Department of Biomedical Engineering

Myocyte Derived Cardiac Spheroids for Post Infarct Cardiac Regeneration

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Abstract

Research has shown that autologous progenitor-like cardiac spheroids, when delivered to an infarcted heart, are able to restore mechanical function. These spheroids are made by isolating and expanding autologous cardiac progenitor cells. Though these results are promising, the process for creating cardiac spheroids is inefficient and time consuming, requiring a large amount of cardiac tissue. For every 10,000 cardiac myocytes in the heart there is only one cardiac progenitor cell; requiring a large amount of initial tissue. This clinical limitation could be overcome if cardiac myocytes, which are more abundant than cardiac progenitor cells, could be used to make cardiac spheroids. Research has shown that mesenchymal stem cells when co-cultured with adult cardiac myocytes cause the cardiac myocytes to behave like a progenitor cell. We found that, when co-cultured with mesenchymal stem cells, cardiac mycoytes could be made to form cardiac spheroid bodies. This was done by isolating adult myocytes from rat hearts and co-culturing them with human mesenchymal stem cells. After two weeks, cultures were observed to form spheroid bodies and the number of spheroids formed were compared to a pure myocyte control. Cardiac specific staining confirmed that the spheroids were made from the myocytes. It was also found that the mesenchymal stem cells, when co-cultured in the same well with the myocytes, form significantly more spheroids than myocytes treated with stem cell conditioned media. Further, no other cell type present in the co-cultures are able to create spheroid bodies when co-cultured with mycoytes or stem cells. The ability to create cardiac spheroid like bodies from adult myocytes offers a way to overcome the limitations of the time needed and the large quantity of autologous cardiac tissue required to currently make these types of bodies.

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Chapter 1: Introduction/Background

The pumping of blood by the heart drives the transport of nutrients and gas throughout the body, a process necessary for maintaining normal organ function and homeostasis. It is for this reason that the health of the heart is extremely important. Any compromise in the well-being of the heart is detrimental to overall quality of life. The heart is a muscle and the contraction of the muscle is what imparts pressure on the blood, driving it to the body. This is the mechanical function of the heart. Function can be compromised when part of the myocardium is damaged by oxygen deprivation caused by occlusion of the coronary arteries. The end result can be death of the downstream cardiac tissue, known as a myocardial infarction (MI). This dead tissue will not endogenously regenerate, resulting in a reduction of cardiac output and a therefore grave health concerns [1] [2].

1.1 Cardiac Disease

Heart disease is a national as well as global threat to public health, it is estimated that by the year 2020 11.1 million people will die from its effects [3]. Each year, in the U.S. alone, 500,000 new cases of severe heart failure (HF) are reported, a condition in which an insufficient amount of blood is pumped throughout the body to maintain normal daily activity [2]. Those who live with heart failure have a significantly reduced quality of life and are unable to perform

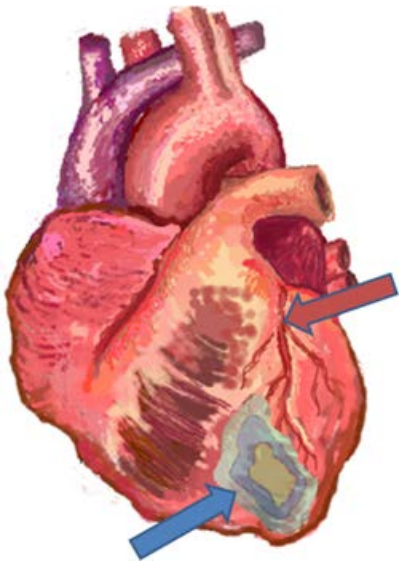

Figure 1: Blockage of the Coronary Artery (red arrow) and area of infarct (blue arrow).

a simple task such as climbing a flight of stairs without losing their breath.

This reduction in cardiac function is caused by necrosis of the myocardium following an infarction[4]. When a coronary artery is blocked for a prolonged period of time the cells downstream die and that region of the heart loses contractile function[4]. The area of infarct is remodeled by the body and becomes a stiff scar [4]. Figure 1 illustrates how a coronary occlusion, indicated with a red arrow, can lead to scarring of the downstream cardiac tissue, shown with a blue arrow. This stiff scar further impedes cardiac output as it is more rigid than the rest of the heart and therefore alters the regular mechanical pumping action during a heartbeat[5]. Figure 2, shows a cross section of an infarcted heart stained with Hematoxylin and Eosin (H&E). The infarcted area is outlined with a yellow dashed line, the discoloration of the infarct indicates the tissue no longer contains eosinophilic structures such as cell cytoplasm or protein. This suggests the tissue is no longer comprised of healthy cells and therefore is damaged, inhibited from behaving as normal cardiac tissue would. **In order to restore normal cardiac function, the region of infarct must be replaced with active contractile tissue**.

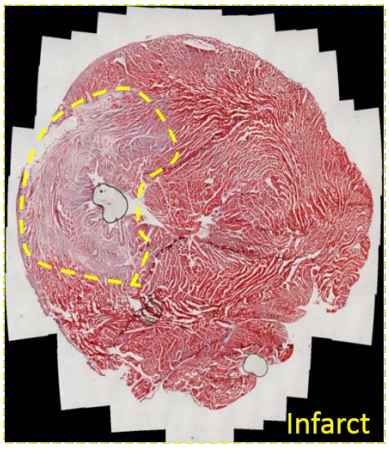

Figure 2: H&E staining on a cross section of an infarcted heart. The discolored area outlined with yellow is the area of infarct.

Current treatments for heart disease primarily focus on returning blood flow to the infarcted region of the heart and do not address the need to replace the damaged tissue. The gold standard for care is to restore blood flow by either bypassing the occlusion with a vascular graft, referred to as a coronary artery bypass graft (CABG), or to mechanically open the blocked vessel with a balloon. Following reperfusion, using one of these techniques, a patient is given anticoagulants to prevent future plaque buildup and/or a stent is implanted to physically suppress occlusion[6].

These treatments do not restore normal heart function as they fail to address the lost mechanical function due to the infarction resulting in a chronic reduction of ejection fraction (EF). Ejection fraction is the percentage of blood that is expelled from the heart during systole as compared to the total volume of blood in the heart at the end of diastole and is a way to quantify the ability of the heart to pump blood. A total allogeneic heart transplant or a left ventricular assist device (LVAD) is needed to restore the lost mechanical pumping force and reduced EF, both options are invasive and have high rates of mortality associated with them [7]. Furthermore, the number of hearts available for transplantation, roughly 2000 a year, is grossly inadequate to satisfy the half a million patients currently suffering from heart failure [2]. LVADs, though more readily available than donor hearts, also have risks associated with them. They do not treat or heal the heart, instead they aid the movement of blood with an implanted mechanical pump. As such, the use of LVADs is not an ideal method to treat HF and is generally used as a temporary measure to bridge a patient to transplant.

Current treatments for myocardial infarction are inadequate as they fail to mechanically restore the heart to its pre-infarct state. Research has been focused on identifying a way to replace damaged myocardium.

1.2 Cellular Therapy

In order to fully restore the lost contractile action of the heart after an infarct, the dead cardiac tissue must be replaced with new functional cells. These cells need to be able to actively contract in rhythm with the surrounding myocardium, as a native cardiac myocyte would. To do this a cell must contain the proteins responsible for contraction as well as the proteins necessary for synchronized beating with the rest of the heart. Some proteins used by the myocyte to achieve contraction are: actinin, troponin, myosin heavy chain, and myosin light chain. The protein connexin-43 forms the gap

junctions between cells that allows for the electrical signal to travel through the heart, triggering a beat. A cell source that is able to mimic the native myocardium without posing serious health risk has yet to be developed [8 9] . The following cells have been explored as therapeutic options: skeletal muscle cells, embryonic stem cells, mesenchymal stem cells (MSC), induced pluripotent stem cells (IPSC), and cardiac progenitor cell-derived cardiospheres (CPCS). Table 1 below outlines some of the advantages and limitations associated with using each of these cells as a therapeutic agent for myocardial infarction.

Table 1: The various cell types which have been researched for cardiac regeneration along with their advantages and disadvantages. Currently, no cell type contains the proteins necessary for synchronized beating without posing a serious health risk.

Cell type	Advantage	Limitation
Skeletal Muscle [10-15]	Contain proteins necessary for contraction	Do not electrically couple with native myocytes Can cause arhythmia
Embryonic Stem Cells [16-20]	Can be induced to differentiate into cardiac myocytes	Risk of teretoma formation
Induced Pluripotent Stem Cells [16, 21, 22]	Can differentiate into mature cardiac myocytes	Risk of teretoma formation. Can be carcinogenic Genetic manipulation Selecting pure population
Mesenchymal Stem Cells [23-25]	Shown to improve cardiac function when delivered to an infarct	Do not contain proteins necessary for contraction
Cardiac Progenitor Cell Derived Cardiospheres [26-29]	Can incorporate with native myocardium without eliciting host immune response.	Low cell number

The major limitation with current cellular based therapies is that they lack the ability to become mature electrically synched myocytes without the risk of becoming cancer or a teratoma [13, 30, 31]. Another limitation is the ability to scale up the production of cells to obtain a clinically relevant cell number.

One attempt at using cells for cardiac regeneration involved transplanting autologous skeletal myoblast cells into damaged myocardium [10]. These are the precursor cells to skeletal myocytes, the objective of their delivery to an infarct is to differentiate them into contractile cells. These cells would then be able to restore some of the lost cardiac function of the infarct. Skeletal myoblast are able to enter the cell cycle and undergo cellular division. The ability to form daughter cells is advantageous for replacing cells that are lost in a heart attack. Skeletal muscle cells, like cardiac myocytes are striated and contain the necessary proteins for contraction such as actinin, myosin heavy chain and troponin [32]. However, unlike cardiac myocytes which are involuntarily controlled, these cells are voluntarily controlled and lack the electrical gap junctions or pacing ability to contract in rhythm with the native myocardium. This can even cause cardiac arrhythmias as the electrical signal that propagates through the heart, to begin a beat, can be disrupted or perturbed resulting in a non-synchronous contraction [32]. For this reason, skeletal muscle cells do not hold promise as a treatment for MI.

Embryonic stem cells (ESCs) are another cell type that was extensively researched for use in repairing damaged myocardium. ESCs are cells taken from a human blastocyst, which have the potential to differentiate into any cell type. They are relevant for cardiac regeneration as they have been differentiated into cardiac myocytes and delivered to a mouse infarct model [33]. Though this method was effective in restoring lost contractile function post MI, the use of ESCs is associated with the formation of teratomas, tumors containing cell types of all three germ lines [34]; [35]. These growths within the body can be detrimental to health, therefore rendering ESCs unfit for human use. There are also ethical concerns associated with destroying an embryo to harvest the ESCs. Another limitation is that the ESCs may instil a rejection response from the host as they the cells of another individual. This would mean a patient would be required to take immune suppressing drugs for the rest of his/her life.

A newer technology that has been applied to the field of cardiac regeneration is the genetic reprogramming of fibroblasts into stem cells, otherwise known as induced pluripotent stem cells (IPSCs) [36]. These IPSCs can be differentiated into cardiac myocytes and subsequently delivered to an infarct[37]. Literature has shown that these cells do improve cardiac function post MI, but like ESCs, there are health risks associated with their use. The genetic manipulation of these cells makes them prone to developing into cancerous tumors, which can severely harm or kill the patient [38]. As such, a large effort must be made to purify the population of differentiated myocytes from the IPSCs. A second limitation is the low efficiency of reprogramming to a stem cell state. These are the two major limitations of using IPSCs for cardiac repair.

A cellular based approach to treating MI offers a means by which the area of damaged myocardium can be restored to functional tissue. The challenge is to find a viable cell source that can improve contractile force without posing a serious health risk to the patient. The first three cell types mentioned in Table 1, skeletal myoblast, ESCs, and IPSCs currently do not fulfill these criteria. They are able to differentiate into contractile cells, but each have inherent health risk associated with them. The skeletal myoblast do not contain the necessary proteins to electrically couple with the myocardium and as such can cause cardiac arrhythmias. A potentially dangerous condition for the patient. The health risk the ESCs and IPSCs pose is that they may develop into tumors when delivered to the heart. This can occur if one of the delivered cells is undifferentiated and begins to proliferate and differentiate into an unwanted tissue type. This is also dangerous for the patient. The last two cell types in table 1, MSCs and cardiac progenitor cell derived spheroids have shown the most promise in current research [39]; [5]. These cell types have been shown to safely reduce infarct size in human clinical trials.

1.3 MSC reduce infarct size Post MI

Bone marrow derived MSC have some ability to mitigate the effects of cell death in the heart following an infarction [23]. When injected in the border region of a mouse infarct model 68% percent of the necrosed area formed new myocardium after 9 days [23]. Further, myocytes within this region were found to be undergoing cellular proliferation, indicated by positive BRDU staining. A new vascular network also formed within the regenerated myocardium [40]. These results translated into human clinical trials with patients suffering from ischemic cardiomyopathy. Autologous mesenchymal stem cells were delivered via transendocardial intramyocardial injection to the infarcted region. One year after the procedure, infarct size had significantly decreased [41]. These findings illustrate that mesenchymal stem cells can promote the recovery of the scar formed post MI back to live tissue. Unlike ESCs and IPSCs, MSCs do not pose any serious health risk to the patient, when delivered to the heart [41]. In this trial, the MSCs proved to be immune privileged, eliciting no response from the host immune cells [41]. Despite these positive results, MSCs fail to significantly increase the reduced left ventricular ejection fraction (LVEF) caused by a MI, a critical parameter of cardiac health [9].

1.4 CPSs are able to improve ejection fraction post MI

Currently, a promising technology for cellular MI therapy is the use of cardiac progenitor cell derived spheroids (CPSs). These cells were directly compared to MSCs in their ability to restore mechanical function when delivered to a mouse model and it was found that the CPSs were able to better restore left ventricular ejection fraction (LVEF) than the MSCs [42].

