organelles, mitochondria undergo fusion and fission processes, are highly motile and possess various morphologies dependent on the health state of the heart (7, 8). For example, mitochondria are fragmented in dilated cardiomyopathy and heart failure induced by lower ATP availability, whereas they are fused in hypertrophy due to aortic constriction (9). Mitochondrial morphology is also linked to alteration in mitochondrial bioenergetics (4, 10). Since their dynamics are closely related to the metabolic status of the cell, mitochondrial morphology and organization can indicate a cell's functionality. In response to these findings, assessing the health of the heart based on mitochondrial morphology seems to be plausible, and targeting mitochondrial metabolism became one of the emerging treatment options for cardiovascular diseases (11). However, in neonatal cardiomyocyte culture, a significant number of mitochondria took abnormal morphology and structures as mentioned above. It is unclear if this is due to physiology or due to external factors like fixation and staining processes. As part of the endeavor to better understand mitochondrial morphology and organization, the possibility of mitochondrial morphology and structure alterations in neonatal cardiomyocytes by fixation and/or staining methods was evaluated in this study.

Since the heart is one of the most important organs in the body, abnormal heart function results in dangerous consequences. Unlike the liver where regeneration occurs after damage, the heart does not regenerate beyond fetal stage (12). Instead, the heart undergoes remodeling after

heart injury which imposes adverse effects on its function. One of the common remodeling consequences is hypertrophy which involves enlargement of cardiomyocytes, enhanced protein synthesis and higher organization of the sarcomere (13). However, the mechanism of this change is poorly understood and requires deeper understanding. One of the suspected players of this inflammatory response is macrophage. After injury occurs in heart, macrophages which are large phagocytic cells found in all tissues that respond to infection or cellular damages play significant roles in cardiac in inflammatory and wound healing processes (14). There are different types of macrophages and their interactions are very complex. Based on the environmental cues, macrophages are activated, recruited to the injury site and engage in inflammatory responses and as well as anti-inflammatory responses (15). It is evident in many studies that macrophages play critical role in cardiovascular system but a lot about their complex functions and mechanisms need to be elucidated. In this study, we specifically looked at the role of specific cytokines in single cardiomyocyte area change to elucidate their functions in cardiac hypertrophy.

Chapter 2

Influence of Fixing and/or Staining Method on Mitochondria Structure in Neonatal Cardiomyocytes

2.1 Introduction

The heart has the largest number of mitochondria compared to other organs. Mitochondria play crucial role in continual contraction of the heart by supplying energy required for the contraction through oxidative phosphorylation. Mitochondria make up a large part of myocytes and are located between myofibrils and just below the sarcolemma (6). Also, intermyofibrillar mitochondria are arranged in highly ordered crystal-like patterns in muscle cells (16). A close relationship between cytoskeletal structures, such as actin, intermediate filament and microtubules, and mitochondria is evident from many studies. Such mitochondrial organization is intended to efficiently transport ATP to nearby contractile unit in cardiomyocytes. Mitochondria are involved in various pathologies including ischemia, cardiomyopathy, and congestive heart failure in which energy deficiency is observed (6,17). Disorganization of cytoskeletal structures can be observed in failing or defective hearts, explaining disruption in efficient energy transfer pathways between cardiomyocytes and mitochondria (18). Since mitochondrial morphology and organization provide important information about metabolic status of the heart, it is crucial to study their spatial relationships among different sub-cellular structures. Quantification of mitochondria and cytoskeletal structure organizations will provide better insight into their relationships in different health states of the heart. In order to establish metrics describing their spatial relationships, normal mitochondrial structures must be analyzed. However, fixed neonatal cardiomyocyte samples contained both swollen and fragmented

mitochondria as well as normal connected mitochondria that resemble normal adult mitochondrial structure. It is necessary to understand the source of this variability in morphology to study their organizations properly. The purpose of this study was to validate the effect fixation and/or staining on mitochondrial morphology and network.

2.2 Materials and Methods

Substrate Preparation

Glass coverslips were sonicated in 95% ethanol for 30 minutes. PDMS and curing agent were mixed in ratio of 10:1. Using Thinky Mixer, PDMS (Ellsworth Adhesives) and curing agent were mixed and degassed. Clean glass coverslips were spin-coated with PDMS and cured in 60C° oven overnight.

Extracellular Matrix Preparation

Silicon wafers for anisotropic patterns were prepared by photolithography techniques. To make anisotropic patterned stamps, PDMS with curing agents was poured onto petri dish containing the silicon wafer and cured overnight in 60c oven. Pattern squares were imprinted on PDMS layer which were then cut out and sonicated with 95% ethanol. PDMS stamps were covered in 100ug/ml fibronectin (Fisher Scientific Company, Hanover Park, IL) for 1 hour. UVO-treated PDMS coated coverslips were stamped using fibronectin coated stamps and submerged in 1% Pluronic acid solution (5g Pluronics F-127, Sigma Aldrich, Inc., Saint Louis MO, dissolved in 500mL sterile water) for 10 minutes to block cell adhesion between fibronectin patterns. Coverslips were washed three times with and stored in PBS (Life Technologies, Carlsbad, CA) at 4 C° until use.

