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Cardiac Tissue Engineering: Using Induced Pluripotent Stem Cells to Repair Damaged Cardiac Tissue

Megan Lenz
College of Saint Benedict/Saint John's University, mklenz@csbsju.edu

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Cardiac Tissue Engineering: Using Induced Pluripotent Stem Cells to Repair Damaged Cardiac Tissue

Megan Lenz

College of Saint Benedict/Saint John's University

CAPSTONE THESIS

Submitted in partial fulfillment of the requirements for the Natural Science Major at the College of St. Benedict | Saint John's University, Saint Joseph & Collegeville, Minnesota.

May 6, 2016

Abstract

Heart Disease is the number one cause of death in the United States and affects many individuals on a daily basis. The purpose of this research was to explore possible tissue regeneration therapies that aim to improve and recover function of damaged myocardial tissue commonly seen as a side-effect of chronic heart disease. Cardiac tissue engineering using induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) is a relatively new method that involves dedifferentiating cells from any tissue of the host into pluripotent stem cells. These cells are then guided to be differentiated into functioning myocardial cells that can be used to repair damaged tissue and restore cardiac function to affected areas. The process and effectiveness of iPSC-CMs as a treatment will be discussed.

Introduction

Heart Disease is the number one cause of death in the United States, accounting for approximately 1 in 4 deaths, and affects many individuals on a daily basis[1]. A common definition for cardiovascular or heart disease is some condition affecting either the blood vessels, tissue, or valves of the heart that often leads to a heart attack or stroke. Some of the most common types of heart disease include but are not limited to coronary heart disease, atrial fibrillation, heart valve disease, congenital heart disease, pericarditis, and heart murmurs. Currently one of the only effective long-term treatment options to recover the damaged cardiac tissue associated with chronic heart disease is a heart transplantation[2]. However, with a critical shortage of donor hearts available and no affordable option for long distance transportation, this option can be hard to rely on. What is needed now is a treatment that aims to replace or regenerate infarcted myocardial tissue. A heart infarction occurs whenever there is an area of cardiac tissue that obtains an insufficient amount of blood, commonly due to events such as a thrombus or embolus, and likely causes local tissue death[3]. One upcoming way to possibly replace or regenerate the diseased or damaged tissue is cardiac tissue engineering in vitro using induced pluripotent stem cells (iPSCs).

Stem cells have an unlimited capacity for self-renewal and give rise to cells that will undergo terminal differentiation[4]. They can be found in most tissues in the human body and aid in regeneration of that tissue's specific functioning cells. For example, hemopoietic stem cells have the ability to terminally differentiate and replace the many different types of cells in the blood. Similarly, the stem cells in the intestine differentiate to renew the lining of the GI tract and the stem cells in the basal layer of the epidermis differentiate to replace the dermal cells of

the skin. On the other hand, there are some stem cells, such as the ones found in the embryo that possess the ability to produce each and every tissue cell in the body[5]. These are considered pluripotent. Pluripotent stem cells are one step above tissue stem cells in that they give rise to the tissue-specific stem cells such as the epithelial and dermal cells described earlier.

Induction of these pluripotent stem cells and the ability to use them to recover heart tissue is the topic of this paper. Induced pluripotent stem cells (iPSCs) are obtained by taking a terminally differentiated cell and de-differentiating it, thus stripping it down of all of the qualities that make it that kind of cell. For example, one has the ability to take dermal cells, de-differentiate and reprogram them until they become "embryonic like" pluripotent stem cells, thus now having *induced* pluripotent stem cells. These new induced pluripotent cells now have the potential to differentiate into any tissue[5]. They can be guided using growth factors or exposing the cell to a certain environment to become any tissue including a cardiomyocyte which is the main functional cell in heart tissue. The once dermal cell can thus be made into a cardiomyocyte. This process can be used for reproductive cloning[6] but can also be used for therapeutic purposes because of their patient-specific capacity. This paper will be focusing on the idea of using iPSCs as a treatment to improve functioning of diseased or damaged heart tissue.

Dedifferentiation

Successful induction of iPSC from adult human fibroblasts was obtained by the Yamanaka lab at Koyota University[7]. The protocol for this process involves introducing four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc to human dermal fibroblasts derived from the facial dermis of a 36-year-old Caucasian male. After 30 days of culturing, some cells showed human embryonic stem cell-like (hESC) qualities. The cells resembled hESCs in physical appearance by having a large nuclei and scant cytoplasm. Figure 1 displayed below contains images and descriptions from the study. Image C demonstrates what a typical non-embryonic cell looks like. This would an example of a terminally differentiated cell such as the dermal cell used in this study. Image D is what a typical hESC looks like and images E and F are the successfully de-differentiated dermal cells into induced pluripotent stem cells or iPSCs. Comparison of these images shows the similar morphology of the iPSCs to a typical pluripotent hESC.

