RICE UNIVERSITY

Self-contained 3D Differentiation of Reprogrammed Amniotic Fluid Derived Stem Cells for Congenital Heart Repair

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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ABSTRACT

Self-contained 3D Differentiation of Reprogrammed Amniotic Fluid Derived Stem Cells for Congenital Heart Repair

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Congenital heart defects (CHD) are the most common type of birth defect and the leading cause of infant death. The most severe defects, such as Tetralogy of Fallot and hypoplastic left heart syndrome, can require immediate surgical intervention soon after birth. Current repair strategies involve surgically implanting inactive patch materials which often require repeat surgeries. Since congenital heart defects can be detected as early as the first trimester, the time between diagnosis and surgery can effectively be used to engineer functioning cardiac tissue. The goal of this study was to create an implantable cardiac patch that could direct the differentiation of induced pluripotent stem cells (iPSC) reprogrammed from human amniotic fluid derived stem cells (AFSC). This differentiation would take place within a closed system, minimizing laboratory handling and maximizing clinical applicability. The resulting cardiac patch would overcome current patch deficiencies associated with arrhythmia, mechanical mismatch, or even heart failure. By creating a three-dimensional system capable of temporally regulating the release of small molecules, autologous induced pluripotent stem cells could be directed to functional cardiomyocytes for use as an implantable cardiac patch for congenital heart defect repair.
repair. Further development of this system could also be used to develop repair strategies for ischemic heart repair.

In order to obtain an autologous cardiomyocyte cell source for CHD, AFSC were readily isolated from amniotic fluid obtained through routine amniocentesis. These cells were classified by previous members in our lab as broadly multipotent, though not sharing the same pluripotency as embryonic stem cells. Attempts to directly differentiate AFSC into cardiac cells resulted in expression of early and late stage cardiac markers, but lack of classic cardiomyocyte contractility. Therefore this study investigated the reprogramming of AFSC to iPSC by modified mRNA transfection and the differentiation of these reprogrammed cells into cardiomyocytes through small molecule inhibitors of the GSK3 and Wnt signaling pathways. Reprogrammed cells were shown to express markers of pluripotency and formed teratomas in vivo. Cardiac differentiation resulted in immature spontaneously beating cells which were characterized through genetic expression, immunohistochemistry and electrophysiology.

By encapsulating GSK3/Wnt small molecule inhibitors within porous silica particles (pSi), reprogrammed AFSC were differentiated into cardiomyocytes with minimal intervention. The release of inhibitors from pSi was tuned by varying the thickness of polymer coatings to coincide with the temporal cues for cardiac differentiation. We evaluated the nanoparticle size, zeta-potential, and release profile in a 2D culture, as well as cell differentiation efficiency, phenotypic analysis and electrophysiology.
Before translating the iPSC-derived cardiomyocyte (CM) differentiation into a three dimensional space, we first investigated an electrospun (ES) gelatin biomaterial and evaluated it for cardiac cell toxicity and the promotion of neovascularization in vivo. pSi containing vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) were conjugated to the ES gelatin and shown to have a sequential sustained release in vitro. Results showed a decrease in cellular toxicity in vitro due to reduced particle internalization and increased neovascularization in vivo.

The results of this research could provide new insights into repair strategies for CHD that would be functional and able to grow with the patient. It can also provide an innovative platform for future tissue engineering constructs as well as help develop cardiac specific toxicity platforms.
Acknowledgments

I would first and foremost like to thank my advisor, Dr. Jacot, for allowing me the opportunity to work in his lab and further develop myself as an independent scientist. I am grateful for the research skills and guidance I have gained and know it will continue to shape my career for years to come.

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<th>Description</th>
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<tr>
<td>CHD</td>
<td>Congenital Heart Defects</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced Pluripotent Stem Cells</td>
</tr>
<tr>
<td>AFSC</td>
<td>Amniotic Fluid-Derived Stem Cells</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen Synthase Kinase 3</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-type MMTV Integration Site Family Member</td>
</tr>
<tr>
<td>pSi</td>
<td>Porous Silica Particles</td>
</tr>
<tr>
<td>CM</td>
<td>Cardiomyocytes</td>
</tr>
<tr>
<td>ES</td>
<td>Electrospun</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>Platelet-derived Growth Factor Subunit B homodimer</td>
</tr>
<tr>
<td>ASD</td>
<td>Atrial Septal Defect</td>
</tr>
<tr>
<td>VSD</td>
<td>Ventricular Septal Defect</td>
</tr>
<tr>
<td>Oct4</td>
<td>Octomer Binding Transcription Factor 4</td>
</tr>
<tr>
<td>Sox2</td>
<td>(sex determining region Y)-box 2</td>
</tr>
<tr>
<td>Klf4</td>
<td>Kruppel-like Factor 4</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Avian Myelocytomatosis Virus Oncogene Cellular Homolog</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>Tcf /Lef</td>
<td>T cell factor/Lymphoid Enhance Factor</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>Ang1</td>
<td>Angiopoietin-1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Stromal Cell-Derived Factor 1α</td>
</tr>
<tr>
<td>T3</td>
<td>Tri-iodo-(L)-thryonine</td>
</tr>
<tr>
<td>ePTFE</td>
<td>Expanded Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PGS</td>
<td>Poly(ploycerol sebacate)</td>
</tr>
<tr>
<td>PU</td>
<td>Polyurethane</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>PGSU</td>
<td>Poly(ploycerol sebacate urethane)</td>
</tr>
<tr>
<td>PEUU</td>
<td>Polyester Urethane Urea</td>
</tr>
<tr>
<td>SIS</td>
<td>Small Intestine Submucosa</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginylgycylaspartic acid</td>
</tr>
<tr>
<td>YIGSR</td>
<td>Tyr-Ile-Gly-Ser-Arg</td>
</tr>
<tr>
<td>GRGDSP</td>
<td>Gly-Arg-Gly-Asp-Ser-Pro</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor 1</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>TTTS</td>
<td>Twin-twin Transfusion Syndrome</td>
</tr>
<tr>
<td>αMEM</td>
<td>α-minimum essential media</td>
</tr>
<tr>
<td>HLA-ABC</td>
<td>Human Leukocyte Antigen- antigens A, B, C</td>
</tr>
</tbody>
</table>
HLA-DR      Human Leukocyte Antigen- antigen D related
Tra-1-81    Tumor Resistance Antigen 1-81
Tra-1-60    Tumor Resistance Antigen 1-60
cTnT        Cardiac Troponin T
MHC         Myosin Heavy Chain
Cx43        Connexin 43
H&E         Hematoxylin and Eosin
Isl1        ISL LIM homeobox 1
Nxk2.5      NK2 homeobox 5
qRT-PCR     Quantitative Real-Time (reverse transcription) Polymerase Chain Reaction
GAPDH       Glyceraldehyde 3-phosphate dehydrogenase
SDS-PAGE    Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBST        Tris-Buffered Saline and Tween 20
MEF         Mouse Embryonic Fibroblast
IWP2        Inhibitor of the Wnt pathway 1
HPLC        High Performance Liquid Chromatography
DLS         Dynamic Light Scattering
SEM         Scanning Electron Microscopy
HUVEC       Human Umbilical Vein Endothelial Cells
VEGFA       Vascular Endothelial Growth Factor A
VEGFR2      Vascular Endothelial Growth Factor Receptor 2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL3A1</td>
<td>Collagen Type III Alpha</td>
</tr>
<tr>
<td>TEOS</td>
<td>Tetraethyl Orthosilicate</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescin isothiocyanate</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>HFIP</td>
<td>Hexafluoro-2-propanol</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha Smooth Muscle Actinin</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction and Overview

In cardiac tissue engineering, the major limitation is a robust cellular source. This stems from the fact that cardiomyocytes are terminally differentiated cells and their replicative capabilities are extremely limited. iPSC provide a potential cell source that can be renewed and have the ability to generate all somatic cell lines. In the case of CHD requiring full thickness cardiac repair, the ideal cell source would be autologous, able to expand and utilized at the time of surgical need. In order to make an iPSC source more translatable to the clinic, cellular reprogramming would need to ensure minimal mutagenesis risk. The differentiation of an iPSC source into cardiac cells can further be translated by controlling the release of small molecules inhibitors of the GSK3/Wnt pathways. By incorporating this differentiation platform within a closed system biomaterial, this would help eliminate handling interactions and be more applicable for clinical translation.
1.1. Project Overview

The overall objective of this research was to develop a cardiac patch platform that directs the differentiation of AFSC derived iPSC within a three dimensional construct. The overarching goal of this research was to create a fully functional and supportive patch material that is capable of repairing full thickness cardiac defects in congenital heart patients.

We hypothesized that 1) a population of stem cells derived from amniotic fluid can be reprogrammed by modified mRNA into a pluripotent state and then differentiated into functional cardiomyocytes, 2) Small molecules can be temporally released from encapsulated particles to differentiate AFSC-iPSC into cardiac cells. 3) Encapsulated particles conjugated to an electrospun biomaterial would reduce cellular toxicity and could be formulated to promote angiogenesis.

1.2. Specific Aim 1

Differentiation of Spontaneously Contracting Cardiomyocytes from Non-virally Reprogrammed Human Amniotic Fluid Stem Cells

This aim was motivated by research presented in the literature, as well as previous work in our lab, that showed the potential for AFSC to be differentiated into cardiac-like cells. Previous works into the direct differentiation of AFSC into cardiac-like cells showed expression of early and some late stage cardiac markers(1, 2). However, no research to date had been able to show functional beating cardiac
cells differentiated directly from AFSC. The molecular and genetic mechanisms are not fully understood as to why direct AFSC differentiation into cardiac cells has not proven, but the temporal regulation of the Wnt/GSK3 signaling pathways has been shown to be critical. Since these pathways are critical in regulating a number of gene transcription events, our first approach was to reprogram AFSC to a pluripotent state using mRNA transfection of Yamanaka factors. Once AFSC derived iPSC were created, we verified pluripotency through immunofluorescence and teratoma studies. Directed cardiac differentiation of AFSC-iPSC then occurred through small molecule inhibition of the GSK3 and Wnt signaling pathways. Successful cardiac differentiation was assessed through immunofluorescence, flow cytometry, protein expression, and calcium/voltage sensitive dyes.

