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Design of a Scalable Method to Produce Cardiogenic Cells from hMSCs through Embryoid Body Formation and PDGF Treatment

A Major Qualifying Project Report

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1. hMSCs
2. Cardiac-myocytes
3. PDGF

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Abstract

This project developed a scalable method to produce cardio-myocytes from human mesenchymal stem cells (hMSCs) for use as a therapy for myocardial infarctions. Embryoid bodies (EBs) formed from hMSCs produced cardiac cells. To increase the production of EBs, a non-adhesive well structure was developed and tested. To increase the percentage of cardiac cells resulting from the process, platelet derived growth factor was used as a supplement. These novel methods will ease the process and increase the yield of cardiac cells.

Executive Summary

The number one killer in the United States is cardiovascular disease with myocardial infraction presenting a very serious problem in all walks of life. A myocardial infarction is a region of dead myocardial cells that no longer function in conjunction with surrounding myocardial tissue. Due to the limited regeneration of the myocardial tissue this results in permanent limited heart function and in many cases leads to death. Current therapies only treat the symptoms without repairing the damaged tissue. However, advances in regenerative medicine offer treatment focused on the regeneration or replacement of the myocardium.

Stem cells are on the forefront of potential therapies due to their ability to proliferate and differentiate into multiple cell lineages. Embryonic stem cells (ESCs) were initial candidates, however, due to political issues and serious concerns about teratomas, researchers have examined adult human mesenchymal stem cells (hMSCs), derived from adult bone marrow. HMSCs have shown similar pluripotent capabilities as ESCs. Using a similar culture technique used to induce ESC differentiation into cardio-myoctyes, known as embryoid body (EB) formation, hMSCs have been shown to differentiate into cardiogenic cells. Current research has shown that the implantation of these cells into a MI canine model has shown improvement in heart function. It has also been shown that growth factors such as platelet derived growth factor (PDGF) can effect hMSC differentiation and proliferation.

The technique used to culture EBs is known as the hanging drop method, which is performed by placing a droplet of cell suspension on a flat surface and then using a specific technique to invert the surface. This produces a hanging droplet of cell suspension and after 3 days of culture causes the formation a 3-dimensional aggregate of un-differentiated cells known as an EB. However, this method has limited scalability and efficiency.

We have designed a new method to mass produce cardiogenic cells from hMSCs. A new method was developed that included non-adhesive wells to optimize the production of EBs and supplemental treatment with PDGF to maximize the cardiogenic output from this process

To optimize the hanging drop method various design alternatives were brainstormed after revising the client statement and creating the objectives, user requirements, functions and constraints for the project and ranking them for importance using pair-wise comparison charts and objective trees. After the design process we narrowed the designs down to two main categories: Optimized Hanging Drop Designs and Non-adhesive Well Designs.

The first step we took was calculating the radius of curvature of a hanging drop using image analysis tools and determined the radius of curvature of a hanging 20ul drop as 2.76 ±0.32mm. This radius of curvature has been used throughout all our designs.

The first preliminary design we considered were the optimized hanging drop designs, where our goal was to reduce the number of drops lost during the maintenance procedure. We supported the drops by creating an indent with the radius of curvature into solid polymers. To further reduce the number of drops lost we installed a pipette channel to introduce the hMSCs from the top. At the interface of the pipette channel and the indent we installed a micro-screen with a pore size of 200um so that the cells initially could pass through the pores to form the EB, but prevented the EB from getting sucked up once EBs formed. Due to unsatisfactory preliminary design results, with no EB formation, we decided to proceed on to the non-adhesive well designs.

The concept of the non-adhesive designs was that the surface tensions at the hanging drop method could be interpreted as repulsive forces and non-adhesive wells with the right radius of curvature would form EBs. Two preliminary designs were created: electrospun wells, and solid polymers.

The electrospun polytrimethylene terephthalate (PTT), a non-adhesive polymer allowed the aspiration of media through its porous 3-D matrix, which had well shapes with 2.76mm as the radius of curvature. There were two possibilities of creating the wells into the electrospun material: heat sinking and negative mold casting. Unfortunately, the material absorbed the media, drying out and leaving the cells without nutrition. No EBs formed with this design.

The second preliminary design was using non-adhesive polymers, which relied on the same concept. Here the main problem was the manufacturing of the wells. We needed to have surface as smooth as possible. Different methods such as drilling, melting and force molding were used, where drilling and melting resulted in very rough surface topographies, not suitable for EB formation. No EBs were formed. Force molding rivets and ball bearings resulted in much smoother surfaces, but the imperfections of the rivet heads with 2.5mm radius of curvature created some micro-cracks within the wells which prevented EB formation. The least imperfections were created with ball bearings, unfortunately no EBs formed here either.

However, to proof the concept of non-adhesive wells, injection molded conical tubes and low attachment coated culture plates were used. The injection molded V- bottom untreated polystyrene conical tubes had a smaller radius of curvature than initially calculated that resulted in homogenous single EB formation in 6 out of 9. The low attachment coated plates purchased from NUNC with a regular U-bottom resulted in EB formation as well, but the morphology of the EBs wasn't as homogenous as the conical tubes. Multiple EBs with low qualities formed.

In order to test our hypothesis of an increased percentage of cardiogenic-like cells migrating from an EB when it has been subjected to PDGF treatment we first had to form

EBs. This was done in the traditional “hanging drop” method. For control purposes we also kept hMSCs from the same passage un-hung culturing them in the traditional fashion. The drops that were hung were fed once a day for a period of three days. The EBs and hMSCs were then plated and treated with media. The experimental slides were treated with the media that had been supplemented with PDGF while the controls were treated with our un-supplemented media. On day seven of our experiment the cells were fixed and prepped for our immunohistochemistry. Our immunohistochemical stain stained for the presence of alpha sarcomeric actinin (a known cardiac marker). Florescent microscopy was then used in conjunction with photomicrographs to determine the number of cells migrating away from the EB and of that number how many expressed alpha sarcomeric actinin.

Our photomicrographs showed that our data would have to come from two subsets of EBs. The EBs marked as “full EBs” were the EB had remained intact with a clearly defined center from which migrating cells could be analyzed, and a second subset we deemed “void EBs” were the EB had been lost in the fixing process but had left the ring of migrating cells behind. Initially our data suggested that the treatment of EBs with the PDGF supplemented media gave an average increase in the number of cardiogenic cells migrating away from the EB from 8% to 16%. However; due to the time constraints of our experiment our “n” number was too low to give a value of statistical significance. A “T” test run on our raw data revealed a “p” value of 0.078.

Though we were unable to definitively prove our hypothesis that treatment of EBs with PDGF supplemented media will increase the percentage of cardiogenic cells migrating away from the EB; we feel that our data does suggest a trend. To achieve a p value so close to statistical significance from such a small “n” number suggests that with further experimentation and larger sample sizes we may be able to achieve results confirming our hypothesis. Additional future work may also include: adjusting the concentration of the PDGF in the media, as well as altering the introductory time and duration spent in the supplemented media.

EB formation can be accomplished though the use of non-adhesive wells and this method is scalable to the need of the user. The topography of the well surface played an important role in EB development. Using precision manufacturing techniques such as injection molding and coating an injection molded plate drastically improved aggregate formation and subsequently EB formation. Shape may also have an important role as a steep well with a smaller curvature form EB singular homogeneous EBs. We recommend a final design that incorporates non-adhesive polymer wells with a steep sloped, small radius of curvature well, which is scalable depending on the number of wells. It may be possible to use this device to optimize the production of EBs from hMSCs and in conjunction with PDGF treatment may increase the output of cardiogenic cells.

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1. Introduction

The number one killer in the United States is cardiovascular disease with mortality rates of almost 2500 Americans each day or an average of 1 death every 35 seconds. More specifically, it has been reported that 13,200,000 American's have Coronary heart disease (CHD) and out of that 7,200,000 Americans experienced a myocardial infarction (MI), also know as an acute heart attack [61]. Myocardial infarction is a region of necrotic myocardial cells that have seized to function in conjunction with surrounding myocardial tissue. This results in severely limited heart function and in many cases leads to death. Due to the limited regeneration of the myocardial tissue much of the effected area is replaced with scar tissue resulting in a loss of functional muscle mass. Current treatments of myocardial disease often only treat the symptoms without repairing the damaged tissue. Recent advances in regenerative medicine, synonymous with tissue engineering, offer alternative methods of treatment focused on the regeneration or replacement of the lost tissue.

Stem cell research has become a significant field in regenerative medicine. Their pluripotent capabilities may someday become an endless source of various cell lineages. Human Mesenchymal Stem Cells (HMSC) have shown the capacity to differentiate into cardio-myocytes in vitro as well as in vivo. Currently laboratory research has had limited success in efficiently producing uniform cardio-myocytes from HMSCs, with a reproducibility that is required for extensive clinical applications. It is our goal to develop a method and device to optimize the production of cardio-myocytes from HMSCs for use in further scientific research.

2. Literature Review

2.1. Biological Background

2.1.1. Stem Cells

In resent years stem cells have gained much attention in the field of tissue engineering. Stem cells are undifferentiated progenitor cells which have the capability of proliferation and differentiation into multiple cell lineages. The differentiation properties of stem cells are of much interest to tissue engineers in the hopes that damaged tissue may be regenerated and replaced. Theoretically, the tissue lost in a patient with a myocardial infarction could be regenerated in the laboratory through the use of stem cells. The regenerated tissue could then be implanted replacing the damaged tissue. Unfortunately much is still unknown about the mechanisms driving stem cell differentiation. Currently there are two types of stem cells being studied for their pluripotent capabilities.

2.1.1.1. Embryonic Stem Cells

Embryonic stem cells are cells that can be found in a developing embryo. ES cells are isolated from the intercellular mass of a developing blastocyst. Four to five days after fertilization of an egg the embryonic stem cells begin to differentiate into the three primary germ layers; the ectoderm, endoderm, and mesoderm. [1] All of the cells found in the body are derived from the three different germ lineages. For this reason embryonic stem cells are a perfect candidate for stem cell research. Their pluripotent capabilities are inherent in the formation of life and may prove to be useful tool in tissue engineering.

Recent studies have suggested that implantation of ES cells into the region of a myocardial infarction increased heart function in mice with myocardial infarctions. [2] Injection or implantation of ES cells was shown to improve mouse heart systolic function by 20% in comparison to sham mice which received only ES media. Diastolic function was also increased to normalized levels eight days after implantation into the infracted region. [2] Such studies imply that ES cells are capable of engrafting and surviving within ischemic myocardial tissue; improving overall cardiac function. Additional studies have demonstrated that cardio-myocytes could be spontaneously derived through in vitro culturing of ES cells. [3] ES cells were cultured and induced to form embryoid bodies (EB). The EBs were then cultured for 7-40 days and were observed for cellular contraction. Contracting cells were found in approximately 8% of plated EB. In addition contracting cells stained positive for myosin cardiac heavy chain α/β and α-sarcomeric actinin; two cardiac specific proteins. [3] Though the means with which these ES cell differentiate is incompletely understood cell

adhesion signals are thought to be a major contributing factor. [4] The potential that ES cells possess gives a glimpse into the future of tissue engineering; a future where damaged tissue can be replaced instead of merely treating the symptoms that they produce.

However, embryonic stem cells have been surrounded in controversy due to the source from which they are attained. Isolation of ES cells ultimately results in the destruction of a developing embryo. At the center of the controversy is the debate regarding when a developing embryo is considered a live human being. Unfortunately the distinction between a developing embryo and a developing human is often difficult to make when working on the microscopic level. In addition the theoretical benefits from embryonic stem cell research often times facilitates ignoring the ethical issues in the hope that future lives may be saved. Regardless, recent political movements have made embryonic stem cells difficult to ascertain, inhibiting the progression of related research. Political actions also halt clinical ES cell experimentation on human subjects, limiting research to laboratories. For many researchers the use of ES cells, as a tool, to understand cell fate is enough reason to carry out further experimental research despite the restrictions. From an engineer's stand point the application of clinical trials is a crucial step in the development of product. As an example, if ES cells were successfully differentiated in vitro into cardio-myocytes their functionality in vivo could never be examined, eliminating the chance of a future treatment for those suffering MI. As a result, embryonic stem cells are not a suitable choice for this MQP as the goal is to mass produce cardio-myocytes. Regardless, much can be learned from ES cells and applied to other pluripotent cells.

2.1.1.2. Adult Stem Cells

Stem cells do not only exist in developing embryos but can in fact be found in adult organisms as well. Within the marrow of adult bones there exists a group of cells with the capacity to differentiate into several lineages before only seen from ES cells. These cells called bone marrow stromal cells (BMSC's) are primitive cells still maintaining the potential for directed transdifferentiation.

Early experiments with these cells like the one conducted by Orlic et al. showed that the cardiac lineage could be isolated with the use of cytotoxcins, more specifically the demethalizing agent 5—azacytidine. When examined these cells showed a fibroblast-like morphology, at first. [5] After a week however these same cells grouped together and began to express the morphology for what is expected of a "normal" cardiac myocyte; including typical sarcomeres, a centrally positioned nucleus, and atrial granules. [5]

2.1.1.2.1. BMC Differentiation

These early experiments helped to show that in fact these BMC's have the potential to become cardiac like cells. For the purpose of this experiment however; the use of such methods, namely the use of cytotoxcins, is not ideal. Fortunately a team based out of the National Heart Center shared this concern. Knowing these cells had the capacity to differentiate when induced to do so by potentially dangerous chemical means, Winston S.N. Shim and his team set out to determined whether the same results could be achieved independent of cytotoxic agents or co-culturing techniques. The marrow stromal cells were first harvested from sixteen patients undergoing coronary surgery. These cells were then cultured in a special media medium containing insulin, dexamethasone, and ascorbic acid, meant to help differentiation. [6] Of the sixteen patient sample thirteen were cultured successfully (the other three having failed due to a limited starting number of cell) showing the spindle structure often associated with mesenchymal cells. Within four days the cells had begun to form colonies of between one and two thousand with approximately five percent of the cultured cells having assumed a polygonal structure associated more with cardio myocyte like cells. In addition to there simple morphology the cells tested early for cardiac troponin I, sarcomeric tropomyosin, and cardiac titin. Showing that BMC's can in fact form cardiac myocyte like cells with out the use of cytotoxins. [6]

2.1.1.1.2. BMC Potential

Now that the science was there to back the cells ability to differentiate the question remaining was, are these cells now capable of increasing the global function of the heart, post myocardial infarct? When primitive BMC's were injected into the border of an acute myocardial infarct, it was found that they do in fact help heart function. In a study done of a twenty seven day period it was found that the injected BMC's were mobilized and homed in on the damaged area. This cytokine-induced cardiac repair decreased mortality by sixty eight percent and infarct size by forty percent.

The experiment done in mice showed that, mice treated with the primitive BMC's post infarct showed a seventy three percent survival rate of on the twenty seven day mark where as the infracted mice that when untreated posted only a seventeen percent survival rate. When the animals were terminated after day twenty seven the treated animals were seen to have a band of newly formed myocardium around the boarder of the infarct. [7] Adult bone marrow stromal cells have been shown to differentiate, become cardiac like cells, and help infracted regions of the heart. However; BMC's are made up of different types of cells. One of these is the mesenchymal stem cell the biological gold vein running through the bone marrow mountain.

These cells have the benefit of being able to be drawn from the same person in which they are to be used. Since there will be no immune response when the cells are re-implanted into the patient they can be passaged in vivo, generating a great many cells from a small sample. Our goal in this project is to take these cells and find a method with which to mass produce them; studies in BMC's give us a good place start.

2.1.1.2. Mesenchymal Stem Cells

Mesenchymal stem cells (MSC) may be a way to circumvent the ethical woes of embryonic stem cell research. MSCs are adult stem cells that also posses pluripotent capabilities. The discovery of MSCs offered an alternative source for regenerative stem cells that did not require the destruction of a developing embryo. MSCs can be found in a variety of tissues including bone marrow, the stroma of the thymus and spleen, and within synovial fluid. [8,9] Bone marrow (BM) is considered to be the best candidate for isolation of MSCs since BM exhibits a high congregation of MSCs while still remaining fairly accessible. [8,10] MSCs can be isolated through the use of gradient centrifugation and cytofleurometric analysis. [11,12]

While ES cells can theoretically differentiate into all of the cells of the body; MSCs can only differentiate into a limited number of cell lineages. Under certain conditions MSCs can be induced to differentiate into several cell lineages. Cell lineages include adipocytes, chondrocytes, tenocytes, and most importantly myocytes. [8,13,14] These tissues are examples of tissue originating from the mesoderm germ layer during embryogenesis. MSCs ability to differentiate into a variety of tissues suggests that they play a role in reparative functions within the body. [8] This theory is plausible as MSCs can be found in a variety of tissues; these tissues may serve as reservoirs for the reparative cells until they are needed by the body. In addition their pluripotent capabilities would facilitate the regeneration and replacement of the damaged tissue. [4]

2.1.2. MSC Restoration of Cardiac Function In Vivo

Research has shown the reparative function of MSCs in vivo through injection or implantation. Recent studies have shown that injection of MSCs into an infarcted portion of the heart increased overall cardiac function by upwards of 27%. [12] Identification of MSCs was achieved through 5-bromodeoxyuridine (5-Bru) treatment prior to injection into the infarcted area. [12] Improved heart function was measured using echocardiography before and 4 weeks after implantation of MSCs.

It is still unclear whether heart function improved due to MSCs differentiating into cardio-myocytes, an increase in native cardio-myocyte proliferation, or the replacement of scarred tissue with a more elastic one. This is an example of Active vs. Passive treatment of

MIs. Passive treatment involves treating the symptoms associated with the MI (poor heart function), while active treatment involves the treatment of the root problem; dead cardiac myocytes. In in-vivo studies it is hard to identify weather cardiac function is improved by passive or active means. If cardiac improvement is the result of substitution of scarred cardiac tissue with a more mechanically favorable cell type (in this case MSCs), this would be an example of passive treatment; the symptoms are improved even though the missing cardiac muscle has not been functionally replaced. While passive treatment may be a means to an end, it is our goal to effectively replace the MI with functional cardio-myocytes derived from MSCs.

While any one of these scenarios may result in increased cardiac function MSC differentiation into cardio-myocytes has been suggested by Wang et al. [12] Differentiation was suggested by staining brU positive MSCs with anti-troponin monoclonal antibodies. [12] Other experiments too have shown the potential reparative qualities of hMSCs. In an experimentally induced current block of mouse neonatal cardio myocytes several different cell types were added to determine their comparative regenerative value. Forty eight hours after the 200-450 um channel block was implemented, hMSCs, human skeletal myoblasts, rat cardiac fibroblasts, and no cells were added into their respective samples. Having given the cells twenty four hours, it was seen that in every culture type save for one there were still two distinct beats for the independent sections. However; in the sample infused with hMSCs there was restored synchronization showing that the hMSCs had repaired at least in part the conduction gap in five of the eight samples. [15]

The reparative capabilities of hMSCs is not only limited to the restoration of a current gap but can be induced to help restore the global heart function after a myocardial infarct which is of coarse the goal. In a study done with New Zealand rabbits thirty four individuals were randomly divided into two infarct groups; one would receive a cell treatment of hMSCs cultured from the bone marrow of adult males, and the other would receive an equal volume on PBS solution. Following the transplantation of these two different cell types a four week observation stage was allotted to note any improvements or differences found in the two groups. During this observation period two of the cell treated animals died, while seven of the control animals died, showing a dramatic difference in myocardial restoration. [12]

This evidence suggests that the implanted MSC cells had begun to differentiate into cardio-myocytes in vivo. Since the goal of this MQP is to produce cardio-myocytes for implantation it is important that we understand the factors that may induce MSC differentiation into cardio-myocytes.

2.1.3. Cardio-myocyte Identification

Cardiac myocytes can be identified in different fashions and by different attributes. All of these attributes however; can be divided into two categories; the first set of traits indicative of cardiac myocyte cells is based on contractibility and morphology. The second set of traits to look for when identifying cardiac myocytes are specific markers such as alpha sarcomeric actinin and myosin heavy chain.

Cardiac cells contract in unison when in their native state, giving the appearance of a uniform beat in a healthy individual, thus contracting cells are often looked for when trying to make the determination whether experimental cells demonstrate cardiac like characteristics. Another trait seen in the morphology of native cardiac cells is the way the sarcomers a line giving a distinct look of thin lines across the cell. These sarcomers are native only to cardiac muscle tissue and are there for a great indicator of a cardiac lineage in experimental cell samples. [16] These well defined striations can be seen in the figure below.

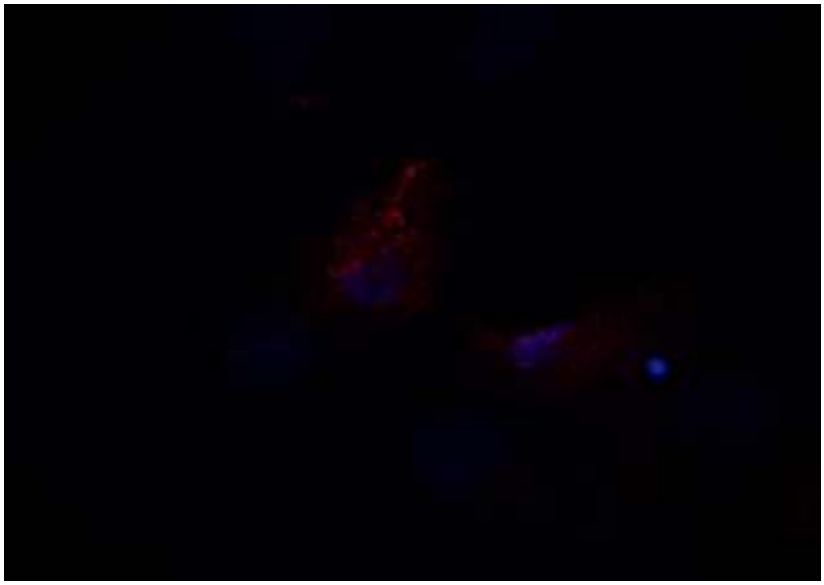

Figure 1: Microscopy of Native Heart Cardiac Myocyte

Finally a slightly more vague indicator of a cardiac lineage is the over all morphology of the cells. Cardiac cells generally take the shape of long striated rectangles. Though the morphology of these cells seems straight forward it can be confused with the spindle shape hMSCs sometimes take on. [17] An example of a native cardiac myocyte can be seen in the figure below.

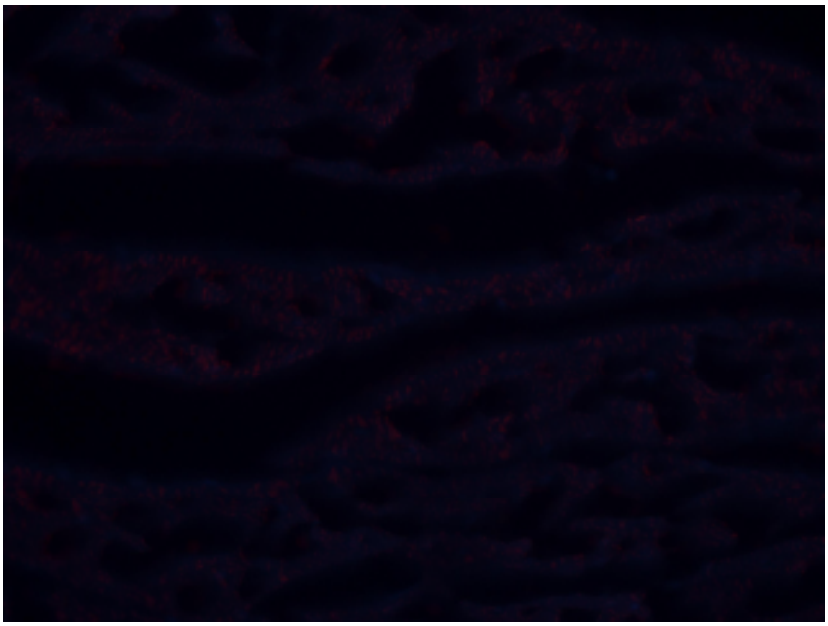

Figure 2: Native Healthy Cardiac Myocyte Tissue

The more precise strategy for determining the lineage of an hMSC derived cell is to use chemical markers. The two types of markers most commonly tested for are alpha sarcomeric actinin and myosin heavy chain. Alpha sarcomeric actinin can be stained for using quantum dots and shows definitively whether or not a cell is expressing the alpha sarcomeric actinin a definitive marker for cardiac myocytes. Myosin heavy chain is another distinct marker for cardiac myocytes used for contraction and is there in another marker for testing the lineage of experimental cell samples. [6]

Another less used strategy for the identification of cardiac myocytes is there distinctive electrical current. Cardiac myocytes have a unique electrical impulse that is designed to use ionic channels and intracellular Ca2+ signaling in pacemaker cells to ensure the proper setting of pacemaking in the native healthy heart tissue. This same cycle of Ca++ can be measured in experimental sample cells and can indicate a cardiac lineage from the electron gradient. [18, 19]

2.1.4. MSC Differentiation In Vitro

Although the mechanisms behind MSC differentiation are not yet fully understood, studies have identified a variety of methods that may promote MSC differentiation into cardio-myocytes. A common method is to use chemical and protein additive in order to induce stem cell differentiation. Limited success has been associated with the addition of chemicals such as 5-azacytidine (5-aza-C) and dimethyl sulfide (DMSO). [20] Although these chemicals may induce differentiation of stem cells down specific cell lineages, they are not ideal for use in this MQP. Use of DNA demethylating agents such as 5-aza-C and

DMSO are not ideal for clinical application due to their epigenetic properties. The effects of using a demethalizing agent will also make FDA clearance a much more complicated process. In cases of tissue or cell implantation it is important to limit the factors that may lead to cells behaving in an irregular fashion. DMSO and other epigenetic chemicals may lead to complications when treated tissue is implanted. An alternative choice is to use additives which may signal differentiation into cardio-myocytes rather than altering the DNA to induce them.

2.1.4.1. Platelet Derived Growth Factor

Platelet derived growth factors (PDGF) stimulates cell growth and proliferation in a variety of cells. [21] PDGF is a dimeric molecule consisting of disulfide-bonded polypeptide chains; and can exist in either a homodimeric states (AA) and (BB) or a heterodimeric state (AB). [21] It is known that PDGF-B is expressed heavily in the capillary and arterial tissue during embryonic development and for a short time post natal but then down regulated as the tissue "matures". This suggests that the presence of PDGF can potential play a role in the differentiation of embryonic cells. The structure of the protein PDGF-B has been crystallized for use in experimentation. [22]

In addition cardiac myocyte like cells have been shown to up regulate the expression of PDGF-A, and PDGF-B when cultured simply form bone marrow cells (BMC's). The cells expressed traditional expected markers for cardiac cells including troponin T, alpha and beta myocin heavy chain, and connexin 43. In addition to showing the up regulation of PDGF in these cells the experiments also concluded that the supplementation of cultured media with PDGF shortened the time to alpha myocin heavy chain expression by half. [23] The expression of this cardiac cell marker is shown a full week early when in the presence of PDGF. [24]

It has been seen that the different components of platelet derived growth factors work together to influence human mesenchymal stem cells into proliferation. [24] Treatment of MIs with PDGF has shown signs of improving heart function by reducing the size of the infarct overtime [25]; suggesting that PDGF plays a role in reparative functions in the heart. In addition, the injection of PDGF into infarcted region of the heart produced no toxicity in the surrounding tissue. [26] Genetic "knock-outs" for PDGF receptors in mice have shown that the PDGF family is necessary for normal embryonic development. [25] "Knock-out" mice either died during development or suffered from developmental abnormalities. In addition to driving mesenchymal proliferation, PDGF has been shown to direct the migration, differentiation and function of a variety of specialized mesenchymal and migratory cell types, both during development and in adults. [27] PDGFs general role in tissue repair and cell

differentiation makes it a perfect candidate for studying its effect on MSC differentiation into cardio-myocytes.