NRVM Harvest and Culture

1-2 day-old neonatal Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were sacrificed, and their left ventricles were harvested per UC Irvine IACUC approved animal protocol (Protocol No.2013-3093). The hearts were cut into small pieces, washed with Hank's Balanced Salt Solution (HBSS) (Life Technologies, Carlsbad, CA) and treated with 1mg/mL trypsin (Sigma-Aldrich, Inc., Saint Louis, MO) while rotating at 75 rpm for twelve hours at 4°C. Trypsin solution was removed and M199 culture medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 10mM HEPES, 0.1 mM MEM non-essential amino acids, 3.5 g/L glucose, 2 mM L-glutamine (Life Technologies, Carlsbad, CA), 2 mg/L vitamin B-12, and 50 U/mL penicillin (Sigma-Aldrich, Inc., Saint Louis, MO) was added to neutralize the tissues. Then, tissues were digested with 1mg/mL collagenase (Worthington, Lakewood, NJ) solution at 37 °C, and cardiomyocytes were released into solution. Cardiomyocytes were isolated by two rounds of centrifugation at 1200 rpm for 10 minutes with resuspension of cell pellet with cold HBSS in between. Final cell pellet was suspended in 10% FBS M199 media. To increase purity of the culture, two 45-minute pre-plating and one 40-minute pre-plating steps were performed before seeding cardiomyocytes on to the substrates. The cardiomyocytes were seeded at the density of 1M cells/3mL for confluent monolayer and 45000 cells/3mL for sparse culture. Cells were incubated in 10% FBS M199 media at 37°C and at 5% CO_2. 24 hours after seeding, dead cells were washed out and media was changed to fresh 10% FBS M199 media. Media was changed to 2% FBS M199 media 48 hours after seeding to limit fibroblast proliferation.

Live Staining Mitochondria

For live staining of mitochondria, 100nM MitoTracker Red CMX Ros (Life Technologies, Carlsbad, CA) was diluted with 2% FBS M199 media to appropriate concentrations. 72 hours

after seeding, cells were incubated in warm MitoTracker-media mixture in incubator at 37C°. After 30 minutes, cells were washed with warm PBS twice and incubated in fresh 2% FBS media at 37C° for 2 hours before fixing.

Fixing

All samples except those used in fixative and/or permeabilization reagent concentration and/or incubation time variation studies were fixed in 4% PFA with 0.05% Tx-100 for 10 minutes. 4% Paraformaldehyde (PFA) with 0.05% Triton X-100 (Sigma Aldrich, Inc., Saint Louis MO) was prepared from diluting 16% PFA (Fisher Scientific Company, Hanover Park, IL) and warmed up in a water bath at 37C°. For PFA concentration variation experiments, appropriate amount of PBS was added to produce target concentrations. Moreover, for PFA spike experiment, 500ul of 4% PFA was added to cells in culture media for 2 minutes prior to fixing procedure. Samples were washed three times in warm PBS and were incubated in PFA/Triton-x solution for appropriate time frame. Finally, cells were washed with warm PBS three times with 5-minute incubation in between the washes and were stored in PBS.

Immunostaining

For immunostaining, primary antibodies including sarcomeric a-actinin (Mouse Monoclonal Anti-a-actinin, Sigma Aldrich, Inc., Saint Louis, MO), 4',6'-diaminodino-2-phenylinodole (DAPI) (Life Technologies, Carlsbad, CA), Alexa Fluor 488 Phalloidin (Life Technologies, Carlsbad, CA) were diluted 1:200 in PBS and used to stain sarcomeres, nuclei and actins, respectively. Different dilutions of COXIV and GRP75 antibodies (Abcam) were used to stain mitochondria. Secondary staining was performed using tetramethylrhodamine-conjugated goat anti-mouse IgG antibody (Alexa Fluor 633, 750 Goat Anti-Mouse, Life Technologies, Carlsbad, CA). Samples were incubated in primary and secondary stain solution for 1-2 hours

each and were washed three times with PBS to remove excess stain in between. Same washing procedure was done after secondary antibody incubation. Lastly, they were mounted onto glass microscope slides (VWR, Randor, PA) using Prolong Gold Antifade Reagent (Life Technologies, Carlsbad, CA) as a mounting media and were sealed with nail polish. The sealant was cured overnight at room temperature.

Imaging and Image Analysis

IX-83 inverted motorized microscope (Olympus America, Center Valley, PA) mounted with a digital CCD camera ORCAR2 C10600-10B (Hamamatsu Photonics, Shizuoka Prefecture, Japan) and an UPLFN 40x oil immersion objective (Olympus America, Center Valley, PA) was used to image stained samples. Image analysis was done using customized MATLAB software.

2.3 Results

The aim of this study was to elucidate mitochondrial morphology and structures in engineered neonatal cardiomyocytes. To successfully analyze their organizations, mitochondrial images of good quality are required. However, this was hindered by poor staining and morphological variations of mitochondria in fixed cardiomyocyte samples. In an effort to generate suitable images for accurate structural analysis and to have better understanding of neonatal mitochondrial morphology, this chapter consists the troubleshooting of fixation and mitochondrial staining methods in both fixed and live samples.

MitoTracker Red CMXRos Live Staining

MitoTracker Red CMXRos was used to stain mitochondria in both live and fixed cells. Live staining with 100nM, 50nM and 25nM MitoTracker Red CMXRos followed by 4% PFA with 0.05% TritonX-100 fixation-permeabilization produced images with swollen and

fragmented mitochondria (Figure 1). Reducing MitoTracker Red CMXRos concentration to 10nM improved overall mitochondrial morphology and network. Although, lower dye concentration allowed maintenance of connected and defined mitochondrial network, the fluorescence signal was reduced (Figure 2A-D). Furthermore, fixative concentration and incubation time were varied to produce mitochondrial morphology that resemble closely with that of the example image (Figure 2F). However, none of the tested conditions produced significantly improved overall mitochondrial morphology. Also, PFA spiking followed by PFA fixation did not improve staining (Figure 2E).