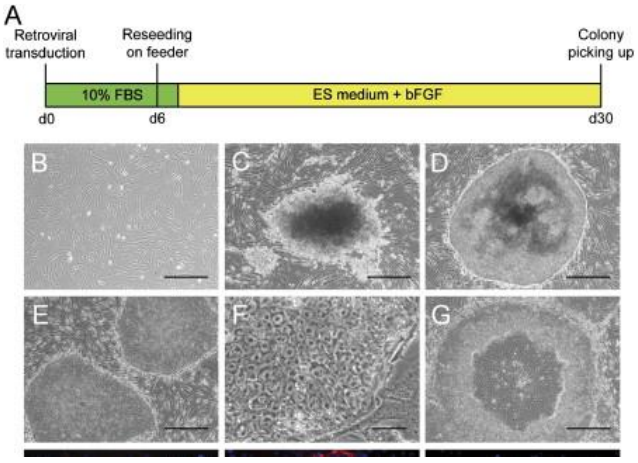

Figure 1.
Induction of iPS Cells from Adult HDF
(A) Time schedule of iPS cell generation.
(B) Morphology of HDF.
(C) Typical image of non-ES cell-like colony.
(D) Typical image of hES cell-like colony.
(E) Morphology of established iPS cell line at passage number 6 (clone 201B7).
(F) Image of iPS cells with high magnification.
(G) Spontaneously differentiated cells in the center part of human iPS cell colonies.

Takahashi et al. (2007)

In addition to sharing similar morphology, the iPSCs expressed hESC-specific surface antigens SSEA-3, SSEA-4, tumor-related antigens (TRA)-1-60, TRA-1-81, TRA-2-49/6E, and NANOG protein. Wetstern blotting also showed evidence of dedifferentiation when it revealed protein levels of OCT3/4, SOX2, NANOG, SALL4, E-CADHERIN, and hTERT that were similar to hESCs. Lastly, the dedifferentiated cells showed high telomerase activity with a calculated doubling times of 46.9± 12.4, 47.7± 6.6, and 43.2 ± 11.5 hours. These times are equivalent to earlier reported population doubling times of hESCs. Because of similarity in morphology, surface antigens, gene expression, and telomerase activity, the adult human fibroblasts were determined to be dedifferentiated into iPSCs that mimicked the function of hESCs.

Differentiation

After dedifferentiating the iPSCs, they now have the potential to be differentiated into one of many lineages of cell types. This paper is interested in the differentiation of the iPSCs into functional cardiomyocytes. There are several methods of differentiating iPSCs into functioning cardiomyocytes, three of which being the embryoid body method[8], the matrix sandwich method[9] and the small molecule method[10].

The embryoid body (EB) method is one of the first methods of differentiating iPSCs into cardiomyocytes and is primarily based on giving cardiac specific growth factors to the pluripotent cells and allowing them to join together to make a three dimensional body[8]. The iPSCs were introduced and cultured with differentiation medium (EB20) that consisted of 80% DMEM/F12, 0.1 mmol/L nonessential amino acids, 1mmol/L l-glutamine, 0.1 mmol/L β-mercaptoethanol, and 20% FBS that was pretested for cardiac differentiation to create three

dimensional embryonic bodies (EBs). This was used to initiate cardiac differentiation from the human iPSCs. The EBs were cultured with their medium changed regularly. In as little as 10 days, some of the EBs had recorded contraction rates which suggested differentiation to a functioning cardiomyocyte. The contraction forces were by no means comparable to the force generated by an original cardiomyocyte but the point of this observation is that the EBs have started to differentiate into a beating cardiomyocyte. Labs have started to develop new methods for maturation[11] of rat and human engineered heart tissue by chronic electrical stimulation[13], by cyclic stretch[14], or using vascular loading and vascular co-culture[15].

Gene expression patterns of these EBs were tested on day 60 when cardiogenesis reached a stable plateau. As seen in Figure 2A taken from this study, multiple cardiac genes were tested for expression using a quantitative RT-PCR machine in the iPSCs and EBs: transcription factor Nkx2.5 (NKX2-5), several myofilament protein genes including cardiac troponin T (TNNT2), α-myosin heavy chain (MYH6), α-actinin (ACTN2), myosin light chain 2 atrial isoform (MYL7), myosin light chain 2 ventricular isoform (MYL2), atrial natriuretic factor (HPPA), and phospholamban (PLN). At day 60, the undifferentiated iPSCs showed low expression of cardiac genes, shown with a dull mark, while the cardiomyocyte differentiated EBs showed a significant upregulation of the full range of cardiac genes, shown with a bight mark. Also, Figures 2B and 2C show that the pluripotent genes OCT4 and NANOG were downregulated in the differentiated cardiomyocyte EBs which suggests that the embryoid body method of differentiating cardiomyocytes from iPSCs is a successful method.