Various culturing method have also been used to induce cells to differentiate into cardio-myocytes. Co-cultures of cardio-myocytes and MSC have shown increased differentiation of MSC into myogenic cells. [28] Co-culturing of cardiac tissue and MSC closely resembles the conditions found when MSC are implanted into the heart. Co-culture studies examine the role of cell to cell interaction in MSC differentiation into cardio-myocytes. Direct co-culturing may induce MSC differentiation through direct cell to cell contact. [28] The conditions produced by the proximity of MSC cells and cardio-myocytes during wound repair may be crucial to the reparative functions of MSCs. It is unknown whether it is the cell to cell contact that induces MSC differentiation or if signaling molecules (independent from cell contact) are responsible. The problem with co-cultures is that it is difficult to identify the mechanism behind its function. A multitude of extracellular signaling molecules may be produced by cells during normal cellular activities. Identification of the specific molecules that direct MSC fate may be difficult, if not impossible, though quantitative analysis due to the blanket effect of co-cultures. Regardless, it is important to keep in mind that cell to cell interaction may play a significant role in cardio-myocyte differentiation from MSCs.

2.1.5. Embryoid Bodies

Yet another method that may increase stem cell differentiation into cardio-myocytes is the development of embryoid bodies (EB). Embryoid bodies derived from ES cells are an in vitro model of early embryogenesis and are often used to study ES cell differentiation in vitro. Under certain environmental conditions individual ES cells will conglomerate and form a spherical body resembling the intercellular mass of a developing embryo. Upon formation, the EB will begin to spontaneously differentiate into a variety of cell lineages. [29] In addition ES cells within an EB tend to differentiate into more advanced stages of embryogenesis, including cells such as cardio-myocytes. [29] As mentioned earlier in the paper, Kehat et al demonstrated the ability to derive cardio-myocytes from ES cells when cultured to form EBs. RT-PCR analysis showed an increase in cardiac specific markers such as Myosin Heavy Chain (MHC), Myosin Light Chain (MLC), and Cardiac Troponin T. [3] There was also a noticeable drop in OCT-4 levels in comparison to undifferentiated ES cells. Oct- 4 is a transcription factor which plays a role in maintenance of cell pluripotency [30], thus a decrease would indicate that the ES cells are losing their pluripotent capabilities due to cell differentiation.

Mesenchymal stem cells have also displayed the capabilities of EB formation under certain conditions, in addition the capacity to spontaneously differentiate into variety of cell lineages. EB formation may provide a preparatory step before the differentiation of stem

cells. During EB formation transcription factors like Oct-4 are increased until about the forth day after the initial formation. An increase in Oct-4 may suggest that the MSCs may be able to somewhat dedifferentiate, obtaining the more pluripotent capabilities of embryonic stem cells. Not much research has been performed on the EB differentiation into cardio-myocytes through the use of MSCs, however the EB formation step may prove to be a useful method of increasing cardio-myocyte differentiation in conjunction with culture additives such as PDGF.

2.2. Engineering Background

2.2.1. Tissue Engineering

Tissue engineering is an interdisciplinary integration of technique and principles from engineering with the field life sciences in order to develop and produce engineered tissue to replace, regenerate, or improve the function of natural tissue; or as Langer and Vacanti have previously defined, tissue engineering is “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function”. [31] This definition, however, is too broad and lacks substance as it pertains to our project. The expansive field of tissue engineering encompasses the formation of many tissue types, induced by many stimuli, using different cell types, and created under a variety of conditions. A newer term, regenerative medicine, synonymous with tissue engineering reveals more about the intention of our project and is a step forward in the clarification of the specificity of our project. To clearly define our project, principles from regenerative medicine and tissue engineering will be used to manipulate pluripotent stem cells; in this case adult hMSCs, through differentiation and proliferation to repair, replace, or improve the function of natural native tissue of the heart.

The differentiation and proliferation of pluripotent stems cells is primarily based in principles and techniques that fall within the field of life sciences. However, engineering plays a crucial role in conjunction with life science to develop innovative uses of these principles and the optimization of these techniques. One key aspect is the necessity of mimicking in vivo conditions in an in vitro setting to not only maintain normal cellular activities while cells differentiate and proliferate, but also provide a predictable environment for analysis. This environment must maintain different variables based on specific needs. To mimic the in vivo environment it must be able to control and monitor operating variables such as pH, temperature, pressure, nutrient supply, and waste removal. [32] Applications of this technology in commercial uses are tissue engineering bioreactors, industrial fermentation processing, wastewater treatment, food processing, manufacturing of biopharmaceuticals and recombinant proteins (e.g. antibodies, growth factors, vaccines and antibiotics). [32] In

normal cell culture under laboratory conditions this is done through the use of an incubator and common cell culture techniques. By providing a comprehensive level of monitoring and control over specific environmental factors in cell cultures, controlled studies can be preformed to better understand what specific chemical, physical, or biological variables effect which function in engineering a defined tissue. [32]

Bioreactors play a large role in controlling and monitoring environmental factors for the engineering of tissues in three dimensional culture systems. As defined by Heberer et al. bioreactors are ‘‘devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g., pH, temperature, pressure, nutrient supply, and waste removal)’’. [32] It is these bioreactor designs that allow for the control necessary for the optimization and scalability of proliferation and differentiation in tissue engineering. Each design is different in the way it controls, monitors, and mimics the in vivo environment, which can be accomplished in a variety of ways, from perfusion pumps to dynamic stirred or rotational vessels to static cultures. [33]

The functions and principles of bioreactors, more than the specific designs, will be the focus for controlling and monitoring the environment to optimize the proliferation and differentiation of cardiogenic hMSCs. A high degree of controllability will be essential to determine effects of specific stimuli on the differentiation and proliferation of hMSCs. This requires a flexible system that tightly maintains conditions that mimic the in vivo environment. Also, once a method for the creation of cardiogenic cells from hMSCs has been developed for use in a controlled system, it will be necessary to prove the application in not only a laboratory but also for commercial mass production. The culture system should allow for automated steps in the process, which is essential for not only controlled, reproducible, statistically relevant studies but also for the control and reproducibility that are crucial in commercial manufacturing for clinical applications. [34]

2.2.2. Current Embryoid Body Formation

The first step to control the differentiation of hMSCs in cardiogenic cell and also their proliferation is the formation of embryoid bodies. For the de-differentiate of hMSCs it has been reported that they need to be cultured in such as way that they form cell aggregates, known as embryoid bodies. [35] A high degree of controllability and reproducibility is necessary to optimize this crucial step in the process of differentiating hMSCs into cardiogenic cells. This can be accomplished by two directly related means; the first is through biological additives, which are then incorporated into the second means, a controllable culture system.

There are different techniques used to produce embryoid bodies in an array of different culture systems. These culture systems are commonly used for the formation of EBs using ESCs, not hMSCs. However, cell aggregation, which leads to EB formation, is a physical characteristic of the culture method and will produce similar results with different cell types. EB culture is usually performed either in liquid suspension, hanging drop, or semi-solid methylcellulose culture. Both the hanging drop and methylcellulose culture techniques have been developed in an effort allow the formation of EBs while limiting aggregation events. [36] Agglomeration of cell aggregates in EB formation has been reported by researchers as adversely affecting the efficiency of the EB formation and the cellular yield as a function of initial cell seeding. [37, 38] This becomes a problem in culture systems were cell aggregates are not separated during the first four days, the initial period of EB formation. [38] When ES cells are seeded into a semi-solid methylcellulose culture they tend to remain stationary and develop EBs from single cells, preventing the agglomeration of cell aggregates. However, it is can be very difficult transfer EBs encased in the semisolid Methylcellulose (MC) matrix with pipettes, and it is also possible for the MC matrix to prevent mass transfer. [39] For the HD culture, different cell seedings are applied to the lid of culture dish as a droplet of medium and cells with a volume of 20–50 ul. The lid is turned over and the droplets of ES cells hang from the lid due the surface tension. The cells are held in suspension and because there is no place for cell adhesion and the round structure of the droplet, there is the aggregation of cells at the bottom of the droplet and agglomeration is prevented. [39] However, the hanging droplets are very unstable on the lid and even under controlled conditions there is a high chance of disturbing or loosing the hanging droplets and the EBs they contain. In this culture method it is difficult to observe the different stages of EB formation and transfer of the EBs via pipettes is difficult and sloppy. [39] Although these techniques provide relatively good environments for EB formation, do to logistical problems and limited expansion potential for larger production; other culture systems for EB formation have been studied by researchers.

Research in this field of study has taken on two different aspects, static culturing and dynamic culturing of ES cells into EBs. The dynamic culturing has shown promise as a technology that can more readily incorporate bioreactor principles of mass transfer, control, and automation. These systems fall into two categories, stirring vessels and rotational vessels. The first of these technologies, stirring vessels, has been used more frequently in large-scale production of cells due to it high success rate in mass transfer. However, the use of this system alone does not address the problem associated with agglomeration and produces large cell clumps instead of uniform EBs. [40] Recent studies have shown promise in using polymers to micro encapsulate cell aggregates in conjunction with stirring vessels to prevent agglomeration. Dang et al. reported that micro-encapsulation of ES cells in agarose

hydrogel permitted EB formation but prevented agglomeration. The study showed that if cell aggregates are separated during the first four days of formation, a period where E-cadherin cell-adhesion molecule expression is high, and then released from the encapsulation, the EBs did not fuse together to form larger EBs. [38] This study found that cell expansion was much higher when cell aggregates where not allowed to agglomerate providing more evidence of increased efficiency from separated cell aggregates during EB formation.

The second category of devices used for EB formation in dynamic culture is rotating vessels. These devices can be broken down into two similar methods or designs, slow turning lateral vessel (STLV) and high aspect rotating vessel (HARV). Both systems rely on the creation of microgravity, or constant free-fall, through rotation of the device to suspend cells a medium and form EBs. The difference in the two designs is in the structures used in the creation of microgravity and their results. The STLV was very successful at creating small EBs of homogeneous size with little or no necrosis in the center. Comparison with static Petri dish culture showed that after 7 days post seeding of hESCs, the STLV revealed nearly 4 times more EBs in than the static cultures. [40] Unlike the STLV the HARV did not effectively produce EBs but instead produce large clumps of cells, which showed more agglomeration of cell aggregates than the static culture. [40]

The two dynamic methods studied that have shown success incorporating bioreactor functions such as efficient mass transfer, reproducibility, control, and automation into production of EBs are the STRV and micro-encapsulation in conjunction with a stirred vessel. These systems have shown potential in producing controlled and reproducible EBs in large quantities, however, they lack in other areas. They are problematic due to their high degree of complexity, increased potential of failure due to the addition of complex variables, that transfer of EBs to adhesion environments, and constant monitoring of their dynamic nature due to increase in cell densities. To avoid these pitfalls while still retaining the efficiency in EB formation, researchers have looked into static systems that rely on non-adhesion and separation of cell aggregates for the formation of EBs.

2.2.3. Potential Future Techniques for Embryoid Body Formation

2.2.3.1. Non-Adhesive Environments for Embryoid Body Formation

Non-adhesive materials prevent cell adhesion by surface interactions with cell proteins. Surfaces can interact with cells by one of three methods, either by blocking protein adsorption and receptor-mediated cell adhesion by either their extreme hydrophilic or hydrophobic nature or by allowing adsorption of cell adhesion-mediating proteins, but causing these proteins to become very rigid and reorganization-resistant, which makes it so that their specific amino acid sequences are not accessible for integrin receptors on cells.

[41] It has been shown in previous studies that in a non-adhesion environment, such as suspension in a hang droplet, in a semi-solid gel, or in a rotating vessel, cells can aggregate and undergo EB formation. [37, 38, 40] This idea of a non-adhesive environment can then be applied to materials that don't allow for cellular adhesion such as polymer plastics, hydrogels, and natural materials. Surfaces that prevent cell adhesion have been produced using various natural and synthetic molecules. Some molecules discussed by Bacakoval et al. in a review paper on the principles behind cell adhesion that are classified as preventing cell adhesion are the anti-adhesive protein albumin, hydrogels based on hyaluronic acid or poly(hydroxyl ethyl methacrylate), polyvinyl alcohol (PVA), polyacryl-amide, dextran, poly(ethylene glycol) (PEG), and poly(ethylene oxide) (PEO). [41] The non-adhesive materials can then be shaped to form structures that mimic physical constraints that have been reported to promote cell aggregates, a prime example being a hanging water droplet. The application of these two properties, non-adhesion and physical constriction, can then be used to produce structures that efficiently prevent agglomeration and effectively promote the formation of uniform, homogeneous EBs.

The use of non-adhesive materials to form embryoid bodies from embryonic stem cells through the creation of well structures is a technique that has been previously studied by other researchers. [39, 42] Embryoid body formation of ES cells is very similar to the EB formation of hMSCs, both rely on the same physical constraints and it can be assumed that structures that promote ES cell aggregation will also promote the aggregation of hMSCs. Studies have been conducted on two aspects of this culture technique, the creation of wells out of a non-adhesive material and a well structure that is then coated with a non-adhesive material.

2.2.3.2. Non-Adhesive Polymer Machined Well Structures

Many materials, usually polymers, naturally prevent cell adhesion and need to be treated in order to promote attachment. Many polymers also have good characteristics in addition to non-adhesive properties that make them well suited for a cell culture environment. An excellent example of a polymer that can be machined to produce wells is polyoxymethylene (POM). The performance of POM, a non-adhesive polymer thermoplastic, was studied and shown to have excellent qualities as a component in a cell culture system or bioreactor for hMSCs. POM has physical and mechanical properties that make it an excellent candidate for the creation of cell culture wells used to produce EBs from hMSCs. As reported by Penick K. J et al., POM is an exceptional material to prototype and fabricate small laboratory apparatus or for use in a cell culture environment or more specifically a bioreactor. [43] White POM has negligible porosity and low moisture absorption around about 0.9% at saturation, which means that it is naturally hydrophobic and will not promote

cell adhesion. [43] A study performed by Penick K. J et al. reported no cell attachment to the POM polymer and no adverse effects to cellular proliferation and differentiation of hMSCs. [43]

This material has an exceptional balance of machining characteristics, tensile properties (76 MPa ultimate at 23°C), stiffness, shear strength, toughness, and thermal properties with a melting point of 175°C. A high melting point means that it can be withstand autoclave temperatures, and in this case POM also retains its shape and physical integrity at elevated temperatures. [43] POM can be machined; milling for the creation of wells and after drilling and tapping it holds the machined threads well. It is reasonably wear-resistant, has good impact strength, low friction, is a good electrical insulator, dimensionally stable, and chemically resistant to solvents. It also has a good history as being used as a biocompatible material as a component of tilting disk cardiac valve prostheses [44, 45] and as an orthopedic implant material. [46] It has also been deemed safe for food contact use by the FDA. These are all qualities and characteristics a suitable material should posses if it is to be used in a cell culture system structured to promote the formation of EBs. The material should be able to be sterilized, should be biocompatible, should have surface properties that don't promote cell attachment, and should have physical and chemical properties that allow for the creation of different structured wells.

2.2.3.3. Non-Adhesive Polymer Coated Well Structures

A study performed by Koike M et al. researched coating a well structure with a non-adhesive polymer, another method for producing wells that promote EB formation. This study compared the use of a 2-methacryloyloxyethyl phosphorylcholine (MPC) phospholipid biocompatible polymer and normal cell culture polymers in the formation of embryoid bodies from ES cells. This study determined well shape and cell adhesion as a function of EB formation. Three types of plates, round bottom (U-bottom) polystyrene (PS) 96-well plates, flat-bottom 96-well polystyrene plates, and polypropylene (PP) plates, were used to test the different curvatures on EB formation. To study cell adhesion as a function of EB formation a round bottom PS 96-well plate was coated with MPC and a flat-bottom 96-well PS plate was coated with MPC, which were then compared to the other plates used in the study. [39]

In the MPC coated plates cell aggregates were formed on the first day. Then the size of the cell aggregates slowly decreased until the 5 day when EBs were about 550 um in diameter and contained approx. 22,000 cells on average. In the uncoated round bottom PS 96-well plate, uncoated flat bottom PS plate, and the PP plate there were little or no cell aggregates and resulted in no formation of embryoid bodies. [39] On the non-adhesive polymer coated plate there was a formation of cell aggregates and this lead to EB formation. In the cases where the cells were allowed to attach there was no formation of EBs. This

reinforces the idea that EB formation is inversely related to cell attachment, which shows that EB formation can be mediated by non-adhesive polymers.

Koike M et al. also showed that the efficiency of EB formation is related to surface shape of the non-adhesive material. In the round bottomed PS 96-well plates coated with MPC there was generally the formation of one EB per well. However, in the flat bottomed PS 96-well plated coated with MPC there were multiple EB with heterogeneous sizes formed in each well. [39] These observations have also been reported by Kurosawa H et al. who used a conical shaped tube too form cell aggregates and then EBs of out mouse embryonic stem cells. This data seems to show that if cells can be concentrated in a small area, due to a curved surface around a center point such as a hanging water droplet and gravities pull, the cells will then signal each other to form one EB. When they are allowed to spread out over a wide flat area they tend to form multiple EB of varying size. This phenomenon can also be seen in static cell suspension culture systems were cells are allowed to spread out over a large flat area and have shown that they form a very heterogeneous diameter size population, as reported by Dang et al. [37]

Another important observation to come out of this study was that the attachment potential, necrotic center growth, and EB cell density growth was effected depending on the cell density used in each well to start the EB. Three different cell densities were used to start the EBs, 4000 cells, 1000 cells, and 200 cells. [39] Dang et al. reported that there is a maximum on the number of cells in a single EB, which is around 28,000±9000 cells. [37] Out of these three densities the 4000-EB experienced the maximum cell number at the earliest time, 3 days, but it also showed the largest necrotic centers due to the failure of mass transfer. Both the 1000-EB and the 200 EB didn't reach the maximum cell limit for an EB as purposed by Dang et al. and reach their peak cell number at the later time than the 4000-EB. However, they didn't experience as much trouble with mass transfer and necrosis at their centers. [39] The most shocking observations was that the larger 4000-EB and 1000-EB weren't able to generate cardio-myocytes as efficiently as the 200-EB because when place on a adhesive surface for attachment they weren't able to spread out as far as the small EB. The 200-EB was completely flattened on the attachment plate much faster than the 1000-EB and the 4000-EB never completely flattened out. [39] These observations have clear implications for hMSC development into cardiogenic cells, which also need to form EBs in a non-adhesive environment and then transferred to an adhesive environment once the EBs are formed. This shows that production efficiency can't just be increased by putting more cells into the EB or growing EBs as large as possible. Further experimentation is necessary to determine the appropriate EB diameter and cell number.

Another polymer used as a non adhesive coating for culture systems is Poly (N-isopropylacrylamide (PIPAAm). The more common use of this material is in areas like drug

delivery tissue engineering, but some research has been conducted on growing tissues on PIPAAm coated tissue culture dishes with controlled cell adhesion and detachment with no cell adhesion. [47] Thermo-sensitive polymers gained a lot of interest in the field of biomedical engineering in the past decade. PIPAAm is a thermo-responsive polymer and its most characteristic property is that the surface property of the polymer can be changed by the change of temperature from hydrophobic to hydrophilic. [47]

PIPAAm has a low critical solution temperature (LCST) at 32°C, meaning that at temperatures below 32°C, PIPAAm molecules are highly hydrated, thus PIPAAm-grafted surfaces are hydrophilic. Above 32°C, extensive PIPAAm dehydration occurs, resulting in a transition to hydrophobic surfaces. [48, 49] This change is fully reversible with temperature. The hydrophilic/hydrophobic properties of this polymer can be used to form EBs if the material surface can be modified for non cell-attachment.

In a study by Akiyama et al., researches grafted ultra-thin poly(N-isopropylacrylamide) layers on to tissue culture polystyrene surfaces (TCPS) to investigate cell adhesion and detachment control of endothelial cells. The study examined the relationship of the different thicknesses of PIPAAm-grafted layers on TCPS and the temperature-dependant cellular behavior in terms of cell adhesion with respect to the varying PIPAAm thickness. [47]

To polymerize and graft PIPAAm onto the TCPS, researchers used uniform electron beam irradiation polymerization immediately after spreading a homogenous layer of PIPAAm and rinsed the polymerization. Electron beam irradiation polymerization is essential for the design of the nanometer-thick layers. Findings of the total reflection Fourier Transform Infrared Spectroscopy (FTIR) revealed that amounts of the grafted polymers were 1.4±0.1 $\mu g/cm^2$ (n=4) and 2.9±0.1 (n=4) $\mu g/cm^2$, thus abbreviate as PIPAAm-1.4 and PIPAAm-2.9 respectively. Researchers analyzed these two thicknesses at temperatures of 37°C and 20°C, and they altered the surface wettability of PIPAAm-1.4 and -2.9 with temperature. PIPAAm-1.4 surfaces were more hydrophobic ($\cos \theta = 0.21$ at 37°C and $\cos \theta = 0.35$ at 20°C) than PIPAAm-2.9 ($\cos \theta = 0.42$ at 37°C and $\cos \theta = 0.50$ at 20°C) [47]. In conclusion, the Endothelial Cells adhered to the thinner surface of PIPAAm-1.4, whereas researchers observed no cell adhesion with PIPAAm-2.9. Therefore, the thicker the PIPAAm graft, the less cells will adhere to the surface at 37°C. No cell adhesion occurs because the outermost regions of the PIPAAm-2.9 layers are hydrated more than PIPAAm-1.4, so cells do not attach to the surface of the PIPAAm-2.9 layered dish. Cells more easily adhere to less hydrated grafts. [47]

There are similar studies done investigating the cell adhesion and detachment as well as swelling of the PIPAAm hydrogels. However, no research has been done on forming EBs using PIPAAm so far. The properties of this thermo-sensitive polymer give it the potential of being used in the formation of EBs. The differentiation of cardiogenic cells and

the formation of EBs may be combined into a single step device capable of acting like a non-adhesive environment in one instant and then an adhesive environment under other circumstances. A microwell coated with PIPAAm may remove the process of transporting EBs from the different environments thus adding more efficiency to the overall production of cardiogenic cells from hMSCs.

This technique is embodied in a technology know as cell sheet engineering and is a potential aspect that can be added to our project. After forming homogeneous spherical EBs, the next step is to transfer cardiogenic EBs onto an adhesive surface to grow cardio-myocytes. This section will focus mainly on the transfer of EBs from a non-adhesive environment to an adhesive environment without losing any of the formed EBs. The transfer phase will be considered once the formed EBs have proven to express cardiogenic markers.

To produce cardio-myocytes, the formed EBs from hMSCs are plated and grown in an adhesive environment. Conventionally, proteolytic enzymes such as Trypsin have been used in the detachment of adhered cells. Trypsin degrades the cell adhesion molecules, and it breaks the protein bonds between the cells and also between cells and the surface of the grown environment. [50] The disadvantage with trypsin is that it might degrade highly differentiated cell functions irreversibly. [21] A solution involving non-invasive cell detachment is possible using the previously mentioned thermosensitive polymer poly(N-Isopropylacrylamide) (PIPAAm). This thermosensitive polymer has a low critical solution temperature (LCST) at 32°C; therefore, at 37°C, the surface is highly hydrophobic, allowing the various cell types to adhere, spread, and proliferate at 37°C. [52] The cell monolayer can be detached completely by spontaneously reducing the temperature below 32°C, without the use of trypsin. [52] By keeping the cells in a monolayer and preventing the exposure to trypsin, the cell sheet can be transferred and reattached on another surface promoting proliferation. [51] This technique using PIPAAm, where the cells are non-invasively attached and de-attached is called cell sheet engineering. [51, 52] To mimic the thick heart tissue, several harvested monolayers of myocardial cell sheets can be layered on top of each other. [52]

The thermo-sensitive property of PIPAAm and its ability to change hydrophobic/hydrophilic properties will be important for myocardial regeneration using hMSCs. Maintaining and transferring cardio-myocytes in a monolayer/multi-layer without interrupting the integrity and cell-cell interactions can be done with PIPAAm. Once the EB formation process is successful, cell sheet engineering should be investigated closely.

2.2.3.4. Electrospun Scaffolds in Tissue Engineering Applications

First patented in 1934, electrospinning is a century-old polymer processing technique that started to gain importance in the field of tissue engineering as a scaffold fabrication tool

[US patent no. 1,975,504; 1934. The most advantageous property of the electrospun scaffold is that it is composed of randomly aligned nanofibers ranging 10-2000nm in diameter. [53] The created nanofibers can be used in the application of filtration, drug delivery, separation membranes, medical devices and tissue engineering. [53] We are considering electrospun nanofibers to form EBs. The created cell and protein-resistant polymer mesh would increase the EB production because EBs would not be lost during the process of media change; this is possible by draining the media from through the polymer mesh.

In comparison to phase separation and template synthesis, electrospinning is a flexible and simple procedure to create nanofibers. [54] It is flexible because almost any polymer can be used to fabricate continuous nanofibers and the equipment needed are only a high voltage power supply, a spinneret with a metallic needle, a syringe pump, a grounded collector, and a copper plate. The formation of nanofibres through electrospinning is based on the uniaxial stretching of a viscoelastic solution. [54]

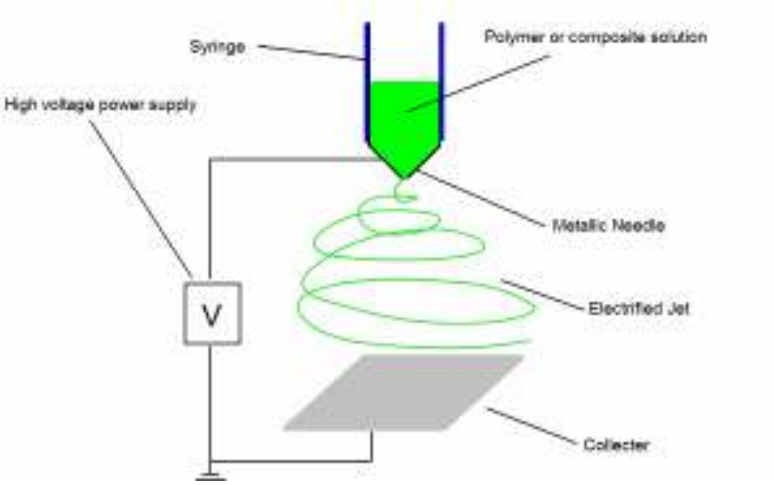

Figure 3: Schematic of an electrospinning setup.

The electrospinning process starts with the desired polymer loaded into the spinneret, and a syringe pump pushes the polymer solution to the tip of the metal needle. A high voltage is applied between the solution depot and the collector unit, where the voltage can be up to 30kV. [53] The positively-charged polymer creates a jet stream and is drawn to the ground collecting plate, which is usually a metal (copper) screen, plate, or a rod located 5 to 30 centimeters away. As the jet travels, electrostatic repulsion stretches the fibers and divides them into many more fibers, and the solvent of the solution evaporates, which leaves the charged polymer on the grounded collector. [54] A nanometer-scale polymer can be

produced as long as there is a flow of polymer from the solution depot. The created fibers can range from 50 nanometers to 10 microns. [53]

Several parameters control the resulting electrospun material. One parameter is named system parameters, according to Ramakrishna et.al [53], which includes the polymer and solution properties such as molecular weight, polymer composition, viscosity of the polymer, conductivity, and surface tension. Other parameters are processing parameters, including the electric potential, flow rate and concentration, distance between the solution depot and the grounded collector, temperature, humidity, air velocity and motion of the collector. All of these variables may affect the morphology, fiber diameter, and pore size of the nanofibers. [55] Mixing solvents also may create smaller diameters and narrower distributions of nanofibers. [55] Some of the solvents commonly used are ethanol, dichloromethane, water and DMF (N,N-dimethylformamide). [55] The electrospun material needs to be sterilized before the clinical use. The sterilization technique is related closely to the polymer used. Some sterilization techniques used are autoclave (heat sterilization), bleach, UV-ozone and ethylene oxide (EO) gas sterilization.