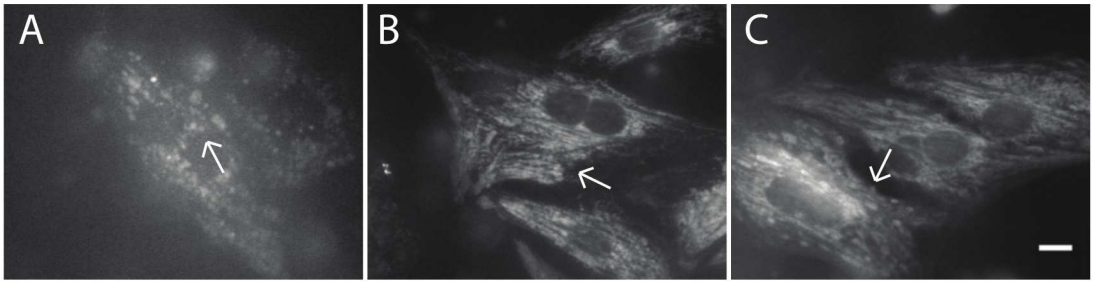

Figure 1. Effect of MitoTracker CMXRos Concentration on Mitochondrial Morphology. Mitochondria stained with 25nM (A), 50nM (B), 100nM (C). Arrow indicates swollen or fragmented mitochondria. Scale bar 10µm.

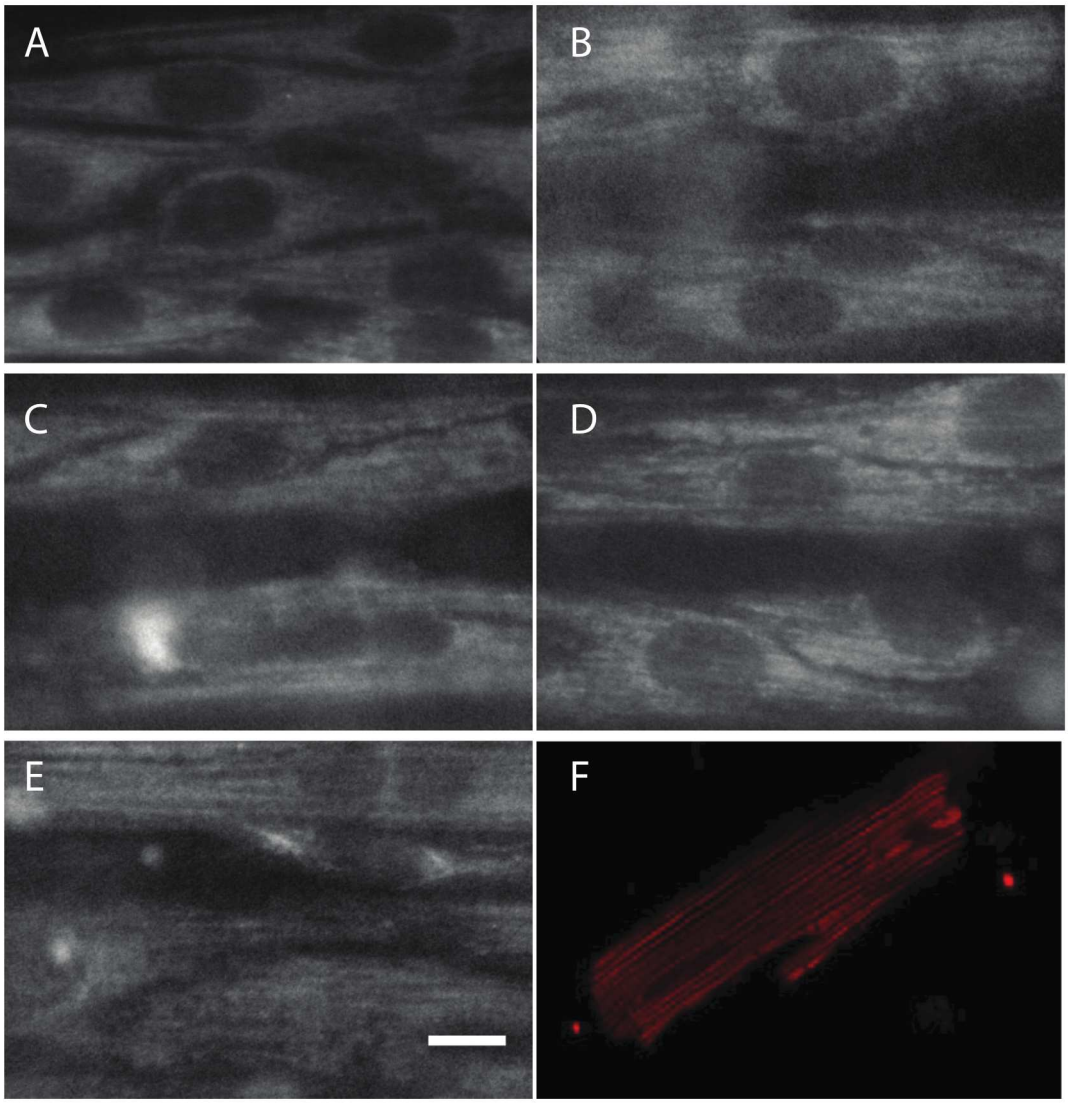

Figure 2. Effect of PFA Concentration and Incubation Time Variations on Mitochondrial Morphology. Mitochondria stained with 10nM MitoTracker CMXRos fixed with 2% PFA (A-B) or 4% PFA (C-D) for 5 minutes (A, C) or 10 minutes (B, D) while permeabilization solution TritonX-100 concentration remained constant at 0.05%. Scale bar 10μm (A-E). (F) Data from Hom et al. Example image of mitochondria in **adult** rat cardiomyocyte visualized using TMRE. Scale unknown (25).

Anti-GRP75 Staining

Glucose related protein (GRP75) is a member of heat shock protein 70 family localized in mitochondria. A previous study showed that the antibody against Grp75 co-localized with mitochondria specific dye rhodamine 123 (19) in fixed REF-52 cells, a rat embryo fibroblast cell-line, showed targeted mitochondria staining. However, anti-Grp75 antibodies did not stain mitochondria in neonatal rat cardiomyocytes in any of the concentrations tried (Figure 3A-B).