Progenitor cells are undifferentiated stem like cells that are able to differentiate to one specific tissue type. CPSs, a type of progenitor cell, are made by isolating a population of autologous cardiac progenitor cells from a cardiac biopsy and then growing the cells in culture, after which, they form multicellular spheroid bodies[12 43, 44]. Unlike mature cardiac myocytes, some of these cells express Ki67, a mitotic indicator located in the nucleus, and are not striated nor rectangular. When implanted into a rat heart these cells demonstrated the ability to contract and expressed cardiac specific proteins [42]. This translated into an ability to improve cardiac ejection fraction in a rat infarct model as compared to an infarcted heart injected with just medium [28]. When these cells are delivered to a human heart following a MI, ejection fraction is also significantly improved and negative cardiac remodeling is reduced [11]. This was demonstrated in a human clinical trial called Cardiac Stem Cells in Patients with Ischemic Cardiomyopathy, SCIPIO. A right atrial biopsy was taken from 20 post infarct patients who were electing to receive a CABG. The cardiac progenitor cells were isolated from the biopsy and expanded. These cells were then delivered with the vascular bypass graft and left ventricular ejection fraction (LVEF) was used as a way to measure cardiac function. LVEF is the percentage of blood that is ejected from the left ventricle using the quantity of blood at the end of diastole as the basis for comparison. After 1 year, LVEF, on average, improved 8% and following 4 years, LVEF improved 12%. Though these gains are modest, they demonstrate the ability of CPSs to return mechanical function to a damaged human heart.

Despite these promising results, there is a major limitation associated with the creation of the cardiac spheres. The population of C-kit positive cells, a marker for cardiac progenitor cells, within normal cardiac tissue is very small, for roughly every 10,000 myocytes it is estimated there is one cardiac progenitor cell [45]. C-kit is a surface protein that acts a receptor for stem cell factors that can trigger proliferation or differentiation [46]. This is significant as adult cardiac myocte cells do not possess c-kit, and it's presence on a cell suggest the ability of that cell to proliferate or differentiate. The presence of

C-kit on a cardiac cell is one parameter used to define it as a cardiac progenitor cell. As these cells are rare in the heart, a large amount of biopsied tissue is required in order to derive a clinically significant number of CPS. The process of isolating the progenitor cells from a cardiac biopsy wastes a substantial number of differentiated cardiac myocytes. If there was a way to use the entire biopsy, the amount of tissue taken from a heart could be drastically reduced. Additionally, CPSs are cultured for 65 days before being harvested for use, a relatively long period of time for a MI patient waiting for treatment [9].

One advantage that CPSs may have over other cells intended for cardiac regeneration is they are 3D structures unlike other cells which are cultured on a 2D surface. The 3D morphology of the CPSs is more like the environment that cells in the heart experience than a 2D culture. One reason for this is the entire cell is in contact with other cells around it, this may change the signaling that the cell experiences. Another difference between 2D and 3D culture is the cells within the spheroids are anchored to material that is closer in stiffness to normal cardiac tissue. Tissue culture plastic is stiffer than heart tissue, this can play a role in cellular behavior. CPSs hold promise as agents for cardiac regeneration, but a more efficient way of deriving a cell type similar to these spheroid bodies is needed.

1.5 Myocyte Proliferation and Reprogramming

Until recently, it was thought that cardiac myocytes were terminally differentiated and unable to undergo mitosis. Recent literature has suggested that this is not the case. It was found that a baseline rate of 1% of myocytes in the heart regenerate [47]. Additionally, it was discovered that following an infarction, some of the surrounding myocytes reenter the cell cycle [40]. These two findings suggest that cardiac myocytes are not terminally differentiated, but instead, are able to proliferate and form daughter cells. This is relevant for treating heart failure as a proliferating myocyte is similar to a cardiac progenitor cell, a cardiac cell that is not fully differentiated. As such, a proliferating myocyte may form

spheroid bodies similar to CPSs, which have been shown to improve LVEF post-infarct. Being able to use myocytes to form these spheroid bodies would be advantageous as they are far more abundant in the heart than cardiac progenitor cells, requiring a smaller biopsy to harvest the same number of cells.

For a myocyte to undergo cellular division it must alter the arrangement of its organelles as well as its general shape. The sarcomere within in the cell disassembles and the cell loses its striated appearance. Furthermore, the cell changes from being long and rectangular to globular and round. Within the nucleus, the protein Ki-67 acts to aid in DNA replication. These changes in appearance and protein expression can be used as clues to determine if myocytes are proliferating and exhibiting behaviors similar to that of a stem cell.

One important question is why are CPSs well suited for regenerating damaged myocardium? The hybrid nature of CPSs, containing both proteins associated with stem cells and differentiated myocytes may offer some clues. The stem like proteins they express, such as ki-67, a protein used in DNA synthesis and associated with cellular mitosis, suggest they are able to proliferate. Proliferation is advantageous in the context of myocardial regeneration as more of the dead or damaged cells can be replaced. Proteins found in mature myocytes, like actinin, are needed for the sarcomere of the cell to assemble. It is the sarcomere that is responsible for mechanical contraction of the cell and therefore is required for beating to occur. The presence of proteins associated with stem cells as well as mature myocytes is one reason CPSs may be effective as therapeutic agents for myocardial repair.

1.6 Stem cells have the potential to reprogram cardiac myocytes

Research done by Rodriguez et al. showed that in co-culture, mouse cardiac myocytes and adipose derived stem cells (ADSCs) fused with one another [48]. This is not a novel phenomenon as most types of stem cells can fuse with somatic cells, such as neurons. Myocytes that fused with the ADSCs expressed C-kit and Ki67, suggesting these cells were moving to a more progenitor-like state and proliferating, similar to that of cardiac progenitor cells [48, 49]. Also similar to cardiac progenitor cells, the fused myocytes began to form multicellular spheroid bodies. These spheroid bodies, like CPSs, also expressed GATA-4, NKX-2.5, and MEF2C. Human DNA could not be found in the spheroid bodies, showing they were exclusively derived from the murine cardiac myocytes, similar to the way that CPSs are exclusively derived from autologous cardiac cells. The stark resemblance in gene expression and phenotype of these stem cell induced cariospheres to CPSs suggests that they would have similar positive implications if used for MI therapy. Rodriguez et al even reported witnessing their spheroid bodies differentiate into myocytes when delivered to the heart, though have yet to publish further information or data about this [48].

Rodriguez et al. also co-cultured MSCs with the isolated mouse myocytes. Like co-culture with ADSCs, the myocytes expressed GATA-4, MEF2C, NKX2.5 and Ki-67. It was not reported if the MSC-myocyte co-culture yielded spheroid bodies.

The major limitation with the study done by Rodriguez et al was a low rate of myocyte reprogramming. The group reported only 0.1% of myocytes appeared to take on the appearance and phenotype of a cardiac progenitor cell when in co-culture with ADSCs [48]. This could be a result of poor myocyte health after isolation or a limitation of the ADSCs. The potential of this study is a way to create a cell type similar to that of CPCS using mature myocytes, which are significantly more abundant in the heart than progenitor cells. A more effective way to derive these stem cell induced spheroids is required as only 0.1% of myocytes were reported to take on the phenotype of a CPSs.

1.7 Potential of MSCs for more efficient creation of spheroids.

Human mesenchymal stem cells (MSCs), when delivered to an infarcted heart, have been shown to reduce infarct size [24]. Despite these positive findings, MSC treatment is not an ideal therapy. Restoration of cardiac contraction to the pre-infarct state is not achieved as the mesenchymal stem cells do not appear to differentiate into cardiac myocytes. This suggests that the MSCs are mitigating the damaging effects of an MI without actually directly replacing the lost tissue. This effect could be attributed to either paracrine factors, cell to cell contact without membrane fusing or fusing of the cellular membranes [48].

Although it has been shown that the presence of mesenchymal stem cells in proximity to an infarct can lessen the negative impact of a MI, the exact reason for this is unknown. Several possibilities are: MSC cardiac differentiation, proliferation of the native myocardium, or the release of cardiac preserving paracrine factors. Cardiac differentiation is unlikely as literature shows that MSC in direct contact co-culture with cardiac myocytes do not take on a cardiac lineage [50]. It is possible that the reduction in scar size, observed with MSC therapy, can be attributed to myocyte proliferation induced by the MSCs. MSCs have been shown to secrete Insulin like growth factor, (IGF) [51]. IGF is an important growth factor in inducing proliferation of myocytes in human development and cardiac myocyte proliferation in adult Zebra Fish [52, 53]. This gives reason to suspect that IGF may have the potential to induce cardiac myocyte proliferation of mature cells. MSCs may also benefit the native myocytes by releasing anti-apoptotic factors, aiding them in surviving the harsh post-infarct environment.

The result of prolonged co-culture of MSCs and cardiac myocytes has not been studied. It remains to be known if MSC myocyte co-culture will give rise to cardiac spheroid bodies. Further, if cardiac spheroids do form, this method of deriving spheroid bodies may be more efficient than culture

with adipose derived cells? Evidence suggests that MSCs have more potential to induce myocyte reprogramming than adipose derived stem cells as they secrete significantly larger amounts of Insulin like growth factor (IGF) [51] .IGF has been shown to direct the proliferation of ventricular myocytes in development, and is a required factor in cardiac repair of zebrafish hearts [52]; [53]}. This fact could allow MSCs to overcome the obstacle of low efficiency of cardiac spheroid development observed with ADSC co-culture, reducing the amount of culture time needed to derive a clinically relevant number of cardiac spheroids.

Chapter 2: Hypothesis and Aims

Based on the prior research described in the last section, we hypothesized that after prolonged direct contact with MSCs, myocytes will form cardiac derived spheroids, a potential cell source for regenerating infarcted tissue. In order to test this hypothesis, cardiac myocytes were isolated from whole rat hearts and cultured with mesenchymal stem cells. Following two weeks of co-culture, spheroids were counted and compared to that of a pure culture of myocytes, MSCs, and cardiac fibroblasts. Spheroids will be stained for actinin to verify a cardiac lineage, and Ki-67 to determine if proliferation was taking place within the spheroids. Further, the necessity of direct cell-to-cell contact between the MSCs and myocytes will be assesed by attempting to form spheroids without MSC/myocyte contact.

Specific Aim 1: Determine if cardiac myocytes co-cultured with MSCs form cardiac spheroids

For the first aim, we hypothesize that co-culture of myocytes and MSCs will yield cardiac spheroids containing proliferating myocytes. To test this, cardiac myocytes were isolated from adult rat hearts and cultured with MSC. After two weeks cultures were examined for cardiac spheroid formation and the total number of spheroids was counted. Further, cells within the spheroids were stained for the proliferative marker Ki-67. Some samples cultured for one month in an effort to track the growth of the spheroids following formation.

Specific Aim 2: Evaluate the role of non-myocyte cardiac cells in cardiac spheroid formation

For this aim we hypothesize that both myocytes and stem cells must be present in culture for cardiac spheroids to form. We postulate that both myocytes and MSCs are necessary elements in cardiac spheroid formation, co-culture with other cell types will not yield cardiac

spheroids. To test this, myocytes and stem cells were cultured alone and after two weeks of culture examined for cardiac spheroid formation. The number of spheroids were counted and compared the number of spheroids formed in a co-culture of MSC and myocytes. Further, MSCs and myocytes were each co-cultured with the non-myocyte cells extracted during the cardiac myocyte isolation process. These cultures were also examined for cardiac spheroid formation and compared to a MSC/myocyte co-culture.

From the results of these experiments it can be determined if MSCs or myocytes can form cardiac spheroids when either cultured alone or with non-myocyte cardiac cells.

Specific Aim 3: Determine the role of MSC in cardiac spheroid formation

For the last aim, we hypothesize that for spheroid formation, direct co-culture between the myocytes and MSCs is advantageous. Furthermore, we hypothesize that the ratio of myocytes to MSC is proportional to the number of spheroids that will form in culture. To test if stem cell secreted paracrine factors were responsible for spheroid formation myocytes were exposed to conditioned medium which has been used in culture with MSCs for 24 hours. Myocyte media was changed every 2 days and following two weeks of culture, spheroid number was counted and compared to a control group of myocytes and MSC grown in direct contact.

To test the effect that myocyte stem cell ratio has on colony formation, varying proportions of myocytes to MSCs were used in direct contact culture. Following two weeks of culture, spheroid number was counted and statistically compared between groups.

Chapter 3, Aim 1: Determine if co-culture of MSC with myocytes form cardiac spheroids

The first aim was to establish if direct contact co-culture of MSCs and cardiac myocytes would result in the formation of cardiac spheroids. This is a novel method for making spheroids which hold promise as therapeutic agents post MI. The procedure for isolating the myocytes and co-culturing them with the MSCs is described below.

3.1 Method: cardiac myocyte isolation

The treatment of the rats used in this study was reviewed and approved by the WPI Institutional Animal Care and Use Committee. The rats were retired breeders and predominantly female. As they were retired breeders the rats were all adults, 27 rats were used throughout the course of this study. The Sprague Dawley rats were euthanized and hearts were harvested within 5 minutes and placed in a 50 ml conical tube filled with 20mililiters of KB solution (Appendix A). Each conical tube was then sprayed with ethanol and introduced into a class II bio-safety cabinet, and the content of the tube was emptied into a petri-dish. Forceps were used to gently squeeze the heart in order to remove any remaining blood. The atria were removed with surgical scissors and discarded. The ventricles were moved to a new petri-dish with 20ml fresh KB and subsequently cut into roughly 1cm wide strips. Attention was given as to cut the strips along the length of the fibers of the heart. These strips were incubated in a 50ml conical tube with 20ml fresh KB and 200ul Liberase DH (Sigma, 11988468001; Appendix A) for ten minutes at 37°C. The contents of the tube were then poured into a new petri-dish and pulled apart with two forceps until the tissue pieces were small enough to fit through a 25ml pipette tip. Using a 25 ml pipette, KB, liberase DH, and tissue solution was triturated for 7min. The tissue

was then allowed to settle on the bottom of the tube and the top 14mls were removed and centrifuged at 500rpms for 5 minutes. The supernatant was aspirated and the cell pellet was suspended in 5mls KB; this solution was then agitated and centrifuged again in the same manner. The cell pellet was then re-suspended in 1ml KB and a hemocytometer (Marienfield Neubauer-improved, Ref 06 500 30; 0.0025mm^2) was used to determine the total cell count using the formula below, Equation 1. Each round of triturating yielded roughly 50,000 to 1 million cells. This large range of cellular yield can be attributed to the batch to batch variation of the liberase as well as variation in liberase incubation time. Further, the amount of mechanical force applied to the cells from trituration can alter the yield as well.