Electrospinning is a flexible technique to create nanofibers because almost any material can be used , even natural polymers, such as collagen Type I (mesenchymal stem cells), gelatin, fibrinogen and hyaluronic acid (synovial fluid, cartilage and dermis). Synthetic materials like PCL, PLA, PGA, et cetera, are being used more commonly than natural polymers to avoid the limitations of natural polymers. [53] The electrospun materials can be used in regeneration applications involving blood vessels, skin, cartilage, and bone. In research on bone regeneration, mesenchymal stem cells have been used by Yoshimoto et al [53] on a PCL scaffold.

Even though the goal of most current research groups in the field of tissue engineering is to improve cell attachment and biocompatibility, our goal is to create nanofibers that are cell and protein resistant by forming EBs in the electrospun meshes. There has been a series of polymers reported that inhibit cell adhesion, such as Polystyrene PS, Polypropylene (PP), poly(trimethylene terephthalate) (PTT), and Polyethylene terephthalate (PET). These materials are known to be hydrophobic and are cell and protein resistant. In current research, Polystyrene has been used to create nanofibers that have superhydrophobic surfaces. A surface is considered superhydrophobic when the contact angle of the hydrophobicity measured is greater than 150°. [56] Surface properties are varied, by increasing the solution concentration and decreasing the extrusion rate of the polystyrene, to create superhydrophobic nanofibers with a uniform fiber diameter of 140nm when the polymer was added solvents like N,Ndimethyl formamide (DMF) or tetrahydrofuran (THF). The contact angles ranged from 143 to 153 for different solvent concentrations. [56]

Once nanofibers are formed with polymers that have cell and protein resistant properties, our hypothesis is that the hanging-drop method can be mimicked to form EBs by creating well shapes with the curvature of the hanging droplets. Once the well shapes are created, the droplet with 15,000 cells and the medium can be placed into the bottom of the well. There are two possible ways to produce the well shapes into the electrospun material. The first way is to apply the predetermined curvature on a copper plate that will be used as the ground collector plate and deposit the nanofibers on top of the dents, creating the well structure, once pealed off the copper plate, which functions as the negative. The second production method considered is producing a non-adhesive electrospun sheet and heat-sink the curvature dents in to the material by heating the tip of the copper plate. The advantage of heat-sinking is that the created well shapes become more durable; however, heat-sinking would require a two-step process. In either design, a plastic will be used as a backbone with holes for the well shapes to fit through to support the electrospun sheet. might look like the water droplet in FIGURE 1.4 (B) where the hydrophobic polymer repels the medium and does not allow the cells to interact with the polymer. This mimicks the hanging-drop method by reducing the cell-surface contact result in in homogeneous spherical EB size and shape. The predetermined curvature of the hanging droplets will allow the cells to form aggregates similar to the hanging drop method.

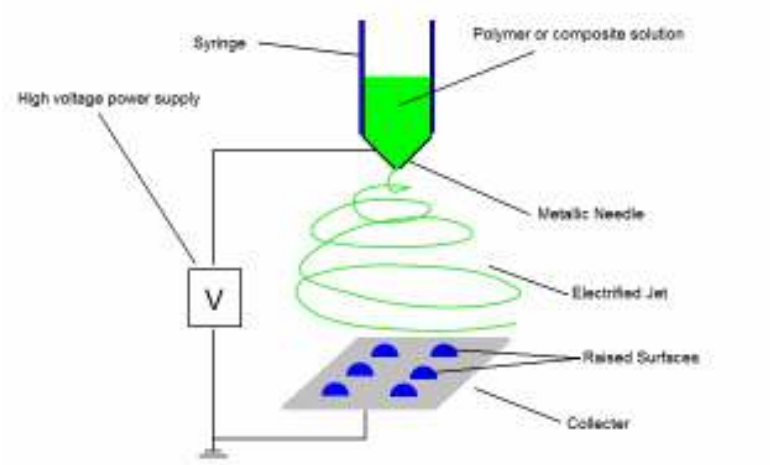

Figure 4: Process of Electrospinning to Create Wells.

The medium can be drained through the porous mesh that is created by the deposit of random of polymer nanofibers on the collector plate. Since the pore sizes can be adjusted by varying the solution/polymer parameters and the system parameter, a fresh medium can be used after applying suction from the outside of the well and draining the old medium. Since the pore sizes will be smaller than individual cells but big enough for the medium to flow through it, few or no cells will be lost. More importantly, more media can be

removed using the electrospun mesh with less time used and less risk of losing the EB, increasing the efficiency of the method. The elongated nanofibers produced by heat-sinking may improve the media suction. Adding a suction device for the electrospun material may increase the EB production while decreasing the time spent on an individual well during maintenance.

As a conclusion, electrospinning polymers that show cell and protein resistant characteristics are of great importance to the project. The non-adhesive polymers woven into porous meshes would increase the homogenous spherical EB production and reduce the total maintenance time as described above. The manufacturing process for the electrospun materials is easier than machining solid polymers.

2.2.3.5. Micro-wells Designed for Embryoid Formation

To mimic and control a cellular microenvironment, mircowells fabrications are on the rise. With microwell fabrication cell shape, proliferation and differentiation as well as cell-cell interactions can be controlled. There are different techniques to create microwells, such as soft lithography and photolithography. [57-59] Soft lithography is favorable due to its simplicity, cost effectiveness, and flexibility in material choice. Soft lithography techniques include microcontact printing and microfluidic patterning. [57, 58]

A recent study done by Mohr et al. describes a three-dimensional microwell system for long term hESC culture and homogenous sized and shaped embryoid formation using chemical and physical constraints via microcontact printing. [59] Microwells are created by forming poly(dimethyl)-siloxane (PDMS) stamps to shape the prepolymer polyurethane (PU). The created wells then are shielded with gold, and triethylene glycol terminated self-assembled monolayers are coated. [59] The most common non-adhesive material in the microwell fabrication is poly(ethylene glycol) PEG, also known as poly(ethylene oxide) PEG. [57, 60] This hydrophilic material resists protein absorption, resulting in its non-adhesive characteristics. PEG is used as a triblock copolymer and in interpenetrating polymeric networks (IPN) as well. [57] For cell adhesion in the bottom of the square 50 and 120μm deep wells, Matrigel was coated to form hESCs [59]. In this study, the microwells are used to control the shape and the size of the hESCs by preventing the expansion of the cells across multiple microwells, provided by triethylene glycol terminated alkanithiols. [59]

We could use PEG for our project as non-adhesive polymer, since it is widely used as the non-adhesive material in microwells. Designing microwells with different shapes and depths can be advantageous for the embryoid formation. Mimicking the shape of the hanging drops and shaping the PEG microwells accordingly could be beneficial for the EB formation.

2.3. Market Analysis

There is an excellent potential for engineered cardiogenic tissue in the tissue engineering market and for clinical use. Sadly, cardiovascular disease (CVD) has been the number one killer in the United States every year but 1918 with mortality rates of almost 2500 Americans each day or an average of 1 death every 35 seconds. [61] More specifically, it is reported from 1999–2002 that 13,200,000 American's have Coronary heart disease (CHD) and out of that 7,200,000 Americans experienced a myocardial infarction (MI), also know as an acute heart attack. CHD is the single largest killer of Americans, with a coronary event rate of 1 every 26 seconds and a mortality rate of about one death every minute. About 40% of the people who experience a coronary attack in a given year will die from it, causing 1 of every 5 deaths in the United States in 2003. [61] Data reported from a FHS study of the NHLBI estimates that 25% of men and 38% of women will die within 1 year after having an initial recognized MI. [61] These numbers represent an extremely large demand and need for cardiogenic tissues and the solutions that they offer. However, it is necessary to look at cardiac disease and the market demand for engineered cardiogenic tissue not only from incident and mortality statistics but also from a financial view to gage the lucrative potential of developing a regenerative medicine solution.

In conjunction with the statistics there is a huge market for cardiovascular treatments. As stated in the Heart Diseases and Stroke Statistics 2006 update by the American Heart Association, the total cost of coronary heart diseases is roughly 142.5 billion dollars. The estimated direct cost of myocardial infarction is about $75.2 billion dollars. [61]

2.3.1. Current Treatments

Currently there is a variety of medications, procedures and medical devices available to prevent and treat MI. The commonly prescribed cardiac medications are anticoagulants, antiplatelet agents, Angiotensin-Converting Enzyme (ACE) Inhibitors, beta blockers, calcium channel blockers, Digitalis Preparations and Statins. [61]

Anticoagulants, also known as blood thinners help prevent clotting in the blood vessels. Commonly prescribed anticoagulants are Dalteparin, Enoxaparin and Heparin. Beta-blockers are mainly used to prevent future heart attacks by lowering the blood pressure for patients who have had a heart attack. The decreased heart rate and cardiac output lowers the blood pressure and makes the heart beat with less force. [31] Calcium channel blockers aim the same thing, but the way that the blood pressure is controlled is by interrupting the calcium flow into the heart. Amlodipine, Diltiazem, Nifedipine and other medications are commonly prescribed. Vasodilators also known as nitrates are used when the patient is having chest pain (angina), but cannot tolerate ACE inhibitors. Digitalis

Preparations are used to relieve heart failure symptoms, especially when the patient isn't responding to ACE inhibitors and diuretics. Increases the force of the heart's contractions, which can be beneficial in heart failure. Lanoxin is prescribed as digitalis preparations, which are also known as Digoxin or Digitoxin. Statins are prescribed to lower LDL and raise HDL cholesterol and lower triglyceride levels. [61] The leading drug companies are Pfizer, AstraZeneca, GlaxoSmithKline and Bayer.

Generally performed cardiac procedures and surgeries are angioplasty, atherectomy, stent procedure, transmyocardial revascularization, bypass surgery, minimally invasive heart surgery, radiofrequency ablation, artificial heart valve surgery, heart transplant and cardiomyoplasty. Angioplasty is with 1,244,000 procedures the second most common performed procedure in the United States. [61] This small invasive procedure is performed by guiding a tiny balloon via catheters through blood vessels to open the blocked coronary arteries by inflating the balloon so that the blood flow of the heart increases. Stents, biocompatible wire meshes are implanted after angioplasty to improve blood flow to the heart muscle and prevent restenosis. A series of research is done on drug eluting stents that provide time and pH dependant drug release. One of the commercially available drug eluting stents is Taxus Express^2 by Boston Scientific, which elutes Paclitaxel. Another promising drug eluting stent commontly is sirolimus eluting Cypher by Cordis. These two companies are the biggest rivals in the 5 billion dollar world market. [62] Similar procedures to angioplasty are laser angioplasty and atherectomy, where the plaque is removed by laser beams and rotating shavers. The angioplasty procedure usually takes about 30 minutes to an hour and often requires an overnight stay at the hospital. A bypass surgery, also known as coronary artery bypass graft or open heart surgery is one of the most effective procedures to manage clogged arteries. The way this procedure works is by taking arteries or veins from other parts of your body –called grafts – and using them to reroute the blood around the clogged artery. [63] A patient may undergo as many surgeries as needed. [61] In 2003, 467,000 bypass surgeries with a mean cost of $83,919 have been recorded. [61] An alternative to the open heart surgery is the minimally invasive heart surgery. In the bypass surgery, the heart is stopped and the blood is pumped using oxygenators. This can be avoided by the minimally invasive heart surgery. Hospital stay of several days is necessary for both of the procedures. There is an in-hospital death rate of 2.2%. [61]

To aid the heart maintain or recover its regular function medical devices such as left ventricular assist devices, pacemakers, and implantable defibrillators are being used. Left ventricular assist devices are big chambers that pump the blood out to the body. Artificial pacemakers are implanted to help the heart beat in its regular rhythm by sending electrical pulses. Implantable defibrillators are used in patients with a risk of fibrillations. This device helps prevent sudden cardiac death by delivering electrical shocks to the heart. [61] In 2003,

an estimated 64,000 inpatient implantable defibrillators and 197,000 inpatient pacemaker procedures were performed in the United States. [61]

When the irreversible heart damages are too severe and the above mentioned procedures and devices are of no use, then a heart transplant is needed. This means that the diseased heart is replaced by the donated human heart, but the organ candidates list is very full due to high demand. In 2004, 2,016 heart transplantations were performed, but there are lots of other people waiting for a donated heart. The biggest problem with donated organs is the biocompatibility. There are certain criteria that the donor and the candidate need to have. Regenerative medicine reduces the problems of biocompatibility and the wait for the organ.

2.3.2. The Stem Cell Potential and Tissue Engineering

By mass producing cardiogenic stem cells that can proliferate we will be able to help patients with first time myocardial infarction, as well as patients that are under risk of a recurrent heart attack. Giving the patients under risk of death a second chance would expand the whole market size. [61] Implanting cardio-myocytes to gain heart function would decrease the number of other procedures and surgeries. The number of death due to cardiovascular diseases would decrease in general. Since the treatment cannot prevent cardiovascular diseases the size of the drug industry would not change. Drugs such as anticoagulants, blood pressure controllers will still need to be taken after the treatment to prevent any other possible myocardial infarctions. It is usually recommended to take aspirin, beta-blockers, ACE inhibitors and statins for the rest of your life. [62]

Implanting cardiogenic cells would be the one of the first procedures performed after a heart attack. This would decrease the need for more complicated procedures or surgeries currently used, but medical devices such as stents might still used to prevent recurrent blockage. By downsizing the surgeries invasiveness the hospitalization costs would go down. The treatment could be administered via catheters which would reduce surgery costs and shorten the hospital stay and thus lower the hospitalization cost. The after-care cost would decrease as well. The doctor cost and other follow up tests will always be necessary.

The potential market for engineered tissues for use in regenerative medicine is still in its infancy, however this doesn't mean that there aren't lucrative opportunities in the industry. A technical report composed by The European Science and Technology Observatory (ESTO) and the European Commission Joint Research Centre (DG JRC) Institute for Prospective Technological Studies reviewed the human tissue-engineered products and market as of 2003. Commercial tissue engineering is still in its initial phase, the first tissue-engineered product was just approved 10 years ago in 1996 for marketing in the USA. A total of 113 companies worldwide have been identified as being active in the industry of tissue

engineering and out of these companies 80 are biotechnology companies (71%), 24 are medical device companies (21%) and 9 belong to the pharmaceutical sector (8%). [61] The majority of the US companies are active in the area of structural applications, ex. skin, bone, heart valves, arteries, myocardial particles, which is still expanding. [31]

Three categories of tissue-engineered products, skin, cartilage, and bone, have emerged and have had commercial successes. Currently there are about two dozen tissue engineered skin substitutes on the market in Europe and the USA with a total estimated annual sales world-wide around 17 to 20 million. [61] Annual sales for tissue-engineered cartilage products are about twice that of skin and have been estimated around 40 million. Several companies are active in this field of tissue engineering, the most important being Genzyme Biosurgery, Fidia Advanced Biomaterials, Verigen, co.don, BioTissueTechnologies and TETEC AG. [61] The largest industry of the three is bone replacement and repair, which is estimated 300 million, but this includes autologous, allogeneic, xenogeneic and synthetic bone materials. The most interesting company in this market, Osteocel, is developing a product derived from human mesenchymal stem cells from bone marrow for promoting new bone formation. [61]

Even though the industry is very specialized and in its infancy, there is plenty room for growth. Market estimates for tissue-engineered products have shown good potential and have ranged from 80 billion for the USA alone to 400 billion worldwide with cumulative spending since 1990 exceeding 3.5 billion, increasing at about 16% per year. [31, 64, 65] Even moderate estimates have been reported as high as 3.9 billion by 2007. [61] These numbers combined with the evidence that several US companies are active in the field of producing cardiogenic tissue, some of which are Genzyme Biosurgery, Diacrin, BioHeart, Osiris, gives promise that there is a large demand for a efficient/optimized method for developing cardiogenic engineered tissue for regenerative medicine and clinical use.

2.3. Summary

Our goal to optimize the differentiation of hMSCs into cardio-myocytes is a two stage process, exhibiting concentrations in both engineering and biological technologies. The two stage process consists of the formation of EBs from hMSCs and then inducing their differentiation into cardiomyotes. A crucial step in this process is the formation of embryiod bodies, prior to induction of differentiation. It is our intention to form EBs from hMSCs through experimentation with various culture systems. In order to increase the yield of uniform EBs it is our intention to develop a system utilizing non-adhesive well technology. This technique offers itself as a potential method for efficient EB production in a controlled and reproducible fashion. In conjunction signaling molecules may be used at various stages

in order to facilitate this process. The results attained from these test with then be compared to those of current EB formation techniques; such as the hanging droplet method.

The second stage, consisting of the differentiation of EBs into caridiomyocytes, will be supplemented via cell signaling molecules in an adhesive environment. The purpose of the second stage is to effectively induce the formation of cariodmyocytes from EBs with a greater efficiency then previously seen. The use of co-culturing systems will also be comparatively studied for their effects on cell differentiation.

3. Project Approach

The completion of the background literature review was the first step in the initiation of the project. One a comprehensive understanding of the project was attained the next step was to begin clarifying and defining the project. This was accomplished by clarifying the initial problem statement, forming a project hypothesis, defining any assumptions, and defining the specific aims of the project.

3.1. Clarification of the Initial Problem Statement

After input from our client/advisor/customer and considerable research it was concluded that the basic problem statement and goal of our senior level design project was as follows: *"Manufacture a device/method to produce myocardial cells from HMSCs."* This problem statement, the start of our design process, was still very vague and really just a simplified description of the process we would undergo for the duration of the project. It gave us a place to start and a basic idea of where this project would take us, but left open a variety of options and lacked specific direction. Our first step in the design process would be to more clearly define this statement.

At this point we conducted our first set of design meetings with our entire design team, which was composed of two biomedical engineering majors and two biology/biotechnology majors. These preliminary meetings were designed to assess and clarify the basic problem statement we had been given. The first step in this process was to determine the current methods that were being used to produce myocardial cells from HMSCs. After researching the problem in current literature and discussions with our advisor pertaining to his current research, it was determined that there were a variety of methods currently under investigation that fit our basic definition of the project. However, we could not remain on the fence and the need for clarity and direction pushed us to decide on one method we would use for further research.

It was decided, based off of the current research being done by other investigators and discussions with our client/advisor, that we would use embryoid body (EB) formation as

the backbone for producing myocardial cells. Once this method was decided upon, research was conducted into the details of how this process worked. It was determined from a review of the current literature, that cardiogenic cells were created from this method by simply forming EBs in a non-adhesive environment, hanging droplet, and then plating these EBs on an adhesive environment, tissue-culture treated four chamber slide. It was also determined that biological agents, use by other investigators in different studies, could effect/direct the differentiation of progenitor stem cells, such as hMSCs, into cardiogenic cells.

From these findings, the basic statement we were given at the start of the project was further clarified with specific criteria. The clarified statement that came out of these discussions and research is as follows: *"Design and develop a scalable method to increase production of cardiogenic cells from hMSCs by optimizing embryoid body formation and utilizing PDGF treatment."* This statement embodies the basic statement we were given at the start project, but is modified to include the specific aims decided on from team discussions.

3.2. The Two Front Approach

It became evident from our literature research and these preliminary meetings that this problem statement could be broken down into two separate yet integrated problems. The first problem would be to optimize embryoid body formation, determined to be essential to the current method of producing cardiogenic cells from hMSCs. The second problem would be to increase the yield of cardiogenic cells contained within the embryoid bodies with PDGF treatment.

However, to define the problem statement as two different problems is not correct. The two different aspects of the problem statement are highly integrated and rely on mutual cooperation. Even so, to simplify the approach to the problem it was decided that the problem statement and the design process should be attacked on two fronts, engineering design and biological analysis. These two fronts would not be integrated battle front, instead of separate entities working alone to solve the same problem.

The design team was then separated into two functional groups that would use their separate background knowledge and expertise to attack the problem from different angles but yet work in harmony with each other. To accomplish this goal it was decided that team members, Tom Hayes and Cem Saracel, bioengineers, would compose the "engineering team", where as Gharam Han and Mike Riccio, biology/biotechnology majors, would compose the "biology team." These two teams would work separately on the design process, conducting separate meetings, but would come together as a whole to observe the big picture.

This process relied on a continual feedback loop and open communication between the engineering and biology team. The two teams would work separately on the two aspects of the project and then come together, to have an overall team meeting. During this meeting the teams would describe the process that each group had conducted on its own, and summarize any design criteria, decisions, or ideas that had been developed. It is during these meetings that the big picture of the project would be discussed and from these discussions the direction of the two teams would be decided for the following period of separate attack.

3.3. Project Hypothesis

The main goal of this project as defined by the clarification of the problem statement is to design and develop a scalable method to increase production of cardiogenic cells from hMSCs by optimizing embryoid body formation and utilizing PDGF treatment. The current method of producing cardiogenic cells from hMSC embryoid body formation is limited in both its scalability and cardiogenic yield. This limits the use of adult stem cell derived therapies in regenerative medicine for patients with heart disease.

It is hypothesized that the design of a scalable method to optimize the production of embryoid bodies from hMSCs will result in a higher yield and a more cost and time effective production embryoid bodies. It is also hypothesized the treatment of these EBs with PGDF will result in a higher yield of cardiogenic cells by directing the differentiation of cells within the EB down this lineage. It is then hypothesized that the combination of these two aspects, a scalable, optimized method of EB production and PGDF treatment, will result in a highly efficient production of cardiogenic cells that is more successful than the current method.

3.4. Project Assumptions

The main hypothesis of the project is that the combination of a scalable, optimized method for the production of EBs from hMSCs and PDGF treatment used to direct cell differentiation within the EB will result in a more efficient method of cardiogenic cell production compared to the current method. Therefore, the following assumptions were made:

- Embryoid body formation is the best method to produce cardiogenic cell from hMSCs
- The hanging drop method is not the optimized method of EB production

3.5. Specific Aims

The goal of this project is to design and develop a scalable method to increase production of cardiogenic cells from hMSCs for use in regenerative medicine for patients with heart disease by optimizing embryoid body formation and utilizing PDGF treatment. The specific aims of the project are as follows:

- Produce EBs with the same or higher efficiency as the current method
- Design a method that is scalable to consumer needs
- Design a method of EB production that produces standardized, reproducible EBs
- Increase cardiogenic output from the EBs
- Conduct and design experiments to assess the hypotheses

4. Design

This section explains the design process undergone to design and develop a scalable method to increase production of cardiogenic cells from hMSCs by optimizing embryoid body formation and utilizing PDGF treatment. This process is dictated by three groups; the client, the design team, and the user. The client, the person or persons whom want the product to be developed, for this project was Professor Glenn Guadette. He provided the initial problem statement and was the motivating force behind the project. The users, the individuals who will be using the product that is developed, for this project are graduate students Jacques Guyette and Dan Filipe, Professor Guadette, and anyone else in need of a method to produce cardiogenic cell from hMSCs. The design team for this project consisted of four individuals, Tom Hayes and Cem Saracel, biomedical engineering majors at Worcester Polytechnic Institute, and Gharam Han and Mike Riccio, biology/biotechnology majors at Worcester Polytechnic Institute.

The design team was further broken down into two teams, the engineering team and the biology team. These teams would work together in parallel on the project. The engineering team, consisting of Tom Hayes and Cem Saracel, would perform the engineering heavy aspects of the project, or primarily the engineering design process.

The engineering design process is a step-by-step program beginning with a simplistic problem statement and ending with the generation of a large volume of information and a specific solution to the initial problem. This process is clearly outlined in the five-stage prescriptive model in Figure 13. This process begins with the client statement which defines the problem statement. The first stage is to clarify this statement by defining and clarifying the project objectives, identifying constraints, and defining functions. After the problem statement has been defined and clarified the process of designing can begin. The first stage

of deign is to develop a conceptual design by developing specifications and design alternatives. Once this step is completed, the next stage is to analysis and evaluate the conceptual designs to determine the preliminary design. The final stage is forming a detailed design, which involves refining, optimizing, and communicating the final design. To successfully accomplish the specific aims of the project the engineering design team followed the engineering design process.

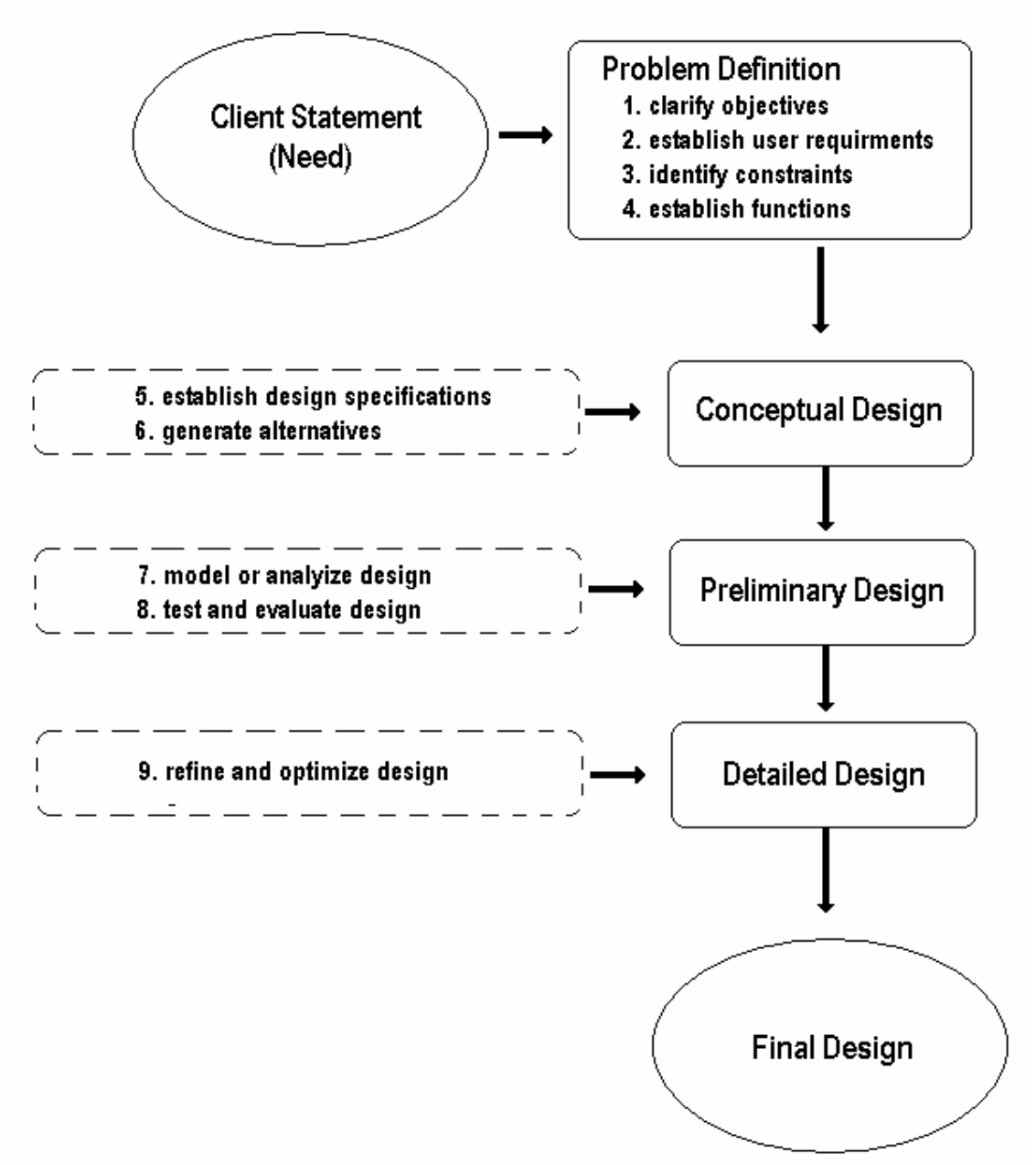

Figure 5: Five-stage prescriptive model of the design process (reproduced based off information from Engineering Design: a project-based introduction, Dym and Little)

4.1. Clarification of Design Goals

The first step to the engineering approach to the problem was to clarify the project statement in terms of engineering criteria. Both the engineering and biology team played a role in developing this initial understanding of the big picture of the project. This helped to keep both teams working in parallel and working towards the same ends. During this next step the problem statement would be to broken down into engineering specific criteria to give direction to the engineering process.