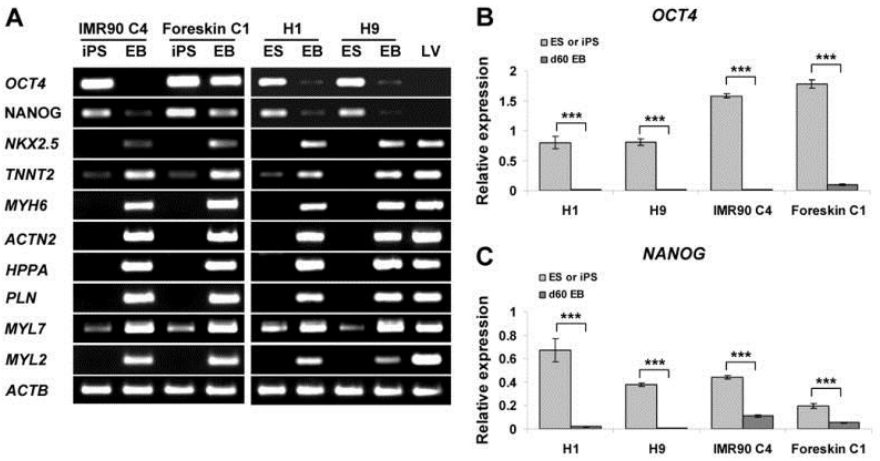

Figure 2: Cardiac and Pluripotency Gene Expression in Cardiomyocytes Derived from iPS and ES Cells. (A) RT-PCR analyses of pluripotency genes, *OCT4* and *NANOG*, and cardiac genes in undifferentiated iPS and ES cells, day 60 EBs, and adult left ventricular myocardium (LV). (B and C) Quantitative RT-PCR analyses of total *OCT4* (B) and *NANOG* (C) expression in undifferentiated iPS and ES cells compared to differentiated contracting areas from day 60 EBs. Error bars represent SEM (n=3), *** indicate $P < 0.001$ comparing gene expression in undifferentiated cells and d60 EBs using t-test.

Zhang et al. (2009)

The matrix sandwich method is another method of differentiating pluripotent stem cells into cardiomyocytes that involves a more effective protocol by using extracellular matrix (ECM) in combination with growth factors known to promote cardiogenesis[9]. The process reported by a lab at the University of Minnesota used pluripotent stem cells cultured as a monolayer on Matrigel, a layer of ECM preparation, and overlayed with another layer of Matrigel to create a "matrix sandwich". This promoted an epithelial- to –mesenchymal transition as seen in gastrulation with the generation of N-cadherin mesenchymal cells. Figure 3 taken from this study displays below the addition of the N-cadherin mesenchymal cells seen in this method. The addition of growth factors known for cardiogenesis (Activin A, BMP4, and bFGF) to the matrix sandwich generated cardiomyocytes with high purity and yield that formed contracting sheets by day 15. Using RT-QPCR results, the contracting cells showed significant upregulation of early mesoderm genes T and MESP1, cardiac transcription factors of GATA4, ISL1 and NKX2-5, and cardiac myofilament proteins TNNT2, TNNI3, MYL7, and MYL2. In pluripotent genes

OCT4 and NANOG, the contracting cells showed significant downregulation which suggests that the pluripotent stem cells were differentiated into cardiomyocytes.

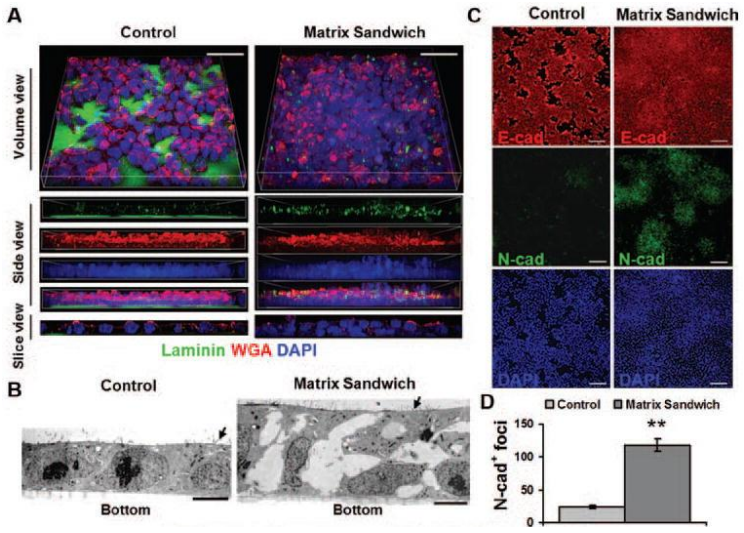

Figure 3
(A) Confocal imaging of iPSCs (DF19-9-11T) seeded as single cells on Matrigel coated surface and propagated in mTeSR1 for 4 days without Matrigel overlay (Control) or with a Matrigel overlay (Matrix Sandwich) for the last 24 hours. 3D reconstructed z-series of images labeled with an antibody to laminin, fluorescent-conjugated WGA for glycoproteins, and DAPI for nuclei. Scale bars are 20 μm. **(B)** Electron micrographs of control cultures demonstrate confluent monolayers compared to matrix sandwich cultures which are multilayered with an upper epithelial layer and mesenchymal cells below. Arrows indicate microvilli. Scale bars are 5 μm. **(C)** Epifluorescence images of control and matrix sandwich cell culture 24 hours after Matrigel overlay immunolabeled with E-cadherin and N-cadherin antibodies. Scale bars are 100 μm. **(D)** Comparison of average number of N-cadherin$^+$ foci (per 1.8 cm^2 surface area) in control and matrix sandwich cell cultures (DF19-9-11T). Error bars represent SEM, N=3. Data were compared using Student's t-test with ** indicating significantly different, $P < 0.01$

Zhang et al. (2012)

The third method of differentiation of iPSCs into functioning cardiomyocytes is accomplished serum-free by modulating Wnt/β- catenin signaling[10]. This method involves culturing a monolayer of iPSCs with the addition of growth factors important in cardiovascular development such as fibroblast growth factor 2 (FGF2), transforming growth factor β (TGFβ), superfamily growth factors Activin A and BMP4, and vascular endothelial growth factor (VEGF). Appropriate application of Gsk3 inhibitor followed by β- catenin shRNA or a chemical Wnt inhibitor provides the opportunity of producing a high yield of virtually pure functional cardiomyocytes from human iPSCs without cell sorting or selection. At day 4 of culture, gene expression of cardiac transcription factors NKX2.5 and ISL1 can be detected with cardiac proteins detected at day 5. Cardiac Troponin T (cTnT) can be detected as soon as day 8 of

culture. The formation of differentiated functional cardiomyocytes can be established by visual observation of contracting regions, with the first beating observed between days 8 and 10.