Equation 1: Cell counting with a hemocytometer

$$\frac{\#\text{ of Cells counted}}{\#\text{ of boxes counted on hemocytometer}} \times \#\text{ of mls pellet resuspended in} \times 2 = \text{Total cell \#}$$

Using total cell number, additional media was then added to attain the desired cellular density of 20,000cells/ml. 200ul of the diluted cell suspension was plated into a 4well chamber slide for a total cell number of 5,000 myocytes per well. CC2 coated four well chamber slides were used as vessels for culture (Nunc Lab-Tek s6690). The coating on these slides is proprietary, but stated to mimic polylysine and “provide binding sites optimal for fastidious cells” on the Nunc Lab-Tek webpage. Each well was 1.7cm^2 in area, equating to a myocyte density of roughly 2,900 myocytes/cm^2. Wells were washed with two washes of Phosphate Buffered Saline (PBS) after 30mins of incubation. The washed myocytes were then incubated with 500ul of serum free Dulbecco’s Modified Eagle’s Medium (DMEM Bio-Whittaker 12-604F) with 1%Penecillin/streptomycin added for one day before co-culture was begun.

3.2 Method: Myocyte co-culture with MSC

A solution of hMSCs was then prepared for plating with the myocytes as follows. A T-75 flask (Becton Dickinson Labware, #35-3136) were seeded with 500,000 hMSCS (Lonza, #PT-2501) and allowed to reach 70-90% confluence. Cells were then washed using Phosphate Buffered Saline without Calcium and Magnesium and 5ml of Trypsin (Clonetics CC-5002) was added to the T-75 flask. The flask was then incubated for 5mins at 37C° and 5% CO2. 5mls of 10% FBS in DMEM was then added to the flask to deactivate the trypsin and the contents of the flask was removed and centrifuged for 5mins at 1000rpms in a 15ml conical tube. The cell pellet was then suspended in 2mls of media and 10uls were removed, added to 10 microliters of Trypan Blue solution and subsequently loaded into a hemocytometer. Equation 1 was used to calculate total cell number. Based on total cell number a quantity of cell suspension was added to the wells of the 4 well chamber slides such that 5,000 hMSCs were seeded per well. Following co-culture, cells were grown in an incubator at 37C° and 5% CO2 for time points of two weeks and one month. 10% FBS in DMEM was used to culture the cells and media was changed every two days. A 4 well chamber seeded with 5,000 myocytes/well was cultured without adding MSCs as a control. In each well 5,000 mycoytes were plated and media was changed, one day after plating and every other day after that. The medium used was DMEM with 10% FBS 1% P/S. This experimental group establish if a pure culture of myocytes would give rise to spheroids. MSCs were also culture alone at 5,000 cells per well with 10%FBS 1%P/S. Media was changed every other day to mimic the treatment of the MSCs in co-cultures. This experimental group was used to establish if MSCs alone could generate spheroid bodies.

3.3 Method: Colony Counting

After two weeks of culture, wells were examined for spheroid formation. A spheroid was defined as 5 or more cells in intimate contact. An inverted microscope was used to examine the wells. The number of spheroids in each well was counted first by one person, and then by 2 additional independent observers to confirm there was no bias in the counts.

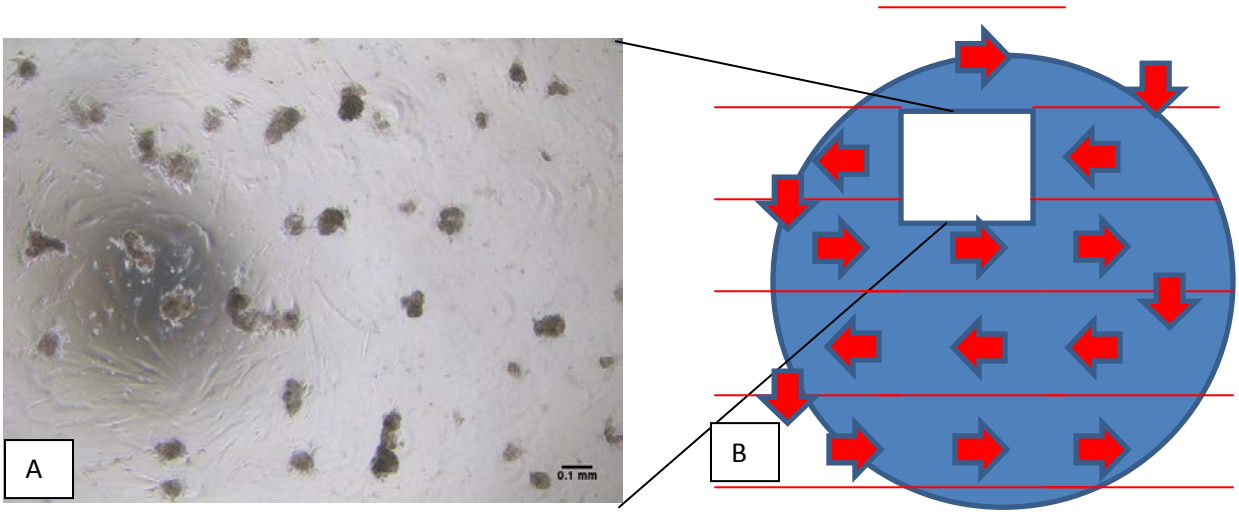

Figure 3 : Wide field image of a co-culture taken with an inverted microscope (A). The pattern used to count all of the colonies within the well (B).

Figure 3 above portrays the technique used to ensure the entirety of the well was counted. An inverted microscope was used to inspect the culture surface for spheroids (A) based on the above definition. The scanning technique shown in (B) was implemented. This systematic approach begins by scanning the top row and then using a reference at the bottom of the field of view to shift down one field. The observer then scans horizontally and again shifts down the width of the field of view. By using this approach, all colonies can be counted effectively and accurately.

As spheroids were defined as 5 or more cells in contact, a method for counting them was developed. This was done to standardize what can and cannot be counted as a spheroid allowing for comparison between wells and experiments. 5 cells was chosen as an arbitrary number and nuclei were not used a way to count cells as some myocytes are bi-nucleated while others are mononucleated. Size of the spheroids was not used as the shape of the spheroids is not uniform or consistent. Clusters of cells were identified in the wells and then the outline of the individual cells within the cluster were used to count total cells in contact. 5 or more cells in contact defines a spheroid. This process is illustrated in Figure 4. After two weeks of co-culture, the number of spheroids counted in the control group of pure myocytes was compared to the number of spheroids counted in the myocyte/MSC co-culture group. A Student's T-test was used with a null hypothesis that the number of spheroids between the two groups was the same. A p-value of 0.05 was used.

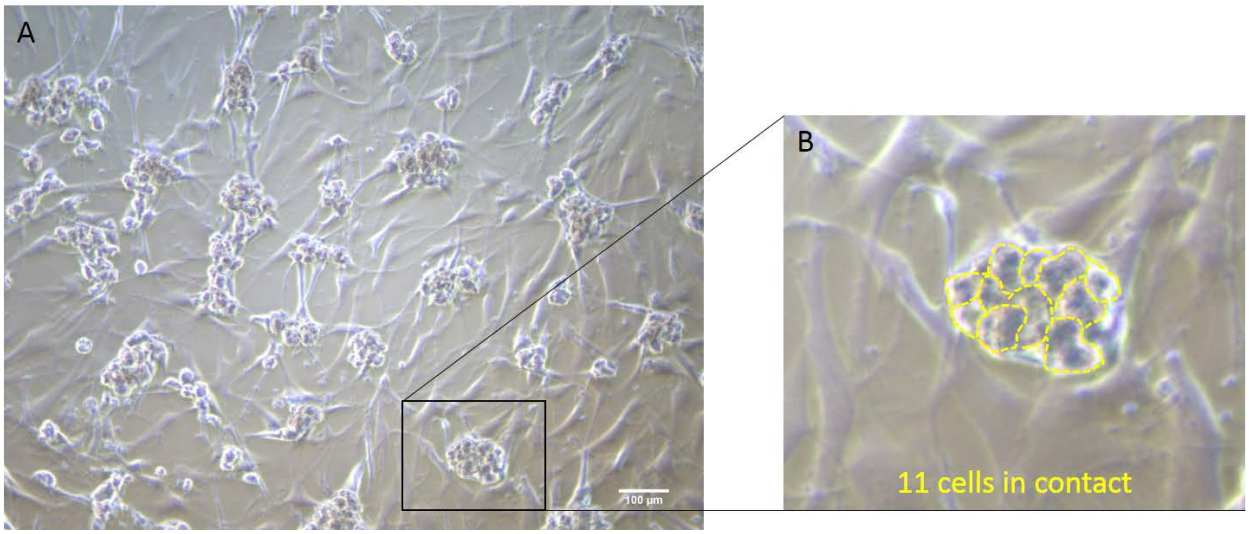

Figure 4: Method for counting cells in contact. Clusters of cells are identified (A.) then the outline of cells are used to count the total number in contact (B). 5 or more cells in contact define a spheroid.

3.4 Method: Immunohistochemistry

Immunohistochemistry (IHC), targeted protein fluorescent tagging, was used to detect the presence of two proteins, Ki-67 and alpha-actinin. The presence of the cytoskeletal protein alpha actinin indicates cells have a cardiac lineage, while Ki-67 is a protein, found in the nucleus, associated with the replication of DNA and is expressed in cardiac progenitor cells.

All immunocytochemistry was done using the same protocol. Slides were immersed in -5°C acetone for 10 minutes to fix the sample. Three washes in PBS for 5 minutes each followed in order to remove the acetone. Samples were then blocked in the serum of the species in which the secondary antibody was made (ki-67 goat, actinin rabbit) for 30 min at a concentration of 5%. After blocking primary antibody (Mouse monoclonal anti-actinin 1:100, Sigma-Aldrich, # A7811; Rabbit monoclonal ki-67 1:50, sc-23900 Santa Cruz Biotechnology) was placed on the positive controls and the samples were left untouched 12-15 hours at 4C. Negative controls were incubated alongside the positive controls in the serum that the blocking step was done with. PBS washing was done three times for 5 minutes to remove all non-absorbed primary antibody. Both the positive and negative controls were then incubated in the secondary antibody for 1hr at room temperature (RT) at a concentration of 1:400 (AF488 for actinin, Invitrogen Alexa Fluor 488 Rabbit anti-Mouse, # A11059; AF568 KI-67, Invitrogen Alexa Fluor 568 Goat anti-rabbit A11079). The purpose of the negative control is to determine if there is non-specific binding of the secondary antibody to the cells. A fluorescent signal in the negative controls would indicate this. Three washes with PBS for 5 minutes were done. Samples were then counterstained with Hoechst (1:6000) for 5 min and washed for a final three times for 5 min each with PBS. Cytoseal 60 was used to coverslip.

3.4.1 Click-it EdU Cell Proliferation Assays

In order to detect newly synthesized DNA in the myocyte/MSC co-cultures a Click-it EdU proliferation assay was run (Life technologies, C10340). EdU (5-ethynyl-2'-deoxyuridine) was thawed and a stock solution was prepared in accordance with the instructions on the company webpage (Click-it EdU Manuals and Protocols). A co-culture of 5,000 myocytes and 5,000 MSCs in a 4 well chamber slide was used for this experiment. Using the stock solution of EdU a 10 micromolar solution of EdU was made with DMEM and 10% FBS 1% P/S. This culture media was prepared immediately before being incubated with the co-culture. New 10 micromolar EdU media was placed on the co-cultures daily from day 0 of until day 5 of co-culture. After day 5 of co-culture the cells were fixed in 4% paraformaldehyde for 10 minutes and then washed three times with PBS for 5 minutes each. The cells were then incubated with 0.5% Triton-X-100 in PBS for 20 minutes. Following Triton-X-100 treatment, cells were washed 3 times in PBS for 5 minutes each. A working solution of Alexa Fluor azide 647 was made in accordance with the instructions on the Life Technologies website (Click-it EdU Manuals and Protocols). 100ul of this working solution was added to the cells and incubated in a dark environment for one hour. Following this step, three washes with PBS for 5 minutes each were done. A nuclear counterstain with Hoechst was then done by incubating a Hoechst solution in PBS (1:6000) and incubating it for 5 mins on the cells. Three washes with PBS were then done. The cells on the 4 well chamber slide were then mounted with cytoseal 60 (Richard Allan Scientific, 8310-4) and cover-slipped.

3.5 Method: Imaging

Live cell imaging was done with an inverted microscope (Leica DMIL). Cells were imaged through either the bottom of a 24 or 96 well plate or a 4 well chamber slide. Different magnification objectives were used to image the cultures, the objectives included, 4x, 10x, 20x, 40x. Images were taken using visible light. The Leica Application Suite software was used for capturing and processing the images.

To image the fluorescently stained cultures, a Leica Upright Fluorescence Microscope DMLB2 was used. Alexa fluor 488 fluorescent tags were imaged with a Chroma L5 FITC filter cube, Alexa fluor 566 secondary was imaged with a Chroma N3 Texas Red filter cube, Alexa fluor 647 secondary was imaged with a QDOT 655 cube, and Hoechst nuclear staining was imaged with a Chroma A4 DAPI filter cube. Oil immersion objectives were also implemented to take 40x images. The Leica Application Suite software was used for capturing and processing the images.

A Leica confocal microscope was used to make 3 dimensional movies of the co-cultures by incrementally imaging sections along the vertical axis and compiling them together. Images were taken at 40x. This scope was synced with Leica software to capture and process the images.

3.6 Method: Tracking area of cardiac spheroids

To track the change in area of cardiac spheroids from 2 weeks to one month, live cell imaging, as described above, was implemented. Cardiac myocytes and MSCs were co-cultured in a 24 well plate. This differs from the 4 well chamber slide that was previously used. Myocytes were still seeded at

quantity of 5,000 cells/well though the area of the well was 1.91cm^2 equating to a new cell density of roughly 2,600 myocytes/cm^2 as compared to 2,900 myocytes/cm^2 with the 4 well chamber slides. At two weeks, wells were examined for spheroid formation and any cardiac spheroids found were imaged in order to measure their surface area. Image J software was used for the analysis, the outline of the spheroids were traced using the software. The total area was found by setting the scale in image J using a scale bar to define the pixel to distance ratio. With this information image J is able to calculate the total area of an outlined region. All of the spheroids for the time point were then averaged. This same procedure was done at one month and the averages for the two groups at each time point were compared using a Students T test with a p-value of less than or equal to 0.05. The null hypothesis was that spheroid size was the same at 2 and 4 weeks.