It was decided that cardiogenic cells would be created from HMSCs by EB formation and PDGF treatment, as has been previously described. The engineering team decided that it would focus on EB production. This would involve optimizing the formation of EBs, to create an efficient, scaleable method of EB production to mass produce EBs for cardiogenic differentiation. The start of this process was to determine and define, qualitatively, the requirements of the project based on examination of the problem statement and preliminary meetings. Through initial team discussions and discussions with the client, Professor Glenn Guadette, a list of requirements in the form of goals, objectives, functions, and constraints was formulated by the design team.

It was determine that the primary goal of the design team would be to design and develop a scaleable method to optimize the production of EBs from HMSCs. The biology team would then focus on directing the differentiation of the cells contained within the EBs with PDGF treatment. These initial statements were then broken down into a set of goals for the end product.

Goals:

1. Increase EB production
2. Increase cardiogenic output from the EBs
3. Optimize EB production
4. Produce a high level of reproducibility

These goals helped to clarify the direction of the engineering process and the overall project. However, further clarification would be needed before these goals could be obtained. The next step, undertaken by the design team was to create a list of basic attributes. Through preliminary meetings and discussions with the client a list of project objectives, functions, and constraints was created.

Table 1: List of Basic Design Attributes

Objectives:	*Constraints:*	*Functions:*
1. Scalable	1. Fits in/under a sterile laminar flow hood	1. Produces homogeneous sized EBs
2. Cost Effective/minimal cost	2. Must fit in incubator	2. Produces a non-adhesive environment for EB growth
3. Efficiently produces cardiogenic cells	3. Cost	3. Easy transfer of EBs to adhesive environment
4. Ease of use	4. Time	4. Decreases maintenance time for EBs
5. Safe/non-toxic	5. Doesn't adversely effect cellular activities	5. Decreases number of lost EBs
6. Time Efficient	6. Durable	6. Reduce contamination and maintain sterility
7. Reusable	7. Within Scope of the Project	7. Ability to test and control multiple variables
8. Easy to Manufacture		8. Reusable parts can be sterilized
9. Reliable/Reproducible		9. Resists external stimuli (shaking or spilling)
10. Structurally sound		10. Ability to visually see EBs 11. Integrates with cell culture equipment/techniques

4.1.1. Objectives

To add direction to the design process it was necessary to create a list of objectives that would help the design team better understand to project and move forward with the design process. While the engineering team was focused on creating criteria to direct the design process, the biology team worked in parallel learning the details of the current "hanging drop" method used to create EBs. The biology team practiced producing EBs with the current method and delivered feedback about the pitfalls, limitations, and specific details involved in the process. The design team used this feedback along with the basic set of objectives, created previously after initial team and client discussions, to revise the project objectives. This revision involved the inclusion of objectives that were deemed important after closer examination of the problem and the detailed breakdown of the objective list into separate categories and subcategories to formulate an indented objective list.

Table 2: Indented Project Objective List

1. Ease of use
    a. Compatible with existing instruments
    b. Simple Design
    c. Easy to handle
    d. User friendly
2. Cost Effective/minimal cost
3. Efficiently produces cardiogenic cells
    a. Efficiently produces EBs
    b. Produces homogeneous EBs
    c. Reduce Lost EBs
    d. Integrates with the addition of PDGF to increase cardiac output
4. Scalable
5. Structurally sound
6. Safe/non-toxic
7. Time Efficient
    a. Reduce Time
    b. Increase EB Production
    c. Decrease Loses
    d. Increase EB cardiac output

8. Reusable
9. Easy to Sterilize
10. Easy to Manufacture
11. Reliable/Reproducible

The design team had decided on eleven main objectives, as is listed in the indented objectives, that would adequately satisfy the needs of the project. These main objectives were ease of use, cost effective/minimal cost, efficiently produces cardiogenic cells, scalable, structurally sound, safe/non-toxic, time efficient, reusable, easy to sterilize, easy to manufacture, and reliable. However, some objectives needed to be further clarified.

It was decided that the objectives of being easy to use, efficiently producing cardiogenic cells, and being time efficient were initially to vague and needed further clarification. It was determined that the device should be a simple design and compatible with existing cell culture instruments and equipment to limit the complications of learning the new system and reducing the potential of complex parts interfering with efficient EB production. On the same hand the device should be user friendly and easy to handle to minimize the potential of human error affecting its success. There were many aspects involved in the efficient production of cardiogenic cells from hMSCs, but four main factors were selected. It was decided that the most important factors would be to efficiently produce EBs used in the production of cardiogenic cells and to induce some standardization and reproducibility by producing homogenous EBs. Also, there would have to be a reduction in EBs that were lost during processing and, importantly, this device would need to integrate with the PDGF treatments being assessed by the biology team. Finally, the object of being time efficient was broken down into subcategories because it was decided a reduction of time was only one important aspect of this objective. It would also be important to increase the production of EB, reduce the lost EB and cells, and increase the cardiogenic output from each EB.

After the creation of these objectives, it was recognized that some objectives were more important than others and that understanding these objectives in terms of their importance would be beneficial to the design process. To determine which objectives were more important to the design process a technique involving Pairwise Comparison Charts was used to construct a weighted objective tree. The principle behind the Pairwise Comparison Charts are that they provide a direct way of evaluating the importance of objectives on the same level by comparing each of the objectives on the same level. This involves scoring the objectives compared to each other with a score of 1 given to the more important objective, while the less important objective receives a 0. In the case were both objectives are equally important to the design process, a score of ½ is assigned to both. The sum of each objective's scores compared to all the other objectives determines the rank of

that objective. An objective with a score of 10 would be deemed more important than an objective with a score of 5 and so on. However, all the objectives are important to the design process, which means that no objective can receive a score of zero, or a score of no importance. In order to alleviate this problem the scores are normalized by adding one to each score and then dividing by the highest score. The results from this process can be seen in Tables 3-6, which show the detailed Pairwise Comparison Charts.

Table 3: First Level Objectives Pairwise Comparison Chart

1 – more important ½ - same 0 – less important	Efficiently produces cardiogenic cells	Ease of use	Cost Effective/ minimal cost	Scalable	Structurally sound	Safe/non-toxic	Time Efficient	Reusable	Easy to Sterilize	Easy to Manufacture	Reliable	**Score**	**Normalize Score**	**Normalize Rank**
Efficiently produces cardiogenic cells	X	1	1	½	1	1	1	1	1	1	1	**9.5**	**10.5**	**10.5/66**
Ease of use	0	X	1	0	½	0	½	1	1	½	1	**5.5**	**6.5**	**6.5/66**
Cost Effective/minimal cost	0	½	X	0	½	0	½	1	½	½	0	**3.5**	**4.5**	**4.5/66**
Scalable	½	1	½	X	1	½	1	1	1	½	½	**7.5**	**8.5**	**8.5/66**
Structurally sound	0	½	½	0	X	0	1	1	1	½	½	**5**	**6**	**6/66**
Safe/non-toxic	0	1	1	1	½	X	1	1	½	1	½	**7.5**	**8.5**	**8.5/66**
Time Efficient	0	½	½	0	0	0	X	1	1	1	½	**4.5**	**5.5**	**5.5/66**
Reusable	0	0	0	0	0	0	0	X	0	0	0	**0**	**1**	**1/66**
Easy to Sterilize	0	0	½	0	½	½	0	1	X	½	0	**3**	**4**	**4/66**
Easy to Manufacture	0	½	½	0	½	0	0	1	½	X	0	**3**	**4**	**4/66**
Reliable/Reproducible	0	1	1	½	½	½	½	1	1	1	X	**7**	**8**	**8/66**

The most important objective identified by this process was to efficiently produce cardiogenic cells, which validates the process because this was identified by the design team as one of the most important goals of the project. This was then followed by both

scalable and safe/non-toxic, the two objectives the tied for second in importance. These were followed by reliable, ease of use, structurally sound, time efficient, cost effective/minimal cost, easy to manufacture, easy to sterilize, and reusable, listed according to importance respectively. Tables 4 – 6, below, analyzed the sub categories of the main objectives.

Table 4: Efficiently Produces Cardiogenic Cells: 2nd Level Pairwise Comparison Chart

2nd Level: Efficiently produces cardiogenic cells	Efficiently produces EBs	Produces homogeneous EBs	Integrates with the addition of biological factors/ increase output	Reduce Lost EBs	Score	Normalize Score	Normalize Rank
Efficiently produces EBs	X	1	1	½	**2.5**	**3.5**	**3.5/10**
Produces homogeneous EBs	0	X	1	½	**1.5**	**2.5**	**2.5/10**
Integrates with the addition of PDGF to increase output	0	0	X	0	**0**	**1**	**1/10**
Reduce Lost EBs	½	½	1	X	**2**	**3**	**3/10**

The objective of efficiently producing cardiogenic cells was further clarified by being broken down in to four sub categories. Table 4 is the Pairwise Comparison Chart used to determine the importance of these objectives to the design process. The sub-objective efficiently produces EBs was determined to be the most important objective of this category, followed by reduce lost EBs, produce homogeneous EBs, and integrates with the addition of growth factors, PDGF, to increase cardiogenic output, respectively.

Table 5: Ease of Use: 2nd Level Pairwise Comparison Chart

2nd Level: Ease of Use	Compatible with existing instruments	Simple Design	Easy to handle	User Friendly	Score	Normalize Score	Normalize Rank
Compatible with existing instruments	X	½	1	½	**2**	**3**	**3/10**
Simple Design	½	X	½	½	**1.5**	**2.5**	**2.5/10**
Easy to handle	0	½	X	½	**1**	**2**	**2/10**
User Friendly	½	½	½	X	**1.5**	**2.5**	**2.5/10**

The design team also broke the objective ease of use into four sub-categories in order to further clarify this objective. As seen in Table 5, for the design to be compatible with existing cell culture instruments was deemed the most important aspect of being easy to use. For the device the objectives that it should be user friendly, a simple design, and easy to

handle were also determined important, respectively, to the design process but less important than being compatible with existing instruments.

Table 6: Time Efficient: 2nd Level Pairwise Comparison Chart

2nd Level: Time Efficient	Reduce time	Increase production	Reduce loses	Increase EB cardiac output	Score	Normalize Score	Normalize Rank
Reduce time	X	0	0	0	**0**	**1**	**1/9**
Increase production	1	X	1	½	**2.5**	**3.5**	**3.5/9**
Reduce loses	1	0	X	0	**1**	**2**	**2/9**
Increase EB cardiac output	1	½	1	X	**1.5**	**2.5**	**2.5/9**

Table 6 shows the results from the objective time efficient, which was also broken down into four sub-objectives. The increased production of EBs was determined to be the most important aspect of the objective of time efficient. However, increased EB cardiac output, reduced loses, and reduced maintenance time, were also deemed important aspect aspects of this objective, respectively.

Once the objectives and the sub categories had been weighted a picture began to emerge of the direction the design process would follow. The next step was to compile the weighted objectives and then organize them into an objective tree, which can be seen in Figure 6. The weights given for each objective on the same level were further modified so that all objectives scores for one level added up to one. This structuring was based according to the normalized score received during comparison evaluation. The objective tree was also set up so that each objective was assigned two weight values. The first value is the weight in comparison to objectives on the same level, where as the second value is in relation to all the objectives, including sub-category objectives.

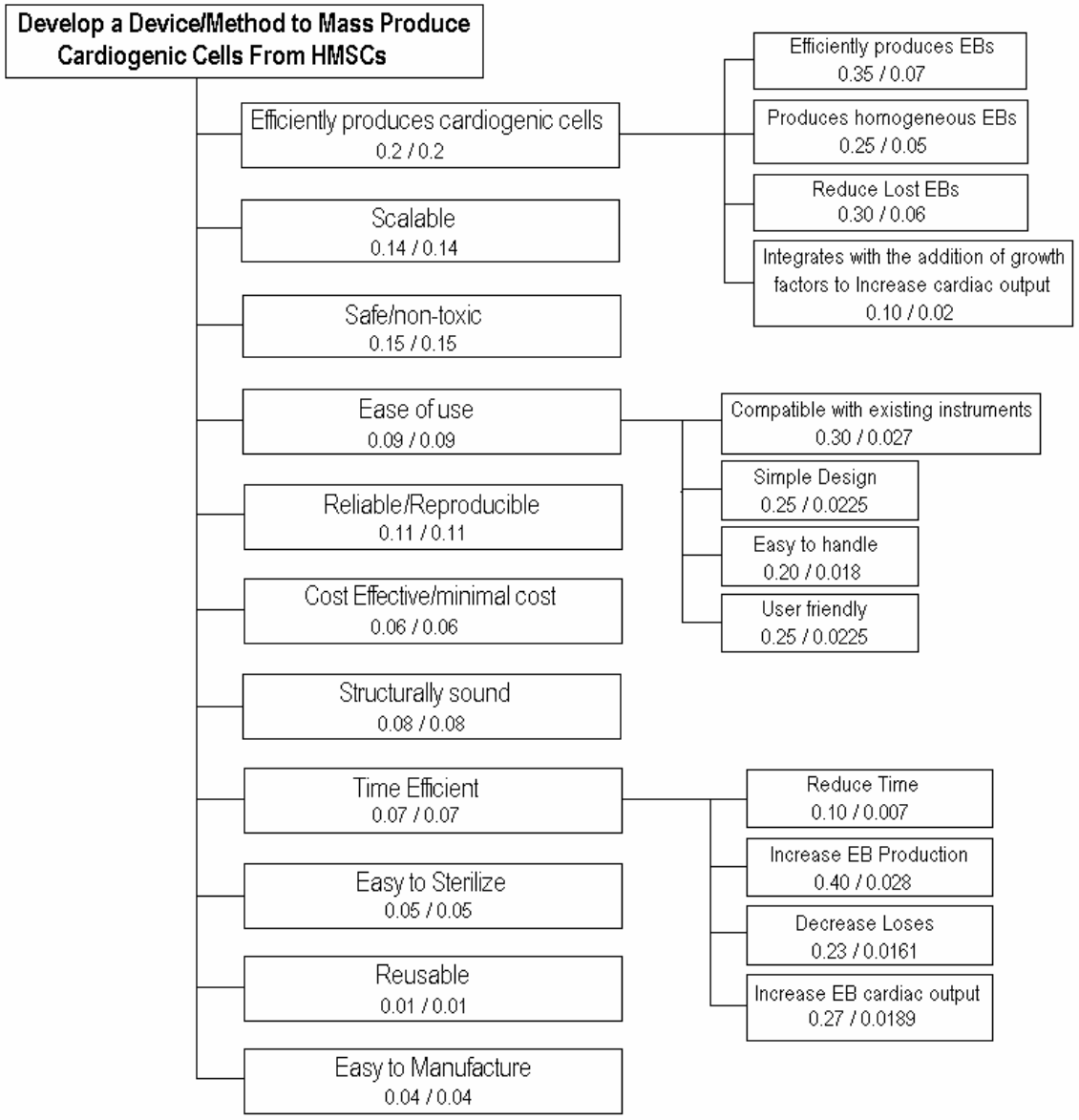

Figure 6: Weighted Objective Tree

4.1.2. Revision of the Client Statement

The creation of objectives, constraints, and functions helped to clearly define the problem statement. Then after weighting the objectives is was more clearly understood were the design process would be headed. From this process it was determined that a design that could be scalable depending on need and efficiently produced EBs that could then be differentiated into cardiogenic cells was essential to optimizing the production of cardiogenic cell from hMSCs. However, it was also determined that device should be safe to the user and the cells, reliable, and produce reproducible results. The design needed to integrate with

current techniques being used and existing instrumentation in order to be comfortable with the user. Finally, the design needed to be easy to manufacture and cost effective or it would not be a better choice for consumers. From this analysis of the problem statement the design team was then able to formulate the following revised client statement.

“Design and develop a scalable and cost effective method to produce cardiogenic cells from hMSCs by both, optimizing embryoid body production with an easy to use, safe, and easy to manufacture device and directing differentiation with PDGF treatment.”

4.2. Conceptual Design

The first steps of the design process give direction to the project by establishing goals, objectives, basic functions, and constraints. Based on this set of criteria the next step is to create conceptual designs. Through discussions between the design team potential solutions to the problem are formulated. These initial conceptual designs help to organize and develop ideas, while providing a platform to describe various means intended to satisfy specific design functions.

4.2.1. Brainstorming and Initial Ideas

Integrated brainstorming sessions were held between the engineering and biology team. This was the first step in creating conceptual designs after discussions with the client. It was decided that the main focus of the initial design would be on EB growth, which was the current method for production of cardiogenic cells from hMSCs. The treatment of EBs with PDGF determined to be not involved with EB formation would remain involved in the design process but would be down played in the initial ideas. Background research had reviled several different methods for the production of EBs, which would all be explored throughout this process.

However, before the design team began the process of developing conceptual designs it was important to understand the mechanisms behind EB production. The current method employed by the client was the hanging droplet method, which involves placing droplets of cell suspension on the cover of a 96-well plate and flipping it over. This allows the droplets to hang upside down as seen in Figure 7. The cells within the droplet are contained within a non-adhesive environment, which with no surface to attach to the cells remain in suspension. Then due to the cell to cell interactions within the droplet, the cells start the process of aggregation, or clumping of the cells. As the cell clumps become heavier due to the continued aggregation events, gravity pull the cells in suspension towards the bottom of

the droplet. This forces the cells closer and closer, speeding up the aggregation events. This process is represented in Figure 8.

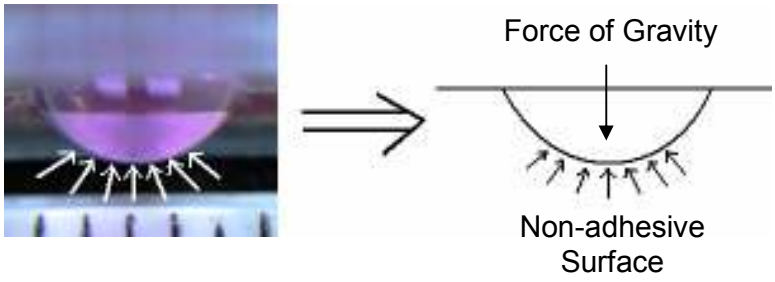

Figure 7: Hanging Droplet Produces a Non-adhesive Environment

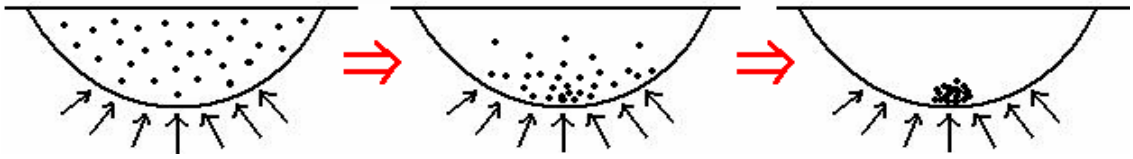

Figure 8: The Process of Aggregation in EB Formation

The shape of the droplet is important to the production of EBs. As the aggregation events continue the force of gravity pulls cells to the bottom of the droplet. The spherical shape of the hanging droplet, as seen in Figure 7, constricts the cells to signal point of aggregation. As the cells settle to this point at the bottom of the hanging droplet they form a large aggregate. The next step of the process is still unclear to researchers, but due to the non-adhesive environment, the three dimensional structure of the aggregate, and the cell to cell interactions without and cells to matrix interactions, the cells formed a three dimensional structure known as an embryoid body, or EB.

From this analysis, the production of an EB can be broken down into three major steps; the aggregation of cells in suspension due to a non-adhesive environment, the coalescing of the smaller aggregates and cells to a singular point due to droplet shape, and the transformation of the large aggregate into a EB due to the non-adhesive environment at the bottom of the droplet. The most important aspect gained from this analysis was that a non-adhesive environment as crucial to EB formation. Without this environment cells would attach to a surface, begin differentiation, and not undergo the cell to cell interactions necessary to EB formation. However, it was also determined that the aggregation of cells to a singular point was an important, thus the shape of this non-adhesive environment would also be important.

With this basic understanding about EB formation the design team began discussing ideas about how to optimize the formation of EBs and formulating conceptual designs. These initial design ideas can be organized into three categories, optimizing the already proven technique of the hanging droplet method, bioreactor designs, and non-adhesive well design. The following are the initial designs proposed for the project.

4.2.1.1. Optimizing Hanging Drop

These systems, based on the proven method of hanging droplet, were the combination of the design team's ideas to improve the hanging drop production of EBs. In the hanging drop method droplets are placed on a flat surface and then that surface is flipped to allow the droplets to hang. These hanging drops provide a non-adhesive environment for the cells and create aggregation events that lead to EB formation. The first step of brainstorming was to attack the limitations and pitfalls of this technique.

Hanging Drop with Droplet Support

This system, depicted in Figure 9, involved the incorporation of supports on a flat plate. Drop sized divots on the surface of the plate would be used to stabilize the hanging droplets of media and cells. This would ease the transportation of plates containing hanging droplets and prevent the sliding of droplets during flipping.

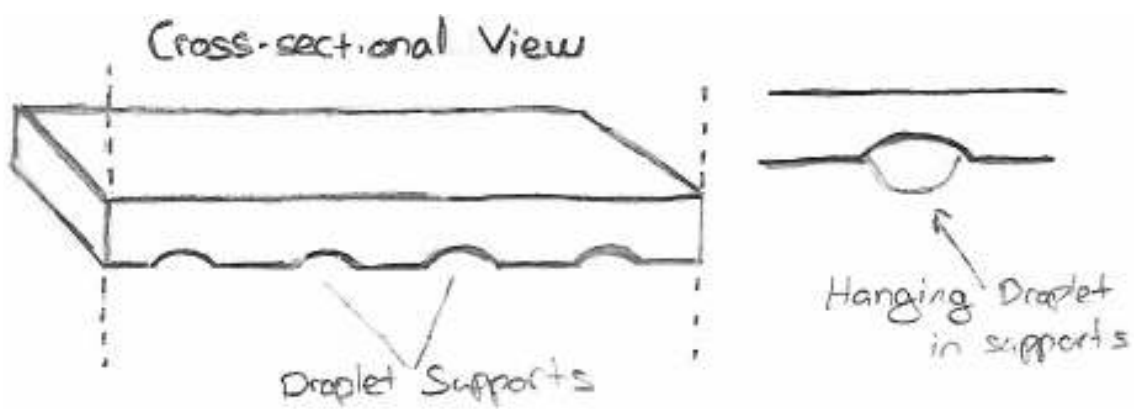

Figure 9: Hanging Drop Surface with Droplet Supports

Pros:
- Provides droplet stability
- Prevents droplet sliding during flipping
- Easy to manufacture/simple design

Cons:
- Doesn't eliminate flipping step
- Supports may get in the way

Top-Loading Hanging Drop with Micro-Screen

This device produces EBs with the same hanging drop principle as the current method but eliminates the flipping step. As seen in Figure 10 droplet size wells are structured into the bottom of the plate that will contain the droplet of media and provide stability. A channel is located above the droplet support that is attached to an adaptor that fits a standard micro pipette tip. This allows for media to be removed and added from the above the droplet support without having to flip the plate.

The interface between the droplet support and the channel contains a micro-screen with a very small porosity. This mesh has pores large enough that cells can fit through but small enough that EBs can not. This prevents accidental removal of the EB formed at the bottom of the droplet contained in the droplet support from being sucked into the media channel or pipette tip. Media and cells can be pipetted into the droplet support from above the plate eliminating flipping, while the media within the droplet can be changed without having to flip the plate or suck up the EB. Various other designs can be seen in Appendix 1.

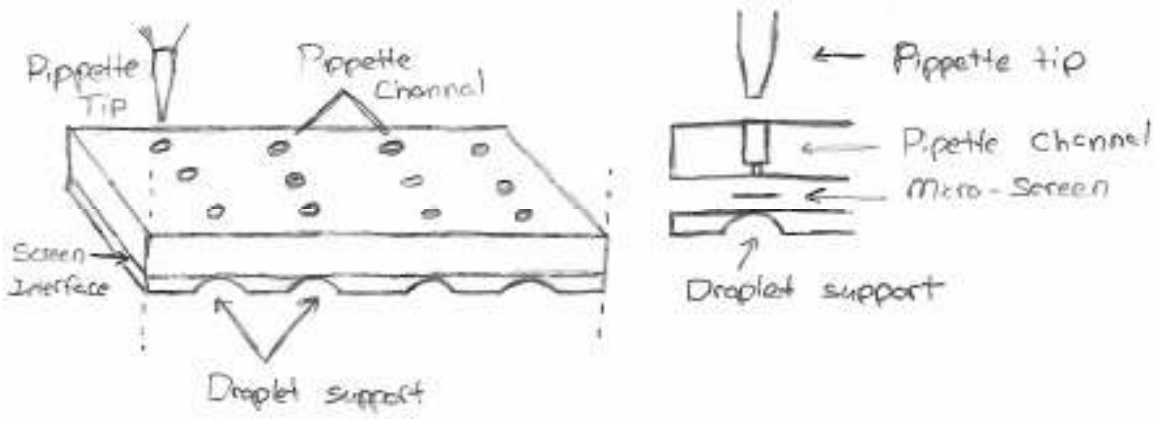

Figure 10: Top-Loading Hanging Droplet with Micro-Screen

Pros:
- Provides droplet support
- Eliminates flipping
- Mimics proven hanging drop technique
- Integrates with existing equipment
- Simplistic design

Cons:
- Droplets are still exposed
- Untested mechanics

Automated Piston/Pump Hanging Drop

The device employs the same technique as hanging drop, a hanging droplet of cell suspension, to form an EB from a cell suspension of hMSCs. The difference between this device and the other conceptual designs in this category is that it is fully automated. Using a piston or pump and a set of one-way valves media is added and removed from a closed

cylinder system with a micro-mesh screen, as seen in Figure 11. The initial cell suspension of hMSCs is added to the system through the removable top section and is distributed onto the mesh screen. Then the top portion of the cylinder system, through which the hMSC cell suspension was added, is closed. The piston then moves the cell suspension through the mesh to the bottom of the cylinder, open end, and forms a hanging drop. After the cells have aggregated into an EB the media is removed from the chamber via the piston and a suction pump. This old media is held in a chamber consisting of a chamber that can be emptied and sterilized. The micro-mesh prevents the newly formed EB from being removed along with the media. After media removal, fresh media is added to the cylinder. This media is contained in a refillable reservoir that can be removed from the unit and sterilized. The addition of the new media to the system with help of the piston or pump moves the EB and media back into the handing droplet position.

This system would consist of many devices all connected together into an integrated system. This system would fit within a special incubating unit or a smaller version would fit in a standard incubating system. The only manual labor involved in EB formation would be the first step of filling the chambers with cell suspension and replacing the top section.