Anti-COXIV Staining

Another primary antibody used to stain mitochondria was cytochrome c oxidase (COXIV), which is the last enzyme in the mitochondrial electron transport chain located in inner mitochondrial membrane. It is one of the most widely used mitochondrial markers for various mitochondrial detection assays (20). In order to optimize visualizing mitochondria using this antibody, the primary antibody concentration and incubation time were varied. It was found that 10ug/mL anti-COXIV visualized mitochondria better than 5ug/mL anti-COXIV and 2-hour incubation time was required to produce the optimal staining of mitochondria in neonatal cardiomyocyte (Figure 3C, Dii). Yet, the quality of mitochondrial morphology and networks within the same sample were inconsistent. Abnormal morphologies such as swollen and fragmented forms were observed. Further verification of the mitochondrial morphology population is necessary.

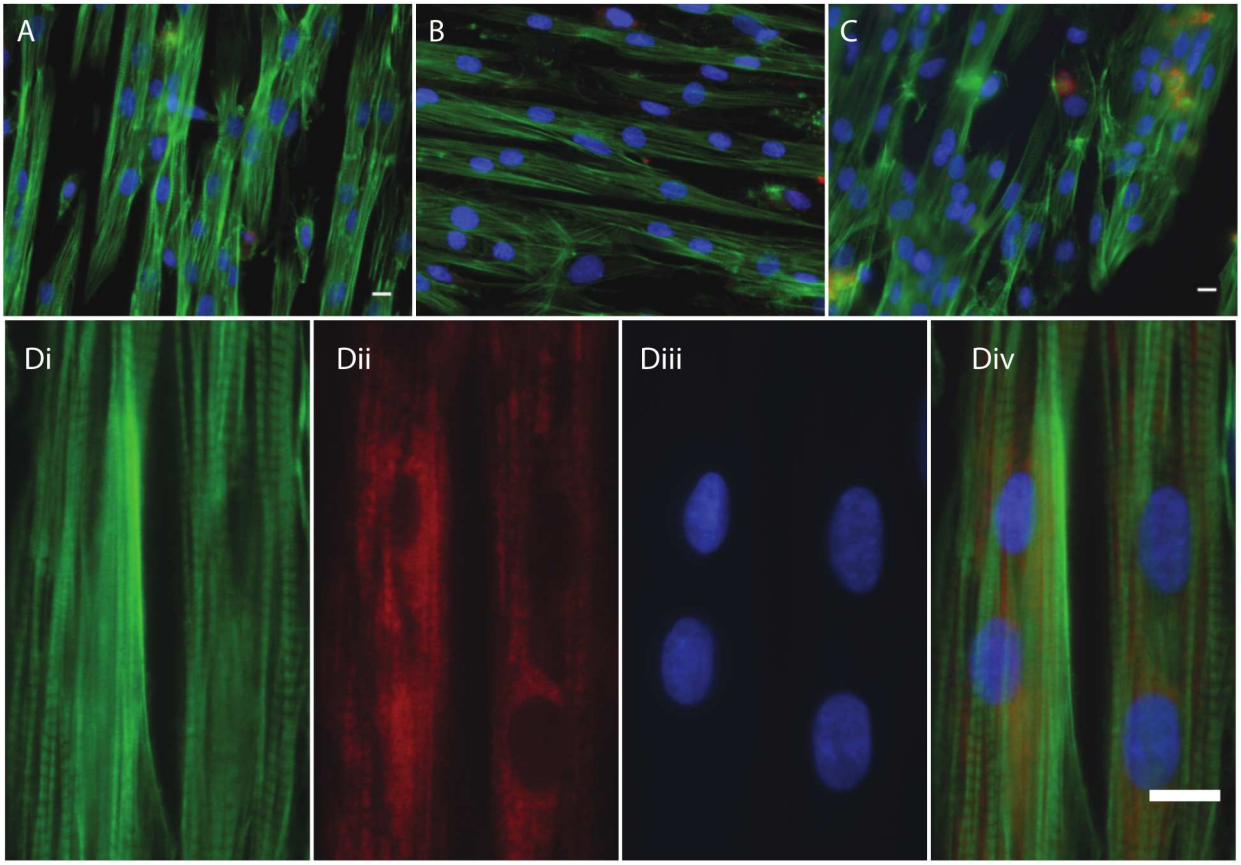

Figure 3. Immunostain Images of Mitochondria and Cytoskeletal Structures. Mitochondria (Red) stained with primary antibody against GRP75 with 1:100 dilution (A) and with 1:200 dilution (B). Anti-COXIV primary antibody used to stain mitochondria (Red) with 1-hour (C) and 2-hour incubation times (Dii). (Div) Merged images of (Di-Diii). Actin (Green) and nuclei (Blue) stained with Phalloidin and DAPI. Scale Bars 10µm.

2.4 Discussion and Future Directions

It is well established that mitochondria serve a critical role in sustaining proper heart function. Many studies show that mitochondria are involved in cardiac diseases, and their structure and morphology have close associations with cardiac function and pathologies (21). For example, fragmented mitochondria were present in dilated cardiomyopathy, MI heart failure and

myocardial hibernation (2, 22, 23). However, it is still not fully elucidated how changes in cell structure and mitochondrial morphology influence mitochondrial bioenergetics and dysfunctions observed in cardiac pathologies or vice versa. Therefore, studying correlations of mitochondrial organization with contractile components will provide better scope in understanding abnormal or pathological heart where both structural and energetic changes occur and developing metrics will facilitate understanding of their structural relationships. Prior to analyzing mitochondrial organization in fixed neonatal cardiomyocyte culture, presence of variable mitochondrial morphology should be evaluated.