1.3. Specific Aim 2

Controlled Release of Small Molecules for Cardiac Differentiation of Pluripotent Stem Cells

The timing and exposure of GSK3 and Wnt inhibitory molecules are critical to the overall cardiac differentiation efficiency of iPSC. The engineering of an encapsulation platform for GSK3 and Wnt inhibitory molecules would allow for a self-contained differentiation requiring minimal laboratory handling. The dual release mechanism was accomplished by blending two formulations of small molecule loaded particles. The first release was from pSi that were tuned to release the GSK3 inhibitor within 24 hours of exposure to iPSC. The Wnt inhibitor was
loaded within pSi then further coated with poly(lactic-co-glycolic acid) (PLGA) in order to delay release. Full characterization of the encapsulated particles was done through SEM, size and zeta potential, loading efficiency and release profile. This 2D delivery system gave insight into cardiac differentiation cues as well as expected local drug concentrations. AFSC-iPSC showed to have little cellular toxicity and high viability in response to particle localization. Also the timing and release of the inhibitory molecules from particles was sufficient in directing cardiac differentiation in AFSC-IPSC. Cardiac differentiation was analyzed through spontaneous contraction, immunofluorescence of cardiac morphologies, gene/protein expression and action potential analysis.

1.4. Specific Aim 3

Electrospun patch functionalized with nanoparticles allows for spatiotemporal release of VEGF and PDGF-BB promoting in vivo neovascularization.

In order to translate the pSi cardiac differentiation platform into a three dimensional space, we investigated the internalization and resulting apoptosis signaling in neonatal rat cardiac cells. Freely dispersed pSi were compared to pSi crosslinked to ES gelatin patches. Our findings showed that there was significant reduction in overall particle internalization in ES gelatin patch groups compared to pSi groups. As a result, apoptosis was shown to be decreased in the ES gelatin patch group as well. In order to highlight the dual release capabilities of this platform,
VEGF and PDGF-BB were loaded into pSi conjugated to ES gelatin scaffolds and assessed for neovascularization capabilities in vivo. Through histological and genetic analysis of explanted patches, results showed that the VEGF and PDGF-BB released from the ES gelatin patch worked in a synergistic manner increasing localized angiogenesis in a subcutaneous mouse model. Collectively, ES gelatin with conjugated pSi showed a delivery platform that reduced cardiac cell toxicity and was able to promote neovascularization when loaded with growth factors.
Chapter 2

Background

2.1. Congenital Heart Defects

Based on clinical studies, CHD affect around 1% of live births (3-5). Ultrasound procedures allow for approximately 39% of major CHD, such as Tetralogy of Fallot (Figure 2.1) or hypoplastic left heart syndrome, to be diagnosed in utero during the second trimester (6), while the diagnosis of acute CHD, such as small atrial septal defects (ASDs) or ventricular septal defects (VSDs), may not occur until birth or later. Typically a trivial cardiac lesion that has eluded detection will naturally close during infancy (7). Also, many CHD do not manifest themselves until there is mixing of oxygenated and deoxygenated blood after birth. This results from incomplete closure of fetal circulatory vessels, such as the ductus arteriosus. Therefore the actual reported percentage of births with CHD is likely to be slightly higher than reported (3).
The heart is a complex organ and a myriad of diseases can result from lesions or malfunctions. As suggested by the statistical occurrence of CHD, the degree of severity in CHD is very diverse. There are a number of risk factors that can lead to CHD, including but not limited to, maternal diabetes and maternal lithium exposure, phenytoin and alcohol use. In cases of maternal diabetes, the risk of having a newborn with a structural heart defect increases by 30% (8). Family history of CHD can also play a role, with about 1-4% of babies born to parents with CHD are affected (3). The exact causes for the majority of CHD are unknown, but are proposed to be linked to causative genes in developing fetuses (9). From the perspective of tissue engineering for CHD, corrective action attempts to treat abnormalities associated with a given defect. A brief overview of clinically relevant lesion specific CHD will help to better understand the engineering challenges in

Figure 2.1 - Tetralogy of Fallot
creating a viable construct. Table 2-1 summarizes some specific CHD with associated causes and current treatment options.

Table 2-1 Congeital Heart Defects (10)

<table>
<thead>
<tr>
<th>Defect</th>
<th>Estimated % of Total Congenital Heart Defects in the US (3, 11)</th>
<th>Causes</th>
<th>Treatment Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventricular septal defects</td>
<td>20%</td>
<td>Hole in ventricular septum</td>
<td>Septal baffles (12-14), patch closure materials (15)</td>
</tr>
<tr>
<td>Tetralogy of Fallot</td>
<td>10%</td>
<td>1. Large ventricular defect 2. Severe right ventricular outflow tract obstruction 3. Overriding of the aorta 4. Right ventricular hypertrophy</td>
<td>Right ventricular outflow tract reconstruction (16), VSD closure device (17, 18)</td>
</tr>
<tr>
<td>Patent ductus arteriosus</td>
<td>10%</td>
<td>Ductus arteriosus remains open after birth</td>
<td>PDA occlusion device (19), occlusion spring coil (20)</td>
</tr>
<tr>
<td>Atrial septal defect</td>
<td>5%</td>
<td>Hole in atrial septum</td>
<td>Septal baffles (21, 22), patch closure materials (23, 24)</td>
</tr>
<tr>
<td>Transposition of the great vessels</td>
<td>5%</td>
<td>Abnormal spatial arrangement of great vessels</td>
<td>Arterial switch operation (25), septal occlusion device</td>
</tr>
<tr>
<td>Atrioventricular septal defects</td>
<td>3% (26)</td>
<td>Hole in atrial and ventricular septum, tricuspid and mitral valves might not form separately</td>
<td>Patch closure materials, atrioventricular valve repair (18, 27)</td>
</tr>
<tr>
<td>Aortic valve stenosis</td>
<td>5%</td>
<td>Narrowing across aortic valve</td>
<td>Surgical or balloon valvuloplasty (28), bioprosthesis (29)</td>
</tr>
<tr>
<td>Ebstein anomaly</td>
<td>1%</td>
<td>Abnormal tricuspid valve and atrialization of ventricle</td>
<td>Valve reconstruction, Bioprosthetic or mechanical valve, surgical or transcatheter placement (30, 31)</td>
</tr>
<tr>
<td>Tricuspid atresia</td>
<td>1%</td>
<td>Absence of tricuspid valve</td>
<td>Valve reconstruction, Bioprosthetic or mechanical valve, surgical or transcatheter placement (31, 32)</td>
</tr>
<tr>
<td>Truncus arteriosus</td>
<td>&lt;1%</td>
<td>Pulmonary artery and aorta fail to separate</td>
<td>Right ventricular outflow tract reconstruction, VSD closure patch, valve reconstruction (33)</td>
</tr>
<tr>
<td>Coarctation of the</td>
<td>10%</td>
<td>Mild-severe</td>
<td>Patch for aortoplasty (34),</td>
</tr>
<tr>
<td>Condition</td>
<td>Incidence</td>
<td>Description</td>
<td>Treatment</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>-----------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Interrupted aortic arch</td>
<td>1%</td>
<td>Underdeveloped/ completely occluded aorta</td>
<td>Surgical reconstruction (36), patch for aortoplasty (34)</td>
</tr>
<tr>
<td>Hypoplastic left heart syndrome</td>
<td>1%</td>
<td>Varying degrees of underdeveloped aorta, aortic valve, left ventricle, mitral valve, and left atrium</td>
<td>Left heart total repair, shunts (37), aortoplasty (34)</td>
</tr>
<tr>
<td>Anomalies of the pulmonary veins</td>
<td>1%</td>
<td>Mild-complete obstruction of pulmonary veins</td>
<td>Venous connection reconstruction, patch closure materials (38, 39), stents (40)</td>
</tr>
<tr>
<td>Anomalous left coronary artery from the pulmonary artery</td>
<td>&lt;1% (41)</td>
<td>Coronary artery carries deoxygenated blood from pulmonary artery instead of the aorta</td>
<td>Coronary artery anastomosis, saphenous vein bypass graft, modified Takeuchi procedure (41)</td>
</tr>
</tbody>
</table>

### 2.2. Congenital Heart Defect Tissue Engineering

Tissue engineering is an emerging field in regenerative medicine and can be defined a number of ways, but the overall goal is to generate or promote the growth of new tissue to restoring native structure and function of damaged or degenerative tissue. Current ideology on tissue engineering suggest certain key components in making a successful construct, Figure 2.2. The components of an ideal tissue engineering construct can be simplified into the scaffold, cells, biomechanical/biophysical stimuli, and biochemical stimuli (drugs and growth factors) (42).
A major issue in tissue engineering is that restoring native tissue requires a heterogeneous host of tissues. In the example of dermal tissue engineering, there are a number of suitable collagen/elastin based constructs that can mimic the native “barrier” function of the skin, but nothing has been shown to restore other dermal characteristics such as sweat glands and hair follicles. This heterogeneous concept of tissue alludes to the challenge of neovascularization. Tissues are three dimensional and therefore the exchange of oxygen, carbon dioxide, and other nutrients must take place throughout the entire volume of tissue. Cells beyond the limit of diffusion (200µm) will not survive without this proper exchange.

In congenital heart disease, common abnormalities requiring surgical intervention involve a lesion or hole in septal heart tissue requiring closure. Small defects (<5mm) often do not require surgical intervention and will spontaneously close (44). However, when the hole is too large, surgeons will place patches or
occlusion materials in a patient to help bridge the native heart tissue and allow for restored structure. In terms of an ideal tissue engineering construct, restoring structural support is only part of the challenge. A biomaterial may meet the mechanical properties needed to withstand the fatigue of a contracting heart, but if the material is inert it will not completely restore native function. Vascularization is also a limiting factor in current cardiac patches (45). Unless the cardiac patch is prevascularized or able to have neovascular ingrowth, the patch will be diffusion limited and therefore limited to the overall thickness (46). Another requirement in restoring native function is the synchronized beating of an implanted cardiac patch with the native heart pace (47). Following the restoration of natural function is the ability of living tissue to grow and expand. This is particularly important in pediatric patients, where their continued growth can lead to implant failure, requiring reoperations (21). This necessity to grow and expand presents a unique engineering challenge which is not relevant in adult applications. A number of CHD cases require cardiac wall repair, the most common being ventricular/atrial septal wall repairs using “baffles” that direct the flow of blood between desired chambers while sealing blood from other chambers (48). This differs from full-thickness cardiac wall patches where the goal is to augment free wall structures. Examples of full-thickness cardiac patches needed during surgical intervention include: ventricular outflow patch for Tetralogy of Fallot, complete left heart remodeling in hypoplastic left heart syndrome, and a right ventricular patch in Ebstein anomaly. To date, no commercially available patch can completely restore native structure and function after implantation.
Figure 2.3 - Schematic of tissue engineered system for repair of congenital heart defects

2.2.1. Cell Sources for CHD Tissue Engineering

A tissue construct can require an enormous amount of cells to fully populate the entire volume of an actual implant. The cells within a construct make up the majority of mass and play key roles in integrating with existing native tissues and help maintain tissue homeostasis. The two main classes of cells that have been considered in cardiac tissue engineering are terminally differentiated cells and stem cells. Ideally the cells would come from an autologous source, but this can prove to be difficult in the case of a developing fetus. Xenogeneic cells from a nonhuman species have limitations in clinical applications due to the significant differences in antigens between species. Allogeneic cell sources have potential in terms of ability to
create a predesignated supply of cells; however complications with immune response and tissue rejection are possible.