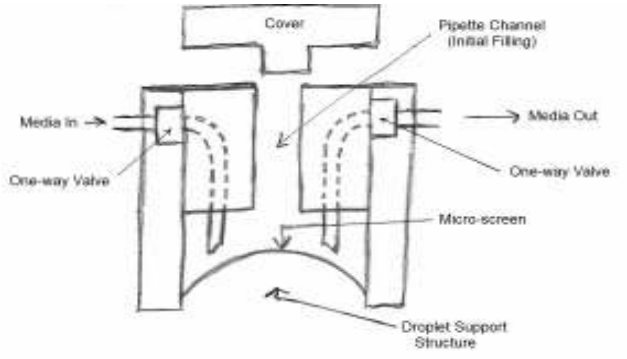

Figure 11: Automated Pump Hanging Drop

Pros:
- Automated system removes human error
- Very low maintenance time
- No manual EB maintenance

Cons:
- Complicated design
- Expensive design

4.2.1.2. Bioreactor Design

Bioreactors are used in many applications and are the best method used to move tissue growth into mass production. Different types of bioreactors have been used by

researchers in current literature to form EBs with limited success. Some of our first conceptual designs involved the use of these bioreactor systems to optimize the production of EBs. However, after discussions with the client, the design team concluded that bioreactor design would not satisfy all of the goals of the project. The following are brief descriptions of the initial conceptual designs developed by the design team.

Spinning Flask Bioreactor / Rotating Bioreactor

This device works on the principle that if a constant flow is introduced into a cell suspension of hMSCs, the cells will be unable to attach to a surface and form aggregates. The aggregation events will happen at a faster rate than in the hanging droplet method, due to more interaction of cells in constant motion, and would result in faster EB formation. However, limitations such as uncontrollable EB size and EB removal would have to be assessed. This design, as seen in Figure 12, is composed of a flask filled with cell suspension and a rotary blade system connected to an electric motor to induce and maintain a low shear flow. This system would be small enough to fit in a standard sized incubator, common to most laboratories.

A rotating bioreactor works using the same basic principle as the spinning flask bioreactor. The cell suspension is kept in constant motion, however, in this design cells are kept in constant free fall by a horizontal rotating vessel, as seen in Figure 13. The cells that are in constant free fall are unable to attach to the surface of the rotating vessel and thus undergo aggregation events and form EBs.

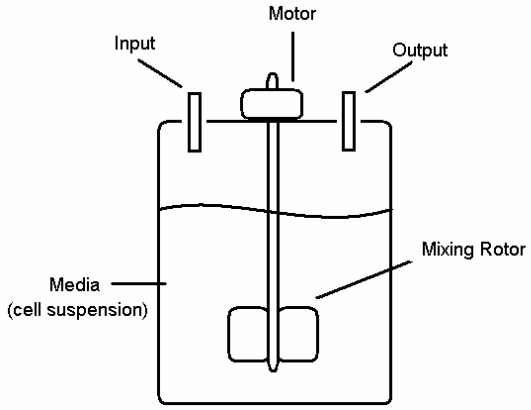

Figure 12: Spinning Flask Bioreactor

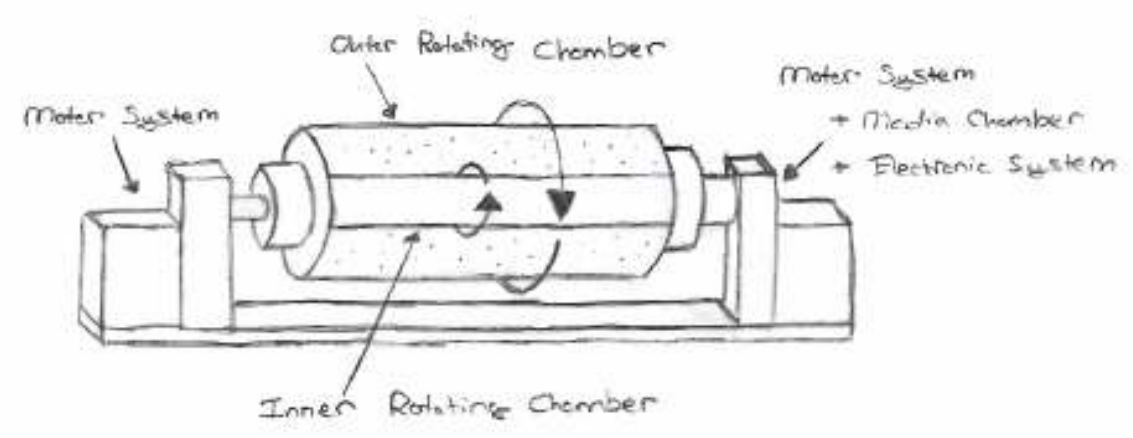

Figure 13: Rotating Bioreactor

Semi-permeable Membrane Bioreactors

These designs encompassed the use of a semi-permeable membrane, permeable to the media and nutrients in a cell suspension, but impermeable to the cells. This membrane would also have to been cell resistant, which means that the cells would be unable to attach to its surface. These systems consisted of two major designs, a flow reactor system and a "tea-bag" approach. In the flow system, as seen in Figure 14, a constant low shear flow is maintained through the semi-permeable, cell resistant membrane. The media is able to pass through the membrane but the cells are not. The cells collect against the membrane and due to its cell resistant nature are unable to attach to the surface and thus undergo aggregation events. As an adjunct to this design an adhesive membrane can be located opposed to the flow, so that if flow was reversed the cell would have an adhesive environment to attach and differentiate.

The "tea-bag" approach is similar in that is also involves the use of a semi-permeable membrane, but in this design there is no flow. The hMSCs are placed into holding wells composed of this cell resistant, semi-permeable membrane, which are then placed into a container filled with just media, Figure 15. The nutrients within the media are able to cross the membrane barrier but the cells are confined to the wells. Due to the non-adhesive nature of the membrane and the force of gravity, the cells undergo aggregation events in this non-adhesive environment and develop into EBs.

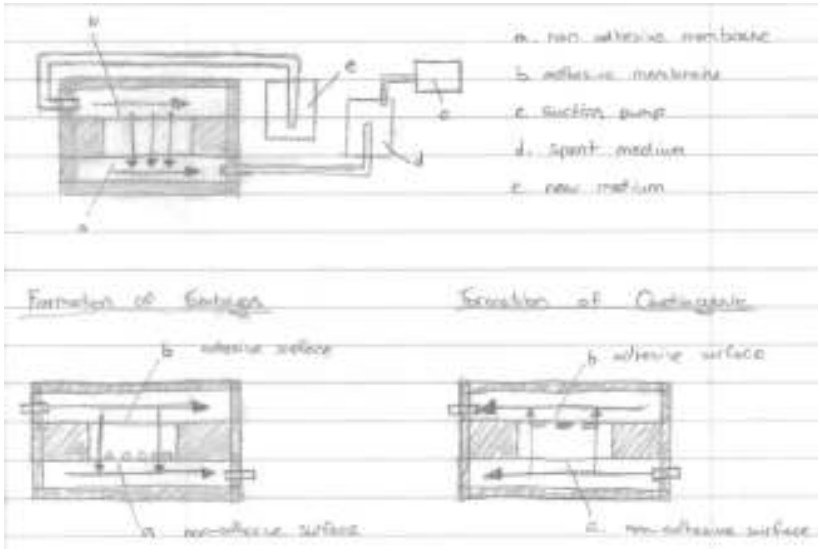

Figure 14: Semi-permeable Membrane Flow Bioreactor

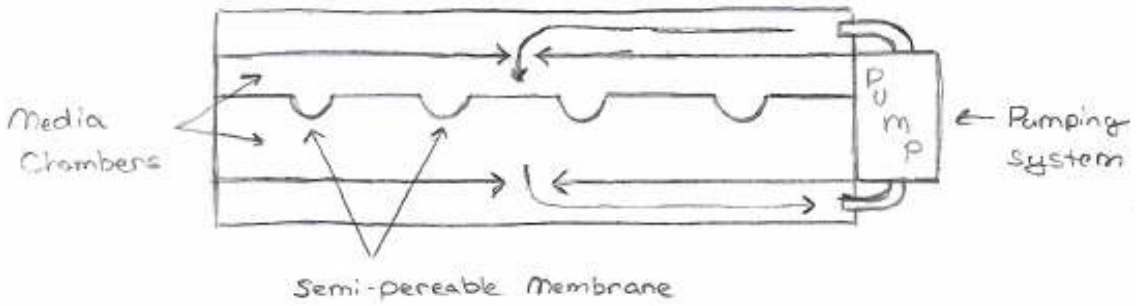

Figure 15: Semi-permeable Membrane "Tea-Bag" Bioreactor

4.2.1.3. Non-adhesive Well Design

Non-adhesive well design was based off of the principles gathered from analyzing the hanging droplet method, most importantly the cell attachment resistant environment and the droplet shape. The ideas behind these designs are that if a non-adhesive environment can be produced by a polymer or coating, one in which the cells will be unable to attach, the cells will undergo aggregation. Then if this environment mimics the shape of a hanging droplet, the cells will follow the same processes as in the hanging droplet method and form EBs. The following are the initial ideas developed using these principles.

<u>Solid Polymer Wells</u>

This design involves the use of a solid polymer scaffold with wells created into its surface. The cell suspension will be placed within the wells in the polymer, which has a cell attachment resistant surface, as seen in Figure 16. The wells in the polymer scaffold are shaped to mimic the shape of a hanging droplet. The whole polymer scaffold is structured to

similar dimensions as a standard 96 well plate and has a cover to help maintain sterility. This device would be used under a laminar flow hood and stored in a standard incubator.

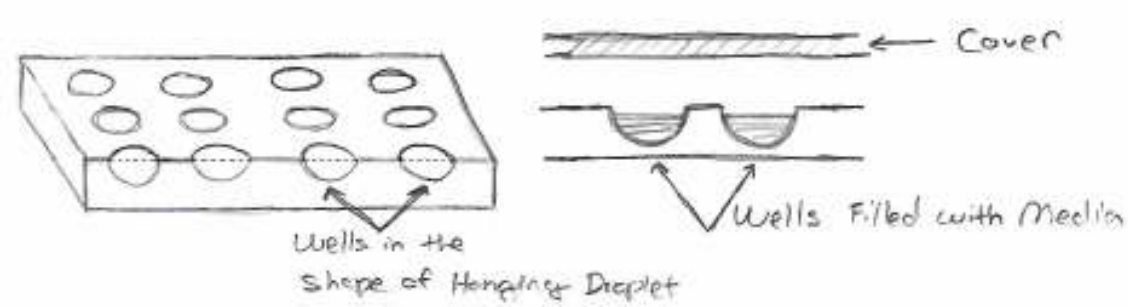

Figure 16: Solid Polymer Wells

Pros:
- Simple design
- Easy to manufacture
- Eliminates flipping

Cons:
- Hard to remove EB
- Manual media removal

Electrospun Polymer Wells

This design is based off of the same principles as the solid polymer wells; however, the nature of the electrospun polymer gives certain advantages. The electrospun polymer would be cell attachment resistant to produce a non-adhesive environment for the cell suspension, but would be composed of a fiber matrix instead of a solid polymer surface. This fiber matrix would have a porosity small enough to act as a semi-permeable membrane. The electrospun polymer would be permeable to the cell suspension media and nutrients, but not to the cells. This characteristic would allow for the following design, Figure 17.

The cell suspension would sit within wells shaped to mimic a hanging droplet used in the hanging droplet method. Due to the non-adhesive environment caused by the electrospun polymer and its shape the cells would form aggregates and then EBs. This electrospun matrix containing the wells, would sit on top of a 96-well plate that had been modified to attach to an aspirator or some other suction device. When the media needed to be changed, the suction would be applied to the bottom of the wells to remove the media. However, due to the porosity of the fiber matrix, the EB would remain in the well. New media could then be added. A 96-well plate cover would fit over the device to help maintain sterility. The device would be used in a laminar flow hood and stored in a standard incubator.

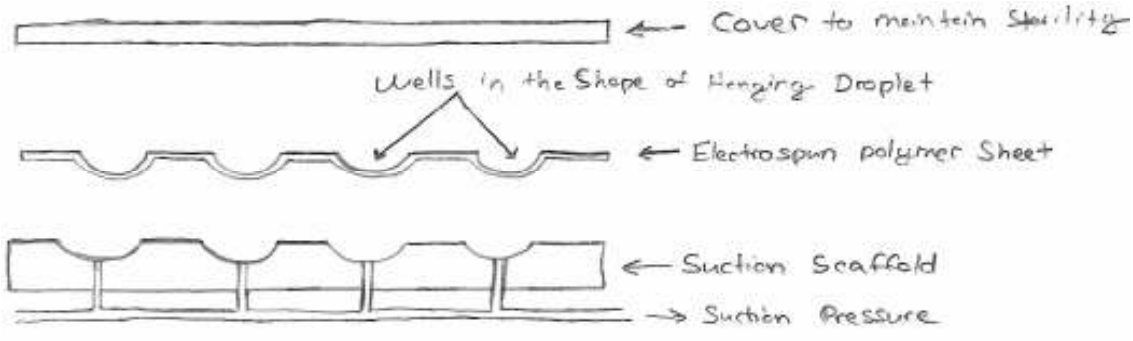

Figure 17: Electrospun Polymer Wells

Pros:
- Eliminates flipping
- Eliminates media removal that can cause EB lose

Cons:
- Harder to manufacture
- Untested media removal technique

Coated Wells

This design involved the use of a very hydrophobic polymer such as a hydrogel or polymer such as 2-methacryloyloxyethyl phosphorylcholine (MPC), which contains a phosphorylcholine moiety. These coatings would mimic the environment of a hanging droplet by either being applied to a standard round bottom 96-well plate or a solid polymer scaffold with wells that are structured to represent a hanging droplet shape. As seen in Figure 18, this design would be very similar to a standard 96-well plate used currently in cell culture. The coating would be covalently attached to the surface of the scaffold material. This design could be used with existing culture techniques and instruments and would have dimensions that allow for its use in a laminar flow hood or common incubator.

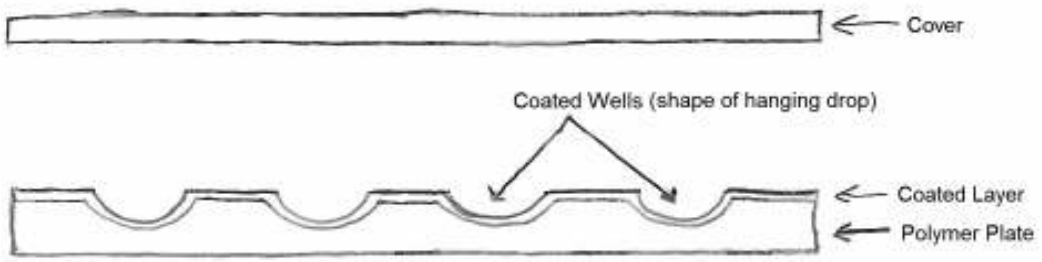

Figure 18: Coated Well Scaffolds

Pros:
- Eliminates Flipping
- Integrates easily with existing technologies
- Prevents evaporation

Cons:
- Hard to manufacture
- Hard to remove EBs

4.3. Preliminary Design

The conceptual design process had left the design team with various designs, all potential solutions to the problem statement, each with its own advantages and disadvantages. At this stage in the engineering process many of the designs were just ideas based off of data from the literature or from the design team's observations of EB formation. Further testing would be necessary to evaluate the potential of the designs. However, due to the limited amount of time and resources for the project, not all the designs could be modeled and tested. The design team decided to use selection matrices to organize and rank the conceptual designs to determine which designs would be tested and what the order they would be tested.

4.3.1. Selection Matrices

Selection matrices were used to quantify which designs fulfilled the engineering criteria the best and thus were more important to the design team. First the designs were judged against the constraints of the project. A design that satisfied a constraint would receive a "Y" and would then move on to be scored against the objectives. If a design was deemed not to satisfy a constraint then it would receive an "N" and would not move on to the second round of scoring against the objectives. This was done to eliminate any designs that the design team felt would not satisfy the constraints, which by definition constrain the project into a strict workspace. For example, the design team determined qualitatively that the bioreactor designs created during the conceptual design stage would not be within the scope of the project. When they were scored in the first selection matrix it can be clearly seen that they did not satisfy all constraints. The results from this initial selection matrix can be seen in Table 7.

Table 7: First Level Selection Matrix - Constraints

Alternative Design Selection	Fits in/under a sterile laminar flow hood	Must fit in incubator	Time	Cost	Doesn't adversely effect cellular activities	Durable	Feasible
Electrospun Wells	Y	Y	Y	Y	Y	Y	Y
Hanging Drop w/ Droplet Supports	Y	Y	Y	Y	Y	Y	Y
Solid Polymer Wells	Y	Y	Y	Y	Y	Y	Y
NiPAMM Coated Wells	Y	Y	Y	Y	Y	Y	N
Hydrogel Coated Wells	Y	Y	Y	N	Y	Y	Y

Top-Loading Hanging Droplet w/ Screen	Y	Y	Y	Y	Y	Y	Y
Automated Piston Wells	Y	Y	N	N	Y	Y	N
Semi-permeable Membrane Flow Bioreactor	Y	N	N	N	Y	Y	N
Semi-permeable Membrane "tea-bag" Bioreactor	Y	N	N	N	Y	N	N
Spinning Flask Bioreactor	Y	N	N	Y	Y	Y	Y
Roatating Bioreactor	Y	N	N	N	Y	Y	Y

This first level selection matrix helped the design team to identify the designs that would not move on to further evaluation and be tested. From this matrix the designs that were eliminated were the spinning flask bioreactor, the semi-permeable membrane bioreactors, the automated piston wells, and the coated wells. The designs that were selected to move forward to the second selection matrix were the electrospun wells, hanging drop w/ droplet supports, the solid polymer wells, and the top-loading hanging droplet with screen.

However, a note should be made here that after testing had begun on the other designs it was found that coated wells had been created by two companies, Corning and ThermoFisher Scientific. Upon re-evaluation, it was determined that this technique was now cost effective and feasible because the team could buy the plates from a third party instead of making them in the laboratory. These plates were then purchased to test the efficiency of growing the cells on a polymer coated surface.

After determining the designs that would not be eliminated from the preliminary design stage, the next step was to determine that importance of each design compared the other designs. This was done so that the design team could organize order of testing for the designs. To accomplish this goal a second selection matrix was used that weighted the designs against the project objectives. A score of 1 to 3 was given to each design for a particular objective. This score represented how well the design satisfied this objective, with 3 being the highest. This score was then multiplied by the weight of that objective to receive a weighted score for every objective. Finally, weighted scores were added to give a total score for each design. The results from this process can be seen in Table 8.

Table 8: Second Level Selection Matrix – Objectives and Constraints

Design Constraints	Design 1: Electrospun Wells		Design 2: Hanging Drop w/ Droplet Supports		Design 3: Solid Polymer Wells		Design 5: Top-Loading Hanging Droplet w/ Screen	
C: Fits in/under a sterile laminar flow hood	Y		Y		Y		Y	
C: Must fit in incubator	Y		Y		Y		Y	
C: Time	Y		Y		Y		Y	
C: Cost	Y		Y		Y		Y	
C: Doesn't adversely effect cellular activities	Y		Y		Y		Y	
C: Durable	Y		Y		Y		Y	
C: Within Scope of Project	Y		Y		Y		Y	
Design Objectives (weight %)	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**	**Score**	**Weighted Score**
O: Efficiently produces cardiogenic cells (20)	2	40	3	60	3	60	3	60
O: Efficiently produces EBs (7)	2	14	2	14	2	14	2	14
O: Produces homogeneous EBs (5)	2	10	1	5	3	15	1	5
O: Reduce Lost EBs (6)	3	18	1	6	3	18	2	12
O: Integrates with the addition of biological factors to Increase cardiac output (2)	3	6	3	6	3	6	3	6
O: Ease of use (9)	2	18	1	9	3	27	2	18
O: Compatible with existing instruments (2.7)	3	8.1	3	8.1	3	8.1	3	8.1
O: Simple Design (2.25)	2	4.5	2	4.5	3	6.75	2	6.75
O: Easy to handle (1.8)	3	5.4	1	3.6	3	5.4	2	5.4
O: User friendly (2.25)	2	4.5	2	4.5	2	4.5	2	4.5
O: Cost Effective/minimal cost (6)	1	6	2	12	2	12	2	12
O: Scalable (14)	3	42	1	14	3	42	2	28
O: Structurally sound (8)	2	16	2	16	3	24	2	16
O: Safe/non-toxic (15)	3	45	3	45	3	45	3	45
O: Time Efficient (7)	3	21	2	14	2	14	2	14
O: Reduce Time (0.7)	3	2.1	2	1.4	3	2.1	3	2.1
O: Increase EB Production (2.8)	3	8.4	2	5.6	3	8.4	3	8.4
O: Decrease Loses (1.61)	3	4.83	1	1.61	3	4.83	2	3.22
O: Increase EB cardiac output (1.89)	2	3.78	2	3.78	2	3.78	2	3.78
O: Reusable (1)	2	2	2	2	3	3	2	2
O: Easy to Sterilize (5)	2	10	3	15	3	15	3	15
O: Easy to Manufacture (4)	2	8	2	8	3	12	2	8
O: Reliable (11)	2	22	2	22	2	22	2	22
Total (426.9)		**319.61**		**281.09**		**372.86**		**319.25**

From this selection matrix it can be seen that the solid polymer wells were determined to be the design with the most potential of satisfying the criteria established for the project. However, the design team wanted to perform further testing to better evaluate the potential of the designs. This testing would be organized according to the results seen in

Table 10. The first designs to be tested would be the non-adhesive wells, which included the electrospun wells and the solid polymer wells. Then if further design evaluation was necessary the top-loading hanging droplet with micro-screen and the hanging droplet with droplet support would be tested.

5. Methodology

5.1. PDGF Treatment Evaluation

5.1.1. hMSCs Culture

hMSC's were cultured in hMSC culture medium (DMEM+10%FBS+1%P/S; Cambrex Bio Science). Cell cultures were incubated at 37° with 5% CO2 until cells were 70-80% confluent. Cell passage number varied from passage 3 to passage 10. [see Appendix 2]

5.1.2. Hanging Drop EB Formation

To form EBs for PDGF treatment the "Hanging Drop" method was used hMSCs cultured in hMSC culture medium were trypsinized (Sigma) and re-suspended in EB culture medium (DMEM+20%FBS+1%P/S; Cambrex Bio Science) at a concentration of 7.5x105 cells/mL. The lid of a sterile 96-well plate was inverted; with the inside face facing upwards. Using a 200 µl "wide orifice" pipette tip (VWR), multiple 20 µl cell suspension samples were applied to the inside face of the 96-well plate lid as separate 20 µl droplets. The bottom portion of the 96-well plate was also inverted and DMEM was applied to the chambers to prevent evaporation. The plate lid was then carefully, but quickly, flipped producing hanging 20 µl cell suspension droplets. The lid was then placed back on top of the now inverted bottom portion of the plate and was incubated at 37° with 5% CO2 until day three. The droplet medium was changed every day with fresh EB culture medium until day three, when the EBs were transferred. [see Appendix 2]

5.1.3. EB Transfer and PDGF Treatment

EBs that had been cultured for three days and given ample time to form were then transferred into 4-well chamber slides. A micro pippetor and 20 µl "fine-tipped" pipette tips were used to obtain and transfer each 20 µl droplet containing the EB to a chamber of the four-well chamber slide. Each chamber received two EBs and 300-500 µl of either normal EB culturing medium or Platelet Derived Growth Factor-ββ (PDGF) supplemented medium (DMEM+20%FBS+1%P/S+ 10ng/mL PDGF). Control slides containing hMSCs were also prepared. Each chamber received 3x105 cells and 300-500 µl of either EB culture medium or PDGF supplemented medium. Slides were left to incubate at 37° with 5% CO2 until day 7. [see Appendix 2]

5.2. Staining

5.2.1. Fixation

Before the cells could be stained for immunohistochemistry they had to first be fixed to the four well chamber slides. This was done in the same fashion for all slides. First the media was removed and discarded. It was then replaced with a wash of parafamaldahyde for fixation. After allowing the cells to sit for a period the parafamaldahyde was then replaced with a solution of thirty percent sucrose in which the cells where stored until the immunohistochemistry could be done. [see Appendix 2]

5.2.2. Immunohistochemistry

The slides were first brought up to room temperature by being left to sit at room temperature for thirty minutes. The thirty percent sucrose was then removed and the slides were given a five minute wash with phosphate buffered solution (PBS). After the cells were rinsed they were washed with a detergent (Triton X) for ten minutes. The detergent was removed form the slides and they were again washed in PBS. After the cells were washed the slides were given a universal blocking agent and left to sit for thirty minutes. The blocker was then removed and the cells were given a wash in PBS for five minutes. After the blocker was removed the slides were given the primary antibody (mouse alpha sacromeric actinin) in the following fashion. Wells one and three were given a 175ul wash of one to three hundred dilution of the primary and wells two and four were keep moist with a wash of PBS, the slides were then left to sit for thirty minutes. At the conclusion of this time the cells were once again washed three times with PBS for five minutes each. Once the slides were washed each well was exposed to a universal secondary antibody derived from rabbit IgG, and once again left to sit for thirty minutes. At the conclusion of this thirty minute period the cells were once again given three washes of PBS for five minutes each. After this series of washes the slides were given a 175ul wash of one to five hundred dilution of Quantum Dot solution, and left to sit for thirty minutes. The slides then received a PBS wash for five minutes. When the PBS was removed the cells were then exposed to a 175ul sample of nuclear dye (Hoechst Stain) at a one to six thousand dilution. This Hoechst dye was left on the cells for twenty minutes. When the Hoechst dye was removed the cells received a final five minute washing in PBS. After this wash the “four well” portion of the slide was removed and the slides were prepped for dehydration. The dehydration process was consisted of six, thirty second washes in dH2O, 30% ethanol, 70% ethanol, 95% ethanol, 100% ethanol, and Taluine respectively. After the cells were removed from the Taluine they were then cover slipped using Cytoseal and left over night to dry for observation. [see Appendix 2]

5.2.3. Quantification

In order to generate quantitative data from our slides we devised a means with which to impartially photograph the stained EBs. The wells were first scanned for EBs beginning with the bottom left hand corner. After an EB was located it was subjected to a series of pictures. The EB was divided into four quadrants a top left, top right, bottom left and bottom right. The quadrant area was established based on the presumed approximate center of the EB. The pictures were taken at a 20x zoom to maximize the area in all four quadrants. Then for consistency the two lower quadrants were photographed in the same location under a 40x magnification. At each quadrant and magnification, two pictures were taken and an overlay image was constructed using ImageJ, forming one combined image. This image was then used to differentiate the positively stained cells. The pictures then underwent further observation at which time the cells in each quadrant were counted and a positive or negative stain was confirmed. These counts were used to establish the ratio of cardiogenic to non-cardiogenic cells.

5.3. Design Formation

5.3.1. Curvature Analysis

Before any of the design alternatives involving embryoid body formation in wells could be tested, the curvature of the droplets formed by the method of hanging droplet had to be analyzed. This was achieved by analyzing images taken of hanging droplets. First, the cover of a 96-well plate was used to create 20ul and 50ul droplets (n=22 and n=11, respectively). Pictures of these droplets were taken with a scale in the background using a high resolution camera. The images were transferred onto a computer and analyzed using the software Photoshop. In Photoshop, a perfect circle was fit on the image of the curvature. After determining how many pixels spanned 1mm in the pre-imposed background, the curvature was calculated by determining the number of pixels in the radius of the fit perfect circle. The results were compiled in MS Excel and statistically analyzed by determining the mean radius of curvature, the standard deviations and the percent deviations.

5.3.2. Electrospun Poly(trimethylene terephthalate) Wells

The method of electrospinning, depositing an electrically charged polymer on a collector plate, was used with PTT, a material chosen because of its hydrophobicity, biocompatibility, availability, and its ability to be electrospun. The electrospinning was performed at Biosurfaces Inc., a company in Ashland, MA. The electrospun PTT was kindly provided to us as 4” by 6” flat electrospun sheet or as a 4” by 6” sheet with wells. The wells in the second sheet were produced by electrospinning directly onto a negative mold of our

wells. This electrospinning plate well mold was created by hammering ball bearings with a radius of 2.5mm onto a copper plate, producing raised bumps with our desired radius of curvature. Well shapes were created into the flat polymer sheet by heat sinking. Two metal plates, created with the same method as the electrospun plate well mold, with the mold of our desired well shape and size were heated with an iron. The PTT polymer was then pressed between the plates until the fibers stretched and adopted the final form.