In fixed neonatal rat cardiomyocyte culture, combinations of fragmented, swollen and connected mitochondria were observed. It is unsure if this is normal morphology for immature neonatal cardiomyocytes or abnormal morphology induced by fixation and staining processes. So, the primary goal of the study was to determine if alterations in fixation and/or staining methods will change mitochondrial structures. Since mitochondria are dynamic organelle, it is probable that they are sensitive to the changes in their environments including sudden changes in pH, temperature and osmolarity during fixation process. As observed in other studies, a significant number of abnormal morphologies such as swelling and fragmentation of mitochondria was observed in cells treated with 4% PFA with 0.05% triton-X fixation/permeabilization method disregarding the type of staining vehicle used (Figure 2). It is known that osmolarity of PFA solution differs significantly from that of physiology, and this difference can cause changes in cell structures (24). However, varying PFA concentration and incubation time did not improve the variations and alterations in mitochondrial morphology in samples because this method still exposed the cells to experience sudden changes in osmolarity. In the effort to minimize sudden osmolarity change, cells in the culture media were spiked with

small amount of 4% PFA followed by standard PFA/TritonX fixation-permeabilization method. Unfortunately, this method also did not notably improve mitochondrial staining. Spiking the cultures in media with PFA and reducing MitoTracker dye concentration slightly enhanced mitochondrial morphology and networks. As seen in Figure 2F, normal mitochondria in adult cardiomyocyte are well organized in "crystal-like pattern" and arranged similarly to actin organization (25, 16). Anti-COXIV primary antibody stained mitochondria with strong signal and acceptable morphology and network (Figure 3Dii). The mitochondrial arrangement and network of the neonatal cardiomyocytes used in this study were comparable to that of the example adult cardiomyocytes but still contained swollen mitochondria (Figure 2F).

In sum, optimization of neither fixation nor staining methods significantly improved mitochondrial structure and network. Since these cells were aligned into anisotropic patterns to approximate adult cardiomyocyte structures, it is possible that they are not actually fully mature enough to have adult mitochondrial organization. In this case, morphological variations of mitochondria in these cells may not be caused by fixation or staining processes but may be normal for neonatal cardiomyocytes. This can be further verified by visualizing mitochondria in live samples with or without using dye or stains. To image live mitochondria, fluorescent protein transfection can be another way to image live cells instead of MitoTracker dye (26). On the other hand, two-photon fluorescence lifetime imaging microscopy (2P-FLIM) uses naturally fluorescent NADH and FAD to image live cells non-invasively. Since mitochondria contain abundant amount of NADH and FAD due to their metabolism, the use of FLIM will eliminate the cytotoxicity induced by addition of fluorescent dyes (27). Furthermore, two-photon microscopy reduces photo-bleaching and photo-toxicity, so normal mitochondrial morphology can be preserved better (28).

Once quality images of mitochondria are validated, orientation and co-orientation order parameters can be utilized to quantify correlations of mitochondria and other cytoskeletal structures in heart (29). Better understanding of spatial correlation among mitochondria and contractile elements will clarify their dynamics and structure alterations occur in both physiological and pathological heart.

Chapter 3

Influence of Macrophage Phenotype on Cardiomyocyte Morphology in Cardiac Hypertrophy

3.1 Introduction

Myocardial Infarction (MI) is one of the most common diseases among current generations. The disease is caused by reduction of oxygen supply in heart following obstruction in the coronary artery. Inflammatory cascade, including cardiomyocyte necrosis, neutrophil and macrophage infiltration and cardiac remodeling occur following ischemia. Remodeling of the left ventricle of the heart is usually adverse, resulting in left ventricular dilation and function reduction (30). Cardiac hypertrophy is another undesirable cardiac remodeling following infarction resulting higher risk of heart failure and even sudden death (13, 31). It is associated with thickening of the heart muscle wall and enlargement of cell size in cellular level. Evident in numerous studies, macrophages play significant roles in inflammatory responses and remodeling after heart injury (32). For example, it is known that macrophages are the main source of different types of MMPs and TIMPs, which are required for deposition and degradation of ECM respectively (33). Also, they participate in cardiac remodeling by activating fibroblasts and endothelial cells for scar formation and angiogenesis (34). Therefore, a better understanding of macrophages in cardiac remodeling is crucial in developing MI therapies which will prevent adverse remodeling post infarction. There are different macrophage phenotypes observed in inflammatory response. According to environmental cues, macrophages are activated into different macrophage populations, and they have different functions and play different roles at different time points along the progression of the inflammatory responses and cardiac

remodeling. Macrophages are plastic and can change phenotype in response to micro-environmental cues. Due to their outstanding plasticity, there exits wide spectrum of macrophage population. Inactivated macrophages, M0, are often fully polarized and classified into classically activated M1 and alternatively activated M2 (35). M1 is pro-inflammatory and secrete pro-inflammatory cytokines such as TNF-α and IL-6 and causes tissue destruction and cardiomyocyte hypertrophy. On the other hand, M2 is anti-inflammatory and secretes anti-inflammatory cytokines such as IL-10. They are involved in healing process like scar formation and angiogenesis (32, 36, 37, 38). Studies have shown that when either type of macrophage is depleted in the process, the healing process is impaired (39). Therefore, a fine balance between the different macrophage phenotypes is essential for an optimally remodeled heart after injury. However, much must be elucidated between macrophages interactions, behaviors and how they affect cardiac remodeling. In this study, the paracrine influence of macrophage in cardiac hypertrophy was investigated by observing cardiomyocyte area changes in response to different types of cytokines released by specific macrophages.