Comparison to Embryonic Stem Cells

Mesenchymal stem cells[16], cardiac progenitor cells[12], cardiosphere-derived cells[17], and human embryonic stem cells (hESCs)[15, 18],[14] have all been administered as a therapy for reducing myocardial infarction damage and has shown improvement in cardiac tissue function. hESCs are stem cells derived from an embryonic origin and are thus pluripotent in nature. There are similarities in the function of hESC- derived and iPSC- derived cardiomyocytes[10] because both of the cells are pluripotent and have the potential to differentiate into one of the many cell lineages in the human body. The difference between the two comes from where the original cell is derived from. As stated before, iPSCs are cells taken from anywhere in the body and are dedifferentiated to an induced pluripotent state to produce functional cardiomyocytes[22]. On the other hand, hESCs are already pluripotent because they are embryonic and thus already have the potential for differentiation into cardiomyocytes. Because of the associated use of human embryos for regenerative therapy, hESCs are linked to major ethical concerns and are likely to be overlooked. hESCs are also not patient-specific in comparison to iPSCs that use cells from the patient themselves to regenerate a specific type of tissue. An individual that used regenerated tissue derived from hESCs would risk an adverse immune/inflammatory reaction and would likely have to take immunosuppressants for the remainder of their lifetime. Despite the ethical and immune problems, hESCs are very similar in

function to iPSCs when used as a regenerative therapy and are useful in determining effectiveness of iPSCs for therapy.

A lab at Duke University created a tissue-engineered cardiac patch (Figure 4A) made from hESC- derived cardiomyocytes to test its functional maturity for possible future use in regenerative cardiac tissue therapy[18]. After 20-30 days of differentiation, hESC- derived cardiomyocyte populations were used to make 3D cardiac patches with elliptical pores to facilitate nutrient transport and enable properly aligned cardiac tissue (Figure 4B and 4C). Using the immunostaining process for cardiac markers, the cells revealed aligned cardiomyocytes exhibiting cross-striated patterns of troponin T (cTNT), myosin heavy chain (MHC) and α- actinin (SAA), which suggests well-developed sarcomeric structures (Figure 4D and 4E). Immunostaining also for N-cadherin (Figure 4F) and connexin-43 (Cx43) demonstrated the presence of intercellular gap and adherens junctions, indicating functional electromechanical coupling between cardiomyocytes. Using quantitative RT-PCR data, it was shown that expression of pluripotency specific genes OCT4 and NANOG were significantly downregulated during the differentiation process and nearly disappeared in the cardiac patches. On the other hand, early cardiac markers ISL1 and GATA4 increased with cardiac differentiation suggesting complete cardiac differentiation. More evidence of cardiac function came from optical mapping of transmembrane potentials which revealed continuous action potential propagation in the cardiac patches. Because of evidence revealing successful electromechanical coupling, downregulation of pluripotent genes with upregulation of cardiac-specific genes, and action potential propagation among the hESC- derived cardiomyocytes, it can be suggested that hESCs

are successful in creating mature functioning cardiomyocytes that could possibly be used in cardiac tissue regenerative therapies.

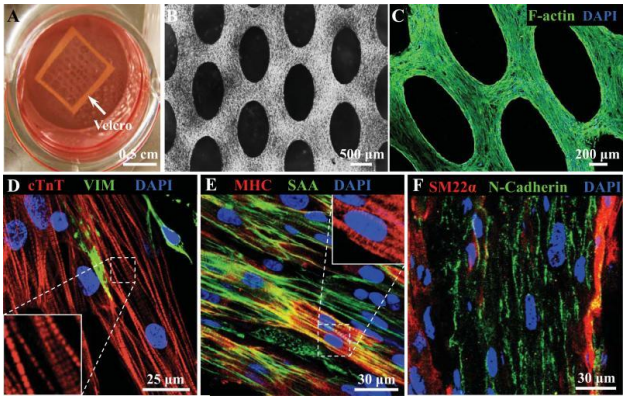

Figure 4

Structural properties of human cardiac tissue patches A) Representative 2-week old cardiac tissue patch anchored within a Velcro frame. B-C) Staggered elliptical pores within the patch (B) are surrounded by densely packed and aligned cells (C). D) hESC-CMs in 2-week old cardiac tissue patches are aligned and show cross-striated patterning of cardiac Troponin T (cTnT). Fibroblasts positive for vimentin (Vim) are interspersed among hESC-CMs. E) hESC-CMs also exhibit cross-striated pattern of myosin heavy chain (MHC) and sarcomeric α-actinin (SAA). F) Cardiac patches show evidence of mechanical coupling (N-cadherin) between hESC-CMs as well as presence of smooth muscle cells (SM22α). Data shown for patches made with 70% hESC-CMs.

Zhang et al. (2013)

Administered IPSCs to cardiac tissue

Because of the high proliferation rate of iPSCs, direct injection into damaged tissue that needs to be repaired could cause tumor formation. To avoid this issue, many protocols involving administration of iPSC- derived cardiomyocytes (iPSC-CMs) onto damaged or infarcted heart tissue form the cells into a cardiac patch.