3.7 Method: Low cell count co-culture

In order to detect if myocyte number increases as a result of co-culture, roughly 1 to 5 myocytes were co-cultured with MSCs. This was done in order to count the myocytes over time. 10 microliters of standard post isolation myocyte suspension was diluted with 5mls of KB solution. After the solution was agitated to ensure a homogeneous mixture, 10 microliters of the diluted suspension was placed in the well of a 96 well plate. One hour following plating wells were washed with PBS. The myocytes were then incubated at 37^{o}C for one day. After one day of culture MSCs were added to the wells, 10 MSCs were added to each well. This number was chosen to ensure an MSC was in proximity with the myocyte, but not to alter the ratio of myocytes to MSCs substantially. Prior to adding the MSCs to the wells, the number of myocytes in each well was counted using an inverted microscope. The co-cultures were grown for 10 days, allowing enough time for the MSCs to manipulate the myocytes, and the total number of myocytes was counted again. Co-cultures were grown with DMEM with 10% FBS and 1% penicillin/streptomyosin. A culture of pure myocytes, plated in the same fashion, was run alongside the

co-cultures as a control. This control was used to examine if myocytes in pure culture would increase in number. The change in myocyte number was then compared using a Student's T-test with a null hypothesis of the two groups being the same. A p-value of 0.05 was used.

3.8 Results: Co-culture yields cardiac spheroid formation

After two weeks of direct contact myocyte/MSC co-culture, round multicellular spheroid bodies spontaneously formed within the wells. For consistency, spheroid bodies were defined as 5 or more cells in intimate contact resting on the feeder layer of MSC. Using this definition, the total number of cardiac spheroid bodies formed in the myocytes/MSC wells was compared to wells containing solely myocytes. Figure 5 shows that in the control group of just myocytes an average of 1.5 colonies formed while in the co-culture group an average of 64.5 colonies formed per well. This suggest that spheroid formation is not something that myocytes spontaneously do when cultured alone.

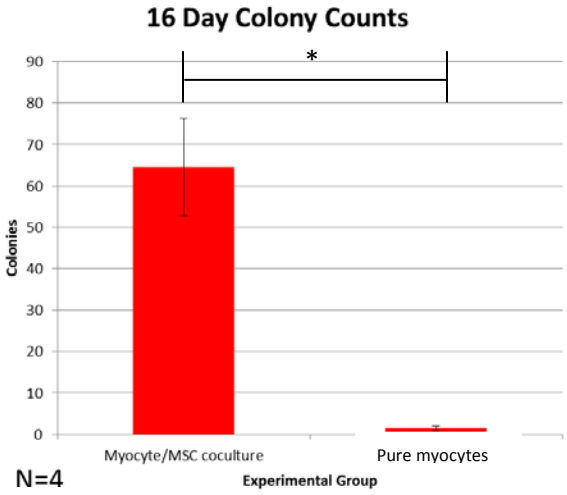

Figure 5: Average number of colonies counted per well following 16 days of culture. Co-culture yields a significantly greater number of spheroids as compared to lone myocytes using a Students T-test with a p-value of 0.05.

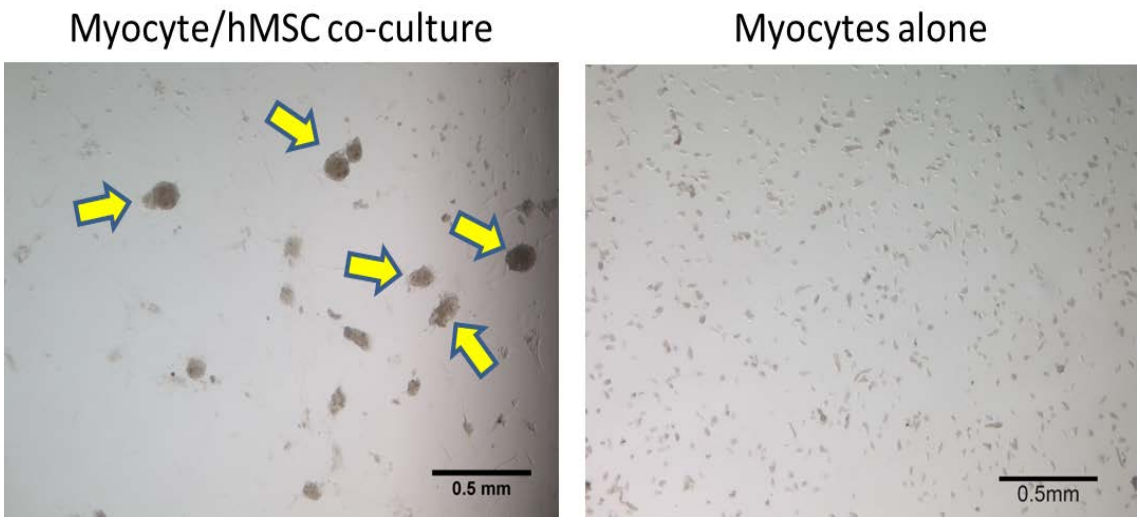

Figure 7: Spheroids have formed in the myocyte/MSC co-culture (A), indicated by yellow arrows. The pure culture of myocytes is void of spheroids (B).

Qualitatively the pure cultures of myocytes appeared different than the cells in co-culture (p-value of 0.05. The null hypothesis was that the number of spheroids in each well after 14 days was the same). Figure 7 (A) shows the co-culture of myocytes and MSCs. Large spheroid bodies have spontaneously formed in the culture. These spheroids are starkly different in appearance to the surrounding cells. They are darker, larger, and round while the surrounding cells are lighter in color and smaller. The yellow arrows indicate where the spheroid bodies have formed. Very few of the myocytes in culture remained by themselves. The pure culture of myocytes shown in Figure 7 (B) is void of any spheroids, it can be seen in the image that the cells are predominantly separate. Figure 6 (A) shows a 40x image of a spheroid body derived from Myocyte/MSC

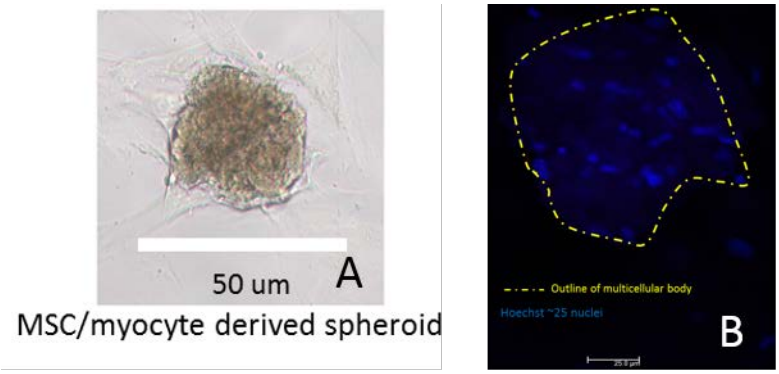

Figure 6: 40x magnification of myocyte/MSC derived spheroid body. The spheroid is round and granular in appearance (A). : Hoechst staining, which stains cellular nuclei blue, shows spheroids are multicellular (B).

co-culture. The spheroid is round with a granular appearance.

Within the spheroid there appear to be smaller round forms that make up the entirety of the sphere. Hoechst staining revealed that these bodies were in fact comprised of many cells, as seen in suggesting these smaller round forms are individual cells figure 6 (B).

3.9 Results: spheroids stain positive for alpha-actinin

After finding that myocyte/MSC co-culture resulted in the formation of spheroids, actinin staining was done to determine the origin of the cells within the spheroids. Alpha-actinin is a protein specific to cardiac myocytes and, therefore, the presence of this protein indicates a cardiac lineage[1]. Figure 8 below shows a cardiac colony that has been stained for alpha-actinin (green). A substantial proportion of the cells in the spheroids appear to stain positive for actinin, while cells outside of the spheroid did not stain positive for alpha-actinin. Therefore, it can be concluded that the spheroid bodies are primarily derived from the isolated cardiac myocytes and it is appropriate to call this assembly of cells a cardiac spheroid body. The mesenchymal stem cells are likely not responsible for the positive signal as they do not differentiate into cardiac myocytes when in myocyte/MSC co-culture, and therefore do not produce actinin [48]. Additionally, cells within the colonies exhibited an appearance resembling myocytes and not stem cells. Cells were darker in appearance compared to a MSC and some even retained striation like a mature myocyte. Based on positive actinin staining and phenotype it can be

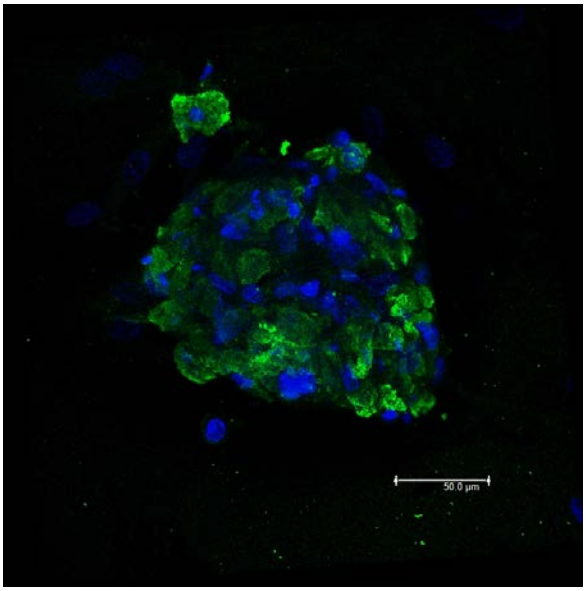

Figure 8: Spheroid bodies stain positive for actinin. Green signal indicates positive actinin staining while blue staining shows the location of the nucleus. Green signal is predominantly found in the spheroid bodies suggesting spheroids are predominantly comprised of myocytes

determined that the spheroid bodies are largely made up of myocytes.

3.10 Results: Myocytes in co-culture lose striation

Within 2-3 days following the beginning of co-culture, cardiac myocytes lose their striated appearance, as well as their rectangular shape. Figure 9 is a series of images taken over time from when the myocytes are isolated until fourteen days after co-culture, though are not of the same cell. These images capture this transition from a rectangular striated cell into a round amorphous shape.

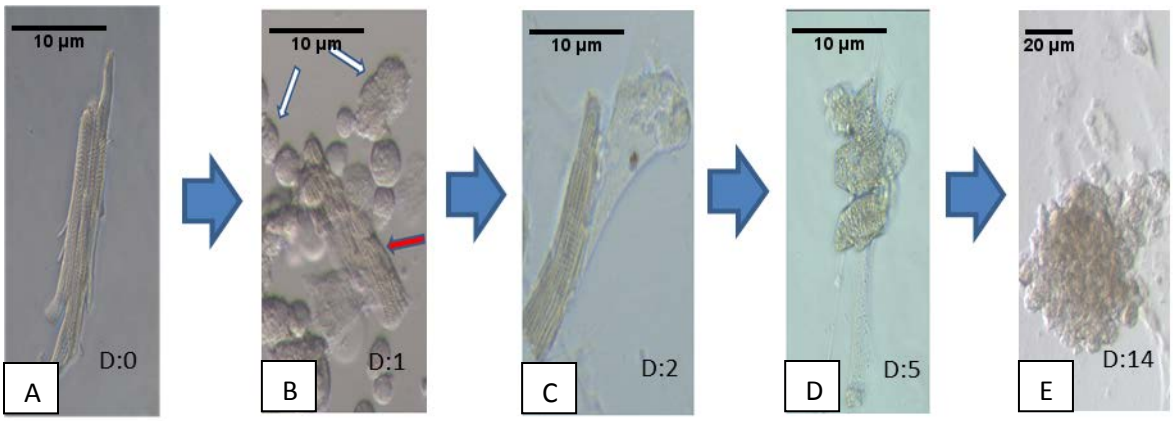

Figure 9: Representative images of myocyte appearance over time during cell culture (not the same cell). From day 0 to day 14. At day 0 myocytes are elongated, rectangular and striated (A). Day 1 stem cells are added to the culture (B). Day 2, stem cells attach and localize with the myocytes (C). Day 5, myocytes begin to lose striation, rectangular shape, and ball up (D). Day 14, large multicellular spheroid bodies have formed (E).

These types of changes in cellular behavior can offer some insight when trying to determine what changes are occurring within a cell. At Day 0, the myocytes is long and rectangular with a clear appearance. Myocytes with this appearance adhere to the culture surface, remaining attached between media changes an indication of cellular viability. The stem cells are circular and detached when first introduced to the culture of myocytes. After a day, stem cells attach to the culture surface and elongate, as well as orient themselves in contact with the heart cells, resting in physical contact. After 5 days of co-culture, myocytes begin to round out and lose their striated appearance. A few myocytes retain their

striated rectangular appearance, these cells are typically not co-localized with a MSC, which may be the reason for this. After the loss of striation, clusters of multiple cells begin to appear on the stem cells, and after two weeks in culture, these pairings of cells become large spheroid bodies. The cells within these bodies are small and round, containing no patterned structure within them.

3.11 Results: Spheroids increase in area over time

The surface area of the cardiac spheroids was calculated by tracing the outline of the spheroids in Image J and using the software to calculate area. Surface area was averaged with other spheroids in the well. This average was compared over time and as depicted in Figure 10, the average area rose from roughly 30,000um^2 to about 350,000um^2. This difference is statistically significant based on a Student's T-test with a p-value of less than or equal to 0.05. The null hypothesis was that the surface area between the two time points was the same. Surface area was chosen to be used as an indicator of size as it is a more robust measurement than diameter. Taking the diameter assumes a circular shape, while surface area is independent of shape.

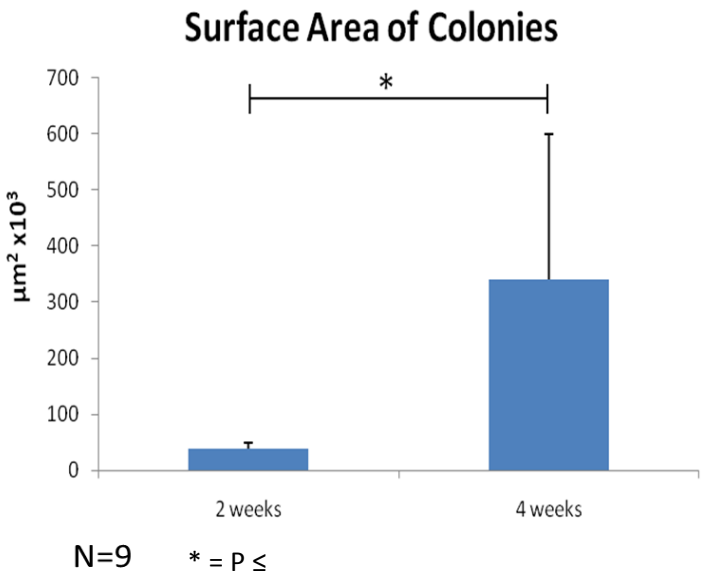

Figure 10: The average surface area of the colonies increases significantly between two and four weeks based on a Student's T test with a P-value less than or equal to 0.05. The null hypothesis was that there was no difference in area between the two time points.