5.3.3. Polyoxymethylene Wells

As determine from research of current literature, the solid polymer POM was proven to show non-adhesive properties that were beneficial to our design. To recreate the well shape and size of the hanging drop in the POM sheet we initially bought a 6” by 12” polymer sheet with a thickness of 3/8” from Small Parts Inc. The polymer sheet then was cut into equal smaller pieces of 1.5” by 6” by 3/8” to create the wells into the material using various methods.

5.3.3.1. Drilled Wells

The first method to create the wells in the material was by drilling. WPI’s machining shop was used to drill eight wells into the POM sheet with the previously measured curvature of the hanging drop. A drill bit with a radius of 3mm was chosen to drill the wells, since this radius was the closest to the measured radius. Different well depths were drilled to create different volumes.

5.3.3.2. Melted Wells

A soldering iron was used to melt wells into one of the smaller solid POM polymer sheets. The shape and size of the tip of the soldering iron was rounded by hammering until it had approximately the same radius of curvature as the desired measurements. By applying the rounded tip of the soldering iron into the material and then turning the iron on and letting it heat up, four wells were created with minimal applied heat.

5.3.3.3. Force Molded Wells

Force molding of wells was preformed by using both rivets and ball bearings. The steel rivets consisted of a smooth cylindrical shaft with a larger round shaped head. The closest radius of the rivet head available was 2.5mm. To ease the well creation, the polymer and the rivets were preheated in boiling water for 10 minutes. The idea behind this pretreatment was to get near the glass transition temperature of the POM polymer and so ease the manufacturing process. Round and smooth rivets heads with the least manufacturing imperfections were chosen to create the four force molded wells. Wells were

created by hammering the rivet onto the POM polymer sheet, leave divots or impressions of the rivet head.

The same method was used to create the wells with ball bearings. Ball bearings are widely used in the industry where perfection is very important. All ball bearing beads are manufactured very precisely with as little imperfection as possible. We used these smooth, perfectly round chrome stainless steel ball bearings with a radius of 2.5mm to force mold four equal sized wells into the solid polymer by hammering the ball bearing onto the surface of the POM sheet. The ball bearings were placed onto the polymer sheet and secured with taped to prevent the ball bearings from moving and then slowly hammered until the desired well depth was reached. This process was repeated until the desired number of wells was created.

5.3.4. Optimized Hanging Droplet

A different approach on EB formation was to optimize the hanging drop method. The major flaws of the hanging drop method are that the drops are unstable on the inverted surface and the difficult technique of flipping easily causes losing of EBs. Three different design alternatives were created to optimize the existing method. The first design alternative increased the surface tension of the drop and inversion surface interface by creating a dent into the POM. Perfectly manufactured round chrome stainless steel ball bearings with a radius of 2.5mm were used to create the dent that stabilized the drops. This was done by hammering the ball bearings unto the POM surface, using the same technique as used in the creation of wells. A second design alternative to improve this design was also created using POM material. A pipette channel was installed, with a drill, above the stabilizing dent in order to gain access to the stabilizing dent without flipping the plate. The radius of the pipette channel was big enough to fit a 200ul pipette tip down to the dent. A micro screen was installed in between the interface the stabilizing dent and the pipette channel. The pore size of the metal micro screen was 200nm, which was bigger than the hMSCs to allow movement of hMSC cell suspension from the channel to the stabilizing dent. However, once the EB formed; it couldn't be sucked through the micro screen because of its larger size.

5.4. Design Testing

5.4.1. Electrospun Poly(trimethylene terephthalate) Wells

5.4.1.1. Material Characterization

The first information needed of the material was its average pore size. A Leica Microscope was used to analyze a 2cm by 2cm sample with 20X magnification. The image

analysis tool, Image J was used to determine the pore sizes. After converting the image quality to 8-bits, the threshold was adjusted so that the pores stood out from the fibers. Image J then automatically measured the pore sizes. Based on the pre-imposed scale of the image the number of pixels was determined and converted to how many pixels there were in 100nm. Using excel the average of the pore size was calculated. Similarly to the pore size analysis, the average fiber thickness was measured using Image J. After determining how many pixels there were on the pre-imposed scale (100nm in 20X) 20 different thicknesses were measured in pixels and converted to nm in Excel.

Next testing of the PTT polymer sheet was to determine what would happen if media with cells was placed on the polymer. Our hypothesis was that the polymer would be hydrophobic enough to repel the media and cells until EBs could be formed in the polymer wells. The electrospun PTT sheet from Biosurfaces was cut into two 1cm2 sections, both with different thicknesses. The thickness of each section was measured using a caliper (0.30mm and 0.85mm). A 20ul of the pink colored media DMEM was placed on each section and observed for 90 minutes on the bench top. Pictures of the before and after the placing the droplet were taken, as well as the pore size, fiber thickness and droplet diameter were measured. The pictures were analyzed using Photoshop.

To further investigate the role in the media absorbance a 1cm by 2cm polymer sheet was placed in a Petri dish with about 5ml of DMEM. The Petri dish was covered and the polymer sheet was left in it for three days. Each day the polymer sheet was checked for absorbance and degradation.

The most significant property of the electrospun PTT polymer sheets was the removal of the old media by applying suction from the outside of the wells, without touching the EBs or any cells resulting in a less number of lost EBs. Our hypothesis therefore was that the media could be aspirated from the outside while the cells were maintained within the wells. To test this, 200ul droplet of DMEM was placed on the PTT sheet and aspirated.

5.4.1.2. EB Evaluation

Sterilized Electrospun PTT well inserts were placed within wells of a standard 96-well plate. Each insert received a sample of hMSC cell suspension at a concentration of $1.5x10^4$ cells/20 µl. The 96-well plate was covered and incubated at 37° with 5% until day 3. Old medium was exchanged for fresh EB culture medium each day. [see Appendix 2]

5.4.2. Polyoxymethylene Wells

5.4.2.1. Unaltered Surface Adhesion Testing

To test the effects of unaltered POM on cell adhesion, hMSC cell suspensions were applied to a piece of POM and cultured. A cell suspension of P7 hMSC's at a concentration of $1x10^5$ cells/mL was prepared using hMSC culture medium. Two 500 µl samples of cell suspension were applied to the unaltered POM surface and incubated in a Petri dish at 37° with 5% CO_2 for two days. After two days the POM plate was stained with DAPI stain to analyze cell adhesion. [see Appendix 2]

5.4.2.2. Well Characterization

The wells created into the POM plates using the various methods described, were characterized using two techniques. The first technique was to physically take basic measurements of well depth, diameter, and estimated radius of curvature. The second technique involved the use of a Leica Microscope to examine the topography of the wells. The microscopic images were analysis to determine surface roughness and to observe surface imperfections.

5.4.2.2. EB Evaluation

POM well plates were first autoclaved to ensure sterility during the EB formation process. An hMSC suspension was prepared in EB culture medium. Due to the varying well sizes in each POM well plate, cell suspension concentrations also varied between $1.5x10^4$ cells/ 20-80 µl for each well. POM plates were incubated in a sterile Petri dish until day three. Old medium was exchanged for fresh EB culture medium each day. [see Appendix 2]

5.4.3. Optimized Hanging Droplet

5.4.3.1. Material Characterization

To confirm the pore size and fiber diameter of the micro screen, pictures were taken using a Leica microscope and analyzed using computer software. Once the channels were made into the optimized hanging drop design and the micro-screen secured in place, images where again taken. These images were analyzed to determine the topography of the channel and if the integrity of the screen was compromised during manufacture.

5.4.3.2. EB Evaluation

After sterilization of the POM plate a cell suspension of hMSCs was prepared using EB culture medium. Each droplet support received a cell suspension sample of 1.5x104 cells/20-80 µl. The POM plate was then flipped and placed upon the provided plate supports, producing hanging drops of cell suspension. The hanging drops were then incubated at 37°

with 5% until day 3. Old medium was exchanged for fresh EB culture medium each day. [see Appendix 2]

After sterilization of the POM plate a cell suspension of hMSCs was prepared using EB culture medium. Cell suspension was loaded through the top of each droplet support at a sample concentration of $1.5x10^4$ cells/20-80 µl. Hanging drops were allowed to incubate at 37° with 5% until day 3. Old medium was exchanged for fresh EB culture medium each day. Cell transfer after day three was accomplished by "shooting" EB formation media through the top of each well causing the droplet to drop into a chamber of a four-chamber microscope slide. [see Appendix 2]

5.4.4. Untreated Polystyrene Injection-molded Conical Tubes

The untreated polystyrene injection-molded conical tubes were first autoclaved to ensure sterility during the EB formation process. An hMSC suspension was prepared in EB culture medium. A cell suspension concentration of $1.5x10^4$ cells/20µl was added to each well. The conical tubes were then incubated in a sterile Petri dish until day three. Old medium was exchanged for fresh EB culture medium each day. [see Appendix 2]

5.4.5. NUNC™ Low Cell Binding Plates

The low cell binding plates were pre-sterilized and sealed in sterilized bags, these were left closed and opened only in the sterile laminar flow hood to ensure sterility during the EB formation process. An hMSC suspension was prepared in EB culture medium. A cell suspension concentration of $1.5x10^4$ cells/20µl was added to each well. The conical tubes were then incubated in a sterile Petri dish until day three. Old medium was exchanged for fresh EB culture medium each day. [see Appendix 2]

6. Results

6.1. PDGF Testing

Our hypothesis throughout the course of our experiments was that treatment of EBs with PDGF B would increase the percentage of migratory cells that expressed alpha sarcomeric actinin, a known cardiac marker. Initially we used canine cardiac tissue as a control to be certain that our staining conditions were suitable for our primary antibody (alpha sarcomeric actinin). To test our hypothesis, we plated EBs formed through the hanging drop method in both media with and without PDGF. After the EBs had been plated we used immunohistochemistry to examine expression of sarcomeric actinin. After the immunohistochemistry was performed, photomicrographs were taken of each EB found.

cells migrating away from EBs were counted and an analysis of the alpha sarcomeric actinin expression was performed.

6.1.1. PDGF Treatment of EBs Suggests an Increase in Migration of Cardiogenic Cells

6.1.1.1. Initial Testing of Alpha Sarcomeric Actinin Assay

Our hypothesis for this experiment was that we would be able to see a strong positive signal in control samples of canine cardiac tissue stained with alpha sarcomeric actinin, showing clear muscle striations, and no signal ina sample that was not treated with primary antibody. Figure 19 below shows the immunohistochemical results for the preliminary experiment. From these photomicrographs we were able to conclude that our staining conditions were suitable for the obtaining a positive stain.

A.) **B.)**

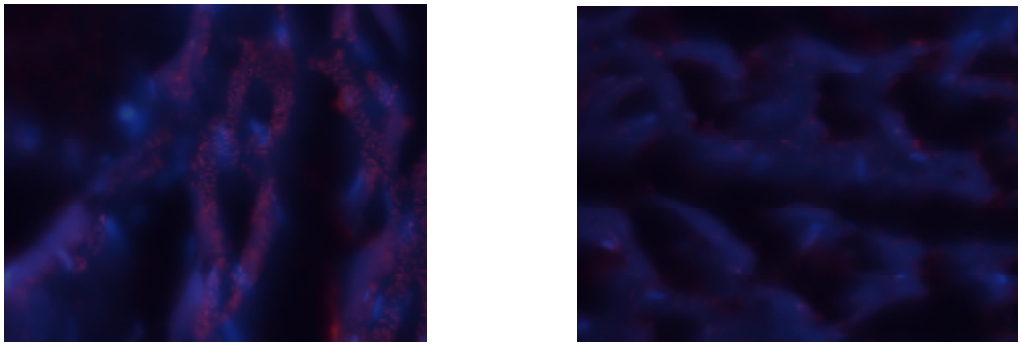

Figure 19: Immunohistochemical Results for the Preliminary Experiment. Control experiment showing cardiac marker alpha actinin as expressed in canine cardiac tissue. Panel A.) shows a sample of canine cardiac tissue that was treated with our primary antibody (alpha actini). Panel B.) shows a sample of canine cardiac tissue that was not treated with the primary antibody. Both tissue samples were treated with secondary antibody and the Quantum Dot marker. Both images were taken at a 40x magnification.

6.1.1.2. Experimental Overview

The cells were segregated into two groups, those that would be used as controls (traditionally cultured hMSCs) and the cells that would be hung to form our EBs. Both groups were given the same time course in which to proliferate and both were fed daily. After the third straight day of changing the media, as explained in the methods, the EBs were plated. While hanging the EBs the control hMSCs were cultured in the standard fashion for control purposes. Both EBs and the control hMSCs were plated into the four-well

chamber slides on the third day as described in the methods section. Figure 20 below diagrams the time structure of our experiment. We were able to conclude that our conditions were suitable for forming EBs for further experimentation.

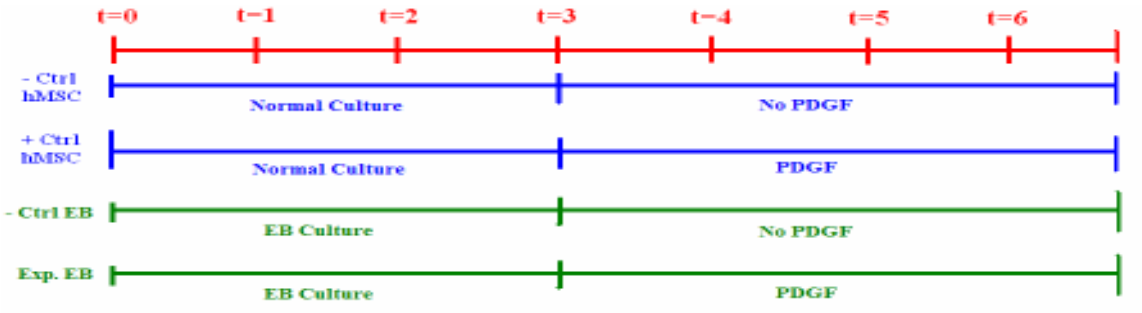

Figure 20: Time Structure for Experimentation. This figure shows the time course of our experiment, the top red bar is indicative of the time in days. The two blue bars show the experiment for our hMSC controls both positive and negative. The green bars show our EB experiment. For an in depth look into the culture media please see our methods section

6.1.1.3. Testing the Effect of PDGF on Cardiogenic Cell Formation

We hypothesized that there would be an increased alpha sarcromeric actinin signal seen in the cells migrating from the EBs treated with PDGF verses the EBs that had been plated in untreated media. The results of our immunohistochemistry are shown below in Figure 21. We observed that the hMSCs in both the treated and untreated media would have no cardiogenic response because there was no spontaneous differentiation without the formation of an EB.

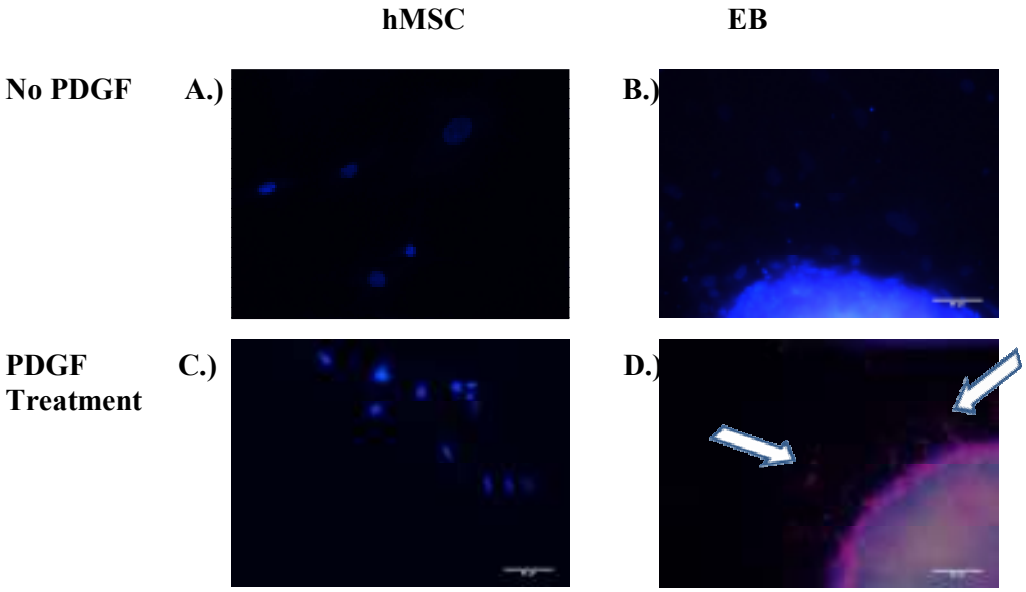

Figure 21: Immunohistochemistry Results of EB Treatment with PDGF. A comparison of the immunohistochemical response of hMSCs and EBs from both the PDGF treated and untreated media. Panel A.) shows hMSCs cultured from untreated media. Panel B.) shows an EB cultured from untreated media. Panel C.) shows hMSCs that were harvested from media treated with PDGF. Panel D.) shows an EB that was cultured from media treated with PDGF. All the pictures shown here were taken at a 40x magnification. The scale bar represents 50um.

6.1.1.4. Photographic Analysis

The hypothesis for this experiment was that in analyzing the number of migrating cells from an EB, we would find that a greater percentage of these cells would express alpha sarcomeric actinin if the EB had undergone the PDGF treatment while plated. Each EB was categorized first as a "full EB" meaning that the EB had remained intact or as a "void EB" referring the ring of cells that had been left behind after an EB had been lost (see Methods for details). In the case of the "full EBs" a picture was taken from each of the four quadrants (top right, top left, bottom right, and bottom left). For the "void EBs" a single picture was taken of the largest cluster of cells. The pictures were then analyzed and the number of cells migrating away from the EBs was counted. A percentage was then calculated showing how many of the total migratory cells were positive for alpha actinin.

Throughout the course of the experiment there was a problem with cell adhesion in both the PDGF treated and untreated EBs. This accounts for the low "n" number as wells as the previously explained void EBs (the ring of cells left behind after an EB is washed away). The EBs that were found however, were all comparable in size, treated and untreated.

Table 9 below shows the results of our raw data both numerically and graphically. The average percentage of cardiogenic cells was about 16% in EBs treated with PDGF, but only 8% in the EBs from the untreated media. These data suggested that treating the EBs with a PDGF supplemented media gave two fold the yield of cells expressing the cardiogenic marker alpha actinin then the EBs that were cultured from the untreated media. However, a statistical analysis showed that our data was not significant. We preformed a "type three two tailed T test" on our cumulative data as can be seen in Figure 22 below the data is depicted both numerically and graphically. Our cumulative data shows that our average percent of cardiac expressing cells did increase as fore mentioned but the results for our T test gave us a value of 0.078 a value not considered to be statistically significant ($p<0.05$). From the statistical analysis of our data we were not able to conclude that PDGF decisively increases the percentage of cardiac-like cells migrating away from an EB. However; due to the above

mentioned problems with EB adherence we feel that to come as close as we did to statistical significance with such a small “n” value suggest a trend in the data. The treatment of EBs with PDGF B is likely to, with further experimentation; prove to be a means by which a greater percentage of cardiogenic-like cells can be generated from EB formation.

Table 9: PDGF Treatment on Expression of alpha sarcomeric actinin

Without PDGF	Positive Cells*	Negative Cells	Total Cells	Percent +/Tot	Approximate EB size
EB 1	2	47	49	0.041	300 um
EB 2	14	37	51	0.275	350 um
Void EB 1	0	34	34	0	300 um
Void EB 2	0	31	31	0	250 um
Void EB 3	5	35	40	0.125	300 um
Void EB 4	6	30	36	0.167	250 um
Void EB 5	1	17	18	0.056	300 um
Void EB 6	0	9	9	0	300 um
Avg #'s	3.5	30	33.5	0.083	293.75 um
With PDGF	Positive Cells	Negative Cells	Total Cells	Percent +/Tot	Approximate EB size
EB 1	6	59	65	0.092	300 um
EB 2	19	53	72	0.264	350 um
Void EB 1	5	14	19	0.263	350 um
Void EB 2	2	10	12	0.166	250 um
Void EB 3	3	13	16	0.188	350 um
Void EB 4	1	5	6	0.166	200 um
Void EB 5	0	12	12	0	250 um
Void EB 6	1	10	11	0.09	250 um
Void EB 7	1	3	4	0.25	200 um
Avg #'s	4.22	19.88	24.11	0.164	277.77 um

*positive cells are cells the were found to express alpha sarcomeric actinin outside of the nucleus of the cell

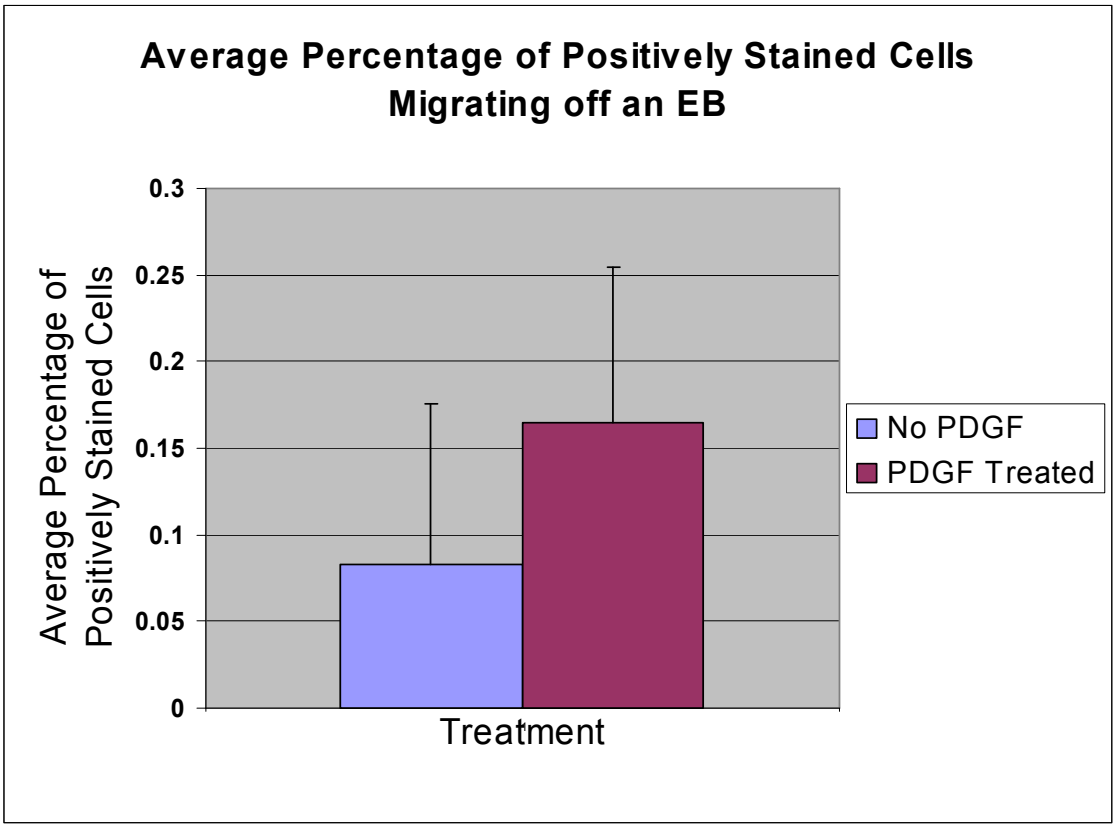

Figure 22: PDGF Treatment Graphical Analysis. This is the graphical representation of this data showing a comparison of the two averages of the expression of alpha sarcomeric actinin for cell migrating off of the EBs.

6.2. Design Testing

6.2.1. Curvature Analysis

The following table, Table 11, represents the summarized results from the curvature analysis of 20ul and 50ul droplets formed in the hang drop method of EB formation. This table gives mean values and standard deviations for the analysis of a 20ul droplet, a 50ul droplet, and the exact value for a half-sphere with a volume of 20ul and 50ul for comparison.

Table 10: Curvature Analysis

	Radius of Curvature (mm)	Height of Droplet (mm)
20ul drop, n=22	2.76 ±0.32 *	1.71 ±0.23
50ul drop, n=11	3.75 ±0.77 *	2.29 ±0.23
20ul Half-Sphere	2.12	

* Radius of curvature was determined from a fit perfect circle using photoshop software

The table that follows, Table 12, is a summary of the raw data gathered from the curvature analysis of 20ul and 50ul droplets formed in the hanging drop method of EB formation. [see Appendix 3] Shown in the table is a mean radius of curvature from a fit perfect circle, the standard deviations, the percent deviations, and the sample population size for a 20ul droplet and 50ul droplet.

Table 11: Summary of Raw Data for Curvature Analysis

20ul Droplets	**Radius of Curvature* (mm)**	**Height of Droplets (mm)**
Mean	2.76	1.71
StDev	0.320	0.237
CV%	11.6%	13.9%
n=22		
50ul Droplets		
Mean	3.75	2.29
StDev	0.773	0.234
CV%	20.6%	10.2%
n=11		

* Radius of curvature was determined from a fit perfect circle using photoshop software

6.2.2. Electrospun Poly(trimethylene terephthalate) Wells

The following section represents the results of the electrospun Poly(trimethylene terephthalate) – PTT and the various test performed, such as microscopic and macroscopic observations, absorbance testing and aspiration testing. In the observations a Leica Microscope was used.

6.2.2.1. Microscopic and Macroscopic Observations

The table below summarizes the results of the average thickness, average pore size, average fiber thickness of the electrospun PTT that has been calculated using image analysis tool Image J and analyzed using MS Excel.

Table 12: PTT Measurements

Average Thickness	0.60 mm
Average Pore Size	39.5 µm
Average Fiber Thickness	3.2 µm

6.2.2.2. Absorbance of PTT

The following table illustrates the results of the absorbance testing of the PTT polymer, where 20ul drops of media were placed on two $1cm^2$ sheets of PTT and were left on the bench top for 90 minutes. After 90 minutes the media got absorbed into the 3-D polymer matrix.

Table 13: Absorbance Results

Sample Size	Sample Thickness	Drop Size	Total Time	Fiber Thickness	Stain diameter
$1cm^2$	0.30mm	20ul	90 mins	3.2 µm	4.48 mm
$1cm^2$	0.85mm	20ul	90 mins	5.26 µm	3.60 mm

A) 

20ul Drop on 0.30mm PTT

B) 

20ul Drop on 0.85mm PTT

C) 

Absorption of media into the matrix (10X)

Figure 23: Absorbance Analysis of PTT. Panel A) and B) show the 20ul drops initially repelled on the PTT polymer sheet. The media was more repelled on the thicker fibers than on thinner sheet. After 90 minutes the media was partially absorbed and partially evaporated, leaving a red stain behind. The stain of the thicker fibers was smaller in diameter. Panel C) shows the fibers and the stain in 10X magnification.

6.2.2.3. Absorbance of PTT

The aspiration of the media was determined by placing a 200ul drop on a PTT polymer sheet and aspirating it with a common lab aspirator. The media was removed within 2 seconds, which proved the concept of pulling media through PTT matrix.

6.2.2.4. PTT EB Formation

The results from EB formation analysis in the electrospun wells can be seen in Table 15. The media was absorbed into the polymer fiber matrix within the first couple of hours of evaluation and no EB formation was observed.