One lab at the University of Minnesota used tissue-engineered cardiac patches as a possible method to deliver cardiomyocytes to injured myocardium to enhance the ability of cells to limit remodeling as well as improve contractility and function after infraction[19]. This lab hypothesized that cardiac patches made from human induced pluripotent stem cell- derived cardiomyocytes (hiPSC-CMs) would decrease the infarction area and increase fractional shortening (contractility) of affected hearts in a span of 4 weeks. hiPSC-CMs were prepared, and cultured to be harvested in 10 days. Aligned cardiac patches were created by combining

the hiPSC-CMs and non-cardiac pericytes on a fibrin gel (CM+PS patch). Fibrin gels are often used as a biological scaffold because the cells are likely to remodel the fibrin matrix and replace it with their own extracellular matrix (ECM) in comparison to other collagen gels[20]. Rats were anesthetized and received a limited left lateral thoracotomy to expose the heart. An induced myocardial infarction was achieved by permanently ligating the left anterior descending coronary artery with a suture. The cardiac patch was applied to the damaged area by applying thin strips parallel to each other and securing with sutures. Cardiac function was assessed via echocardiograph, and multiple fluorescent images.

Echocardiography conducted from week 1 to week 4 postoperatively determined that all groups displayed a reduction in both the left ventricular ejection fraction (LVEF) and fractional shortening (LVFS) relative to baseline. However, the degree of reduction was less in the rats that contained the CM+PS cardiac pads in comparison to the rats that were not given a cardiac patch. The rats that received CM+PS patches also showed smaller left ventricular inner diameter in systole compared to the non-treatment group. In addition to a smaller diameter, the CM+PS patches showed the most significant decrease in infarct scar tissue, as shown below in Figure 5A and 5B from the study. New cardiomyocyte formation was observed from the patch which was shown by the increased angiogenesis of the area as well as proof of collagenous extra cellular matrix (ECM) formation and gap junctions between adjacent cardiomyocytes.

The results from this lab suggest that an engineered cardiac patch using hiPSC-CMs and PSs trapped in a fibrin gel is a successful therapy to improve function of damaged heart tissue. They found that when applied to infracted rat myocardium, the aligned, force-generating patch

remained viable and the CM proliferated. The patch improved cardiac function and reduced infarction size after the 4 week period.

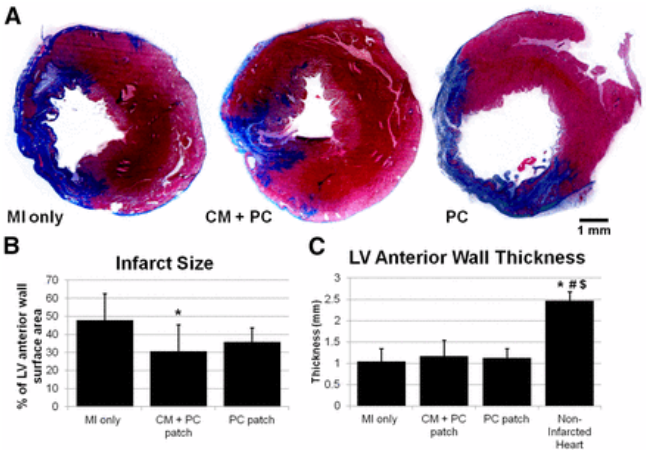

Figure 5.
Infarct size and left ventricular (LV) wall thickness. **(A):** Masson's trichrome images of MI-only, CM+PC, and PC patch hearts, showing representative infarct sizes (blue) and left ventricular anterior wall thickness. CM+PC patch recipient hearts had smaller infarct sizes **(B)** than did the infarct-only control rats, and no reduction in infarct size was seen in the PC patch recipient hearts. No reduction in LV anterior wall thickness **(C)** was observed with either treatment group. Patch not easily visualized in sections. Asterisk indicates comparison with MI-only, number sign indicates comparison with CM+PC patch, and dollar sign indicates comparison with PC patch. Abbreviations: CM, cardiomyocyte; LV, left ventricular; MI, myocardial infarction; PC, pericyte.

Wendel et al. (2015)

Another lab using cardiac patches that show that engraftment of a mixture of hiPSC-CM, endothelial cells, and smooth muscle cells in combination with a IGF-1-fibrin patch into a porcine model of myocardial infraction improved heart function and metabolism without producing arrhythmias[21].

Derivation of Undifferentiated Cells

Jianyi Zhang's lab at the University of Minnesota realized that iPSCs could be differentiated into unlimited lineages of cell types but questioned whether the origin of the dedifferentiated cell mattered in terms of efficiency of the differentiation process, as well as the functionality of the final outcome[22]. This lab believed that the function of the iPS cell-derived cells may be influenced by epigenetic factors that the cells retained from their origin

and thus chose to dedifferentiate cardiac-lineage cells, rather than (for example) dermal fibroblasts, into human cardiac induced- pluripotent stem cell cardiomyocytes (hciPSC-CMs). This study was published in 2015 and reported the first experiments to use iPSCs that have been engineered from cardiac fibroblasts. The fibroblasts used in this study were obtained from the left atrial appendage of three patients that underwent open heart surgery. The tissue was dissociated and single cardiac fibroblasts were collected and dedifferentiated using the CytoTune™-iPS Reprogramming Kit (invitrogen). Once the cells were examined for morphological similarities to embryonic stem cells and tested for expression of pluripotency transcription factors, the hiPSCs were differentiated into cardiomyocytes by the Matrigel sandwich method and treated with different mediums and transcription factors such as Activin A, BMP4, bFGF, RPMI and B27. Differentiation was successful as the pluripotency markers Oct4, Sox2, and Nanog were significantly reduced while expression of early mesodermal and cardiac mesodermal (Mesp1, Nkx2.5, Gata4, Mef2C, and Tbx5) markers had significantly increased. The now induced cardiomyocytes began beating on approximately day 8 of the process and cardiac patches were made by suspending sheets of cardiomyocytes that have been beating for 10 days in fibrinogen.