3.2 Materials and Methods

Substrate Preparation

Same procedures were performed as mentioned in Chapter 2.2 Materials and Methods (p.5).

Extracellular Matrix Preparation

For isotropic samples, coverslips were placed on to 50ug/mL fibronectin solution islands with PDMS sides contacting fibronectin. After 10 minutes, coverslips were rinsed three times and stored in PBS (Life Technologies, Carlsbad, CA).

BMDM Harvest and Culture

Bone marrow derived macrophages (BMDM) were derived from femurs of 6-12 week old C57BL/6J mice. The mice were sacrificed and their femurs were flushed with D-10 media (1X DMEM with 10%FBS, 100U/ml penicillin and 10% MCSF (macrophage colony stimulating factor) to collect cells. Cells were treated with ACK lysis buffer, centrifuged to remove contaminating red blood cells and re-suspended in fresh D-10 media. After 7 days in culture at 37C°, macrophage cells were harvested from the culture plate and seeded at 0.9M cells/6 well plate. Macrophages were stimulated with M1 (10ng/ml LPS and IFN γ) or M2 (20ng/ml IL-13 and IL-4) cytokines 4-6 hours after seeding. After 48 hours, macrophage polarization was confirmed by analyzing TNF-α and IL10 in supernatant by ELISA. The supernatant media was used as macrophage-conditioned media to stimulate cardiomyocytes. For experiments to study direct effect of M1 and M2 stimulating factors on cardiomyocytes, they were prepared without the cells in D-10 or M199 media and incubated for 48 hours at 37C°.

NRVM Harvest and Culture

Same procedures were performed as explained in Chapter 2.2 Materials and Methods (p.5) except that cells were seeded at 75000 cells/well to create sparse NRVM culture.

Cardiomyocyte Stimulation

Macrophage conditioned media was mixed with 2% FBS M199 in 1:1 ratio and introduced to cardiomyocytes 48 hours after seeding. Cells were incubated in 37C° for another 24 hours until fixation.

Fixing and Immunostaining

All samples were fixed in 4% PFA with 0.05% TritonX-100 for 10 minutes. Same procedures were performed for staining sarcomeres, actins and nuclei as described in Chapter 2.2 Materials and Methods (p.5).

Imaging and Image Analysis

Images were obtained using objective same confocal microscope system as mentioned in Chapter 1 but with UPLFLN 10x objective instead of UPLFLN 40x oil immersion objective. Cell purity analysis was done to determine the fibroblast content in samples using customized Image J macro. For cardiomyocyte area analysis, sarcomere images were processed with customized MATLAB code to outline cell boundaries and to calculate cell area.

Statistics

One-way ANOVA with Tukey test was used for pairwise comparison of treatment groups. Significance was determined for p value less than 0.001.

3.3. Results

Neonatal rat ventricular myocytes (NRVM) were seeded sparsely to study single cardiomyocytes in this study. Two days after seeding, cardiomyocytes were treated with different types of media as described in Chapter 3.2 materials and methods section. After 24 hours of incubation in different media conditions, cells were fixed, immunostained and analyzed for cell area and fibroblast content.

Cardiomyocyte Area

The custom MATLAB code used for cardiomyocyte area analysis selected and outlined the boundaries of cardiomyocytes based on sarcomere images. For single cell area analysis, merged, partly included and multinucleated cardiomyocytes were excluded (Figure 4 Di-ii). Due

to the variability of cell size among multiple harvests, all cell areas were normalized to the average M199 cell area from each harvest for area analysis. The results showed alterations in cell sizes after introduction of different media conditions (Figure 4). It was observed that macrophage culture media, D10, caused an increase in cell area when compared to cell area in M199 (Figure 4 A-B). Overall, there were significant increases in cell area in all conditions containing D10 media. The average cardiomyocyte area in M0 conditioned media and M1 conditioned media were not significantly different from that in D10 media (Figure 4A). However, the average area of cardiomyocytes incubated in M2 conditioned media was significantly lower than that in D10 but still higher than cell area in M199 (Figure 4B). Moreover, the effect of macrophage conditioned media on cell size was validated by incubating cardiomyocytes with stimulating factors for M1 and M2 macrophages. The stimulating factors were prepared in both M199 and D10 media to see the possible influence the base media has on cell area. It was revealed that the M1 stimulating factors, LPS and IFN-γ, in D10 media caused a significant cardiomyocyte size increase whereas M2 stimulating factors, IL4 and IL13, in D10 did not affect cell size compared to that of unmodified D10. On the other hand, it showed that M2 stimulating factors prepared in M199 decreased the cardiomyocyte area. Furthermore, LPS which was added in M2 conditions for speeding up cytokine release seemed to have an influence over cell size.