3.12 Results: Cells within cardiac spheroids stain positive for cell cycle markers

To determine if cellular proliferation was occurring within the cardiac spheroid bodies, the presence of the protein ki-67 within the cardiac spheroids at two weeks was examined using a monoclonal antibody. Some cells within the cardiac spheroids did stain positive for ki-67 as shown in image A of Figure 11. Further, the ki-67 signal was co-localized with the blue Hoechst nuclear counterstain. This is where the ki-67 would be expected to be found because the protein acts in the nucleus. As a second means of examining the co-cultures for proliferation was added to the culture media. Edu works by incorporating into the DNA of a cell when the double helix unwinds for replication. Image B Figure 11 shows a cell above the feeder layer of stem cells, this cell has incorporated Edu into its DNA, suggesting the cell has undergone proliferation. The location of the cell, above the first layer of MSCs attached to the culture surface as well as its elongated shape suggest it is a cardiac myocyte. These two processes were done with a pure culture of cardiac myocytes after two weeks of culture, and no positive signal was found.

Cardiosphere cells are positive for cell cycle markers

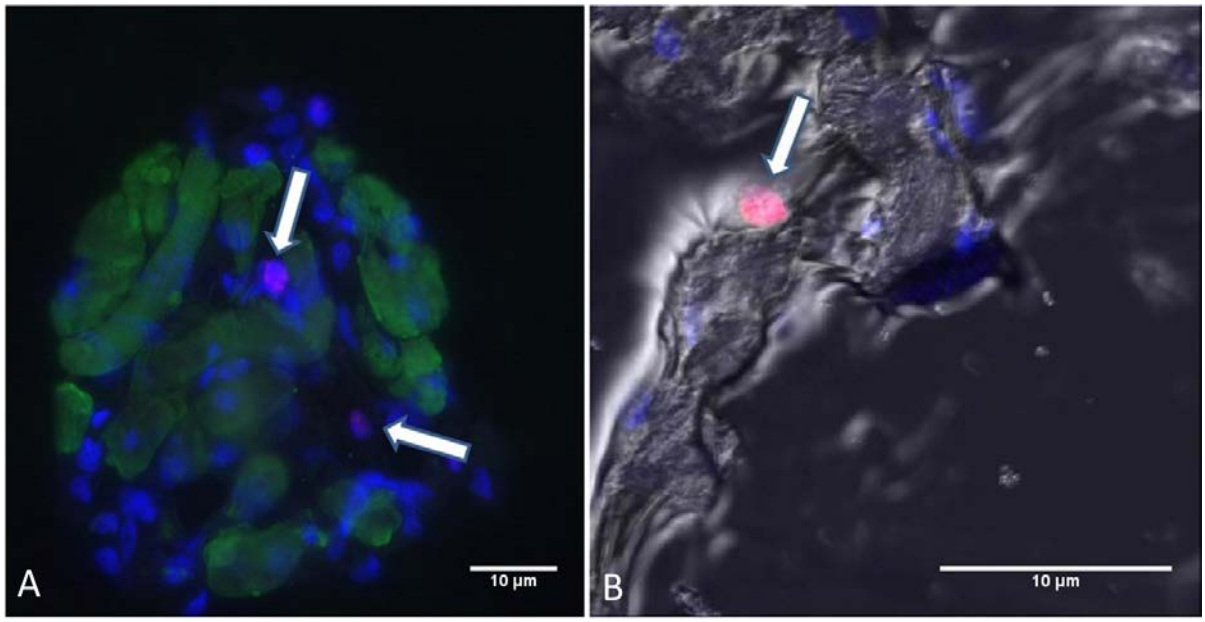

Figure 11: Image A shows nuclei within the cardiosphere that have stained positive for the proliferative marker Ki-67. Image B shows a single myocyte that has incorporated EDU into its DNA suggesting DNA replication. The location of this cell in culture, above the monolayer of MSCs, as well as its appearance suggest it is a myocyte.

3.13 Results: Myocyte number increases in low density co-culture

One to five myocytes were plated in a 96 well plate such that the total myocyte number could be counted and tracked. Half of the wells were co-cultured with MSCs and the other half were used as controls for comparison. The results of low density plating yielded between zero and ten myocytes per well of the 96 well plate. After ten days, the number of myocytes in the co-culture group had risen an average of about one cell, while cell number dropped in the pure culture by an average of roughly half a cell. These results are depicted graphically in Figure 12. This change in cell number is statistically significant based on a Student's T-test with a p-value of less than or equal to 0.05. The null hypothesis was that myocyte number was the same after 10 days in culture. Myocytes were identified from the

MSCs and counted based on their darker granular appearance. The entirety of the well was scanned when counting to ensure no cells were missed.

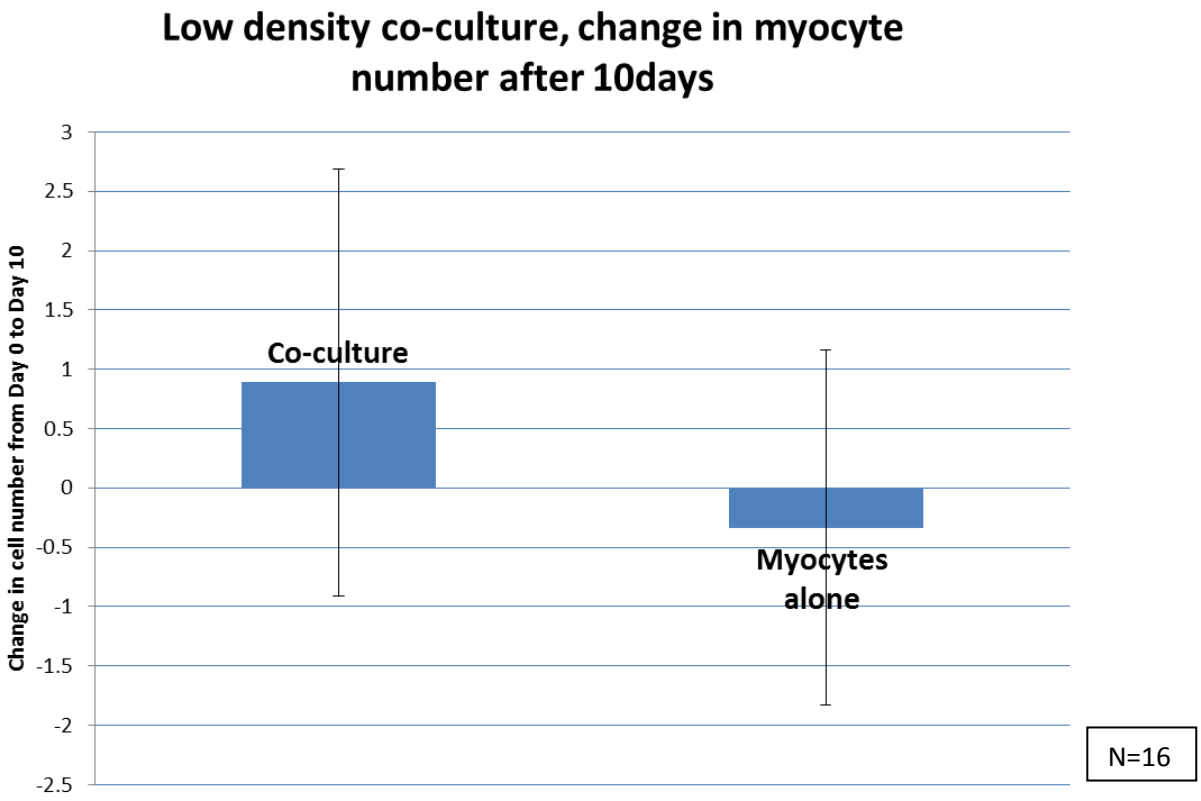

Figure 12: This graph depicts the change in cell number over 10 weeks for myocytes seeded at very low density in a 96 well plate. Myocytes cultured with hMSCs increased in number while myocytes alone decreased. The difference proved to be statistically significant based on a Student's T-test with a p-value less than or equal to 0.05. The null hypothesis was that cell number was the same after 10 days of culture.

3.14 Discussion: Co-culture yields to the formation of cardiac spheroid bodies

As hypothesized, when cultured in direct contact with each other, myocytes and stem cells spontaneously form spheroid bodies. These bodies are not striated or have striated sarcomeres like adult myocytes, nor do they have the clear appearance and elongated shape of a mesenchymal stem cell. Instead they are unique entities which does not form in a pure culture of either parent cell type. The cells within the bodies do not resemble in appearance a myocyte nor a stem cell. These bodies look

similar to those found in myocyte/ADSC (adipose derived stem cells) co-culture and CPSs. They are dark in color, appear to be granular, and are relatively large compared to an individual cell. Additionally, the cells in the spheroids have lost their striation, similar to the cells Rodriguez reported in ADSC/myocyte co-culture and what was observed with CPSs. Further investigation is required to establish the composition and origin of the spheroids. This information will offer clues into the relationship between MSCs and myocytes as well as their potential for clinical use post MI. One major limiting factor of this experiment is the variability in the viability of the myocytes derived from cardiac myocyte isolation. This makes it difficult to compare the number of spheroids derived between myocytes from different isolations.

3.15 Discussion: Cardiac spheroids stain positive for alpha-actinin

Actinin staining revealed that a substantial proportion of cells within the spheroid bodies are actinin positive. This suggests that spheroids are derived mostly of cardiac mycoytes, as mesenchymal stem cells have not been shown to be able to differentiate into myocytes when cultured in-vitro and, as such, do not produce actinin [48]. Because of their cardiac lineage, the spheroid bodies can be called cardiac spheroid bodies. This finding shows that the cardiac spheroid bodies share a similar origin with spheroids formed from myocyte/ADSC co-culture as they are both derived from mature cardiac myocytes. Additionally, the formation of myocyte derived spheroids shows that the mesenchymal stem cells interact with the myocytes, causing them to form the cardiac spheroid bodies when in co-culture with MSCs, but not when myocytes are alone. One limitation of this experiment was not being able to derive what percentage of cells within the spheroid were actinin positive. This cannot be done for two reasons: one, the nucleus of a myocyte does not reside in the cellular body and therefore is difficult to

associate with that respective body, secondly, some myocytes are binucleated so there is not a direct correlation between nuclei number and the number of cells in a spheroid.

3.16 Discussion: Myocytes in co-culture lose striation

The striation of a fully differentiated myocyte comes from the cable like structure of the sarcomere, responsible for cellular contraction. During myocyte proliferation, the sarcomere disassembles and subsequently the cell loses its striation. The loss of striation of myocytes within the spheroids may be a sign that the cells have entered the mitotic cycle. Striation is also an indication of the state of differentiation of the cell. The lack of an organized sarcomere indicates that the cell is no longer a fully differentiated cardiac myocyte. Cells within CPSs and myocyte/ADSC co-culture induced spheroids also are not fully differentiated myocytes, instead expressing some proteins associated with stem cells and some found in mature myocytes [54, 55].

3.17 Discussion: Cardiac spheroids increase in area over time

After the cardiac spheroids form, they significantly increase in surface area over time. Growing the cardiac spheroids over time may make them more effective as a therapeutic agent post MI as larger spheroid contain more material to replace myocardium lost to an infarct. This growth could be attributed to cellular division, hypertrophy, or movement of the MSCs into the cardiac spheroids. The growth of the cardiac spheroids is substantial and unlikely caused by hypertrophy, though the size of individual cells was not quantitatively measured, a step that would be necessary to prove this. Movement of the MSCs into the cardiac spheroids could cause this increase in size. Non-myocyte

migration does not compromise the cardiac spheroids as a potential therapeutic agent for treatment of MI as cells are either taken from the patient or MSCs that have been shown to be immuneprivileged [24]. Staining for cell cycle indicators was done to establish if proliferation was a factor in the increase of spheroid size. One limitation of this study was the use of surface area instead of total spheroid volume. Surface area was calculated from an image looking down on the spheroid and is not as accurate a measurement of spheroid size as the volume of the spheroid would be. Ideally, we would be able to compare the mass of the spheroid from 2 weeks to 4 weeks.

3.18 Discussion: Cells within cardiac spheroids stain positive for cell cycle markers

At two weeks, cells in the spheroids stained positive for the cell cycle marker ki-67, additionally myocytes incorporated EDU into their DNA when exposed to the substance, also an indication of DNA replication. These findings suggest that cells within the spheroid and cardiac myoctes are proliferating, a phenomenon witnessed in the border region of infarcts [40]. These results suggest that myocyte proliferation plays a role in the observed increase in size of the cardiac spheroids. This is relevant as an increase in total myocyte cell number may make the cardiac spheroids more effective as a therapeutic agent for MI. Further, it can be inferred that this is a result of co-culture with hMSCs as myocytes alone do not stain positive for Ki-67. One point of consideration when examining these results is the difficulty in associating a Ki-67 positive nucleus with an actinin positive cell. The nuclei of a myocyte does not reside centrally within the cellular body giving rise to the difficulty of associating the nuclei with the cell body.

3.19 Discussion: Myocyte number increases in low density co-culture

To determine if the positive staining for the cell cycle marker Ki-67 corresponds to daughter cell formation, myocyte number was tracked in low density co-culture. It was found that the total number of myocytes did significantly increase over ten days as compared to a control of myocytes cultured alone, also plated at low density. This suggests that DNA replication in the cardiospheres is a precursor to cytokinesis and subsequently daughter cell formation. This is significant as it suggests that a population of cardiac myocytes can be increased in number through hMSC co-culture. As such, a small biopsy of autologous myocytes could be taken from a patient, and by co-culture with allogenic or autologous MSCs, expanded to a larger quantity of cells and then delivered to an MI. This would provide tissue to restore contractile force, while limiting the damage done to the heart from the initial biopsy. This is a limitation of cardiac stem cells used to create cardiospheres, they require a large amount of initial cardiac tissue. It is necessary to limit the size of any biopsy taken from the heart in order to reduce the reduction in cardiac capacity that this could cause. A limitation of this study was the inability to plate a consistent number of myocytes in each well to being the experiment. This would have facilitated direct comparison between the number of myocytes at the end of the experiment between wells.