2.2.1.1. **Skeletal Myoblasts**

Skeletal myoblasts have been researched as an autologous source for cardiac repair. Skeletal muscle biopsies are taken from the patient and grafted directly to cardiac tissue. Preclinical animal studies have proven skeletal muscle to engraftment, the creation of myotubules, and improved cardiac function post myocardial infarction (MI) (49). Other studies showed direct injection of skeletal myoblast into cardiac infarct tissue may have beneficial effects on cardiac output (50, 51). When considering skeletal myoblasts as a potential autologous cell source for repair of CHD, they are not likely a feasible option for neonatal patients considering their limited muscle mass. Another limitation associated with skeletal myoblast engraftment is their inability to propagate electrical signals. This poses major risks in terms of developing arrhythmias due to the separation of functioning cardiac cells by patches of skeletal muscle cells (52).

2.2.1.2. **Adipose Derived Stem Cells**

Adipose derived stem cells have been considered as a cell source for cardiac repair because this type of cell is readily available, easily harvested, and have a relatively low cost. Adipose tissue includes a heterogeneous mixture of mesenchymal stem cells, hematopoietic stem cells, and endothelial progenitor cells. Therefore a number of basic structural tissues can be developed. Adipose derived
stem cells have been shown in preclinical studies to improve ventricular function in infarction animal models (53, 54). In relation to CHD repair, the same issue exists as with skeletal myoblasts, action potential propagation is hindered and can cause arrhythmias.

2.2.1.3. Resident Cardiac Stem Cells

Resident cardiac stem cells or cardiac progenitor cells have recently been discovered and have warranted great research potential, Figure 2.4. Previously, the heart was considered to lack the ability for self-renewal, therefore a terminally differentiated organ. This likely stems from the fact that cardiac cells are terminally differentiated and are not capable of re-entering the cell cycle under any condition. However, recent evidence has shown that a special population of cardiac cells demonstrate continuous cell division following injury such as myocardial infarction (55). Several studies have shown that resident cardiac stem cells have the capability to differentiate into multiple cell types, such as cardiomyocytes and vascular smooth muscle cells (56, 57). In an animal model, resident cardiac stem cells previously isolated and injected into the site of infarction were shown to reduce the size of infarct and restore a degree of function (55). However the main disadvantages of using cardiac stem cells as a tissue engineering cell source are the limited number of cells per cardiac tissue area, weak proliferation capacity, and harvesting technique.
2.2.1.4. Amniotic Fluid-Derived Stem Cells

Amniotic fluid derived stem cells are broadly multipotent stem cells that are readily isolated from amniotic fluid. Amniotic fluid is also fairly easily obtained through minimally invasive amniocentesis procedures. Populations of cells from amniotic fluid have been shown to express the pluripotency marker, Oct4 (1). AFSC are isolated from amniotic fluid based on adherence and immunoselection for c-kit (CD117) expression utilizing cell sorting protocols. C-kit is a surface receptor shown to play a role in cell survival, proliferation and differentiation (58). Sorted AFSC have been shown to be able to differentiate into numerous cell types such as adipocytes, muscle cells, endothelial and nerve cells, representing cell types from all three germ layers, Figure 2.5 (46).
Figure 2.5 - RT-PCR analysis showing possible differentiation linages of AFSC

Previous studies have investigated the direct differentiation of AFSC to cardiac cells and proven the expression of late stage cardiac markers and functional gap junctions (60). To date, direct differentiation of AFSC to beating cardiac cells has not been shown. Unlike embryonic stem cells, AFSC do not form teratomas when injected into immunodeficient mice. This gives AFSC a distinct advantage for clinical translation.

In terms of a cell therapeutic source for CHD repair, AFSC show great potential for various applications due to their ease of isolation and little to no disruption to the developing fetus. An AFSC cell source could be autologous to a newborn patient, therefore reducing the risk for immune rejection compared to allogenic cell sources.
2.2.1.5. Induced Pluripotent Stem Cells

Induced pluripotent stem cells are somatic cells that can be converted back to a pluripotent state. Through exposure of reprogramming factors, Oct4, Sox2, Klf4, and c-Myc, adult cells can be reprogrammed to express embryonic genes, Figure 2.6 (48).

Figure 2.6 - Reprogramming of somatic cells (61)

These cells are capable of differentiating into tissues other than their original specific lineage. The potential for iPSC to differentiate into cells originating from all three germ layers is of great interest in tissue engineering. However, since iPSC resemble embryonic cells, they too carry the ability to form teratomas when injected into immunodeficient mice.
iPSC have also been proven to be capable of differentiating into functioning cardiac cells through inhibition of the GSK3 and Wnt signaling pathways (62). Inhibition of GSK3 allows for the accumulation of β-catenin which associates with Tcf/Lef and activates gene transcription, preventing embryoid body formation. At this point cells are directed to a mesoderm lineage and inhibition of the Wnt signaling pathway will lead to cardiac differentiation. By optimizing the temporal exposure of small molecule inhibitors of GSK3/Wnt signaling, greater than 85% cardiac differentiation efficiency was achieved (63, 64). Cardiac cells derived from iPSC have been shown to develop spontaneous contractility, but myofilaments do not show directional alignment without maturation.

2.3. Drug delivery in Cardiac Tissue Engineering

Drug delivery and tissue engineering are closely related and have a myriad of applications and techniques. The classes of drugs delivered are many as well, ranging from small molecules, antibiotics, proteins, DNA and RNA. The predominate goal of delivering drugs as part of a tissue engineering strategy are to overcome relatively short half-life/stability issues while in circulation and to maximize therapeutic activity while minimizing toxicity. Applications of drug delivery for CHD tissue engineering are limited, but many repair strategies for myocardial infarction have been investigated. Most cardiac tissue engineering therapies investigate delivery via direct implantation or injection(65, 66), likely due to the highly metabolic and increased fluid mechanics of the heart. However some studies have researched targeting of infarcted myocardium through particles formed through
hydrophobic interactions (67, 68). Aside from the numerous cell-based therapies, drug therapy in cardiac tissue engineering is primarily focused on angiogenic potential, myocardial cell preservation and cellular maturation.

### 2.3.1. Therapies Promoting Angiogenesis

The development of strategies to promote neovascularization fits synonymously with any full thickness tissue repair model. The limit of diffusion hinders most biomaterials past 200µm from having successful cellular growth and proliferation due to limited oxygen and waste exchange. Therefore many growth factors, small molecules and gene therapies are directed at angiogenesis, particularly in myocardial infarction models, in order to regain circulation in ischemic areas.

Various growth factors have been researched to promote angiogenesis in cardiac tissue engineering strategies, i.e. PDGF, VEGF, angiopoietin-1 (Ang1) and Transforming Growth Factor β (TGF-β). These growth factors target cells recruited to sites of infarction and induce neovascularization pathways by attaching to external signaling receptors(69, 70). Factors such as VEGF have been shown to be intrinsically upregulated in serum following the onset of acute myocardial infarction(71). The time course of increased VEGF follows in line with early stage remodeling suggesting that VEGF plays an important role in endogenous angiogenesis, most likely secreted from platelets(72). PDGF has also been linked to later stage angiogenesis remodeling in terms of its effect on endothelial cells. Previous studies suggest that PDGF-BB amplifies angiogenesis through direct action
on endothelial PDGF receptor-β(73, 74). Another approach to endothelial cell regulation has been the delivery of nucleic acids in the form of microRNAs. Studies have shown that delivery of microRNA-24 and microRNA94A in MI mouse models played a critical role in regulating endothelial apoptosis and angiogenesis(75, 76).

The combinatory effect of growth factors has also shown to promote greater neovascularization and maturation compared to single factors alone. When VEGF-A165 and PDGF-BB loaded into alginate hydrogels were directly injected into 1 week post infarcted rat models, vessel maturation and cardiac function were significantly increased compared to either factor alone(77) (Figure 2.7). Other studies have suggested that hypoxia-induced co-expression of VEGF and Ang1 improved post MI function in a porcine model by increasing vascular density, reducing apoptosis and activating cardiac survival pathways(78). Cao et al. showed the combination of PDGF-BB and fibroblast growth factor-2 had angiogenic synergism forming more stable and functional vascular networks compared to either factor alone(79).
2.3.2. Therapies Aimed at Cardiac Preservation

Since the majority of ischemic cardiac treatments come after the onset of a MI, extensive research has gone into cellular cardioprotection and preservation. Cells are targeted to be better conditioned to prevent tissue necrosis and in turn maintain greater cardiac function following MI.

TGF-β has been shown to be upregulated in many experimental models of MI, with isoforms in both early and late stages of infarct healing (80). While TGF-β signaling during MI is not completely understood, some studies have shown that inhibition of TGF-β1 by endothelial nitric oxide synthase (eNOS) gene transfer has cardioprotective attributes such as inhibition of cardiac apoptosis and stimulating
neovascularization if treated prior to MI induction\(^{(81)}\). Other preventative studies have shown that preemptive inhibition of Na\(^+\)/H\(^+\) exchange by the drug cariporide resulted in improved left ventricular recovery following MI possibly resulting from the prevention of reperfusion injury\(^{(82)}\).

Cardioprotective therapies after MI have also have been researched aiming to prevent apoptosis and promote remodeling shortly after ischemia\(^{(83)}\). Previous studies have shown that p38 kinase transfected into the cardiac wall of MI induced mice, resulted in significant rescue of failing myocardium in terms of cardiac output and reduction of infarct area\(^{(84)}\). Other studies have shown that the signaling protein stromal cell-derived factor 1α (SDF-1α) played an important role in cardiac tissue protection and regeneration\(^{(85, 86)}\). Improved cardiac function and increased neovascularization was shown in MI mouse models\(^{(87, 88)}\).

### 2.3.3. Therapies Targeting Myocardial Maturation

Previous research has proven that physical and electrical stimuli can induce cardiomyocyte maturation \(^{(89-91)}\). With the widespread accessibility of iPSC and their effective differentiation into cardiac cells\(^{(63)}\), recent research has evolved to introduce chemical cues for the maturation of iPSC-CMs. As mentioned previously, iPSC-CMs provide a renewable source of cardiac cells whereas native cardiomyocytes are terminally differentiated. However iPSC-CMs functionality resembles immature cardiac cells.
Tri-iodo-L-thryonine (T3) has been researched to promote cardiac hypertrophy in rats and mice when given orally(92). Recent studies have investigated the effect of T3 on iPSC-CMs and have shown signs of cardiac maturation with increased cardiomyocyte size, anisotrophy, sarcomere length and contractile force(93-95).