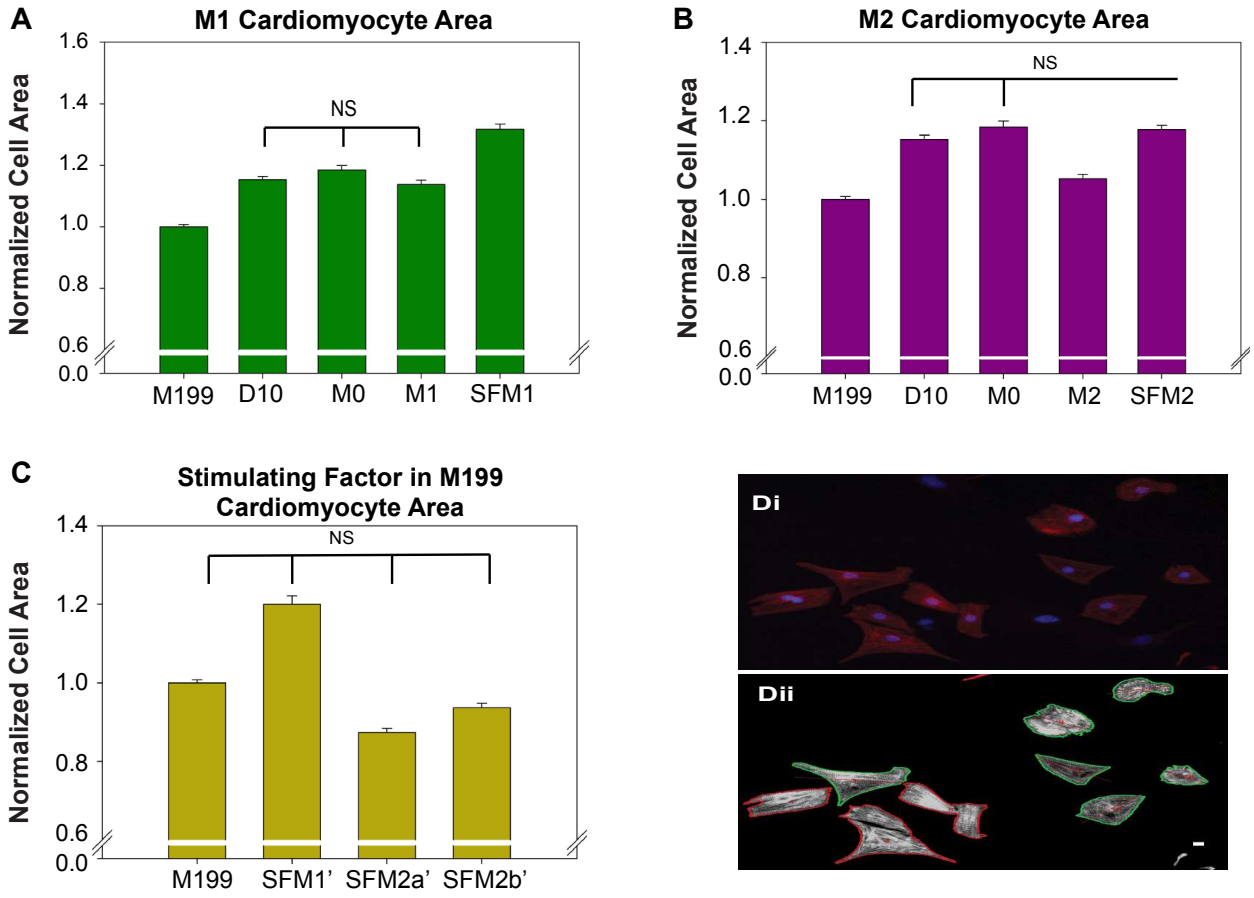

Figure 4. Effect of Macrophage Conditioned Media on Cardiomyocyte Area. Changes in Cardiomyocyte Area Caused by Different Macrophage Conditioned Media and Stimulating Factors. SFM1(LPS and IFN-γ) and SFM2 (IL4, IL13 and LPS) are M1 and M2 macrophage stimulating factors prepared in D10 media. SFM1', SFM2a'(No LPS) and SFM2b'(with LPS) are prepared in M199 media. (A-C) CM area affected by M1 factors in D10 (A), by M2 factors in D10 (B) and by stimulating factors for both M1 and M2 prepared in M199 (C). (Di) Merged sarcomere and nuclei image used to select mononucleated cardiomyocytes. (Dii) Example image of cell area analysis. Green indicate included cells and red indicate excluded cells. N= number of cells (500-3000). Error Bar Standard Error. Scale Bar 10μm.

Fibroblast Content

Fibroblast content in each condition was analyzed through calculating the ratio of fibroblast and cardiomyocyte in each sample (Figure 5A). Although it was expected that macrophage conditioned media have an impact on fibroblast proliferation, no significant differences among different conditions was observed (Figure 5B).

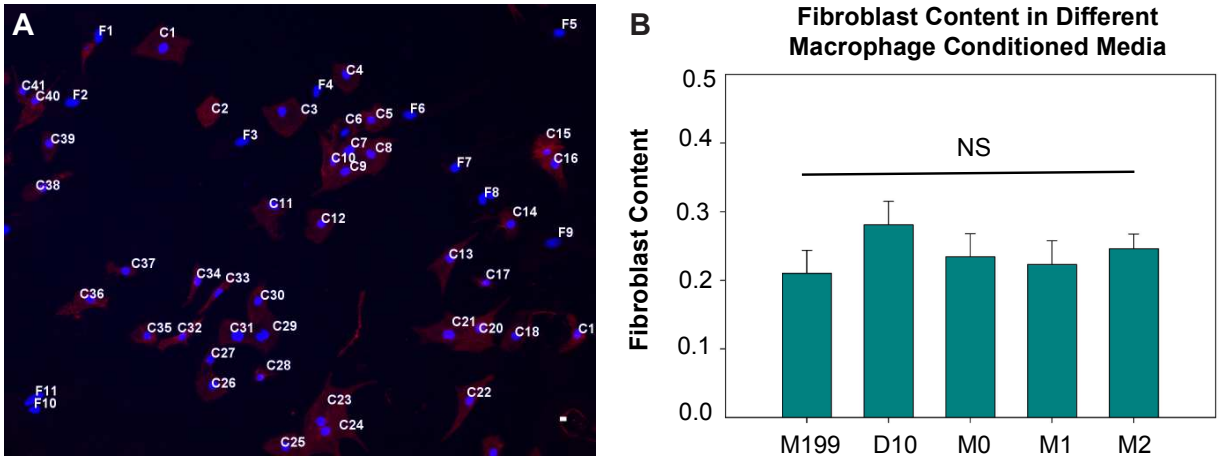

Figure 5. Effect of Macrophage Conditioned Media on Fibroblast Content. Fibroblast Content of Samples in Different Macrophage Conditioned Media. (A) Example image of fibroblast content analysis. (B) Fibroblast content in different macrophage conditioned media. NS indicates no significant difference between experimental groups. Error Bar Standard Error. Scale Bar 10µm.