Myocardial infarction (MI) was surgically induced in 12 week old immunodeficient mice by permanently litigating the left-anterior descending coronary artery with a surgical silk suture. The surviving mice were separated into two groups: ones that were treated with the hiPSC-CM patch and the others that were treated with just a fibrinogen patch. The chest of the mice were closed, the mice were allowed to recover and an echocardiography and staining

procedures were used four weeks after the MI and cell transplantation to evaluated cardiac function.

iPSC-CMs were also derived from different tissues (hUCBiPSC: from human umbilical cord blood mononuclear cell, and hdiPSC: from human dermal fibroblasts) as a way to test the differences in function of induced cardiomyocytes that started from different origins in comparison to induced cardiomyocytes that originated at heart tissue to start with. Results show that hciPSC-CMs have a more cardiac-like Ca2+ handling profile compared to CMs derived from hUCBiPSCs or hdiPSCs.

Overall, this experiment showed that human iPSCs can be successfully generated from cardiac fibroblasts and the hciPSCs can be differentiated into CMs with >92% efficiency. It is thought that the greater than 92% differentiation efficiency likely reflects the epigenetic memory of the cardiac origin of the fibroblast. The mice that received the CM-sheet transplantation showed significantly improved left ventricular contractile function as well as a reduction in the process of apoptosis and increased vascular formation in comparison to the mice that were treated with just the fibrinogen patch. With the help of the data from this experiment, the potential of hciPSC-CM sheet transplantation for treatment of acute myocardial infarction is very promising.

Maturation of iPSC-CMs for higher function

Higher functioning iPSC-CMs could be reached with the proper alignment[23] of the engineered cells onto the damaged heart tissue. Native myocardium are able to function in-sync and follow a specific rhythm because of their extensive mechanical and electrical

alignment. This alignment produces a property of anisotropy: a property of being directionally dependent, as opposed to isotropy, which implies identical properties in all directions. This electromechanical anisotropy is due partly by the aligned myocytes and matrix fibers but most of the synchronal properties of cardiac tissue is due to gap junctions found between the functioning cells. Gap junctions are specialized transmembrane proteins that allow the easy flow of ions between adjacent myocardial cells which in turn, allows synchronized contraction of the heart muscle that functions to propel blood into the pulmonary and systemic circuits. A lab at the University of Minnesota aimed to evaluate the impact of cell and matrix alignment on tissue function in engineered myocardium. They entrapped neonatal rat cardiac cells in a fibrin gel and by appropriately constraining the cell-induced fibrin gel contraction, were able to align the fibrin fibers which aligned the cells in contact with the fibrin. The result was and aligned myocardial equivalent with cells producing their own extracellular matrix. Along with the aligned construct, the lab also created an isotropic construct in order to study the consequences of alignment by comparing their morphology, passive mechanical properties, and twitch force.

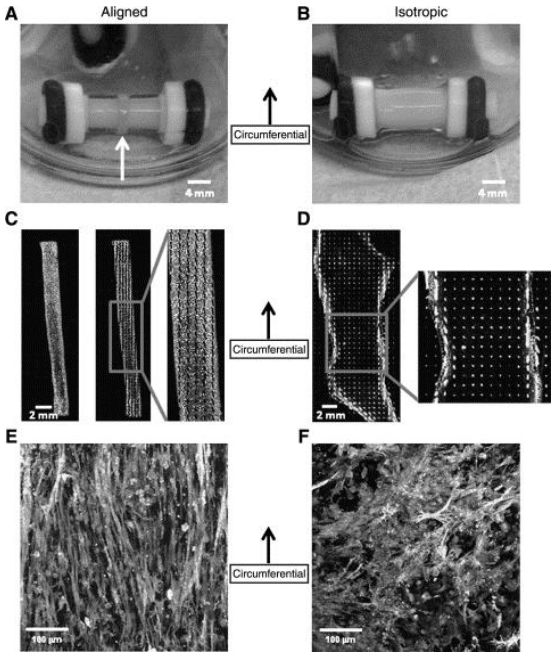

FIG. 6.

Images of isotropic (**A**) and aligned (**B**) constructs after 14 days in culture with corresponding polarized light alignment images (**C, D**). In (**C, D**) the constructs have been cut axially and splayed flat. The grayscale values represent the birefringence of the tissue, a measure of the strength of alignment, and the lines (black in **C**, white in **D**) indicate the local average direction of alignment in the tissue. Note that the aligned construct (**D**) is highly aligned in the circumferential (vertical) direction, while the isotropic construct (**C**) does not possess alignment. Cellular alignment, measured via staining of filamentous actin, matched matrix alignment assessed via polarized light for both construct types (**E, F**).

Black et al. (2009)

Figure 6 shows the aligned (A) and isotropic construct (B) after two weeks of culture. Figure 6A shows that that aligned construct contracted to a thin ring while the isotropic construct remained flat. 6C and 6D show, with polarized light imaging, the matrix alignment, or lack thereof. The aligned construct in 6C shows high alignment in the circumferential direction while the isotropic construct in 6D shows no preferential alignment.