Ascorbic acid has also been shown to enhance differentiation and maturation of iPSC-CMs (96). Ascorbic acid was proven to play a role in intercalated disc formation, collagen deposition, titin expression and metabolic activity(97). This alteration in cardiac cell structure and extracellular matrix can increase overall maturation.

2.4. Biomaterials for CHD Tissue Engineering

Materials intended to replace living tissue pose a number of challenges in terms of host functionality and host response. The ideal biomaterial would not only provide structural support and promote cellular infiltration, but would not illicit any host immune response. In terms of ideal cardiac engineering materials, action potential conductance further complicates matters. For CHD patients, the implanted material must grow with the patient or subsequent re-operations may be necessary to compliment surrounding tissue growth. Previous research has investigated a number of different materials for cardiovascular applications. These materials can be classified into groups based on their compositions whether they are synthetic, natural or combination of the two.
2.4.1. Synthetic Materials

Synthetic polymers for surgical correction of CHD are limited to bioinert materials which can often illicit an inflammatory response and fibrosis. The appeal of synthetic polymers stem from their tunable mechanical, structural and degradation properties. Fabrication of synthetic polymer based scaffolds is very diverse and can involve techniques such as UV polymerization (98), electrospinning (99), or laser sintering (100), to name a few. Polymers such as polyethylene terephthalate and expanded polytetrafluoroethylene (ePTFE) have been used as cardiac patch materials in areas of lower cyclic mechanical stress such as the septal wall (Figure 2.8). However both polymers are not biodegradable and present issues in terms of tissue ingrowth and remodeling. Ideally, corrections for CHD will only require one surgical procedure. In the case of cardiac patches, this requires a delicate balance of new tissue ingrowth aligned with scaffold degradation. Therefore much research has focused on using biodegradable synthetic polymers such as PLGA, poly(plycerol sebacate) (PGS), polyurethanes (PU), and polycaprolactone (PCL) as scaffolds for cardiac tissue applications. The main issue using synthetic polymers as a scaffold material is inferior cell adhesion compared to that of natural polymers. Therefore some strategies involve coating synthetic polymers with other naturally derived materials, as discussed later in the chapter.

Dacron® (polyethylene terephthalate) grafts are sometimes used as septal defect patch material, Figure 2.8. Dacron® is a strong, stable polymer that exhibits minimal degradation in vivo. Once implanted, Dacron® elicits an inflammatory reaction and subsequent fibrosis occurs. This is a structural solution for defective
cardiac tissue often applied to septal repairs, however the material remains inert (101). Another synthetic polymer, ePTFE, is also currently used in repairing CHD. The patch materials developed from ePTFE are arranged spatially to have pores ranging from 20-30 μm, which has shown to inhibit cellular ingrowth (102). Also, ePTFE does not induce fibrosis as Dacron®, and therefore can be used as a patch in areas of blood flow, such as a right ventricular outflow reconstruction (103). Both of these patch materials are far from an ideal engineered cardiac tissue. Since the patches are not remodeled and elicit an inflammatory response (104), deficient mechanical properties as well as hemodynamic changes result. These polymers will not degrade after implantation and will not promote complete tissue remodeling. This is important in pediatric patients that will continue to grow and therefore an inert patch will likely require subsequent surgical operations.

Figure 2.8 - Synthetic materials used for cardiac repair. Left, GORE-TEX Cardiovascular Patch (ePTFE) Right, MAQUET vascular graft, Dacron (105)
Creating a synthetic material that is biocompatible and is able to grow and remodel with a patient is no simple task. Therefore bioresorbable polymer research into a viable patch for CHD has much attention. Like natural polymers, synthetic bioresorbable polymers will degrade over time in vivo resulting from hydrolysis or enzymatic cleavage. The ideal construct will provide mechanical support long enough for native or seeded cells to produce and remodel ECM as well as promote angiogenesis. In CHD patients, this allows for continued growth without the need for additional surgical interventions. PLGA is a biodegradable polymer that is used in many applications in tissue engineering from drug delivery to hard and soft tissue. The appeal of PLGA stems from the naturally metabolized degradation products of lactic and glycolic acid. Zhou et al. (106) showed that a PLGA scaffold wrapped with omentum significantly improved ventricular remodeling and cardiac function.

PGS has been studied frequently in the field of soft tissue engineering. PGS is an appealing polymer that is able to sustain and recover from deformation with minimal loss of elasticity. Therefore the viscoelastic properties of PGS are suggested to fit a mechanically dynamic environment such as the heart. Chen et al. (107) developed a cardiac patch using preconditioned PGS scaffolds in combination with human embryonic stem cell-derived cardiomyocytes. The PGS scaffolds were preconditioned for 6 days in media and cardiomyocytes were seeded and shown to attach without the need for a gelatin coating. The conditioned PGS scaffold was able to sustain beating cardiomyocytes in vitro for longer than 3 months. Recently, Rai et al. (108) created a biomimetic PGS scaffold attempting to chemically modify the
surface of the material in order to enhance cardiomyocyte attachment. This was done by a process involving alkaline hydrolysis and acidification to expose surface carboxyl chemical groups. These groups could then be functionalized with peptides YIGSR and GRGDSP in order to promote cellular attachment. Their results showed that a ligand surface concentration of $10^{-15}$ mL/cm$^2$ was sufficient to support attachment and growth of both rat and human cardiac progenitor cells. The Karp lab has expanded on the mechanical properties of PGS, creating poly(ploycerol sebacate urethane) (PGSU). This co-polymer blend was shown to have tunable mechanics and the ability to deliver localized biomolecules when tested as a cardiac patch material (109).

Fujimoto et al. (102) developed a polyester urethane urea (PEUU) cardiac patch and implanted them into rats with infarcted left ventricular wall. Their results suggested that the scaffold promoted ingrowth of smooth muscle bundles with mature contractile phenotype. Also, 8 weeks after implantation the PEUU patch was largely degraded, suggesting cellular migration and improved cardiac remodeling.

PCL is another synthetic polymer of interest when considering cardiac patch development. PCL is a biodegradable polymer and can be hydrolyzed at the ester linkages forming nontoxic byproducts. Yeong, et al. (100) fabricated a PCL scaffold using a computer-aided selective laser sintering technique. This fabrication technique showed proof of concept for a customizable scaffold design allowing for uniform control of pore size and patterning. By controlling the laser wattage and scanning speed, PCL particles were sintered into a disc shaped scaffold composed of small repeating square pyramid units. In this particular design, they were able to
obtain a compressive stiffness of 345 kPa. Further optimization of scaffold design could lead to tensile strengths closer to native myocardium, which is on the range of 3-15 kPa. It is important for a patch to exhibit stiffness similar to native tissue as very elastic materials could form aneurysms and less elastic materials could present high local stress areas (110).

2.4.2. Naturally derived

A number of strategies involve the use of naturally derived materials as scaffolds for cardiac tissue engineering. The major advantage of naturally derived materials is that they are composed of molecules found in vivo and will degrade into natural metabolic products. Also cellular response can be favorable compared to other synthetic materials in that adhesion molecules which enhance bioactivity are already present within the natural material. In CHD, many surgical procedures have used autologous pericardium as patch material (111). The advantages to this technique are that the material is immediately available, nonimmunogenic, and relatively free of cost. Pericardium has mechanical properties that are inferior to native cardiac tissue and therefore in order to enhance the mechanical properties, some surgical procedures involve glutaraldehyde to fix the material. With this method, aldehyde groups are crosslinked at the amine groups on the lysine and hydroxylysine residues of pericardium collagen. Pericardium is typically crosslinked for 15 to 30 minutes, where the duration of crosslinking can affect the resulting mechanical properties (112). Glutaraldehyde is a toxic solution and therefore adequate washing of the patch material is needed prior to implantation.
Other crosslinking agents, such as genipin (114) and acyl azide (115), have been tested as well. One of the main drawbacks in the use of autologous pericardium as a patch material is the inability of the material to expand or grow in pediatric patients. Also, crosslinking procedures will lyse any native cells, as well as create a chemically different material resulting in possible calcification and fibrous encapsulation (116).

The use of decellularized matrix as a scaffold is appealing because the components were originally functional tissue. Depending on the decellularization process, key ECM molecules can be preserved and therefore may help in promoting new tissue remodeling. Currently there are three decellularized patches approved for cardiovascular patch applications; a pulmonary artery patch material (MatrACELL™, Virginia Beach, VA), a pericardial patch (CryoPatch®, CryoLife Inc, Kennesaw, GA), and a decellularized porcine intestinal submucosa (CorMatrix®, Alpharetta, GA) (117). CorMatrix® however is approved only for use on artery, valve and pericardial tissue repair. Some disadvantages associated with decellularized matrices are immunogenicity, risk of disease transmission, and donor availability (118). Recent studies by Rajabi-Zeleti et al. (119) attempt to renew the use of pericardium-derived patches using an alternative approach to glutaraldehyde fixation. Their group used decellularized pericardium that was enzymatically digested and then reformed into pericardium gels. They showed that after one month of subcutaneous implantation in rats, the pericardium gel based scaffold had low immunological response, enhanced angiogenesis, and cardiomyocyte differentiation compared to control collagen and plain decellularized pericardium.
Crapo et al. (120) show that a small intestinal submucosa (SIS) based gel seeded with neonatal rat cardiac cells created tissue closer to physiological function compared to that of cells seeded on Matrigel. Function was measured in terms of contraction rate and normalized troponin T expression, both higher in the SIS gel group. This difference is attributed to the differing components of each gel. While both gels contain a variety of different ECM components, the main difference is that the SIS based gel contains high concentrations of collagen type I and III, similar to that of myocardium. At a minimum this study proves that myocardial cell attachment and resulting function is complex and must be carefully considered when choosing biomaterials for cardiac tissue engineering.