3.4. Discussion and Future Directions

Although the fact that macrophages are involved in cardiac health maintenance as well as pathologies has been established, the complexity of macrophage interactions limits the complete understanding of immune response in the heart. In order to better understand macrophage role in cardiac hypertrophy and remodeling following heart diseases like myocardial infarction, specific macrophage conditioned media was introduced to cardiomyocyte cultures for 24 hours and observed for changes in cell size. Based on the close association of increased cardiomyocyte size

and cardiac hypertrophy, increased in the cell area was considered as indication of hypertrophy. This study serves as preliminary data for studying macrophage-cardiomyocyte interactions during inflammatory and healing responses. The set-up of this study also simplified the complex macrophage interactions and allowed the direct observation of the interaction between specific types of cytokines and cardiomyocyte size alteration. Studying influence of macrophages in cardiomyocyte size is critical to understand mechanisms behind cardiac hypertrophy and remodeling following heart damages. Overall, introduction of macrophage culture media D10 caused increase in cardiomyocyte area. This is most likely due to higher serum content in D10 media compared to that of M199 (40). There was a decrease in M1 conditioned media (M1) cell size compared to both M1 stimulating factor prepared in D10 media (SFM1) and M199 media (SFM1'). This suggests that stimulating factors for M1 activation, LPS and IFN-γ, caused cell area increase rather than the cytokines released by M1 macrophages. In M1 conditioned media, there were lower concentrations of the stimulating factors in the supernatant due to the factors being bound to the macrophages to induce their polarization. This lower concentration of LPS and IFN-γ and insufficient incubation time can explain the lower cell area observed in M1 experimental group. This result is inconsistent with previous findings, where the involvement of M1 macrophages in inflammatory response and hypertrophy were observed (39, 41). The effect of inflammatory cytokines released by M1 macrophage on cell area was not clear with the setup of this study and needs to be validated.

On the other hand, anti-inflammatory and pro-healing tendency of M2 macrophages were observed. It was shown that M2 stimulating factors, IL13 and IL4, prepared in D10 media (SFM2) did not have any influence on cardiomyocyte area whereas anti-inflammatory M2 cytokines reduced cell areas, overcoming the increase in cell area induced by D10 media. M2

stimulating factors in M199 media (SFM2') caused significantly lower cardiomyocyte are compared to the control. It is unclear why M2 stimulating factors in different media had different effects on cell size. However, it is definite that M2 cytokines reduced cell size significantly but not to the level of "unconditioned" M199 cells. This indicates that anti-inflammatory cytokines not only protect the cells but also alleviate hypertrophy through cardiomyocyte size reduction. The result obtained in this study was parallel with the IL-10 depletion study done by Verma et. al where cardiac hypertrophy, fibrosis and cardiac dysfunctions were exaggerated in IL-10 knock-out mice (42). Such findings show that cytokines are important and critical in inflammatory responses and healing of the heart during and after cardiac injury. Also, it suggests that such responses can be controlled through modifications in cytokine releases, and that this can be very promising in treating or preventing adverse consequences following heart disease to rescue its function. For example, premature introduction of M2 stimulating factors or anti-inflammatory cytokines to induce early onset of M2 activity during remodeling process may be possible ways to overcome cardiac hypertrophy.

Since it is known that M2 macrophages are involved in anti-inflammatory and healing processes in vivo through controlling fibroblast proliferation, it was expected that samples cultured in M2 conditioned media contain higher fibroblast content compared to other conditions tested. Based on this fact, it was expected that M2 conditioned media stimulate fibroblast proliferation in samples (43, 44). However, no significant difference was observed among different conditions.

For future studies, influence of different macrophage phenotypes on cardiac morphologies can be further clarified by observing alterations in subcellular structures of cardiomyocytes in response to different cytokines. Also, scratch-wound experiments can be

performed on confluent cardiomyocyte culture with incubation in different macrophage conditioned media to examine how different types of cytokines affect the healing process in cardiac tissue. This can be a better option to study fibroblast involvement and interactions with cytokines released by macrophages. Moreover, different culture systems can be used to reintroduce complexity to macrophage- cardiomyocyte interactions. Macrophages and cardiomyocytes can be co-cultured using trans-well plates to elucidate the indirect effect of macrophages on cardiomyocyte culture. Furthermore, they can be cultured in the same plate to study direct influences of macrophages as well as that of paracrine factors. Studying interactions among macrophages, cardiomyocytes and fibroblasts is crucial in understanding cardiac inflammation, hypertrophy and remodeling.

Chapter 4

Conclusions

The aim of the first part of the study was to determine if optimization of fixation and/or staining methods was sufficient to obtain normal mitochondrial structure in neonatal rat cardiomyocyte culture. It was shown that mitochondrial morphology and network did not significantly improve with modification of PFA fixation and staining protocols. This suggests that it is possible that fragmented and swollen mitochondrial morphology and structures may be normal for neonatal cardiomyocytes in culture and that it may be due to the not fully adult state of neonatal cardiomyocyte rather than fixation and staining processes.

The later part of the study investigated the effect of macrophages via paracrine pathway in cardiac hypertrophy by examining changes in cardiomyocyte area. Clearly, anti-inflammatory cytokines produced by M2 macrophages alone reduced the cell area, denoting the possibility of anti-inflammatory cytokines as therapeutic agents to mitigate cardiac hypertrophy and to promote beneficial cardiac remodeling.

Furthermore, this work has shown how cell culture methods can be used to study both intercellular structures and whole cell structure in response to varied conditions.

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