3.20 Aim1 Discussion

The data from aim one supports the hypothesis that myocyte/MSC co-culture yields to the creation of cardiac spheroid bodies. It is the similarity to the spheroids derived from cardiac progenitor cells, as well as those made from myocyte/ADSC co-culture is one reason to believe that these cardiac spheroid bodies could be effective as therapeutic agents for cardiac infarction. Both the cardiac

spheroids derived from myocyte/MSC co-culture and those previously documented are comprised of cardiac cells, stain positive for ki-67, and have a similar shape and appearance [48].

One important thing to note from aim 1 is the difference in species between the two cells in co-culture. The myocytes are derived from a rat while the MSCs are human. As the intended aim of creating these cardiac spheroids is to use human cells, it must be confirmed that these results translate when human myocytes are used. Rat and human cardiac myocytes contain many of the same proteins and organelles. This suggests that human myocytes may behave in the same fashion as rat myocytes when in co-culture. Human cardiac spheroids have been generated with cardiac progenitor cells which illustrates human myocytes are able to form spheres [44].

A limitation of this study is the inability to identify the location of the MSCs in the co-culture. This ability would allow us to determine if MSCs were present in the spheroids and contributing to the increase in surface area. Another limitation is not being able to count each individual cell within the spheroid. Using Hoechst staining to count the nuclei would not be accurate as some cardiac myocytes have two nuclei while others do not. Knowing how many cells were in the spheroid would be useful to determine the final number of spheroid derived cells that were generated. The inability to remove the non-myocyte cardiac cells during the myocyte isolation process is also another experimental limitation. These cells could be secreting proteins that affect spheroid formation or the cells could be incorporating into the spheroid itself. This is a variable that must be accounted for to determine if it is the co-culture of myocytes and MSCs that is responsible for spheroid formation.

Chapter 4, Aim 2: Determine role of non-myocyte cardiac cells in spheroid formation

4.1 Methods: Isolation of non-cardiac myocyte cells

In order to separate the non-myocyte cells from the myocytes, 3mls of supernatant were removed during the myocyte counting process, following the isolation of the cardiac myocytes as described in aim 1. This solution contained the cells from the heart that were lighter than the myocytes. As myocytes are relatively heavy cells this suspension of lighter cells was composed of predominantly non-myocyte cardiac cells. These are the cells that are difficult to eliminate from the myocyte/MSC co-culture.

4.2 Methods: Pure cultures of myocytes and MSCs

In order to establish if the cardiac myocytes or MSCs are able to spontaneously form spheroids in the absence of the other cell type, pure populations of each cell were cultured. MSCs were thawed as explained in aim 1 and plated in a 24 well plate, at a density of 5,000 MSCs per well. MSCs were cultured in DMEM with 10% Fetal Bovine Serum and 1% Penicillin/streptomycin, media was changed every other day. Myocytes were isolated in the manner as described in aim 1 and plated in a 24 well plate, 5,000 myocytes/well. Myocytes were cultured with the same media and in the same fashion as the MSCs. Following two weeks of culture, the wells were examined for cardiac spheroid formation.

4.3 Methods: Co-culture of non-myocyte cells with MSCs

After determining cellular density, the cell solution was diluted such that 5,000 non-myocyte cells were plated in each well of a 24 well plate. This equates to 5,000 cells per ½ of a milliliter of media. Co-culturing was done in the same manner as aim 1 with the exception that the original media of the non-myocyte cells was not changed until the MSCs had been added to the culture for 24 hours. This was to prevent unattached cells from being washed away without giving them a chance to interact with the MSCs. After this point, the co-cultures were treated and analyzed in the same manner as aim 1, cardiac spheroids were counted and a Student's T-test was used for comparison.

4.4 Methods: Co-culture of non-myocyte cells with myocytes

To culture the myocytes and non-myocyte cells together, the two populations of cells were isolated as previously described and then plated simultaneously. The cells then sat for 24 hours prior to changing the media, this was done to allow for the detached non-myocyte cells to possibly make interactions with the myocytes. The media was then changed and cultures were run for two weeks at 37^{o}C. DMEM with 10% FBS and 1%P/S was used to culture the cells with, media was changed every two days. After two weeks of culture, wells were examined for spheroid formation.

4.5 Results: Pure cultures of MSCs or Myocytes do not yield cardiac spheroid formation

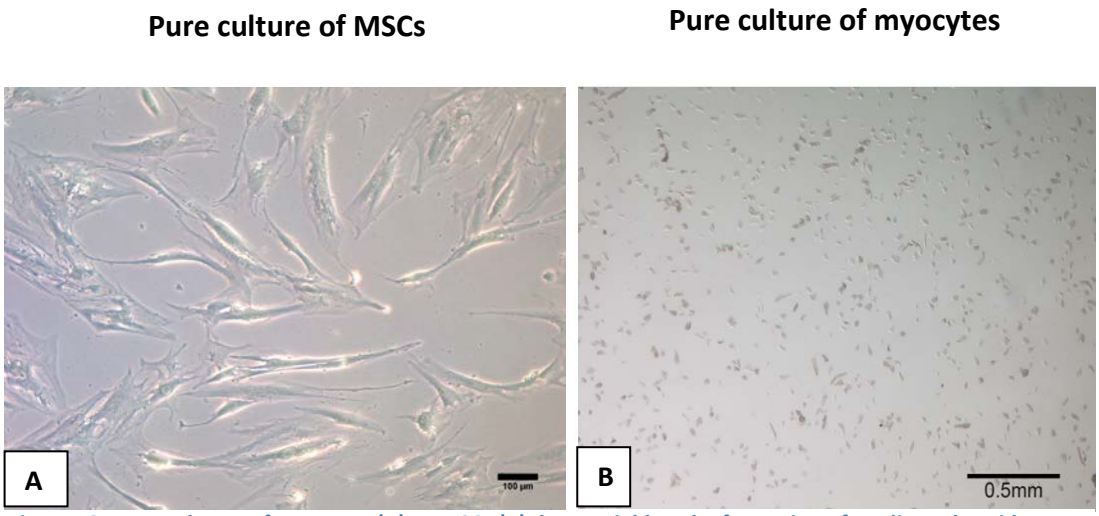

Figure 13: Pure cultures of myocytes (A) or MSCs (B) do not yield to the formation of cardiac spheroids

Cardiac myocytes and MSCs were grown separately to establish if one or both cell types, alone, would be able to form cardiac spheroid bodies. After two weeks in culture, spheroid bodies could not be found in either of the pure cell populations. Figure 13 shows a picture of cells after two weeks of culture. In both circumstances the cells have remained separate and do not shown any grouping as was seen in the myocyte/MSC co-cultures. Further, most cells retain their initial appearance and shape, unlike in co-culture, where the myocytes lose their striation and rectangular shape. These findings were consistent in every well tested. Myocytes or MSCs when cultured alone do not yield to the formation of cardiac spheroid bodies.

4.6 Results: Co-culture of non-myocyte cells with MSC does not yield cardiac spheroid formation

When the non-myocyte cells, extracted from the cardiac myocyte isolation process, were co-cultured with MSCs, cardiac spheroid bodies did not form. Figure 14, shows an image of a MSC/non-myocyte co-culture after two weeks. The culture of cells is absent of any bodies resembling the cardiac spheroids found in myocye/MSC co-cultures. The non-myocyte cardiac cells cannot be found in the culture after two weeks, suggesting they did not attach to the layer of MSCs coating the culture surface. This was confirmed with an inverted microscope used on live cell cultures. This suggests that any non-myocyte cardiac cells remaining after the isolation process wash away during culture and do not play a role in manipulation of the cardiac myocytes. Further, these results show that the non-myocyte cardiac cells do not directly contribute to the formation of the spheroid bodies.

Figure 14: Image of MSCs cultured with non-myocyte cardiac cells after two weeks. Spheroids did not form.

4.7 Results: Co-culture of myocytes with non-myocyte cells does not yield spheroids

After establishing that MSCs and non-myocyte cardiac cells are unable to form spheroid bodies together, it was necessary to test if the direct contact between myocytes and non-myocyte cells could yield spheroid bodies. Myocytes were cultured with the non-myocyte cells taken during a myocyte isolation. Significant colony formation was not seen. Figure 15, to the left, shows an image of the myocyte/non-myocyte co-culture after two weeks. The yellow arrows point out the individual myocytes. Note that the culture is void of any spheroid like bodies. The myocytes also have not become globular or granular as observed in the myocyte/MSC co-culture.

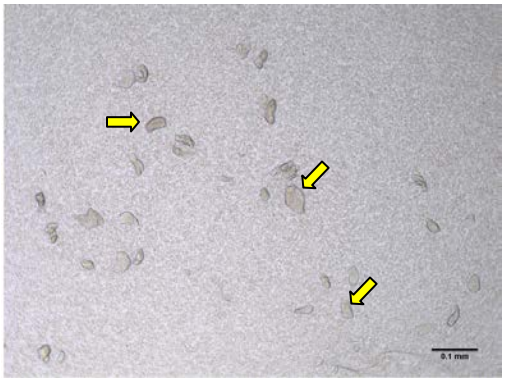

Figure 15: Image of co-culture of myocytes with non-myocyte cells after two weeks in culture. Culture is void of spheroid bodies. Yellow arrows point out the individual myocytes

4.8 Discussion: Pure cultures of MSCs or myocytes do not yield cardiac spheroid formation

The finding that, alone, MSCs or cardiac myocytes cannot form spheroid bodies demonstrates that the interaction between the two cell types is what leads to spheroid genesis. Though the spheroids are heavily actinin positive, suggesting a large presence of myocytes, the MSCs are vital for spheroid

formation to occur. This suggests that the MSCs are manipulating the other cells in culture, altering their behavior and causing spontaneous spheroid creation. As it is extremely difficult to obtain a 100% pure population of cardiac myocytes, it is necessary to establish what cells the MSCs are manipulating, the cardiac myocytes or the other cell types derived and plated during myocyte isolation.

4.9 Discussion: Co-culture of non-myocyte cells with MSC does not yield spheroids

Cardiac progenitor cells are one of the non-myocyte cells that could be present in the myocyte/MSC co-culture. As these cells have been shown to be capable of forming spheroid bodies, it is important to establish if they are the cause of spheroid genesis in myocyte/MSC co-culture. Further, any other cell type, besides myocytes and MSCs, must be eliminated as a reason for spheroid genesis. The lack of spheroids in the non-myocyte cardiac cell/MSC co-culture illustrates that cardiac myocytes are the cell type that drives myocyte/MSC co-culture spheroid creation. Non-myocyte cardiac cells from the isolation process, such as progenitor cells, do not form the spheroids. This is significant as cardiac myocytes are far more abundant than cardiac progenitor cells, which makes them a better option for producing cardiac spheroid bodies for post MI cardiac repair.

4.10 Discussion: Co-culture of myocytes with non-myocyte cells does not yield spheroids

By finding that spheroids will not form when mycoytes and non-myocyte cardiac cells are co-cultured in the absence of MSCs, it can be concluded that the non-myocyte cells are not responsible for the change in appearance and behavior of the myocytes when in myocyte/MSC co-culture. This eliminates other cells besides the MSCs as the reason why the myocytes change morphology and form cardiac spheroid bodies.

4.11 Discussion

As the spheroids are predominantly comprised of myocytes, and also necessary for spheroid formation, this suggests that the stem cells are influencing the myocytes to congregate and form spheroid bodies. Based on the positive proliferative staining found within the spheroids, the hMSCs may also be responsible for the myocytes reentering the cell cycle. These findings support current research about the relationship between MSCs and myocytes [48]. Specifically, that MSCs are able to reprogram myocytes and induce proliferation. What is novel is the finding that the myocytes, in long term co-culture with MSCs, form cardiac spheroids similar to cardiac progenitor derived spheroids [48].

It was also found that non-myocyte cardiac cells cannot form spheroids, nor can they cause spheroids to form when co-cultured with MSCs or myocytes. This eliminates them as a cause for spheroid genesis. The co-culture of MSCs with myocytes is the driving force behind spheroid formation.

A limitation of this experiment was not knowing the specific cell types that comprised the non-myocyte cardiac cells. They could have been fibroblast, resident stem cells or another cell type. Knowing their specific identity would have helped us understand how they could have been interacting with the mycoytes or MSCs in co-culture.

The change of the culture surface from CC2 coated 4 well chamber slides to tissue culture plastic 24 well plates is another variable that should be noted. This culture surface may alter the formation of the cardiac spheroids.

Chapter 5, Aim 3: Determine role of MSCs in spheroid formation

In Aim 1 It was established that the co-culture of cardiac myocytes and hMSCs results in the formation of cardiac spheroid bodies, but the specific role that the MSCs play in this process is not known. The goal of aim 2 was to determine the capacity in which MSCs contribute to cardiac spheroid formation. Two variables were investigated, the necessity co-culture and the correlation between myocyte to MSC ratio and cardiac spheroid formation. Establishing if MSCs must be in direct contact with the MSCs is important when considering the mechanism of cardiac spheroid formation, as well as how the spheroids might be used as therapeutic agents. Knowing how myocyte MSC ratio affects cardiac spheroid formation is relevant in establishing how many MSCs are necessary to make the cardiac spheroid bodies.