Since native cardiac tissue extracellular matrix is predominately composed of collagen, many studies aim to manipulate collagen based scaffolds using growth factors and/or altering properties such as alignment and microstructure (121). Collagen has good cellular attachment and proliferation (122). The main drawbacks to collagen based scaffolds in tissue engineering applications are inferior mechanical and degradation properties. A major requirement for cardiac tissue engineering is the ability to withstand contractile forces of a beating heart. Also as a patch material, collagen patches can be difficult to suture due to their mechanical weakness. However, since collagen is still the main component of cardiac ECM, there is relevance to its use as a scaffold. Miyagi et al (123) produced a collagen based patch that contained covalently immobilized VEGF. This patch was implanted into the right ventricular walls of rat hearts for up to 28 days, showing improved neotissue formation in terms of cell recruitment, proliferation, and blood vessel
density when compared to scaffolds without VEGF. A higher density of VEGF immobilization was also shown to have a greater resulting blood vessel density compared to lower VEGF concentrations. In another study, Serpooshan et al. (124) used compressed collagen type I as scaffolds for myocardial infarction repair. While this is not a congenital disorder, this patch may have applications for remodeling hearts with Tetralogy of Fallot. Their results after four weeks of implantation exhibited limited fibrosis, diminished dilation of the left ventricle, as well as angiogenesis within the patch compared to the control.

Because decellularized matrices and purely collagen based scaffolds both have disadvantages in creating a fully functioning cardiac patch, other naturally derived polymers such as alginate, chitosan, and silk fibroin continue to have research interest (125). Each of these polymers is extracted from living organisms and has shown applications in tissue engineering. Studies have shown that alginate, a natural polymer found in cell walls of seaweed (126), combined with RGD peptide can be formed into scaffolds for cardiac tissue engineering. The RGD is important in cellular attachment to the scaffold, and is immobilized by carbodiimide chemistry. Shachar et al. (127) show the maintenance of key cardiac markers, α-actinin, N-cadherin, and connexin-43, suggesting that alginate-RGD immobilized scaffolds alleviate the need for the addition of ECM proteins or Matrigel. Chitosan is also a promising natural polymer investigated as a component for cardiac patch tissue engineering. It is a linear polysaccharide derived from chitin, which is found in shellfish exoskeletons. Alone, chitosan has been successfully applied to the wound healing market as a clotting agent accelerating hemostasis (128). In tissue
engineering, when chitosan is combined with collagen, the resulting material has mechanical properties superior than collagen alone. Kathuria et al. (129) developed and characterized an elastic chitosan-gelatin cryogel that could be used for potential tissue engineering applications. This material was able to withstand cyclic deformations up to 40% without significant deformation with a Young’s modulus ranging from 36-39 kPa.

Silk fibroin is investigated in a number of tissue engineering applications because it is mechanically strong, noncytotoxic, presents low immunogenicity, and is biodegradable. An investigation into silk fibroin from A. mylitta silk worms shows better cardiomyocyte attachment and functional beating up to 20 days (130). These results are superior than similar scaffolds using silk fibroin derived from mulberry B. mori silk worms. This is proposed to be a result of A. mylitta having RGD domains. Chi et. al, (131) investigated a chitosan-hyaluronan/silk fibroin cardiac patch implanted into the left ventricles of rats. This patch was developed creating an aqueous silk fibroin/chitosan/hyaluronan solution in a 10:1:1 ratio, and then spray-dried into patch form. Their implanted patch showed reduced dilation of left ventricular diameter (4.27 ± 0.29mm), increased wall thickness (1.5 ± 0.13mm), and improved left ventricular fractional shortening (42.8 ± 2.4%).

2.4.3. Natural/Synthetic hybrid

In order to take advantage of the cellular attachment and signaling capabilities of natural polymers while maintaining a way to control and optimize mechanical properties, researchers have investigated a number of materials
combining both natural and synthetic materials. Pok et al. (110, 132) developed a multilayered scaffold composed of PCL, chitosan and gelatin. This scaffold was self-assembled with the stronger PCL core sandwiched between emulsified solutions of gelatin/chitosan (Figure 2.9). By controlling the average molecular weight of the PCL core, the ultimate tensile strength of the scaffold could be controlled in the range of 2-4 MPa. When combined with the gelatin/chitosan, the compressive modulus of the scaffold was close to native cardiac tissue (~15 kPa). This provided for a biocompatible cardiac patch that when seeded with neonatal rat ventricular myocytes resulted in spontaneous beating in a 50 vol.% gelatin:50 vol.% chitosan blend. Aside from layering different polymers, the blending of both natural and synthetic polymers without any chemical linkages show potential in the development of viable cardiac scaffolds. Kharaziha et al. (133) developed a scaffold composed of electrospun PGS and gelatin nanofibers, showing that a 33 wt.% PGS formulation induced optimal synchronous contractions of seeded cardiomyocytes. Recent studies by Martins et al. (134) combine chitosan and carbon nanofibers to create scaffolds that can enhance the electrical properties of a patch material as well, showing a conductivity of 0.25 ± 0.09 S/m. This is important in cardiac tissue where conductivity is necessary to transmit action potentials. Being able to optimize the properties of both natural and synthetic materials suggests a more adaptive and structurally sound scaffold compared to a homogenous construct.
2.4.4. Injectable hydrogels

Another highly researched area is the use of injectable biomaterials for the treatment of myocardial defects. The idea is that an amorphous matrix composed of critical tissue forming components (cells, ECM proteins, small molecules) can be injected directly to the site of deficiency, aiming to preserve and promote cardiac tissue remodeling, Figure 2.10 (135). This strategy is very appealing to myocardial infarction cases where the rapid degradation of cardiac tissue is unrepairable. While CHD do not include myocardial infarction, a number of diseases (coarctation of the aorta, anomalous origin of the left coronary artery arising from the pulmonary artery, etc) (136) run the risk of creating necrotic regions of cardiac tissue due to poor perfusion or inadequate oxygen saturation of the systemic blood (137). As with myocardial infarction, the lack of oxygenated blood due to CHD can manifest itself immediately after birth and upon closure of the ductus arteriosus. Aside from maintaining a patent ductus arteriosus by administration of
prostaglandin E₁, the use of an injectable cardiac matrix in CHD patients could at a minimum help to preserve cardiac tissue until other corrective action can be considered.

Similar to the cardiac patch studies, the variety of biomaterials used for injectable gels can be both natural and synthetic. Injectable gels are typically based on natural materials such as fibrin, collagen, alginate, but there are some gels which incorporate synthetic polymers either as hydrogel copolymers or encapsulation vessels (138, 139). Upon injection into physiological temperatures (37°C), the gelling of components can occur very rapidly, within seconds in most cases. The other key component of injectable materials are living cells aimed to assist in regenerating damaged tissue by paracrine delivery of different signaling molecules such as growth factors, cytokines, hormones, etc. The key advantages to an injectable strategy are: 1) they are minimally invasive and easy to administer through injection, 2) amorphous structure allows for high contourability allowing the gel to fill various defect shapes and sizes and 3) therapeutic agents are easily incorporated and delivered to the defect site.

Fibrin glue has been studied as an injectable material for myocardium repair. Fibrin glue is currently FDA approved as a hemostasis sealant for use during surgical operations (140). When applied as an endoventricular heart patch, Christman et al. (141) showed that the use of a fibrin glue increased cell transport and survival of skeletal myoblasts, decreased the size of infarcted left ventricle, and increased blood flow to the area of myocardial ischemia compared to controls. Other studies focused on the delivery of growth factors with the goal of enhancing
native cardiac cells to survive and proliferate. The Christman group also developed an injectable material composed of decellularized heart matrix (142), which was shown to have endothelial and smooth muscle cell migration as well as arteriole formation after 11 days. Ruvinov et al. (143) show that an alginate based gel can be used to sequentially deliver insulin-like growth factor and hepatocyte growth factor aimed to induce myocardial regeneration. The alginate gel sufficiently prevented proteolysis of the two proteins and when injected into a rat acute myocardial infarction model, infarct expansion was attenuated and increased angiogenesis throughout affected area was observed after 4 weeks. A combination of growth factors and stem cells as a regenerative therapy is popular as well. This concept will not only deliver signaling molecules to native cardiac cells, but provide additional cells that may differentiate and aid in the remodeling process. Wang et al. (138) developed an injectable hydrogel composed of collagen type I, chondroitin sulfate, and a thermosensitive copolymer (Figure 2.10). This hydrogel was shown in vitro to be capable of releasing IGF-1 over a 2 week period in order to enhance the survival and growth of encapsulated mesenchymal stromal cells (MSC). The differentiation potential of the MSCs was maintained within the hydrogel, but the addition of IGF-1 was shown to significantly accelerate MSC growth. The Davies group conducted studies into the effects of injectable gels on cardiac tissue remodeling, showing that injection of PEG based gels following infarction could aid in the immediate healing and remodeling (144). They also showed a temporal remodeling relationship, where the same gels were injected one week post-
infarction had slower degradation rates compared to gels injected immediately post-infarction (145).

Figure 2.10 - Schematic of Injectable hydrogels (146)

The major disadvantage of injectable biomaterials for the repair of infarcted cardiac tissue is that they lack sufficient stiffness compared to the tissue in chronic diseased states. Where healthy adult myocardium has a modulus of approximately 50 kPa, diseased states can range from 200-300 kPa (147). Ifkovits et. al. elucidated the effects of stiffness on ovine infarct models using injectable methacrylated hyaluronic acid hydrogels with varying moduli. The study shows that a higher modulus (~43 kPa) gel applied post-infarction resulted in less infarct expansion and reduced left ventricular dilation compared to a lower modulus (8 kPa) (148). While these injectable gels nearly match the modulus of healthy adult myocardium, the stiffness may not be sufficient to support chronic diseased myocardium. However,
injectable biomaterials may have potential in pediatric cases where years of cardiac
remodeling have not altered the mechanical properties of the myocardium.
Chapter 3

Differentiation of Spontaneously Contracting Cardiomyocytes from Non-virally Reprogrammed Human Amniotic Fluid Stem Cells*

Abstract: Congenital heart defects are the most common birth defect. The limiting factor in tissue engineering repair strategies is an autologous source of functional cardiomyocytes. Amniotic fluid contains an ideal cell source for prenatal harvest and use in correction of congenital heart defects. This study aims to investigate the potential of amniotic fluid-derived stem cells (AFSC) to undergo non-

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viral reprogramming into induced pluripotent stem cells (iPSC) followed by growth-factor-free differentiation into functional cardiomyocytes. AFSC from human second trimester amniotic fluid were transfected by non-viral vesicle fusion with modified mRNA of OCT4, KLF4, SOX2, LIN28, cMYC and nuclear GFP over 18 days, then differentiated using inhibitors of GSK3 followed 48 hours later by inhibition of WNT. AFSC-derived iPSC had high expression of OCT4, NANOG, TRA-1-60, and TRA-1-81 after 18 days of mRNA transfection and formed teratomas containing mesodermal, ectodermal, and endodermal germ layers in immunodeficient mice. By Day 30 of cardiomyocyte differentiation, cells contracted spontaneously, expressed connexin 43 and β-myosin heavy chain organized in sarcomeric banding patterns, expressed cardiac troponin T and β-myosin heavy chain, showed upregulation of NKX2.5, ISL-1 and cardiac troponin T with downregulation of POU5F1, and displayed calcium and voltage transients similar to those in developing cardiomyocytes. These results demonstrate that cells from human amniotic fluid can be differentiated through a pluripotent state into functional cardiomyocytes.