Table 14: EB formation within the electrospun PTT wells

Observations From Experiment 202 - Electrospun Polytrimethylene Terephthalate (PTT) Heat Molded Wells				
Well #	Day One	Day Two	Day Three	Results
1	No EB growth	No EB growth, Media was absorbed into the electrospun matrix	No EB growth, media was dry after being absorbed into the electrospun matrix	No EB growth after three days, Media was absorbed into the electrospun matrix by the second day
2	No EB growth	No EB growth, Media was absorbed into the electrospun matrix	No EB growth, media was dry after being absorbed into the electrospun matrix	No EB growth after three days, Media was absorbed into the electrospun matrix by the second day
3	No EB growth	No EB growth, 20ul was added to the well to feed the cells	No EB growth	No EB growth after three days

6.2.3. Polyoxymethylene Wells

6.2.3.1. Result Unaltered POM Adhesion Testing

To test the surface characteristics of unaltered smooth POM, 50,000 hMSCs were plated. HMSCs did not attach to the POM sheet and formed little aggregates that could be view under the microscope by the second day. DAPI staining showed no aggregations of cells on to the unaltered POM surface.

Table 15: Results of the unaltered POM test

Droplet #	Cell Concentration	Passage #	Media Amount (ul)	Observations
1	50,000	7	80	No cell adherence
2	50,000	7	80	No cell adherence, small aggregates formed
3	50,000	7	1000	No cell adherence, small aggregates formed
4	50,000	7	1000	No cell adherence, small aggregates formed

6.2.3.2. Drilled Wells

Eight wells with 6mm diameter were drilled into POM. The well depth was varying from 1.65mm to 3.34mm. The resulted wells had rough surface topography. Table 14, below, summarizes the created wells and the corresponding depths and radius of curvature.

Table 16: Sizes of the Drilled Wells

Well #	Depth (mm)	Diameter (mm)	Cell Concentration (cells/20ul)	Passage #	Media Amount (ul)
1	1.65	6	7500	6	40
2	1.65	6	7500	6	40
3	2.8	6	7500	6	40
4	2.78	6	7500	6	40
5	2.8	6	7500	6	40
6	2.76	6	7500	6	40
7	3.5	6	7500	6	40
8	3.34	6	7500	6	40

Figure 23 below shows the rough topography of the drilled well due to manufacturing issues. After drilling the smooth POM surface has been altered creating a undesired rough surface.

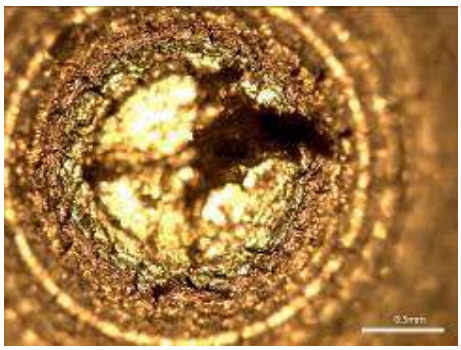

Figure 24: Drilled POM Well Surface Topography

The results from testing EB formation within the drilled wells can be seen in Table 18. There was no observed EB formation in any of the drilled wells tested.

Table 17: EB formation in the drilled wells

Observations From Polyoxymethylene (POM) Drilled Wells				
Well #	Day One	Day Two	Day Three	Results
1	No EB Growth	No EB Growth, 20ul added, when media was added a swirling of color was observed indicating cells producing waste.	No EB growth	No EB growth was observed, no contamination was observed
2	No EB Growth	No EB Growth, 20ul added, when media was added a swirling of color was observed indicating cells producing waste.	No EB growth	No EB growth was observed, no contamination was observed
3	No EB Growth	No EB Growth, 20ul added, when media was added a swirling of color was observed indicating cells producing waste.	No EB growth	No EB growth was observed, no contamination was observed

4	No EB Growth	No EB Growth, 20ul added, when media was added a swirling of color was observed indicating cells producing waste.	No EB growth	No EB growth was observed, no contamination was observed
5	No EB Growth	No EB Growth, 20ul added, when media was added a swirling of color was observed indicating cells producing waste.	No EB growth	No EB growth was observed, no contamination was observed
6	No EB Growth	No EB Growth, 20ul added, when media was added a swirling of color was observed indicating cells producing waste.	No EB growth	No EB growth was observed, no contamination was observed
7	No EB Growth	No EB Growth, 20ul added, when media was added a swirling of color was observed indicating cells producing waste.	No EB growth	No EB growth was observed, no contamination was observed
8	No EB Growth	No EB Growth, 20ul added, when media was added a swirling of color was observed indicating cells producing waste.	No EB growth	No EB growth was observed, no contamination was observed

6.2.3.3. Melted Wells

A soldering iron with a diameter of 3mm round shaped tip was used to create 4 wells into POM. The hot tip heated the material too much resulting in bubbles within the wells. This extra rough well topography was not plated with hMSCs, due to the massive imperfections. Figure 24, below, is a representation of one of the melted wells.

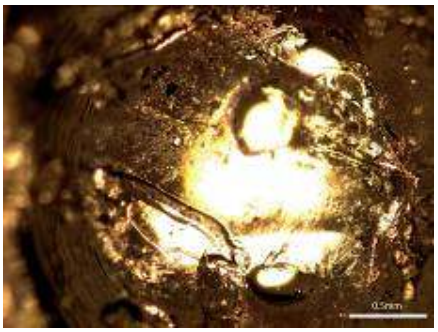

Figure 25: Melted POM Well with Surface Imperfections

6.2.3.4. Force Molded Wells

Rivets and ball bearings with a radius of 2.5mm were used to create smooth wells into POM. The POM sheet was pre-treated in boiling water for 10 minutes, to ease the well creation. A total of 4 riveted wells and 3 ball bearing wells were created. The smoothest well topography was achieved with the ball bearings, since their surface had the least imperfections. No EBs were formed within the wells. After the culturing of hMSCs the surface of the wells were stained with Hoechst Staining. The imperfections created by the rivets resulted in micro-cracks, allowing hMSCs to adhere. Very little cells were attached in the

wells created with ball bearings. The data generated from this evaluation can be seen in Table 19.

Table 18: Comparison of rivet and ball bearing wells

Rivets				
	Well Depth	Radius of curvature (mm)	Surface Topography	Hoechst Staining
1	XX	2.5	Micro-cracks	Some cells
2	XX	2.5	Micro-cracks	Some cells
3	XX	2.5	Micro-cracks	Some cells
4	XX	2.5	Micro-cracks	Some cells
Ball Bearings				
1	XX	2.5	Smooth	Some Cells
2	XX	2.5	Smooth	No Cells
3	XX	2.5	Smooth	No Cells

The results from EB formation within the force molded wells can be seen in Table 20. There was no EB formation observed in any of the wells.

Table 19: EB formation within force molded wells

Observations From Polyoxymethylene (POM) Force Molded Wells				
Well #	Day One	Day Two	Day Three	Results
1	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed
2	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed
3	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed
4	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed

6.2.3. Optimized Hanging Drop

The metal micro-screen at the interface of the pipette channel and the dent for the droplet support was analyzed to verify that the hMSCs were small enough to pass through when they were implanted. Pictures of the micro-screen were taken. The pore size of the micro-screen was 200um. The size of the hMSCs was small enough to pass trough the mesh. Figure 25 shows the micro-screen, which allowed the hMSCs to go through, but prevented EB to pass through once they formed.

Figure 26: Micro-screen Interface. Picture of the micro-screen with pore size of 200um (5X)
The EB formation results, using the optimized hanging drop method, can be seen in Table 21. No EB formation was observed in any of the wells tested.

Table 20: EB formation within Optimized Hanging Drop

Observations From Optimized Hanging Droplet				
Droplet #	Day One	Day Two	Day Three	Results
1	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed
2	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed
3	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed
4	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed
5	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed
6	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed
7	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed
8	No EB growth	No EB growth, 20ul of media was added to feed the cells	No EB growth, 20ul of media was added to feed the cells	No EBs were formed

6.2.5. Untreated Polystyrene PCR Conical Tubes

Ebs formation within untreated polystyrene PCR tubes was also attempted. The results from these experiments can be seen in Table 22.

Table 21: EB formation within untreated polystyrene PCR tubes.

Observations From PCR Tube Experimentation.				
Tube #	Day One	Day Two	Day Three	Results
1	EB Formed	EB Fomred, 200 ul of fresh media was added.	EB Fomred, 200 ul of fresh media was added.	EB Formed
2	EB Formed	EB Fomred, 200 ul of fresh media was added.	EB Fomred, 200 ul of fresh media was added.	EB Formed
3	EB Formed	EB Fomred, 200 ul of fresh media was added.	EB Fomred, 200 ul of fresh media was added.	EB Formed
4	EB Formed	EB Fomred, 200 ul of fresh media was added.	EB Fomred, 200 ul of fresh media was added.	EB Formed
5	EB Formed	EB Fomred, 200 ul of fresh media was added.	EB Fomred, 200 ul of fresh media was added.	EB Formed
6	EB Formed	EB Fomred, 200 ul of fresh media was added.	EB Fomred, 200 ul of fresh media was added.	EB Formed
7	No EB	No EB, 100 ul of fresh media was added to feed cells.	No EB, 100 ul of fresh media was added to feed cells.	No EB
8	No EB	No EB, 100 ul of fresh media was added to feed cells.	No EB, 100 ul of fresh media was added to feed cells.	No EB
9	No EB	No EB, 100 ul of fresh media was added to feed cells.	No EB, 100 ul of fresh media was added to feed cells.	No EB

6.2.6. NUNC™ Low Cell Binding Plates

Low cell binding NUNC™ plates were also used in an attempt to grow EBs in well structures. 100ul of hMSC cell suspension was added to 40 wells and let incubate for 3 days to observe if EBs were formed. The results can be seen in Table 23. At the end of day three multiple fractionated EBs formed in each of the 40 wells.

Table 22: EB formation within NUNC™ Low Cell Binding Plates.

Observations From NUNC Plates.				
Well #	Day One	Day Two	Day Three	Results
1	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
2	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
3	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
4	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
5	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
6	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed

7	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
8	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
9	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
10	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
11	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
12	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
13	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
14	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
15	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
16	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
17	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
18	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
19	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
20	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
21	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
22	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
23	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
24	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
25	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed

26	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
27	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
28	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
29	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
30	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
31	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
32	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
33	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
34	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
35	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
36	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
37	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
38	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
39	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed
40	Multiple, Fractionated Ebs Formed	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple, Fractionated Ebs Formed; 100 ul of media was exchanged.	Multiple EBs Formed

7. Analysis and Discussion

7.1. Design Testing

7.1.1. Curvature Analysis

The design team had determined that reproducing droplet shape was essential to adequately mimic the hanging droplet method with non-adhesive polymer wells. Using

imaging techniques the radius of curvature for two common droplet volumes used in EB formation were found. The radius of curvature for an average 20ul hanging droplet was determined to be 2.76 ±0.32 mm, where as the radius of curvature for an average 50ul droplet was determine to be 3.75 ±0.77 mm. These two curvatures have been proven to form EBs when applied as hanging drops even though there is a high degree of variation between the curvatures of each.

However, the radius of curvature of the 20ul drop differed compared to the radius of curvature of a perfect half sphere with a volume of 20ul, which mathematically is 2.12 mm. The radius of curvature found by the design team was much larger than the predicted valve, but this can be explained by looking at the average height, the distance the droplet hangs away from the surface, determined for an average 20ul droplet. This height was found to be 1.71 ±0.23 mm, which differs from a perfect 20ul volume half sphere where the height would be 2.12 mm. The height of the hanging drop found by our analysis is much less than what was predicted. This means that the droplet is spreading out on the lid, both increasing the radius of curvature and decreasing the height of the droplet.

The reproduction of this curvature and height was difficult due to the small values determined from the droplet analysis. Different techniques were used for the various well designs, but the radius of curvature usually used was 2.5 mm. This curvature was easier to reproduce using various techniques and still fell within the standard deviation of the average radius of curvature found by the design team for the 20ul drop. Also, the imaging technique used to determine the radius of the 20ul hanging droplet involved fitting a perfect circle to the droplet. From the observations of the hanging droplets it was seen that not all droplet curvatures were perfect circles. Some droplets were more elliptical shaped which would result in a shaper curvature. However, it was decided that the perfect-fit radius of curvature of 2.5 mm would be used for experimentation.

7.1.2. Electrospun Poly(trimethylene terephthalate) Polymer

Creating polymer fibers in the nanometer scale, known as electrospinning is gaining importance in the field of biomedical engineering. By electrically charging the polymer solution and depositing fibers in the nanometer scale on a ground collecting plate a 3-D scaffold can be created. The random deposition of the nanofibers ranging from 10-2000nm in diameter creates pore sizes with various polymers. The pore size and fiber thickness can be altered by varying parameters such as viscosity and temperature of the polymer solution, as described earlier in the background section. Biosurfaces, a company in Ashland, MA kindly provided us with a 4” by 6” electrospun sheet of Polytrimethylene Terephthalate (PTT). The thickness varied from 0.28mm to 0.85mm. The average pore size of 39.5nm was small enough to aspirate media through the matrix without removing the EBs. Considering that the

average size of the EBs was around 300-400nm, the EBs wouldn't fall into the fiber matrix and so reduce the number of EBs lost.

The reason for choosing PTT polymer was its hydrophobic properties, supporting the non-adhesive material concept. Heat sinking method was used to create wells with the radius of curvature of a 20ul hanging drop. The heat sinking device consisted of two metal sheets, with shapes and sizes of the drops. The metal sheets were heated with an iron and pressed until the polymer sheet took the desired shape. The temperature of the metal sheets and the amount of applied force varied. The purpose was stretching the fibers while giving the desired shape, so that once the material cooled; it would keep the given shape. Another way to create wells was electrospinning the polymer on the back of a plate with the well shapes, which serves a negative impression.

The next step was testing EB formation by culturing 15,000 hMSCs within the wells. After the first day, the media soaked into the matrix, drying out the cells and leaving them without nutrition and killing them. No EBs formed. To investigate the reason the polymer was tested for absorbance and evaporation. A 20ul drop was placed on the polymer sheet and observed until complete absorption. At the beginning the material showed its hydrophobic properties and the drop was repelled. After 30 minutes the drop lost its round shape. At the end of 90 minutes the drop was completely absorbed by the polymer matrix and a round stain of the drop was left. After analyzing the polymer under a microscope, we concluded that even though the material acted highly hydrophobic, the pores in the matrix caused the absorbance. A possible way to reduce the absorption may be by pre-treating the polymer with phosphate buffered saline (PBS).

The PTT well designs were advantageous in reducing the number of lost EBs by growing the EBs in a well, thus eliminating the flipping process and eliminating the pipetting of EBs by aspirating the old media through the matrix. A suggestion to improve this design would be electrospinning thicker fibers that result in smaller pore sizes, but still allow media aspiration through the matrix. This design would work if the absorption can be eliminated.

7.1.3. Polyoxymethylene Polymer

Solid well designs were considered once the electrospinning design didn't work. Instead of creating wells made out of electrospun fibers, solid polymer sheets were used. A clear 6" by 12" by 3/8" Polyoxymethylene (POM) polymer sheet was purchased from Small Parts Inc. POM was initially used in bioreactor designs and was known for its non-adhesive properties and non-toxicity. To verify that the polymer shows non-adhesive and non-toxic properties with hMSCs, first an unaltered POM sheet was plated with 50,000 hMSCs for cell attachment. After culturing for three days the surface was stained with Hoechst dye, where

no cells were seen. The next step after proving the non-adhesiveness of the polymer was creating wells with the radius of curvature as calculated in the results section.

Four different methods were used to create the wells: drilling, melting and force molding with rivets and ball bearings. First method was drilling wells into the solid polymer with a radius of 3mm. Different well depths were drilled to fit a 20ul of cell suspension. The drilled wells looked not as smooth once they were machined. No EBs formed in the drilled wells, which may be due to the rough surface created by the drilling. It created microscopic and macroscopically visible rough layers. Next method by which wells were created was melting the solid polymer with a soldering iron. The tip of the soldering iron had a radius of 2.5mm. Unfortunately the temperature of the soldering iron was too intense and bubbles were seen within the wells. We determined that the rough imperfections were too severe and did not perform an EB formation test.

Since the first manufacturing methods did not work, we tried to create wells with the least imperfections possible. Rivets had a head size of 2.5mm in radius. Round and homogenous rivets were chosen to create the wells by hammering them into the polymer sheet. Pre-treating POM and the rivets in boiling water to reach POM's glass transition temperature eased the creation of the wells. The resulting wells were much smoother than the machined wells. However, no EBs could form. This may be due to the imperfections on the rivet heads, which created a bad negative imprint on the polymer. Cell colonies were seen in the micro cracks of the riveted wells when stained with Hoechst dye.

Ball bearings were manufactured very precisely for precision tools and had the smoothest surface out of the four methods. The perfectly round ball bearings with a diameter of 2.5mm were hammered into the polymer, creating an imprint on the POM. Since the ball bearings had a smooth surface, the created imprint had the least imperfections. The hammered ball bearings did not seem to alter the properties of the POM. EB formation was tested in the four created wells, but none was formed. To understand the underlying problem, the wells were stained using Hoechst dye, where no cells attachment was seen. The reason to why no EBs formed could not be found. A possible reason may be the radius of curvature.

7.1.4. Untreated Polystyrene Conical Tubes and Low Cell Attachment Plates

It was determined that the manufacturing capabilities available to the design team were not precise enough to produce adequate wells. However, the design team was convinced that EBs could be grown within a non-adhesive well structure. To test this hypothesis further it was decided that the formation of EBs would be evaluated in pre-manufactured materials.

Earlier in the design process the design team had thought about using untreated polystyrene conical tubes, used in PCR tests, to create EBs. This idea was dismissed at the

time because the curvature of the conical tubes could not be altered to fit the curvature of a hanging drop. However, these conical tubes were pre-manufactured by injection molding and had good surface characteristics. The surface was very smooth and hydrophobic with very few imperfections. The conical tube was also capable of holding the correct volume of cell suspension, able to be sterilized, had a cover to help maintain sterility, and the material, polystyrene, has a long history of being used in cell culture. The failure of the previous designs due to inadequate manufacturing techniques made these conical tubes a perfect candidate for proving the concept of EB formation in non-adhesive wells.

The design team decided that not being able to alter the conical tube radius of curvature to mimic the hanging drop radius of curvature was an acceptable trade off for good manufactured surface characteristics. To determine the radius of curvature of the conical tubes in order to compare it to the hanging droplet curvature the bottom curvature of the tubes was imaged using the same technique as was applied to the hanging droplets. The average radius of curvature at the bottom of the conical tubes was determined to be approximately around 1mm. This was smaller than the radius of curvature used for the previous designs. The conical tubes were also v-shaped which resulted in a much steeper well.

Initial testing was then performed on three sterilized conical tubes to determine if EBs would form. The technique used to test these tubes was similar to the techniques used to test the previous design. It involved placing a cell suspension containing media and hMSCs into the tubes and then incubating them for 3 days. After each consecutive day the old media was removed from the tubes and fresh media was added. This test resulted in the formation of EBs in each of the conical tubes. These EBs had good morphology, they were homogeneous, and they were produced in the same amount of time as EBs produced using the hanging droplet. However, this initial test was designed to just determine if EBs could be produced and due to time constrictions these EBs were not further examined with staining.

This first test had shown that EBs could be formed in a non-adhesive wells, so two follow-up tests were performed in the conical tubes to further evaluate the EBs that were formed using this method. These two tests were the same as the previous testing; a cell suspension containing hMSCs would be put into three conical tubes and would be incubated for three days with media change after each consecutive day. The first round of this testing gave good results and the three EBs taken from this experiment were stained for further examination, but contradictory results were seen in the second test. The three conical tubes tested in the second test did not produce any EBs, opposite of what had been seen in the first two tests.

It was unclear why this last round of testing did not produce an EBs. Two possible reasons behind no EB formation in the three conical tubes in this experiment are passage

number and contamination. The first two tests on the conical tubes were done with hMSCs with a passage number of seven and eight, respectively. The last test was done on hMSCs with a passage number of ten, higher than previously used. As the passage number on the cultured hMSCs increases it has been shown by our lab that there is a loss in pluripotency, or the “steminess” of the cells. A higher passage number may also affect the ability of the hMSCs to form EBs in a non-adhesive well. However, during this round of testing there were also problems with contamination of other experiments in the same incubator. The sample within the conical tubes did not appear to be contaminated, but the small volume of media used may have hid that fact that they were contaminated.

However, these experiments rejected the null hypothesis that EBs could not be formed in non-adhesive wells and proved the hypothesis that EBs could be formed in a non-adhesive environment. Six out of the nine conical tubes formed EBs with a homogeneous shape and good morphology during experimentation, which gave this method a success rate of 66%. The three EBs formed from hMSCs with a passage number of eight in the second experiment where then further analyzed by being plated in four chamber slides. These EBs attached very well to the surface of the slide and the dispersion pattern of cell that migrated off of the EB was very good. This further showed that the homogenous EB produced in the conical tubes were of good quality.

To further examine the hypothesis of the forming of EBs within non-adhesive wells, the design team decided to purchase and experiment of low cell binding plates, a new product found by the design team three-fourths way through the project. These plates trademarked as NUNC™ low cell binding (LCB) plates are composed of a standard u-bottom 96-well plate coated in a polymer presenting a phosphorylcholine moiety to the cells. The coating mimics the cell membrane and prevents cells and proteins from attaching. This design is very similar to the conceptual design proposed by the design team during the conceptual design phase of the engineering process. At the time the design team was unaware of this new product and based on time and budget constraints compounded by limited manufacturing capabilities it was determined that creation and evaluation of this design would not be a feasible. However, due to the availability of this pre-manufactured low cell binding plates, distributed by NUNC™ a part of Thermo Fisher Scientific Inc., priced within the constraints of the remaining budget, it was determined that these plates could be used to prove the hypothesis that EBs can be formed in non-adhesive wells.

Time constrictions only allowed for one round of experimentation to be preformed on the NUNC™ LCB plates. The experimentation followed the same standard procedure used in all the non-adhesive well evaluation; a cell suspension containing hMSCs would be put into the wells and would be incubated for three days with media change after each consecutive day. In this experimentation 40 of the 96-wells on one NUNC™ LCB plate were

filled with a cell suspension to evaluate EB formation. This experiment resulted in EB formation within all 40 of the wells used. However, it was observed on the first and second day of the experiment that the EBs were not forming efficiently. Instead of forming tight three-dimensional structures they were forming clouds that were more dense in different locations. By the third day these clouds have turned into EBs, however, there were multiple EBs of different sizes in all of the wells. Some of the EBs, a little more than half, were not of good quality compared to those produced in the hanging drop method or in the PCR conical tubes.

It was unclear as to why multiple EBs formed within all of the 40 wells tested. Contamination was ruled out due to visual observations of the wells and the fact that EBs did form and a high passage number was not used for the experiment. One possible reason may have been due to the larger curvature of the NUNC™ LBC plates with the u-bottom shape. From examination of the bottom curvature, using the same technique as used to determine hanging drop and conical tube curvatures, it was found that the radius of curvature on the bottom of the NUNC™ LBC plates was approximately 4 mm with a height of approximately 4 mm. This radius of curvature was almost twice that of the curvature found for a average 20ul hanging drop and well of twice the size of the conical tubes. Another possible reason for fractured EB formation with the NUNC LBC plates could be due to imperfections in the coating. If the wells were not precisely manufactured micro-imperfects may have stopped the aggregation of the EBs towards the center of the well causing the formation of variable EBs in different locations within the well.

7.1.5. Optimized Hanging Droplet

Designs to optimize the hanging drop method were developed and evaluated by the design team in parallel to the development and evaluation of the non-adhesive well designs. These designs focused on reducing the limitations associated with the hanging droplet method such as maintenance time, droplet instability, and plate inversion. The first of the designs tested was the top-loading droplet support with micro-screen.

This design was developed to stabilize the hanging droplet, eliminate the need to flip the hanging surface to create the hanging droplet, and eliminate the occurrence of accidental EB removal during media change. To evaluate the design a cell suspension containing hMSCs was injected with a pipette through a micro-pore screen into the droplet support well from a channel above the well. This device contained three droplet supports capable of holding one drop each. All three of the droplet supports were filled according to the procedure previously stated and allowed to incubate for three days. Media change was done on the second day by removing the media through the channel above the droplet support and replacing fresh media the same way.

This experiment resulted in the formation of no EBs after three days of incubation and media change. Also, difficulties and limitations of the device were observed while performing the procedure. The micro-pore screen, while providing a way to prevent EBs from being remove from the droplet support during media change, allowed media to enter the channel used to fill and empties the device. This made it difficult for the user to observe if there was any EB formation within the droplet support. As for the lack of EB formation, it was unclear why EBs did not form in the hanging drop within the droplet support.

The cell suspension that was removed from the device during media changing was kept and plated on four chamber slides. After fixation and hoescht staining the slides and their contents were observed using a microscope. Cells could be identified on the slide and appeared to be healthy. This eliminated the possibility that the materials used in the design were killing or harming the cells, and showed that many of the cell remained in suspension while in the device. It was still unclear as to why the cells did not aggregate in suspension and form an EB. We speculate that it may be due to the surface roughness on the pipette channel or micro-screen that prevented appropriate aggregation events.

The second design composed of only droplet supports was developed and evaluated to better understand why there was no EB formation within the top-loading device. This evaluation was done similarly to the normal hanging drop method; a cell suspension containing hMSCs was added to droplet supports, divots on a flat surface, and then the surface was inverted causing the droplets to hang. This device was then incubated for three days with media change of the droplets every consecutive day. However, due to complications from contamination issues in the incubator, this experiment was scrapped and no results were obtained. Then due to time constraints no further experiments could be preformed on this device.

7.2. PDGF Testing

7.2.1. Preliminary Observations

It was our hypothesis that EBs treated with a PDGF B enhanced media would increase the percentage of migratory cells that expressed our primary marker, (alpha sarcomeric actinin) a known cardiac marker. Preliminary results suggested that PDGF increases the percentage of cardiogenic cells migrating from an EB. However; due to a limited number of experimental runs we are unable to conclude this result decisively.

Our low number of experimental samples made it difficult to conclusively see whether or not our PDGF treatment impacted the number of cells migrating form the EB. Our P value from the raw data set was 0.078. To achieve a resulting p value as statistically close to significance as we did with this low “n” allows us to further hypothesis that our preliminary

results suggest a trend in the data that will, with further experimentation, lend itself to a significant value.

Through the course of our experimentation we were able to decisively conclude that the PDGF treatment did not alter the state of the hMSCs, without the intermediate step of embryoid body formation. This result told us that the PDGF treatment did not spontaneously differentiate the hMSCs, further reinforcing the theory that EB formation is a critical step for the generation of cardiac myocytes.

7.2.2. Experimental Difficulties

When examining the EBs we found that they could be sub-categorized into two types, as was mentioned in the results section. Due to adherence problems throughout the course of our experiment we were only able to find data on two "full EBs" for both the treated and untreated media types. The "full EBs" showed a clear center and were easy to analyze with consistency. The "void EBs" were also subdivided for the purpose of analysis. Since the center of an absent EB was impossible to discern on a consistent basis a photo was taken of the largest concentration of cells for that EB, this was done consistently for each "void EB". The adherence problems we experienced throughout our experiments presented us with a larger number of "void EBs". However; because the two subsets were quantified differently it makes examining each one separately difficult. For this reason the quantification of our data came from a combined subset analysis.

7.2.3. The Therapeutic Potential of EB Mass Production and PDGF

For a myocardial infarct to result in a heart attack it takes the loss of function in almost a billion cells. If the two fold increase of cardiogenic cells we saw in our results could be reproduced to statistical significance then our PDGF theory may hold value as a potential therapy. On average we were able to harvest approximately forty EBs form the lid of the ninety-six well plate, if further experimentation showed that our results could be repeated to statistical significance this would give us the same yield as having to perform the test twice and harvest a full eighty EBs. Considering the incredible number of cells damaged by an infarct the absolute number of cardiogenic-like cells would need to be greatly increased. If this increase in the percent of positive cells could be used in conjunction with an engineering method that produced a larger number of EBs the combination could offer a possible therapy in a field that currently only treats the symptoms. An engineering solution could offer us a full ninety-six EBs when an equivalent size experiment was run. The conjoined methods offer a potential therapy that is both scalable and higher yielding.