The passive mechanical properties of each construct was measured using a strain-to-failure test and the twitch force was recorded by stimulating the cells with an electrical stimulus. In both tests, the aligned construct showed a higher functioning cell function. In comparison to the isotropic construct, the aligned group had a greater ultimate tensile strength as well as maximum strength achieved before failure. The aligned group also performed better in the twitch force tests (FIG. 7), with a 181% increase in twitch force associated with electrical pacing, in comparison to the isotropic group.

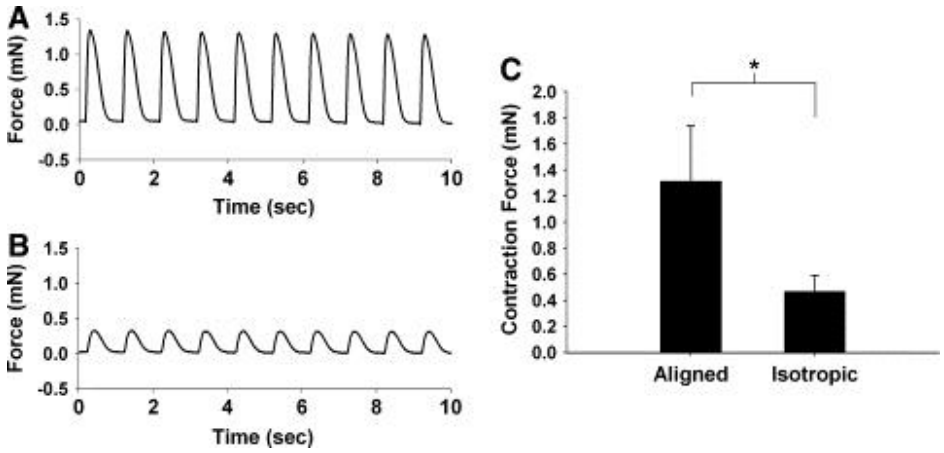

FIG. 7.

Representative force traces during 1 Hz pacing versus time plots for an aligned (**A**) and an isotropic (**B**) construct. (**C**) Pooled twitch force data for 14-day-old aligned (left) and isotropic (right) constructs at 10 mN preload during 1 Hz pacing. $*p < 0.05$ ($n = 14$ for both groups).

Black et al. (2009)

The lab's hypothesis for the improvement of function of aligned construct compared to isotropic construct was that the aligned cells were able to form gap junctions between their cells and, thus, increased their electromechanical function. To test this hypothesis, the lab used Western Blot Analysis to test for Connexin 43 expression, a common protein seen in gap junction formation. As expected, the aligned construct showed a significantly greater expression of Connexin 43 (FIG. 8) in comparison to the isotropic construct and, therefore, suggests a greater number of gap junctions present. The results of this data suggest that the proper alignment of engineered myocardium is imperative to the overall functionality and strength of the resulting tissue.

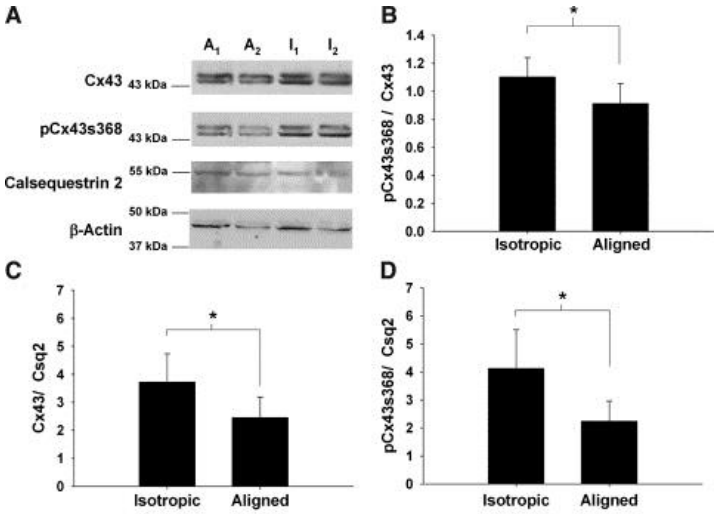

FIG. 8.

(**A**) Representative Western blots for connexin 43 (Cx43), connexin 43 phosphorylated at serine 368 (pCx43s368), calsequestrin 2 (Csq2), and β-actin for two aligned (A_1, A_2) and two isotropic (I_1, I_2) constructs. (**B**) The ratio of pCx43s368 expression to Cx43 expression for aligned and isotropic constructs. (**C**) The ratio of Cx43 to Csq2 for aligned and isotropic constructs. (**D**) The ratio of pCx43s368 expression to Csq2 expression for aligned and isotropic constructs. *$p < 0.05$ ($n = 6$ for all groups).

Black et al. (2009)

Experiment Outline

There has been evidence suggesting the improvement of myocardial tissue following a traumatic heart injury using cardiac patches made from both induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) as well as cardiomyocytes derived from embryonic stem cells (ESC-CMs). Tissue regeneration therapies are a common way of repairing and regenerating heart tissue that had been damaged and caused some degree of heart infarction. Although the process of obtaining ESC-CMs is somewhat simpler than deriving iPSC-CMs, more individuals tend to choose iPSC-CMs for a few good reasons. First of all, when using iPSC-CMs, there is limited worry for tissue rejection from the damaged tissue. This is because the cells used in iPSC-CMs originated from the host's own tissue, saving the individual from taking a lifetime of immunosuppressants potentially needed if foreign ESC-CM's were used instead. Second, using ESC-CMs involves using embryonic cells which could initiate major ethical concerns. Since iPSC-CMs are derived from the host's own tissue, there is limited ethical conflicts and is considered the better option.