3.1. Introduction

Congenital heart defects (CHD) are the most common birth defects and the leading cause of infant death in the United States (149). Autologously derived contractile cardiac cells can be applied to patches for structural defect repair (150), engineered heart tissue(151), cells for cardiomyoplasty (152), and gene editing correction of specific defects(153). With 80% of CHD diagnosed in the second
trimester (154), amniotic fluid presents an ideal source for autologous cells for use in neonatal CHD treatment (46, 152).

Amniotic fluid stem cells (AFSC) are broadly multipotent, but do not directly differentiate into contractile cardiomyocytes (CM). Specifically, AFSC express mesenchymal stem cell markers (CD29, CD44, CD90, and CD105), certain pluripotent markers (SOX2), and are capable of differentiating into all three germ layers(2). While attempts at direct cardiac differentiation have shown gene and protein level similarities (GATA4, Nkx2.5, α-actinin, cTnT), resulting cells ultimately lack contractility(2, 155).

Induced pluripotent stem cells (iPSC) can be differentiated into force-generating CM (64, 151, 152), and studies show that iPSC can be generated from AFSC (156, 157). However, no study has investigated the transformation of AFSC into CM using non-virally attained iPSC as an intermediary.

The objectives of this study were to test whether AFSC can be reprogrammed to iPSC by mRNA delivery and whether non-virally attained AFSC-iPSC are capable of cardiac differentiation. Reprogrammed AFSC were evaluated for pluripotency by protein expression and teratoma formation. CM derived from AFSC-iPSC were evaluated for expression of cardiac genes and proteins, membrane potential fluctuation, calcium handling, and contractile function.
3.2. Materials and Methods

3.2.1. AFSC culture isolation and expansion

AFSC were isolated based on previously published methods from our group (2, 158). Primary human amniotic fluid was obtained from patients in their second trimester undergoing planned amnioreduction as part of a therapeutic treatment for twin-twin transfusion syndrome (TTTS). Amniotic fluid was centrifuged at 1200 rpm for 10 min, and collected cells were plated at 2500 cells/cm² on standard plastic Petri dishes and cultured in a modified α-Minimum Essential Media: 63% αMEM (Invitrogen, Carlsbad, CA), 18% Chang Basal Medium (Irvine Scientific, Santa Ana, CA), 2% Chang C supplement (Irvine Scientific), 15% fetal bovine serum (PAA Laboratories, Dartmouth, MA), and GlutaMAX (Invitrogen) at 37°C and 5% CO₂ in a humidified environment. Media was changed every two to three days, and cells were passaged at 60-70% confluence. At the first passage, a subpopulation of progenitor cells was isolated through fluorescence-activated cell sorting for expression of the membrane receptor CD117/c-kit (BD Biosciences, Bedford, MA). Cell colonies were detached into single cells (Accutase; Sigma-Aldrich, St. Louis, MO; 37°C, 10 min), and c-kit+ cells were collected using a Dako MoFlo sterile cell sorter. All studies of primary human cells were approved by the Institutional Review Boards of both Baylor College of Medicine and Rice University, and subjects gave informed consent.
3.2.2. iPSC generation and culture

AFSC were transfected with mRNA to generate an iPS state using the Stemgent mRNA Reprogramming System (Lexington, MA) (159). Briefly, frozen c-kit+ passage 2 AFSC, were thawed and plated onto 100mm petri dishes. The cells were allowed to expand to 80% confluency and then plated in 6 well plates containing a feeder layer of mitomycin-treated newborn human foreskin fibroblasts (NuFF, Stemgent, Inc., Cambridge, MA). After attachment, transfection of the AFSC was carried out by exposure to reprogramming factors (Oct4, Klf4, Sox2, c-Myc) for 4 hours each day for 18 days. Briefly, AFSC were plated on a feeder layer of NuFF in Pluritron Reprogramming Medium (Stemgent) supplemented with 4ng/mL bFGF (Stemgent) and B18R recombinant protein (eBioscience, Inc., San Diego, CA). AFSC were exposed for 4 hours per day to an mRNA cocktail comprised of OCT4, SOX2, KLF4, c-Myc, LIN28, and nGFP (TriLink Biotechnologies Inc., San Diego, CA) complexed with Lipofectamine (RNAiMAX, Thermo Fisher Scientific, Carlsbad, CA) for 18 consecutive days. At the end of the 18-day transfection, cell colonies were selected based on morphology and the pluripotency expression marker TRA-1-81. Each well yielded approximately 10 iPSC colonies per well that were each 1-2 mm in diameter. Colonies were then continuously split and passaged every 5-7 days onto mouse embryonic fibroblast feeder (MEF, GlobalStem, Inc., Rockville, MD) on treated 6-well plates, and maintained using CDF12 medium as described by Warren et al 18: DMEM/F-12 (Invitrogen, Carlsbad, CA), knockout serum replacement (Life Technologies, Carlsbad, CA), non-essential amino acids (Life Technologies), Glutamax (Life Technologies), 2-mercaptoethanol (Gibco, Carlsbad, CA), 20ng/mL
bFGF (Stemgent), and 1x penicillin-streptomycin. Cells were passaged using 0.1% collagenase type 4 (Worthington Biochemical Corp., Lakewood, NJ) in DMEM/F-12 medium (Invitrogen). Each well of the new passage was seeded with 4 colonies that were broken up physically by scraping with a 10mL pipet and gentle trituration, and yielded approximately 30-40 new colonies that grew to 2-5 mm in diameter before the next passage.

### 3.2.3. Teratoma Formation

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine and conformed to the Guide for the Care and Use of Laboratory Animals as stated by the NIH. In order to characterize the AFSC-iPSC self-renewal and pluripotency properties, a teratoma study was conducted to assess their ability to differentiate into derivatives of the three embryonic germ layers. This work was done by the Bisseg lab at Baylor College of Medicine. Briefly, approximately $1 \times 10^6$ AFSC-iPSC were injected subcutaneously into SCID mice (8-12 weeks of age) and monitored for 8-10 weeks for teratoma formation. Teratoma evaluation was done by histology and H&E staining.

### 3.2.4. Immunocytochemistry

Cell cultures were fixed in 4% paraformaldehyde (Alfa Aesar, Ward Hill, MA) at 4oC for 20 minutes. Fixed cells were then permeated with 0.5% Triton X100 (Sigma-Aldrich) in PBS for 5min at room temperature. Next cells were incubated
with specific antibodies (Abcam, Cambridge, UK) for pluripotency (Oct4, Nanog, Tre-1-81, Tra-1-60) and cardiac marker lineage (myosin heavy chain, connexin 43) at a 1:100 dilution, then in DyLight-conjugated secondary antibodies at a 1:500 dilution (Jackson ImmunoResearch Laboratories) and DAPI with VectaShield (Vector Laboratories, Burlingame, CA). The cell were imaged using an epifluorescence microscope (DMI 6000B, Lieca Microsystems, Wetzlar, DE).

3.2.5. Cardiac Differentiation

By adapting previously published protocols (64), AFSC derived iPSC were differentiated into cardiac cells by small molecule inhibition of the GSK3 and Wnt signaling pathways. Briefly, reprogrammed AFSC colonies were maintained on a feeder layer consisting of irradiated mouse embryonic fibroblast with daily changes of mTeSR1 media (Stem cell technologies, Vancouver, BC). Once sufficient cell numbers were obtained, undifferentiated colonies were dissociated in collagenase type 2 (Worthington Biochemical Corp., Lakewood, NJ) for 5 min then manually dislocated from the feeder layer, dispersed into single cell suspension, then plated as a monolayer of cells onto Matrigel (BD Biosciences, San Jose, CA) at a density of approximately 260,000 cells/cm2. Cells were expanded for 4 days in mTeSR1 media, which then corresponded to day 0. At this point, the media was changed to RPMI media with B27 supplement without insulin and the GSK3 inhibitor, CHIR99021, was exposed to the cells for 24 hours at a concentration of 12µM. At the end of 24 hours, media was replaced with fresh RPMI/B27 without insulin. At day 3, the Wnt inhibitor, IWP2, was added to RPMI/B27 without insulin at a concentration
of 5µM. At day 7, insulin was added to the RPMI/B27 media. The occurrence of beating colonies was monitored through phase contrast microscopy after day 7.

### 3.2.6. Flow Cytometry

Cells were detached into suspension with Accutase (ThermoFisher) and stained with a fluorescently conjugated antibody for cardiac troponin T (BD Biosciences) with dilutions per manufacturer recommended concentrations. FACSDiva software (BD Biosciences) was used for all flow cytometry data collection. FlowJo software (Tree Star, Inc., Ashland, OR) was used for data analysis.

### 3.2.7. Western Blot

Western blot antibodies were purchased from Abcam Inc., electrophoresis and transfer materials were purchased from Bio-Rad (Hercules, CA), and developing materials were purchased from Li-Cor (Lincoln, NE). After 30 days of differentiation, total protein lysates were isolated from differentiated AFSC-iPSC and analyzed using a bicinchoninic acid kit (BCA; Thermo Scientific, Rockford, IL). Extracts were denatured using β-mercaptoethanol and boiling for 5 min, then diluted to equal concentrations of total protein. The samples were electrophoresed by 0.1% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes at 100 V for 1.5 and 1.0 hrs, respectively. Membranes were washed in tris-buffered saline with 0.05% Tween-20 (TBST), then blocked with Odyssey blocking buffer (Li-Cor) for 1 hour. Membranes were incubated overnight at 4°C with mouse monoclonal antibodies against MHC and cTnT (1:300
dilution in Odyssey blocker) and mouse monoclonal antibodies against GAPDH (1:1000 dilution in Odyssey blocker). Membranes were washed and submerged in IRDYE 800CW Goat anti-Mouse IgG secondary antibody with a dilution at 1:1000 in Odyssey blocker for 60min with gentle shaking. Membranes were washed and scanned using an Odyssey CLx scanner set to detect an 800nm wavelength. Western blots were normalized to GAPDH expression. Western blot analyze was performed using Image J (NIH, Bethesda, MD).