If continued proliferation could be seen after these cells were implanted the possibilities would extend even further. Though not established through EB formation other

works have shown that cardiac tissue can show significant regeneration in vitro when provided with PDGF B supplemented media. The future is bright for the field of tissues culture and adult stem cells are on the fore front.

8. Conclusions

This project was accomplished by integrating engineering and biological principles to successfully attack our problem on two fronts. The data generated from our project lead to two basic conclusions; EB formation can be accomplished with non-adhesive wells and PDGF treatment shows promising results towards increasing the cardiogenicity of cells migrating from an EB.

After experimenting with various designs and polymers it was determined that EB formation can be accomplished using a non-adhesive well environment. This method proved to be efficient by producing high quality EBs with similar results compared to the hanging drop method in current and simplifying the process by removing unstable hanging drops and complicated flipping. EB production was also easily scalable with this method depending on the number to wells used.

From the evaluation of our various non-adhesive well designs we were able to conclude that there were some key aspects in the design of the non-adhesive wells. Topography played an important role in the aggregation of cells and the subsequent EB formation. A smooth surface improved the aggregation of cells and lead to large homogenous cell aggregates, where as imperfections provided places for cell to aggregate and adhere preventing the large aggregation events necessary for successful EB formation. The shape of the wells may have also played an important role. It was observed that a steeper sloped well with a smaller radius of curvature produced homogenous, high quality EBs. The larger radius of curvature wells and the flat surface of POM produced smaller, lower quality aggregates.

The treatment of EBs with PDGF resulted in an increase in the cardiogenicity of the cells migrating off of the EB. An increase of 8% to 16% was observed through random sampling and visual analysis. However, when a T-test was used to statistically analyze the data generated from this evaluation a p-value of 0.07 it was attained. This value seems to suggests that the increase from 8% to 16% is not significant, but it is believed that this is due to a type 2 statistically error. The sample size used to generate this data was small, n-values no higher than 10, due to contamination issues and other associated problems. The p-value observed for this data is only slightly off from showing significance even though there was a very small sample size. We believe this promising result indicates that if a larger sample size was used, a higher n-value, that a lower p-value would be attained showing that the increase in cardiogenic cells is significant.

Based of the data generated from this project our final design recommendation is a non-adhesive well design to increase EB output, which can be supplemented with PDGF to increase cardiogenic output. The wells would be designed to incorporate the important aspects developed during experimentation. They would be made from an untreated hydrophobic polymer that had been injection molded into our specific shape. This would reduce cost associated with manufacture as an injection mold could be used over an over to make many plates according to need. This will also reduce the cost of having to chemically attach another polymer as a coating, which means we could focus manufacturing on altering the shape and design of the wells. Current designs use a covalently bonded coating on a standard u-bottom 96-well tissue culture plate to improve EB formation instead of altering well shape. Injection molding has also shown to provide smooth surface topography necessary in a non-adhesive well design. The wells would be designed to have a steep slope, a small radius of curvature, and hold a smaller volume, as this has shown to improve aggregation.

This design satisfies both our client statement and user requirements. It has shown to efficiently produce EBs and can be scalable depending on need. It integrates successfully with current techniques and instrumentation making it easy to use and is safe for both the user and the hMSCs. Using simplistic manufacturing techniques such as injection molding also make it cost effective. We have shown that EB formation can be accomplished efficiently using the scalable method of using non-adhesive wells and supplemental PDGF treatment.

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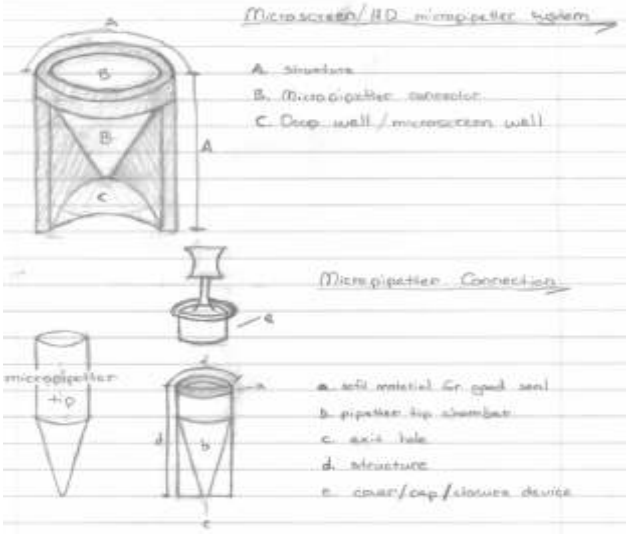
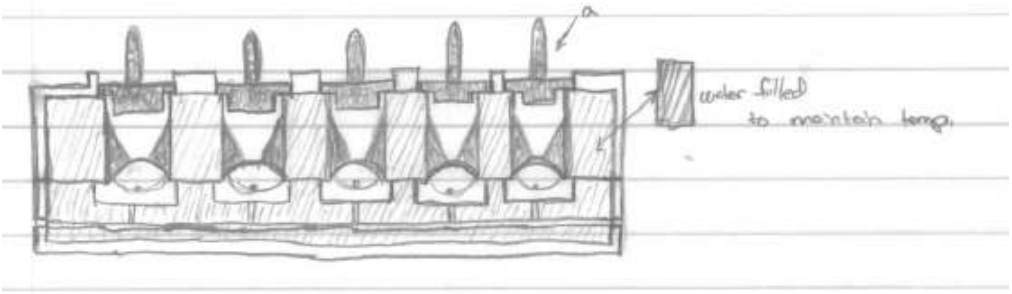
Appendix 1: Design Alternatives

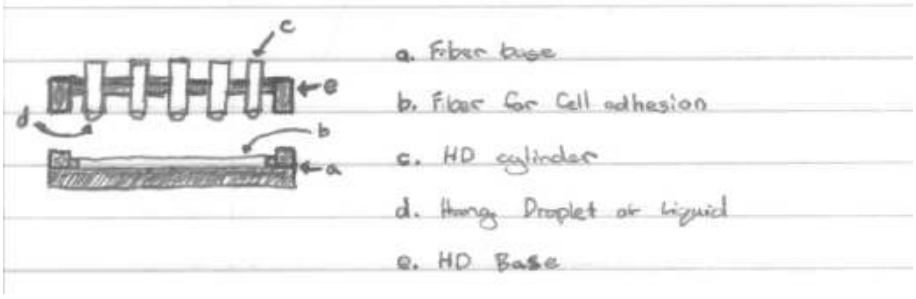
Design Alterative 1.1. Hanging drop with screen and pipette channel



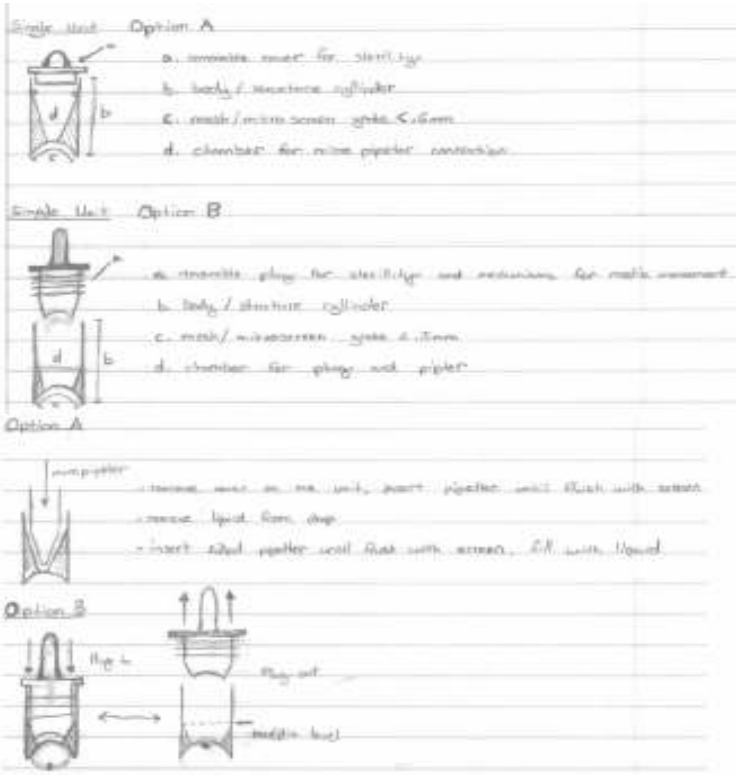
Design Alterative 1.2. Enclosed hanging drop with screen and water chamber



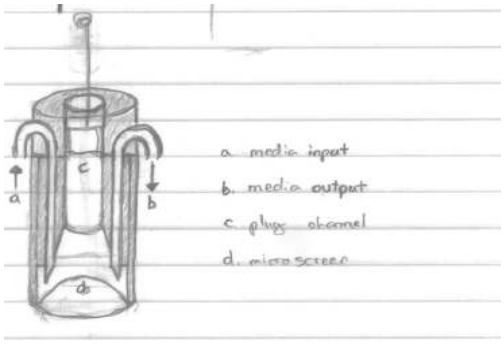
Design Alterative 1.3. Hanging drop with screen and adhesion environment



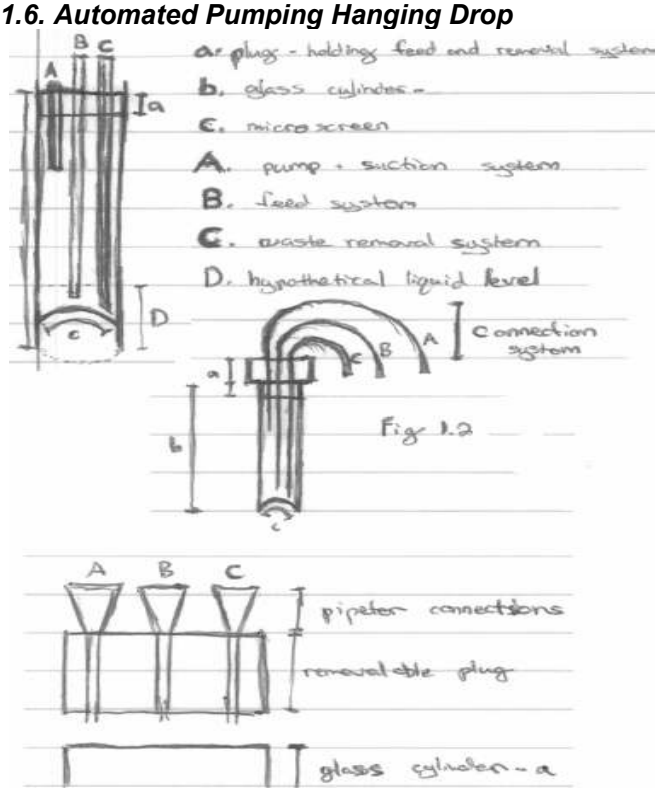
Design Alterative 1.4. Hanging drop with screen and various cover mechanics



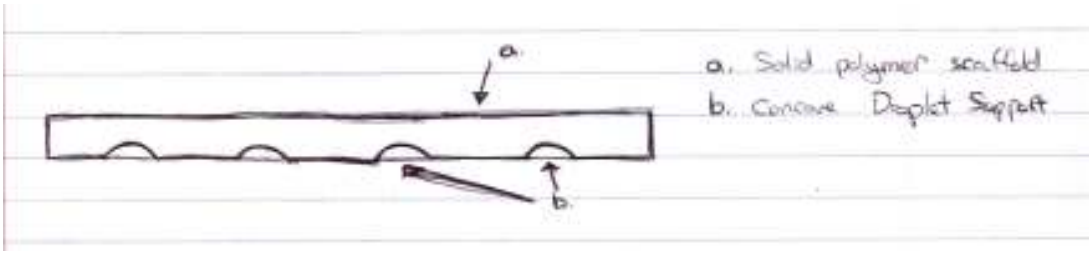
Design Alterative 1.5. Automated Piston Hanging Drop



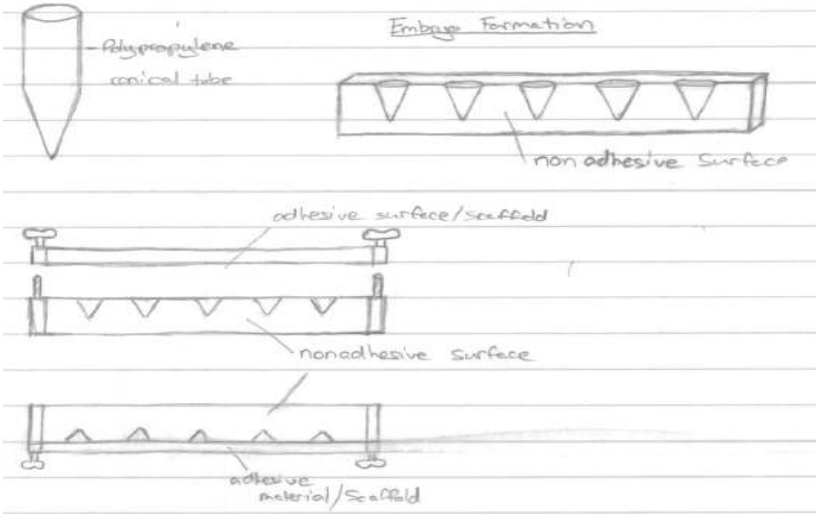
Design Alterative 1.6. Automated Pumping Hanging Drop



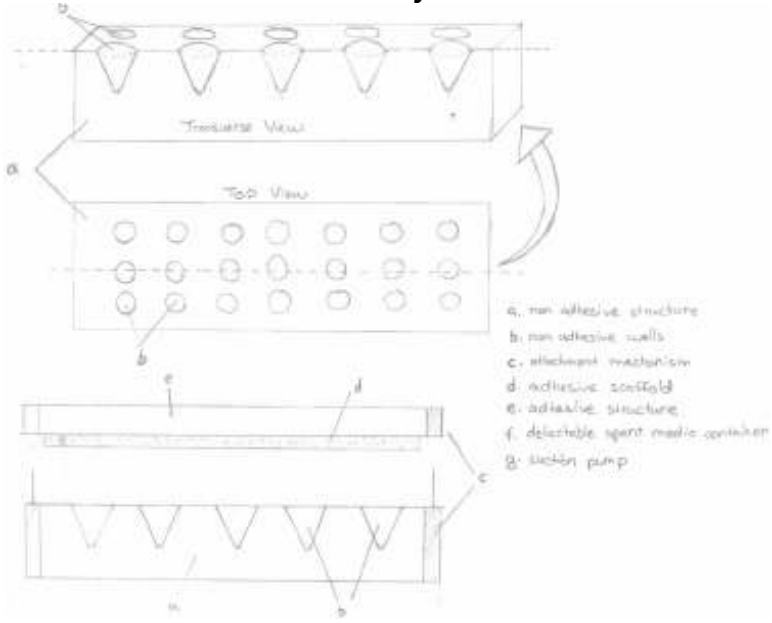
Design Alterative 1.7. Optimized Hanging Drop with Droplet Support



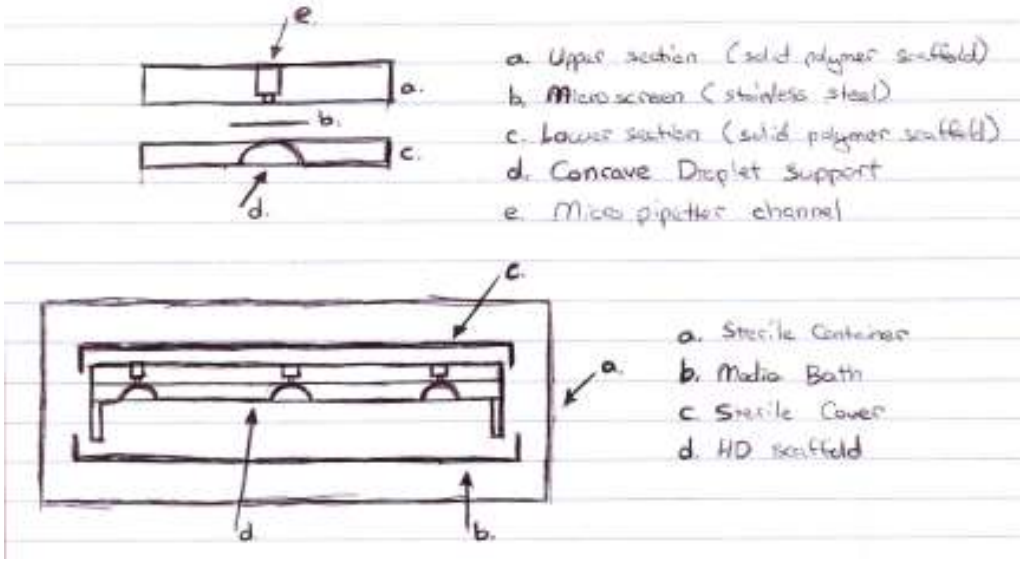
Design Alterative 1.8. Conical Tube Non-adhesive Wells



Design Alterative 1.9. Non-adhesive Solid Polymer Wells



Design Alterative 1.10. Non-adhesive Electrospun Polymer Wells



Appendix 2: Test Protocols

Test Protocol 2.1. Thawing and Plating Frozen hMSCs

Materials:
1. T75 Tissue Culture Flask.
2. ~ 20 mL MSCGM +10% FBS
3. 15 mL conical tube.
4. Pipette-man + Pipettes
5. Pasteur Pipettes
6. Micro-pipetter and tips.

Methods:
1. Prior to thawing cells, warm 20 mL of MSCGM+10% FBS in the 37 C° water bath.
2. Once media is warmed place 14mL of media in a sterile T75 tissue culture flask.
3. Obtain a frozen cryo-vial of hMSCs and thaw in the water bath. *(Watch closely).*
4. Once thawed, immediately transfer the cell suspension to a 15mL conical tube, and deactivate the DMSO with ~2mL of MSCGM media. (*This should be done immediately after the cells have been thawed to prevent cell death.)*
5. Centrifuge the cell suspension at 10000 RPM for 5 min.
6. Aspirate off the DMSO being careful not to disturb the cell pellet.
7. Re-suspend cells in 1mL of fresh media.
8. Transfer cell suspension to the T75 flask, and gently shake for even distribution of cells.
9. Label flask with Date, Name, Cell type, Passage #, and Donor.
10. Store flask in 37 C° incubator with 5% CO_2 .
11. Change media every 3-4 days.

Important Information:

Name	Date	Passage #	Donor

Comments:

Test Protocol 2.2. Passaging hMSCs

Materials:
1. 1 flask of confluent hMSCs.
2. Sterile T75 tissue culture flasks.
2. 4 mL 25% Trypsin solution.
3. 45-50 mL of MSCGM +10% FBS
4. Hemacytometer.
5. 90 ul Trypan Blue
6. Cell counter

Methods
1. Warm MSCGM+10% FBS in 37 ° water bath.

2. Obtain a flask of confluent hMSCs.
3. Aspirate off old media.
4. Add 4 mL of 25% Trypsin and incubate for 5-10 min, or until cell fully lift off the plate.
5. Transfer cell suspension into 15mL conical tube and add 4-6 mL of MSCGM+10%FBS to deactivate the Trypsin.
6. Centrifuge at 10000 RPM for 5 min.
7. Aspirate off Trypsin solution being careful not to disturb the pellet.
8. Re-suspend cells in .5 mL of MSCGM + 10% FBS media.
9. Perform cell count.
    a. Transfer 10 µL of cell suspension into 90 µL of Trypan Blue.
    b. Apply 10 µL of Trypan Blue/cell suspension to each side of the hemocytometer.
    c. Count number of live cells (Clear, not blue) in each of the 5 boxes of the both sides of the hemocytometer.
    d. Perform Cell count calculation.
10. Determine the amount of cell suspension needed to transfer approximately 300,000-500,000 cells.
11. Add 15mL of fresh MSCGM media into each T75 tissue culture flask.
12. Transfer 300,000 -500,000 cells into each flask and shake for even cell distribution.
13. Label each flask with date, new passage number, Donor, and name.
14. Incubate in 37 ° incubator with 5% CO_2 , change the media every 3-4 days.

Important Information:

# of Live Cells	**# Squares Counted**	**Volume of cell susp.**	**Total # cells**

Cells counted x 10^5 x Volume of cell susp. = Total # of cells
Squares

Name	**Date**	**New Passage #**	**Donor**	**# Cells Added**	**Old Plate Information**

Comments:

Test Protocol 2.3. EB Formation

Materials:

1. hMSC cell suspension.
2. 96-well plate
3. DMEM+20% FBS +1% Pen/Strep
4. 90µL Trypan Blue
5. Hemacytometer
6. Cell counter.
7. 20 µL wide orifice pipette tips.

Methods:

1. Perform a cell count and determine the number of cells within the suspension.
2. Calculate the volume of DMEM solution needed to suspend the cell pellet in order to transfer approximately 15,000 cells in 20 µL of solution.
3. Re-suspend cells in appropriate amount of DMEM+20% FBS+1% Pen/Strep solution.
4. Obtain a 96-well plate and flip the lid so the inner surface faces up.
5. Using a wide orifice pipette tip transfer 20 µL of cell suspension onto the lid of the 96-well plate. Place droplets in the center of each circle on the lid, providing one circle's space between each droplet.
6. Flip the bottom portion of the 96-well plate so the bottom if facing upwards.
7. Apply DMEM media to the chambers along the outer perimeter of the plate to avoid droplet evaporation during incubation.
8. Carefully (but quickly) flip the 96-well plate lid, being careful not to let the droplets run.
9. Place the lid squarely on top of the bottom portion of the 96-well plate.
10. Cover notched corner of plate with aluminum foil to prevent evaporation.
11. Label plate with name, passage number, date, and donor number.
12. Place in 37 C° incubator with 5% CO_2.
13. Change Media every day.

Important Information:

# of Live Cells	# Squares Counted	Volume of cell susp.	Total # cells

Name	Date	Passage #	Donor	# Cells Added	Original Cell Susp. Info.

Comments:

Test Protocol 2.4. EB Formation using Electrospun PTT

Materials:

1. hMSC cell suspension.
2. Electrospun PTT well insert.
3. 96-well plate
4. DMEM+20% FBS +1% Pen/Strep
5. 90µL Trypan Blue
6. Hemacytometer
7. Cell counter.
8. 20 µL wide orifice pipette tips.
9. Parafilm

Methods:

1. Prior to experimentation sterilize PTT inserts in the autoclave. (Try to keep inserts dry during sterilization.)
2. Perform a cell count and determine the number of cells within the suspension.
3. Calculate the volume of DMEM solution needed to suspend the cell pellet in order to transfer approximately 15,000 cells in 40-50 µL of solution.
4. Re-suspend cells in appropriate amount of DMEM+20% FBS+1% Pen/Strep solution.
5. Obtain a 96 well plate and place several PTT inserts in the wells of the 96-well plate.
6. Place 40-50 ul of cell suspension into each PTT insert.
7. Cover the 96-well plate with a lid or Parafilm to prevent evaporation.
8. Label plate with name, passage number, date, and donor number.
9. Place in 37 C° incubator with 5% CO_2.
10. Allow to incubate for 3 days while changing the media every day.

Important Information:

1. Did EBs form?
2. If not, why?

Comments:

Test Protocol 2.5. EB Formation Using POM Well Structures

Materials:

1. hMSC cell suspension.
2. POM well plate.
3. DMEM+20% FBS +1% Pen/Strep
4. 90µL Trypan Blue
5. Hemacytometer
6. Cell counter.
7. 20 µL wide orifice pipette tips.
8. Parafilm

Methods:

1. Prior to experimentation sterilize POM well plate by autoclaving or by soaking in ETOH.
2. Perform a cell count and determine the number of cells within the suspension.
3. Calculate the volume of DMEM solution needed to suspend the cell pellet in order to transfer approximately 15,000 cells in 40-50 µL of solution.
4. Re-suspend cells in appropriate amount of DMEM+20% FBS+1% Pen/Strep solution.
5. Obtain sterilized POM Well plate and add 40-50 uL to each well of varying depth.
6. Obtain a piece of Para-film and cover the POM well plate to prevent evaporation.
7. Label plate with name, passage number, date, and donor number.
8. Place in 37 C° incubator with 5% CO_2.
9. Allow to incubate for 3 days while changing the media every day.

Important Information:

1. Did EBs Form?

POM Well Plate					
Machined				Melted	
"Shallow"	"Med Depth"	"Med Depth"	"Large Depth"	"Shallow"	Deep

2. If EBs didn't form, what are some possible reasons.

Comments:

Test Protocol 2.6. PDGF Treatment Testing

Materials:
1. Day three EBs
2. hMSCs
3. Four Chamber Slides
4. DMEM + 20% FBS + 1% P/S Media
5. 10 ng/ml PDGF DMEM + 20% FBS + 1% P/S Media
6. Pipette Tips

Methods:
1. Control Slide Setup.
    a. Obtain a sterile 4-chamber slide.
    b. Obtain EBs that have been growing for three days. (Either from materials testing or hanging drop method)
    c. Transfer two EBs to each to the two chambers to the left and add approximately 1 mL of DMEM + 20% FBS solution to each chamber.
    d. Obtain a hMSC suspension and add approximately 15,000 cells/mL of DMEM + 20% FBS solution to each of the remaining slide chambers.
        i. hMSCs should be the same passage number as the cells used to form the EBs.
    e. Incubate for three days in 5% CO2.

Chamber 1	Chamber 2	Chamber 3	Chamber 4
Control EBs 2 EBs No PDGF 20% FBS	**Neg Control EBs** 2 EBs 20% FBS No PDGF No Primary Antibody	**Contol hMSCs** 15,000 cells 20% FBS No PDGF	**Neg Control hMSC** 15,000 cells 20% FBS No PDGF No Primary Antibody

2. Experimental Slide Setup.

a. Obtain a sterile 4-chamber slide.
b. Obtain EBs that have been growing for three days.
c. Transfer two EBs to each of the three left most chambers of the slide and add approximately 1mL of 10 ng/mL PDGF solution to each chamber.
d. Obtain a hMSC suspension and add approximately 15,000 cells in 1mL of 10ng/mL PDGF solution to the last chamber of the slide.
e. Incubate for three days in 5% CO2.
f.

Chamber 1	Chamber 2	Chamber 3	Chamber 4
Exp. EBs 2 EBs 10ng/mL PDGF	**Exp. EBs** 2 EBs 10ng/mL PDGF	**Exp. EBs** 2 EBs 10ng/mL PDGF	**hMSCs** 15,000 cells 10ng/ML PDGF

Comments:

Appendix 3: Curvature Raw Data

Raw Data from Curvature Analysis 2.1.

20ul Drops	Radius of curvature (mm)	height of drops (mm)	50ul Drops	Radius of curvature (mm)	height of drops (mm)
Drop 1	3.15	2.10	Drop 1	4.43	2.14
Drop 2	2.98	2.04	Drop 2	2.78	2.67
Drop 3	3.75	1.37	Drop 3	3.18	2.44
Drop 4	2.40	2.17	Drop 4	3.35	2.58
Drop 5	2.46	2.06	Drop 5	3.85	2.08
Drop 6	2.75	1.34	Drop 6	3.33	2.26
Drop 7	2.86	1.47	Drop 7	2.98	2.52
Drop 8	2.63	1.92	Drop 8	4.50	2.19
Drop 9	2.84	1.71	Drop 9	4.96	1.93
Drop 10	2.81	1.63	Drop 10	4.73	2.10
Drop 11	2.44	1.73	Drop 11	3.18	2.29
Drop 12	2.52	1.74			
Drop 13	2.45	1.48			
Drop 14	2.54	1.39			
Drop 15	2.87	1.58			
Drop 16	2.94	1.60			
Drop 17	2.46	1.78			
Drop 18	3.18	1.59			
Drop 19	2.84	1.67			
Drop 20	2.58	1.77			
Drop 21	2.53	1.82			
Drop 22	2.69	1.63			