Although there are some good reasons that iPSC-CMs should be used in myocardial tissue regeneration compared to ESC-CMs, there doesn't seem to be any evidence as to what process functionally performs better when repairing heart infarctions. My question that I would like to further explore is if there is a significant difference between using iPSC-CMs or ESC-CMs for heart regenerative therapy after a heart infarction. Both options are cardiomyocytes derived from a stem cell in a pluripotent state, the difference between them comes from how the cell obtained its pluripotency. ESC-CMs are naturally pluripotent as they originated from embryonic stem cells, whereas iPSC-CMs were induced to become pluripotent from introducing specific transcription factors OCT3/4, Sox2, Klf4, and C-Myc to any of the host's tissues. Since both ESC-CMs and iPSC-CMs were originally pluripotent and were differentiated to become functional cardiomyocytes, I would hypothesize that there would be no significant difference between the function of either of them when used to repair a heart infarction.

I would use rat models for this experiment and simulate tissue damage of the heart by inflicting a heart infarction by suturing the left anterior descending coronary artery closed. This process will decrease blood flow to the left ventricle causing a section of the myocardial tissue to become damaged and inhibit function. Using the matrix sandwich method, as explained earlier, the pluripotent cells will be differentiated and two types of cardiac patches would be made: One using iPSC-CMs and the other using ESC-CMs. The matrix sandwich method involves introducing the pluripotent cells to extracellular matrix (ECM) in combination with specific growth factors known to promote cardiogenesis such as Activin A, BMP4, and bFGF. Once the cardiomyocytes have been differentiated, they will be combined on a fibrin gel to create a "cardiac patch" to be placed and aligned directly onto the damaged heart tissue. One group of

rats will be administered the iPSC-CM cardiac patch, while another group will be given the ESC-CM cardiac patch. There will also be another group of rats that undergo the process of the heart infarction but will be given nothing to regenerate the tissue. This group will serve as a control and will be the basis for which the variable groups will be functionally compared.

To gather any conclusion to which there is any significant difference between the function of the pluripotent cells in repair and regeneration of cardiac tissue, much data has to be collected. In this experiment, I will test the function of the left ventricle using echocardiography and collecting data related to the left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), and left ventricular inner diameter (LVID) in systole. In most instances, a transthoracic echocardiogram (TTE) is used to best see the heart how it is functioning. This process is safer than an x-ray because there is no threat of radiation and it also produces a clearer picture. An instrument called a transducer is placed on various locations on the chest and upper abdomen aimed towards the heart. The transducer emits high frequency sound waves, picks up the echo of the sound waves and transmits them into electrical impulses. The electrocardiogram machine takes the electrical impulse information and turns it into 2D or 3D moving pictures of the heart (still pictures can also be taken). If the the patients lungs, ribs, or body tissue is blocking the echo from creating a clear picture, often a liquid contrast can be administered intravenously to better see the inside of the heart. The TTE echocardiogram would be process to measure the ventricular wall size but a different echocardiogram, Doppler echocardiogram, is needed to record the motion of blood moving through the veins and to record data related to the LVEF and LVFS. The movement of blood reflects sound waves to a transducer and an ultrasound computer is able to read the data. The computer can measure

direction and speed of the blood flowing through the heart and blood vessels. This is how the ejection fraction and fractional shortening can be measured in the hearts of rats containing either the iPSC-CM or ESC-CM cardiac patch.

I will also gather data on the infarct size using percentage of scar tissue. Using multiple staining techniques, I will also be able to test the functionality of environment the cardiac patch has made. For example, using trichrome- stained sections, the amount of collagenous matrix can be detected, as well as the amount of gap junction protein Connexin 43 that is present. Production of collagenous matrix and Connexin 43 is a direct indicator to as how well the derived cardiomyocytes are functioning as normal cardiomyocytes. IB4 staining is another important process that can detect the presence of angiogenesis, or blood vessel formation. It can also detect the Ki-67 protein that is present in proliferating tissue. The presence of angiogenesis and Ki-67 protein is evidence that suggests that the cardiomyocytes are growing and interacting with its surrounding cardiac tissue.

Statistical analysis would need to be performed on the data that is recorded in order to determine statistical significance between the two variable groups. If there is a slight difference in values of data from the ESC-CM and iPSC-CM cardiac patch group but not enough to be statistically significant, one could not reject the null hypothesis and the two groups would be considered equal. On the other hand, if one group showed statistical significance from the other, the null hypothesis would be rejected and the two groups would significantly different. This would indicate that there is a superior pluripotent cell that improves functionality of cardiac tissue after a heart infarction.

Cardiac tissue engineering is a promising field that aims to one day reduce the impact of cardiac tissue damage in individuals suffering from chronic heart disease. With the availability of donor hearts so low, researchers need a new method of therapy to replace and/or repair damaged myocardial tissue. Given the ability of iPSCs to supply large quantities of patient-specific functional cardiomyocytes for cardiac repair without the risk of immune rejection, tissue engineering using iPSCs proves to be one of the most innovative and efficient forms of cardiac repair.

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