3.2.8. Quantitative RT-PCR

Total RNA was extracted and purified using a PrepEase RNA spin kit (Affymetrix) and quantified by NanoDrop 1000 Spectrophotometer (Thermo Scientific). cDNA was synthesized from 2 μg of purified total RNA with random primers by Multiscribe Reverese Transcriptase (ThermoFisher). Quantitative real-time PCR was performed using the StepOnePlus system (ThermoFisher) and Veriquest Fast Probe qPCR Master Mix (Affymetrix), according to the manufacturer’s instructions. All data were normalized to mRNA level of housekeeping gene using the $2^{ΔΔ}$ method.

3.2.9. Calcium and voltage transient analysis

The electrical behavior of the spontaneously contracting cells was measured through voltage-sensitive dye (Di-8-ANEPPS) and calcium-sensitive dye (Indo-1) with an epifluorescence microscope (Olympus, Center Valley, PA) and photomultiplier tubes detection system. Staining procedures for cells was the same
for either dye. Cells were first washed with PBS warmed to 37oC to remove any serum contained in the media. Then 2µl of either Di-8-ANEPPS or Indo-1 at a concentration of 2mM was added to 2ml of Tyrode’s solution warmed to 37oC. The solution was then added to washed cells and allowed to sit at room temperature for 30min protecting the sample from light. The cells were then washed three times with fresh Tyrode’s solution and imaged using the epifluorescence microscope. For Indo-1 the detection wavelengths were 405nm and 485nm depending on the binding or calcium ions. Di-8-ANEPPS detection wavelengths were 560nm and 620nm depending of the shifts in membrane potentials. Emission data was collected and analyzed using Ion Optix software (Westwood, MA).

3.3. Results

3.3.1. Non-viral reprogramming of AFSC

mRNA reprogramming of Passage 3 AFSC seeded at 2.6 x 10^4 cells per cm^2 yielded transfected and highly proliferative cells. Each 9.6cm^2 well yielded approximately 10 colonies. Reprogrammed colonies were expanded 5-8 passages and then fixed and stained for pluripotent markers OCT4, NANOG, TRA-1-60, and TRA-1-81, all of which were expressed in all cells (Figure 3.1A). Cells were then subcutaneously injected into immunodeficient mice (NSG strain). Tumors formed after 8 to 12 weeks. Analysis by H&E staining exhibited tissue from mesodermal, ectodermal, and endodermal origin and confirmed pluripotency of injected AFSC iPSC (Figure 3.1B).
Figure 3.1 - Pluripotent characterization of reprogrammed AFSC-iPSC. (A) Immunostaining for the human pluripotency markers OCT4, NANOG, TRA-1-60, and TRA-1-81. Scale bars, 100μm. (B) AFSC-iPSC-derived teratoma exhibiting neural epithelium (ectoderm, right) and gut epithelium (endoderm, bottom) surrounded by muscle and adipose tissue (mesoderm, top left) Scale bar, 100μm.
3.3.2. Small molecule differentiation and genetic analysis of AFSC-iPSC derived CM

Genetic expression of ISL1, NXX2.5, TNNT2, and POU5F1 assessed at each time point was compared to expression at Day 0 (Figure 3.2). Upregulation occurred in cardiac progenitor genes ISL1 and NKX2.5 between Day 0 and Day 8 of differentiation and in late stage cardiac marker TNNT2 between Day 3 and Day 8 of differentiation. Among the days collected, peak expression was observed to be significant at Day 8 for NKX2.5 and TNNT2. Day 8, Day 15, and Day 21 data shows a decrease in expression of ISL1, NKX2.5, and TNNT2 between Day 8 and Day 15. No significant statistical difference was observed in the fold change in expression of POU5F1 between Day 3 and Day 21 of differentiation.
Figure 3.2 - Genetic expression of cardiac differentiation markers. qRT-PCR assessment of genes for committed cardiac lineage (ISL1 and Nkx2.5), cardiomyocytes (TNNT2), and pluripotency (POU5F1). Error bars represent standard deviations of mean values (n=3, p<0.05, *significance compared to D0 other time points).

3.3.3. Expression of late stage cardiac markers

Differentiated CM cultures were stained for visualization of MHC and Cx43. Immunohistochemical analysis shows contractile regions in Figure 3.3A and Figure 3.3B. Higher magnification reveals a distinct repetitive banding pattern of MHC in cellular extensions, indicating immature sarcomeric cytoskeletal structure (Figure 3.3C). Connexin 43 was not organized but expression was cytoplasmic (not shown).
Differentiation efficiency was measured by flow cytometry expression of cTnT at Day 15. The average cTnT expression was showed to be $42.8 \pm 12.3\%$ (Figure 3.3D).

Figure 3.3 - Characterization of spontaneously contracting CM cultures from AFSC-iPSC differentiated for 30 days. (A, B) Immunofluorescent staining of a contractile region for expression of sarcomere protein MHC. Scale bar, 20μm. (C) Magnified area shows striation pattern of sarcomeric cytoskeleton (white arrows). Scale bar, 10μm. (D) Day 15 cTnT expression analyzed by flow cytometry. (E) qRT-PCR assessment of upregulated cardiomyocyte genes DES, PLN, TNNT2, MYH7, and GJA1 and downregulated pluripotent gene POU5F1. (F) Western Blot analysis showing cardiac protein expression of cTnT and MHC.
Gene analysis at Day 30 of differentiation by qRT-PCR showed significant upregulation of PLN, TNNT2, and MHC, genes for contractile machinery, and significant downregulation of pluripotent transcription factor POU5F1 compared to expression at Day 0 (Fig 3E). The human non-muscle alpha-actinin 1 was shown to be downregulated at Day 30. No significant difference was observed in expression of sarcomeric architecture regulator desmin and gap junction formation regulator Cx43, though spontaneous contraction in cultures was observed prior to collection. Protein analysis at Day 30 of differentiation by Western blot showed upregulation of cTnT and MHC, confirming the presence of both thin and thick filament contractile machinery (Fig 3F).

### 3.3.4. Electrophysiology of contractile AFSC-iPSC derived CM

Spontaneously contracting CM cultures generated calcium and voltage fluorescent waveforms that demonstrate fluctuation in the ratio of extracellular to intracellular calcium (Figure 3.4A) and myocardial membrane depolarization (Figure 3.4B) upon contraction. Filtered and amplified fluorescent recordings had average periods of 3.04s (SD=0.36, n=5) and 3.83s (SD=0.15, n=3) for the different beating samples recorded in Figure 3.4A and Figure 3.4B, respectively. Comparison shows a significant difference in beat frequency. In fact, a high degree of variability was observed in spontaneously contracting cultures subjected to identical differentiation, culturing, and environmental conditions.
Figure 3.4 - Waveforms generated from contracting regions as the change in ratio of fluorescence intensity readings over base-level fluorescence. (A) Calcium transient-based fluorescence using Indo1, and (B) Voltage transient-based fluorescence using Di-8-ANEPPS.

3.4. Discussion

Amniotic fluid is the ideal source of autologous cells for use in neonatal CHD treatment because of its capacity to be harvested during CHD diagnosis and the highly proliferative nature of AFSC (46) that enable the external development of tissue in parallel with gestation. Previous studies raise concerns of AFSC immune rejection upon transplantation however, because cells are induced to pluripotency, the antigenic profile is yet to be discovered but hypothesized to be of low immuno-stimulatory nature, similar to ESC and differentiated derivatives(160). These
experiments verify recent findings that iPSC can be generated from AFSC (157) and that AFSC-derived iPSC can be used to generate CM (161). However, we are the first to demonstrate CM generation from AFSC without the use of viruses. Our studies support the feasibility of using amniotic fluid for CHD repair by showing that iPSC can be generated from AFSC using non-viral reprogramming, and that iPSC derived from AFSC non-virally are capable of cardiac differentiation. Previous studies from our group have verified that human AFSC express MSC markers CD29, CD44, CD73, CD90, and CD105, do not express endothelial marker CD31 or hematopoietic marker CD45, and express HLA-ABC but not HLA-DR (162). The work of other investigators has shown that AFSC can be reprogrammed (156, 163) and that pluripotent cells derived from other sources can be differentiated to CM (64, 152, 164). This study integrates these observations, while validating the use of a non-viral method of generating iPSC from AFSC and connecting the findings of others to demonstrate a direct link for differentiating CM from iPSC derived from AFSC.

While other well-established methods include the use of retroviruses, lentivirus, adenovirus, and Sendai virus as DNA delivery agents, mRNA delivery by lipofection was used for transfection to generate iPSC in this study. Lipofection delivery of mRNA is highly efficient, has no risk of transgene incorporation, and does not involve the use of viruses, reducing potential for complication in clinical translation (165). This study is the first to demonstrate the use of lipofection for the transformation of AFSC to a pluripotent state. The achievement of pluripotency in this study, evident by expression of pluripotent markers OCT4, Tra-1-60, and Tra-1-81 in vitro and subcutaneous teratoma formation, is significant because it verifies
the use of non-viral transfection. *in vitro* expression of NANOG supports
dedifferentiation, though not maintenance of pluripotency (166, 167), while
cytoplasmic localization is comparable to patterns in other studies (168).

The use of GSK3 and Wnt inhibition in this study validates cardiac
differentiation for iPSC derived AFSC, but suggests a difference in its efficiency. Of
the existing methods of differentiating pluripotent cells, GSK3 and Wnt inhibition
has shown to produce the greatest yield efficiency of cardiomyocytes in monolayer
culture (169). However, these protocols were developed using embryonic stem cells
(ESC) and iPSC derived from fibroblasts (63, 170). It has been shown that the iPSC
origin may influence the differentiation potential, specifically in regards to the
efficiency and maturity (171). In this study, spontaneous contraction in CM cultures
differentiated from AFSC-iPSC was observed at approximately 21 days of
differentiation, compared to 7-12 days as reported in other studies (64, 152, 164),
though expression of ISL1, N\textit{K}X2.5, and TNNT2 over the time course of
differentiation resembles that of other studies (170, 172). Cardiac immaturity is
further supported by low upregulation of Desmin and indistinct intercalated disc
gap junction formations marked by Cx43, in spite of clear cytoskeletal sarcomeric
banding marked by MHC, obvious upregulation of cardiac encoding genes PLN,
TNNT2, and MYH7, downregulation of pluripotent gene POU5F1, and transcription
of cardiac machinery, evidenced by proteins cTnT and MHC. Alpha-actinin 1 is
shown to have little to no activity in later stages of differentiation, eluding to the
downregulation of non-muscular encoding genes. Immaturity is also evidenced by
the recorded calcium and voltage-mediated fluorescence waveforms of contractile
regions, exhibiting action potentials similar to immature CM because of the slow upstrokes during depolarization and the observed delayed after depolarizations (173), though these could also be explained by impedance mismatch from structural discontinuities. Delayed spontaneous contraction despite ordinary upregulation of NKX2.5 and ISL1, incompletely formed communication structures, and limited upregulation of the sarcomeric architecture regulator DES may imply a delayed developmental progression in the transcription and translation processes involved in the differentiation of CM from AFSC-iPSC using this protocol.