5.1 Method: MSCs conditioned media on cardiac myocytes

The objective of this experiment was to expose a pure culture of myocytes to the cytokines that MSCs release to determine if cardiac spheroids could be made without direct MSC/myocyte contact. Three groups were used in this experiment, myocytes cultured alone as a control, myocytes cultured in MSC treated media, myocytes cultured in 50% MSC treated media and 50% fresh media, and myocytes cultured with MSCs in direct contact. The 50% fresh 50% conditioned experimental group was used to replenish the media with nutrients lost during MSC culture. The control co-culture was done to establish if the isolated myocytes were viable enough to generate spheroids. Cardiac ventricular myoctes were isolated and plated in the same fashion as described in aim 1. MSCs were prepared and co-cultured in a

24 well plate with the myocytes, 5,000 of each cell type were seeded per well. 1milliliter of DMEM with 10% FBS 1%P/S was placed in the wells with the MSCs for 24 hours. Media was removed and placed directly on the plated myocytes for the MSC treated media group and diluted with an equal volume of fresh DMEM for the 50/50 group. Myocytes were incubated with the treated media for 2 days before fresh media was added. For the purpose of counting, cardiac spheroids were defined as 5 or more cells in intimate contact. Each well was counted by three people and the counts were averaged. Counts were taken one day after myocyte plating, prior to MSC co-culture, 4 days after co-culture, 7 days after co-culture, and 14 days after co-culture. Counts were then compared from day 0 to day 14 using a Students T-test with a null hypothesis that spheroid counts were the same. To compare the day 14 spheroid counts between groups a one way ANOVA was ran using a null hypothesis of all groups being the same.

5.2 Method: Co-culture with varying myocyte:MSC ratios

The purpose of this experiment was to establish if there is a correlation between the number of stem cells per myocyte and total spheroid formation. Six groups were compared: myocytes alone as a control, a 1:2 MSC to myocyte culture ratio, a 1:1 MSC to myocyte culture ratio, a 2:1 MSC to myocyte culture ratio, a 5:1 MSC to myocyte culture ratio, and a 10:1 MSC to myocyte culture ratio. The number of cells used was calculated with a baseline of 5,000 myocytes per well and a varying the number of MSCs to achieve the desired myocyte to MSC ratio for each experiment. Respectively the number of MSCs used in each group were: 0, 2,500, 5,000, 10,000, 25,000, and 50,000. The cultures were grown in DMEM with 10% FBS 1%P/S at 37^{o}C. Media was changed every other day. After two weeks in culture, spheroids were counted and compared between groups. A one way Anova was run to determine if significant difference existed between the experimental groups. The null hypothesis was that there was no difference and a p-value of 0.05 was used. A paired Student's T-test was also done to compare the

day 0 spheroid counts to the day 14 spheroid counts. The null hypothesis was that there was no difference in the number of spheroids counted at each time point and a p-value of 0.05 was used.

5.3 Results: Media exchange does not increase spheroid formation

To establish if the intimate contact between myocytes and stem cells is necessary for the creation of cardiac spheroids, myocytes were treated with stem cell conditioned media. To create the stem cell conditioned media, DMEM was cultured with hMSCs for 24 hours, such that the proteins secreted by the hMSCs would be captured and later introduced to the myocytes. To account for nutrient depletion, a 50/50 mix of fresh and conditioned media was used for a second experimental group. As shown in Figure 16 , the media exchange groups did not generate a statistically different number of cardiac spheroids from myocytes cultured with normal media. However, the co-culture group generated a statistically significant different number of cardiac spheroids from the control.

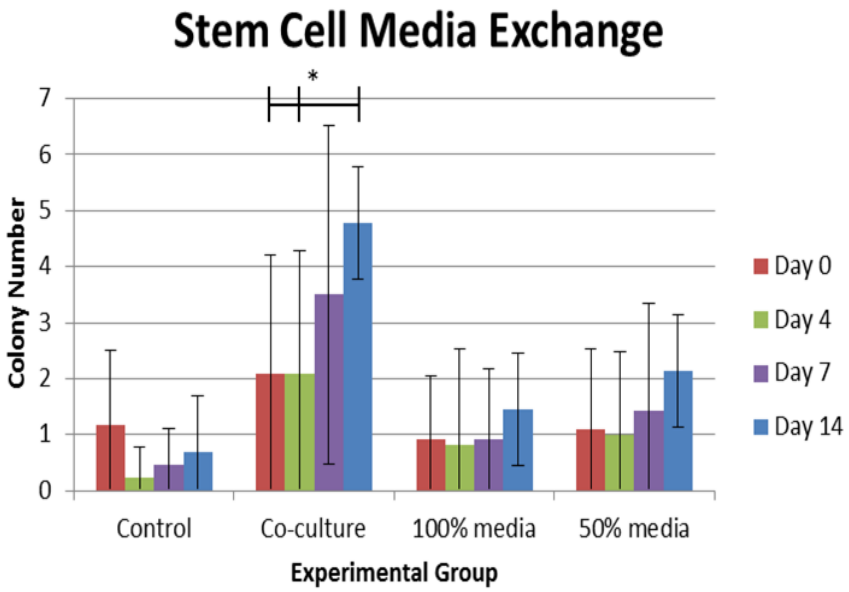

Figure 16: Cardiac spheroid formation in myocytes treated with stem cell conditioned media as compared to a direct contact myocyte/hMSC co-culture. A statistically larger number of spheroids are derived from co-culture as compared to the other groups. A one way ANOVA was done using a p-value of less than or equal to 0.05 (*). The null hypothesis was that colony number was the same between time points. Error bars are one standard deviation.

A point of interest is the upward trend of colony count over the time course of the experiment; this was not observed in the control group. This could suggest that spheroids are forming in the media

exchange groups, but not in a large enough quantity to be statistically significant, like the co-culture group.

5.4 Results: Cardiac Spheroids reside on top of a layer of hMSCs

Three dimensional confocal microscopy was used to create a three dimensional movie of a spheroid, reconstructed from a z-stack of successive images. Figure 17 shows the mass of cells from the side, from which it can be seen that the spheroid grows vertically from the culture surface. The Hoechst stain, blue, visualizes cell nuclei and the actinin stain, green, is specific to myocytes. Hoechst signal not associated with actinin signal denotes location of the MSCs. Almost all of the nuclei associated with actinin signal are located above the nuclei void of actinin signal. The myocytes have assembled above the MSCs located on the culture surface.

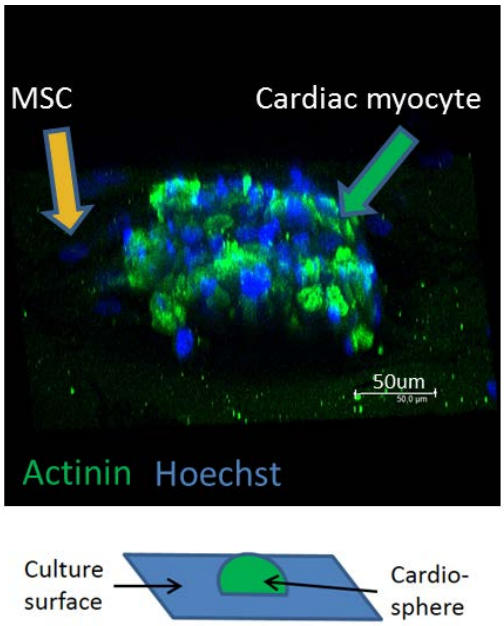

Figure 17: A 3 dimensional image of a myocyte/MSC co-culture. The myocytes are stained for actinin (green) while the MSCs are identified by blue nuclear signal without green signal near. The image illustrates how the myocytes reside on top of the MSCS, depicted in cartoon below the image.

5.5 Results: MSC to myocyte ratio does not increase cardiosphere formation

After establishing the need for direct co-culture, the importance of the total number of MSCs was examined. Using the same number of myocytes, a variable number of stem cells was used in co-culture. Figure 18 shows the results of this experiment, the varying ratios used are depicted on the horizontal axis.

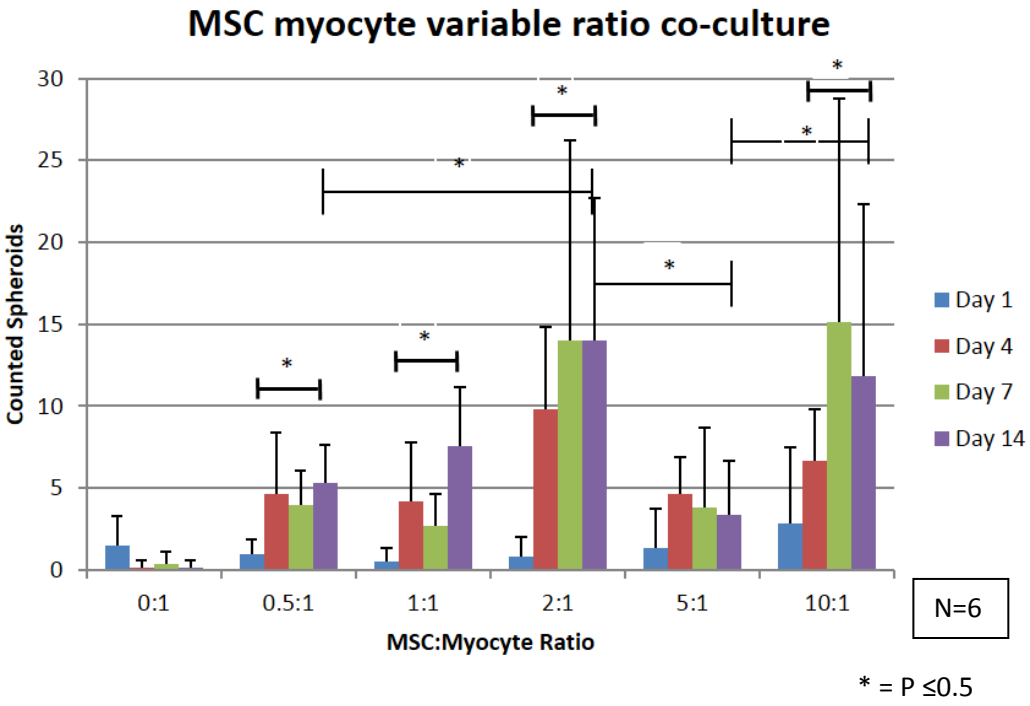

Figure 18: Myocyte/hMSc co-cultures using variable ratios of stem cells to myocytes. The formation of cardiospheres is independent of cell ratio. Total spheroid number increased significantly in all the groups except the 0:1 ratio. A one way ANOVA was done using a p-value of less than or equal to 0.05 (*). The null hypothesis was that spheroid number was the same between time points of a ratio. A second ANOVA was done with the same criteria, but using a null hypothesis that day 14 spheroid number was the same between ratios. Error bars are one standard deviation.

These data demonstrate that within each co-culture group there was a statistically significant rise in the number of cardiac spheroid bodies, with the exception of the 5:1 ratio group. Though there appears to be an increase in spheroid number from day 1 to day 14 in the 5:1 group, it is not statistically significant. In general, regardless of the ratio of hMSCs to myocytes, co-culture yields to the formation of cardiac spheroids in roughly the same number.

5.6 Discussion: Spheroids reside on top of a layer of hMSCs

Actinin staining of the myocyte/MSC co-cultures imaged with the confocal microscope revealed that the cardiac spheroids rested on top of the MSCs. These findings illustrate that the population of myocytes predominantly do not remain on the culture surface. This further supports the finding that the spheroid bodies are comprised of predominately cardiac cells. The interesting observation about the orientation of the cells is that not all of the myocytes are in direct contact with MSCs. Once the cardiac spheroids form, cells expand out away from the MSCs adhered to the culture surface, yet retain their non-striated spherical appearance. This could prove to be helpful when attempting to remove the spheroids from the culture surface.

5.7 Discussion: Media exchange does not increase spheroid formation

The most substantial finding from this experiment was that MSC conditioned media was unable to generate a statistically significant number of cardiac spheroids in a culture of pure myocytes. The inability of the media exchange groups to form a statistically different number of spheroids at the end of the experiment could be a failure to reject the null hypothesis based on too small of a sample size. Stem cell treated media may be giving rise to spheroids, just not as effectively as myocyte co-culture. Only direct myocyte/MSC co-culture led to a significant increase in cardiac spheroid number. Rodriguez et al. reported finding direct contact between MSCs and myocytes, these structure may be the key to the formation of spheroid bodies as they can only form when MSCs and myocytes are in direct contact. These findings suggest it is the direct co-culture that drives the change in appearance and behavior of the myocytes.

Another point of interest with this experiment was the upward trend over time of spheroid bodies in both conditioned media groups. Though not statistically significant, the number of counted spheroid bodies rose over time. This could be because some of the proteins secreted by MSCs have been shown to induce cellular proliferation [39]}. It is possible that the process of changing the media damages these proteins in some way and therefore lessens their function.

The culture conditions in which the medium was exposed to the MSCs does not directly reflect the conditions the MSCs are in when being co-cultured. Specifically, they are not exposed to myocytes. This may change the nature of what is being secreted from the MSCs. Therefore, the composition of MSC secretions in the conditioned media may not be exactly what is in the co-culture media. The half-life of these secretions is also a limited factor. If they secretions are breaking down before being placed on the myocytes, they myocytes may not be experiencing the same secreted factors as they would in co-culture.

5.8 Discussion: MSC to myocyte ratio does not increase spheroid formation

The number of MSCs in culture per myocyte does not directly correlate to more spheroid bodies, further suggesting that the MSCs act as a triggering agent to myocyte reprogramming. If the myocytes required the MSCs for the duration of spheroid formation and growth, it would be expected that more MSCs would mean more spheroids. However, if they are only needed to trigger the reprogramming of myocytes, than a few MSCs could have the same impact on a population of myocytes as a larger group of MSCs, which was what was witnessed in this experiment.

This fact also suggests that MSCs do not directly make up the spheroid bodies. If MSCs were migrating into the spheroid bodies or were initially being incorporated into them, it would be expected that more MSCs would correlate to more spheroid bodies.

To determine if a counting bias contributed to the results, specifically with the 5:1 group, two additional counters were used to verify the data. A counting bias could occur from the counter changing the definition of a spheroid based on what group he/she is counting. Figure 19 shows that this trend was observed when the counting process was replicated.

One limitation of this study was the surface area of the spheroids generated were not examined. There may be a correlation between the ratio of MSCs to myocytes and the size of the spheroids that could impact spheroid number.

5.9 Aim 3 Discussion

In Aim 2 it was shown that direct myocyte to MSC contact is helpful for cardiac spheroid bodies to form. This is significant when considering using MSC derived spheroid bodies for clinical use, as well as the relationship between MSCs and myocytes. The original hypothesis that direct contact is needed is supported.

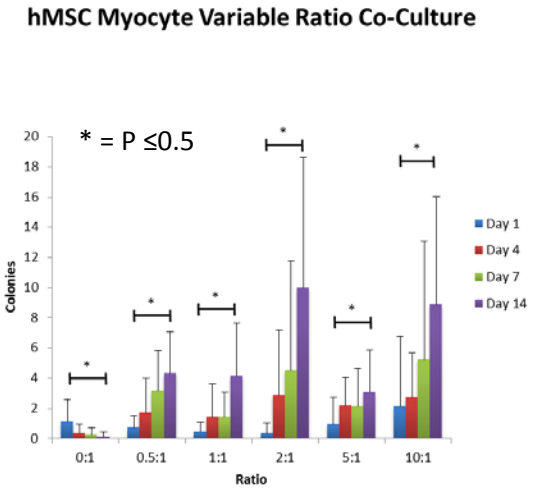

Figure 19: Variable ratio experiment conducted with 3 spheroid counters. Graph appears similar to that depicting data from one counter.

The second hypothesis of aim 3, the number of MSCs in culture will be directly proportional to the number of spheroid bodies that form was not confirmed. No strong trend between myocyte/MSC ratio and spheroid number was found.

These two findings suggest that the MSCs trigger a change in appearance of the mature myocytes that results in spheroid formation. Further, it is not likely that MSCs directly contribute to the population of cells comprising the spheroid bodies or it would be expected that more MSCs would yield more spheroid bodies. These conclusions suggest that a relatively small population of MSCs would be able to produce a large number of spheroid bodies.

Chapter 6: Discussion

In order to mitigate the effects of myocardial infarction, it is necessary to replace the dead tissue with new mechanically active myocytes. Current technology fails to adequately accomplish this. This investigation has shown that isolated cardiac myocytes can be made, through stem cell co-culture, to form cardiac spheroid bodies. These spheroid bodies grow over time and increase in number. For this process to happen the myocytes and stem cells must be in direct co-culture. The relevance of these findings in the context of post infarct cardiac regeneration will be discussed in this section.

6.1 Formation of spheroids

The main objective of this study was to derive a potential cell source for regenerating myocardium after an infarction. The combination of myocytes and MSCs was chosen for several reasons. One, MSCs appear to not illicit a rejection response from the immune system when used in the context of cardiac regeneration[16]. For clinical applications, an autologous source of myocytes would have to be used and therefore would also not be rejected by the immune system. The biggest advantage cardiac myocytes hold in terms of cardiac regeneration is that they contain the proteins necessary for contraction, as well as electrical coupling with native myocardium [32]. Other cell types used to treat MI,

such as stem cells or skeletal muscle, must be cohersed to make these proteins if they are to form beating units in the heart.

hMSCs were chosen as a conditioning agent to the ventricular myocytes based on their ability to reprogram cardiac myocytes[14]. Additionally, when hMSCs and myocytes are implanted together into an infarcted rat heart, they are able to restore more functionality than myocytes or stem cells implanted alone[17]. This suggests that these two cell types are able to work in conjunction to regenerate myocardium. It is known that adult myocytes and stem cells are able to form inter-cell gap junctions, as well as nanotubes, through which cytoplasm is exchanged[14]. Further, hMSCs have been shown to be able to promote proliferation of cardiac myocytes, as well 'protective reprogramming of injured adult myocytes' [54]. It is for this reason that hMSCs were chosen to condition the isolated myocytes, such that they may promote proliferation and potentially cause the formation of cardiac spheroid bodies, similar to those made from cardiac progenitor cells. Cardiac progenitor-derived spheroids are significant as they have been shown to be the most promising cell type for post MI-therapy, though the rarity of cardiac progenitor cells in the heart limits the ability to make a large number of spheroids in a safe and timely manner [Marban, 2010]. If a similar cell type could be generated from mature myocytes, which are much more abundant in the heart, this limitation could be overcome.

When myocytes and stem cells were cultured together, cardiac spheroid bodies did form. These spheroid bodies resembled the cardiospheres formed from the isolation and expansion of cardiac progenitor cells. The cardiac stem cell derived spheroids showed greater potential to mitigate the effects of a MI as compared to all other cell types used for that application [42]. As these bodies are similar in composition to the myocyte/stem cell co-culture derived spheroids, it suggests that cardiac spheroid bodies hold promise as an effective agent to aid the heart in healing post MI. The similarities between

cardiac progenitor cell derived spheroids and myocyte/MSC co-culture derived spheroids is the reason for this belief.

Table 2 lists the common traits between cardiac progenitor cell derived spheroids and those made from myocyte/MSC co-culture.

Table 2: Shared traits between CPS and myocyte/MSC derived spheroids

Similarities between CPS and myocyte/MSC derived spheroids
Round spheroid bodies comprised of granular cells
Autologous cardiac lineage
Spheroid number increases over time
Ki-67 positive

The cells found in the myocyte/MSC-derived spheroids have the potential to restore heart function and can be made with less cardiac tissue than cardiac progenitor derived spheroids. Figure 20 below illustrates the contrast in the availability of the cells used from the cardiac biopsy to make progenitor cell derived spheroids as compared to myocyte/MSC co-culture derived spheroids. They key difference is the abundance of myocytes in the heart as compared to the small amount of cardiac progenitor cells. For every 10,000 myocytes in the heart there is only one cardiac progenitor cell. In the context of making spheroids, this means there are more cells capable of making spheroids using myocyte/MSC co-culture in a biopsy than cells capable of making CPSs.

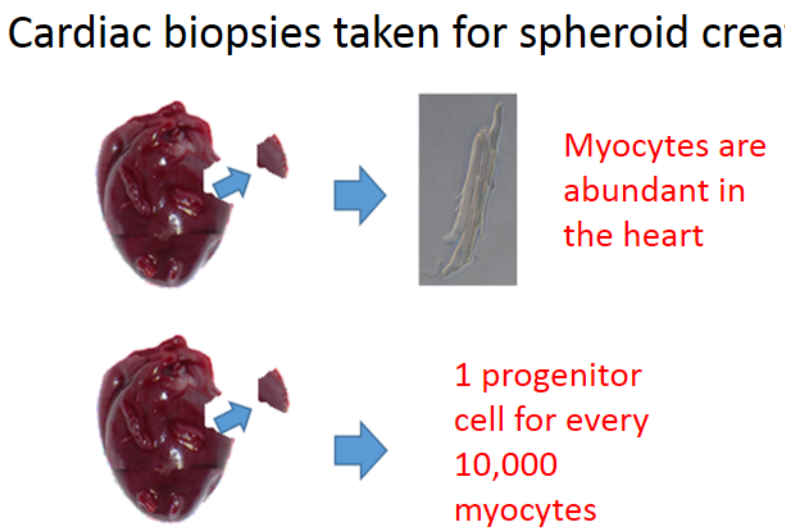

Figure 20: Comparison between the cells used from cardiac biopsies in order to make progenitor cell derived spheroids vs. myocyte/MSC co-culture derived spheroids.

Another point of comparison between our spheroids and those made from cardiac progenitor cells are their size. After 25 days of culture, progenitor derived spheroids are about 200 microns in diameter [Marban, 2006]. Though the myocyte derived spheroids are smaller after 2 weeks, roughly 50 to 100 microns in diameter, after 4 weeks in culture they are roughly the same size as the progenitor cell derived spheroids.

One thing that remains unclear about the formation of the cardiac spheroids is the mechanism by which the MSCs act on the cardiac myocytes. In aim 2, it was found that direct myocyte/MSC co-culture is advantageous for spheroid genesis, offering some insight into how the MSCs communicate with the myocytes. These results suggest that direct co-culture of the two cell types is beneficial for spheroid formation, and therefore, paracrine factors secreted by the MSCs may not substantially contribute to the creation of spheroids. It is possible that the separation of the myocytes and MSCs may change the factors that are secreted by the MSCs. Additionally, the half-life of the secreted cells may play a role in the ability of MSC conditioned media to form spheroids when cultured with mycoytes. If the paracrine

factors are breaking down prior to being exposed to the myocytes, their effect on the myocytes will be diminished. This is another possible explanation as to why direct co-culture yields more spheroid bodies than conditioned media. Literature shows that MSCs and myocytes can form nano-tubes through which cytoplasm is exchanged [54]. This is another possible means by which the MSCs could be influencing the myocytes. The implications of this are that it may be difficult to separate the MSCs from the spheroid bodies for clinical use. Fortunately, this may actually be advantageous as allogenic MSCs, in the context of cardiac regeneration, are immune-privileged and further, have been shown to help mitigate the negative effects of an MI [39].

Determining the role of MSCs in the formation of cardiac spheroids is also important when assessing the production of the spheroid bodies. When considering large-scale production of these cardiac spheroids, it is necessary to know how many stem cells are needed to create the spheroid bodies. The production process must be optimized to limit cost while maintaining yield. It was found that stem cell to myocyte ratio did not significantly impact the number of colonies formed. The lower boundary for this experiment was one stem cell for every two spheroid bodies. Therefore, future experiments should investigate whether colony formation happens below this ratio; in this way the number of stem cells needed to produce spheroids could be minimalized.

6.2 Experimental limitations

One issue that arises when attempting to quantify the number of cardiac spheroids that have formed is determining what is and is not a cardiac spheroid. This can be difficult when multiple myocytes are in close proximity. A standard definition of a cardiac spheroid was adopted such that consistency was maintained from well to well. Also three counters counted each well that was analyzed in order to eliminate possible bias or human error.

A concern when considering the use of these cardiac spheroids for clinical purposes is if the hMSCs make up some of the cardiac spheroid. If they do, the implications of implanting them into a human heart must be determined. As the mesenchymal stem cells will not instigate a response from the host immune system, therefore they are acceptable for therapeutic use if they are forming part of the cardiac spheroids.

A second concern that arises when assessing the feasibility of using cardiac spheroids for clinical use is cell viability within the spheroid. Cells at the center of the spheroid may not be receiving ample nutrients due to the limitation of diffusion through the spheroid body. Diffusion becomes inadequate to maintain cell function around 200um. To avoid this problem the cardiac spheroids could be harvested once their radius reaches 200um. To truly determine if cells within the wells are still viable, the cardiac spheroids would need to be fixed and sectioned, such that the inner cells could be stained. This process is time intensive and technically challenging and, therefore, was not conducted in this study.

6.3 MSCs in the infarcted heart

MSCs have been shown to mitigate the negative impact of myocardial infarction when delivered to the heart, yet the mechanism governing this is unknown. The results of this research offer some clues as to what could potentially be taking place in the heart between the delivered MSCs and native cardiac myocytes. Delivered MSCs could potentially co-localize with the myocytes, as seen in in-vitro co-culture, and reprogram the myocytes in the same manner. This could lead to myocyte proliferation effectively replacing some of the lost cardiac tissue. This phenomenon, as well as the benefit of cytokines secreted by MSCs, could be the way in which MSCs help mitigate the negative impact of MI.

Chapter 7: Conclusion

This study has shown that by co-culturing ventricular myocytes with hMSCs, cardiac spheroids can be created. A similar cell type derived from cardiac stem cells has demonstrated to be more effective at restoring contractile force than any other cell type used for cardiac regeneration. The myocyte/MSC derived spheroids are similar to the cardiac stem cell spheroids. The advantage they offer over cardiac stem cell made spheroids is they require substantially less autologous tissue to create, making them safer for the patient and faster to create in large number. The myocyte/MSC co-culture derived spheroids contain some proteins found in stem cells and some found in mature myocytes.

In aim 2 it was found that myocytes cultured with non-myocyte cardiac cells will not form spheroid bodies. It was also found that MSCs cultured with non-myocyte cardiac cells will not form spheroid bodies. The co-culture of myocytes and MSCs is necessary for spheroids creation.

The results of Aim 3 illustrated that direct co-culture is required for the number of spheroids to statistically increase. There was not a strong correlation between an increase in spheroid number and an increase in the ratio of MSCs to myocytes.

Chapter 8: Future Work

The first step to furthering the development of myocyte/MSC co-culture derived spheroids is to better understand what is occurring within the spheroids in terms of proliferation and cell number. The total number of cells that have undergone proliferation could be tracked by adding EDU to the culture media and tagging that EDU with a fluorescent antibody. Cells that stained positive for EDU would have replicated their DNA while in culture. It would also be useful to count the number of cells within the spheroids. Myocytes can contain either one or two nuclei, so therefore, the number of nuclei in a spheroid will not necessarily correlate to the number of cells in that spheroid. One possible way to count the cells of spheroid is to attempt to dissociate the individual cells from the spheroid with trypsin or another protease, then use a hemocytometer to count the cells.

Another important step in investigating the potential of the spheroids to restore mechanical function to a region of infarct is to investigate the ability of the spheroid cells to differentiate into myocytes. This could be attempted by electrical or mechanical pacing of the cells. This would entail imparting either a cyclical mechanical strain on the cells or a pulsatile electrical stimulation. The goal is to emulate the conditions inside of a beating heart to change the phenotype of the cell.

Future studies should aim to determine the best delivery vehicle for the cardiac spheroid bodies to a rat infarct model. Several delivery techniques should be tested for cellular engraftment to determine the most effective method. Once a way to deliver the spheroids is found, they should be used to treat a rat model of myocardial infarction. LVEF of the infarcted heart could be used as a metric to compare hearts without treatment and a cardio-sphere treated MI. This would gauge the ability of the spheroids to restore the ability to pump blood to the damaged myocardium. Once this has been done, a

similar experiment should be scaled up to a large animal model. If the myocyte/MSC derived spheroids are proved to be safe and effective, human cardiospheres should be made and tested in a human clinical trial. Success in clinical trials shifts the focus of study to up-scaling and optimizing the spheroid production process.

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Appendix A: Making Krebs solution (KB)

Table of chemicals used and their required quantity per one liter KB.

Chemical	Grams/Liter
KCL	1.863
KH_2CL_2	1.361
Glucose	0.285

EGTA	0.190
Taurine	2.5
L-glutamic acid	10.29
HEPES	2.38
ATP	0.276

- 1 liter of deionized water was measured with a graduated cylinder and place in a 2l beaker
- Beaker was put on a heating plate set to 30^{o}C with a magnetic stirrer spinning on the bottom
- All of the ingredients, except for the ATP, were measured out and placed in the 1L of DI water
- Solution was left to warm and stir until clear, (roughly 30min to an hour)
- Beaker was taken into a biosafety cabinet and filtered through a 1L Corning Filter system.
- Filtered contents were stored in at 3-5^{o}C
- Prior to using KB the appropriate quantity of ATP was measured out and put into solution with 5mls of DI water.
- This solution was filtered with a 5ml syringe and a Pall Acrodisk Syringe Filter (0.2um) into the KB solution