A major limitation during this study was the high degree of variability in contractile strength by day 30 of differentiation. Beating cultures were observed to become sparser, less frequent, weaker or stopped altogether. From this there can be speculation of decreasing viability of pacemaker cells or the growth of discontinuous structures due to death or fibrosis. Quantification of cTnT at Day 15 does show a substantial population of late stage cardiac cells however there is still variability in observed differentiation efficiency, delayed spontaneous contraction, and contractile viability. This study suggests that an alternative or modified differentiation procedure is needed for AFSC to be a viable cell source for deriving CM. Although this study is the first to use iPSC non-virally derived from AFSC, possible options for optimizing CM yield and differentiation consistency include inhibition timings, media components (170), and cell density.
3.5. Conclusions

In the present study, AFSC were reprogrammed to iPSC by mRNA transfection, and AFSC-derived iPSC were differentiated into functional CM. Though differentiated CM were immature, as evidenced by delayed contraction, incomplete gap junction formation, and poor upregulation of Desmin, this study is the first to achieve functional CM from AFSC by non-viral means, as evidenced by sarcomere formation within cellular cytoskeleton, upregulation of NKX.2.5, ISL1, and cTnT, expression of cTnT and MHC, and clear calcium handling and membrane voltage propagation. In conclusion, while AFSC can now definitively be said to present a feasible source of functional CM generation, work is needed to improve differentiation efficiency and cardiac maturation.
Controlled Release of Small Molecules for Cardiac Differentiation of Pluripotent Stem Cells

Abstract: Induced pluripotent stem cells (iPSC) have shown to differentiate to functional cardiomyocytes with high efficiency through temporally controlled inhibition of the GSK3/Wnt signaling pathways. In this study we investigated the temporally controlled release of GSK3/Wnt small molecule inhibitors to drive cardiac differentiation of iPSC without manual intervention. Porous silica particles were loaded with GSK3 inhibitor CHIR99021 or Wnt inhibitor IWP2, and the particles containing IWP2 were coated with 5wt% PLGA 50:50 to delay release by approximately 72 hours. Induced pluripotent stem cells reprogrammed through mRNA transfection were cultured with these particles up to 30 days. High performance liquid chromatography (HPLC) suggests a burst release of CHIR99021 within the first 24 hours and a delayed release of IWP2 after 72 hours. Annexin V/PI staining did not show a significant effect on apoptosis or
necrosis rates. Cultured cells upregulated both early (Nkx 2.5, Isl-1) and late (cTnT, MHC, Cx43) cardiac markers, assayed with qRT-PCR, and began spontaneous contraction at 3.0±0.6Hz at 15-21 days after the start of differentiation. Cardiomyocytes had clear sarcomeric striations when stained for β-myosin heavy chain, and showed expression and punctate membrane localization of gap junction protein Connexin43. Calcium and voltage sensitive imaging showed both action potential and calcium transients typical of immature cardiomyocytes. This study showed that the cardiac differentiation of pluripotent stem cells can be directed by porous silica vectors with temporally controlled release of small molecules inhibitors. These results suggest methods for automating and eliminating variability in manual maintenance of inhibitor concentrations in the differentiation of pluripotent stem cells to cardiomyocytes.

### 4.1. Introduction

Current cardiac tissue engineering strategies are limited by a functional cell source(174). Cardiomyocytes (CM) are terminally differentiated cells which do not proliferate(175). Therefore damaged cardiac tissue due to disease or injury cannot repair itself, leading to arrhythmias and heart failure(176).

Induced pluripotent stem cells (iPSC) represent a renewable and scalable cell source for cardiac tissue engineering applications(63). Small molecule inhibition of the GSK3/Wnt inhibitory pathways in iPSC resulted in cardiac differentiation efficiencies of up to 98%(64).
Current procedures for differentiating iPSC into cardiomyocytes involve manual media changes and inhibitor addition. A method of limiting handling interactions would be ideal for clinical translation of iPSC to cardiac cells. Our ultimate goal is to create a self-differentiating material that requires minimal handling interactions. In this study, we combined iPSC with particles that regulated the release of inhibitors of the GSK3 and Wnt pathways.

Porous silica particles (pSi) were tailored to release small molecule inhibitors at desired time points. Drugs loaded into pSi alone without any coatings release in a burst release, with all the drugs dispersed within 24 hours (177). pSi have a high loading efficiency, retain drug bioactivity and can be coated with biocompatible polymers (178). Polymers such as poly(lactic-co-glycolic acid) (PLGA) have been used widely in drug delivery platforms because of their tunable release kinetics based on co-polymer ratios and exposure of the hydrolysable chemical backbones (179-181). pSi have been shown to load and deliver hydrophobic small molecules for therapeutic purposes (182, 183). Amniotic fluid derived stem cells (AFSC) have been widely researched for treatment of congenital heart diseases (2, 46, 158), however AFSC cannot directly differentiate into functional beating cardiomyocytes (2). Our laboratory has previously reprogrammed AFSC by modified mRNA transfection and subsequently differentiated AFSC-iPSC into functional beating cardiac cells (184). Using small molecule loaded pSi, AFSC-iPSC could effectively differentiate into cardiac cells. Differentiation was evaluated in vitro for cardiac differentiation. Cells were cultured in transwells to maintain consistent inhibitor exposure released from particles as well maintain cellular waste/nutrient
exchange. Differences between cardiac cells derived from direct exposure to GSK3/Wnt inhibitors and those exposed to particles containing the same inhibitors were evaluated through differentiation efficiency, phenotypic/genotypic analysis and electrophysiology.

4.2. Methods

4.2.1. Cell source

Cells were isolated from second-trimester human amniotic fluid and reprogrammed to induced pluripotent stem cells (iPSC) through mRNA transfection as previously reported (184). Pluripotency was assessed through RNA expression of pluripotency markers OCT4, nanog, and Tra-1-81 with teratoma formation in a nude mouse as previously reported (184). Reprogrammed AFSC were then passaged onto a mouse embryonic fibroblast (MTI-Globalstem, Gaithersburg, MD) feeder layer (1.85x10^4 cells/cm^2) for further expansion and maintained in knockout serum replacement induced pluripotent stem cell media (DMEM/F12, 20% knockout serum replacement, 1% NEAA, 1% Pen/Strep, 0.1% beta-mercaptoethanol, 4ng/ml bFGF) (Thermo Fisher Scientific, Waltham, MA). At 60-70% confluency, colonies were passaged to new mouse embryonic fibroblast feeder layers, every 7-10 days. All studies of primary human cells were approved by the Institutional Review Boards of both Baylor College of Medicine and Rice University, and subjects gave informed consent.
4.2.2. **Porous silica particle synthesis**

A tannic acid template and the Stöber process were used to synthesis pSi as per previous studies (185). Briefly, 272mg Tannic acid (Sigma Aldrich St Louis, MO) was dissolved in 50ml ethanol while continuously stirring. Then 25ml ammonium hydroxide (Sigma Aldrich) was added and stirred for 1min. Tetraethyl orthosilicate (TEOS) (300µl) (Sigma Aldrich) was added to the mixture dropwise and stirred continuously for 3 hours. The resulting particles were centrifuged and washed in 1:1 ethanol:water for a total of 6 washings. The particles were then resuspended in 15ml of deionized water and filtered twice through 40um centrifuge tube filters. Particles were centrifuged again; the water was removed and then resuspended in 1ml isopropyl alcohol. The particles were then dried overnight in a vacuum oven at 60°C and -36Pa until dry. Once dry, 4mg of pSi were rehydrated in 0.5ml inhibitor loading solutions, consisting of CHIR99021 (Tocris bioscience, Avonmouth, Bristol, UK) or IWP2 (Tocris bioscience) re-suspended in DMSO at 600ug/ml for 20min at 37°C with constant mixing. The particles were then washed and lyophilized for further use.

4.2.3. **PLGA encapsulation of porous silica particles**

Particles loaded with IWP2 were further encapsulated with a PLGA coating in order to delay the release kinetics using a Solid-in-Oil-in-Water method (S/O/W). An oil solution consisting of dissolved 5wt% 50:50 PLGA (Lactel absorbable polymers, Pelham, AL) in dichloromethane was homogenized with the prepared loaded pSi. The solid in oil emulsion was then added dropwise to a water solution
consisting of 2.5% polyvinyl alcohol. The solution was allowed to stir continuously for 6 hours, washed and lyophilized until ready for use.

### 4.2.4. Particle characterization

Dynamic light scattering (DLS) and Z-potential of pSi were performed using a Zetasizer ZEN3600 (Malvern, Worcestershire, U.K.). Moreover, samples were prepared for Scanning electron microscope (SEM) by drying overnight on a stage and sputter coating with a 5nm thick layer of Pt/Pl. SEM were taken using Nova NanoSEM 230. PLGA-pSi and pSi size distribution were also measured with the software ImageJ (NIH Image). TEM samples were prepared by drying nanoparticles onto 300 mesh carbon-coated copper grids and then analyzed. FITC-labeled pSi has been used to verify the presence of the silica inside the PLGA shell by confocal microscopy.

### 4.2.5. Inhibitor loading efficiency and release studies

All supernatants after initial loading of inhibitors were stored at -20°C. Supernatants were analyzed by reverse phase HPLC. Inhibitor release studies were done sampling particle suspension inhibitor release up to 6 days. Briefly, drug release solution was prepared using 1mL of 1x PBS, and 1mL of 1% bovine serum albumin. The lyophilized pSi were suspended at a concentration of 3mg/mL of drug release solution and incubated at 37°C with constant stirring. At 1, 3, 5, 12 and every 24 hours thereafter, suspensions were centrifuged and 1ml drug release solution was taken and replaced with fresh drug release solution. Agilent Zorbax
Eclipse Plus C18 (100x4.6mm, 3.5um pore particle size) (Agilent Technologies, Santa Clara, CA) column was used for analysis. Samples flowed through the column at 1.0 ml/min and with a mobile phase consisting of acetonitrile in 0.1% trifluoroacetic acid: H2O in 0.1% trifluoroacetic acid (V/V). Samples were analyzed at 281nm.

4.2.6. Cellular toxicity to particles

pSi without any inhibitors loaded were added to iPSC cultures at a density of 2.5ng/cell to assess apoptosis and necrosis. Annexin V and propidium iodide staining were used to quantify apoptosis/necrosis. Cells were lifted at 0 and 72 hours, washed and double stained with Annexin V-FITC and propidium iodide (Affymetrix ebioscience, Santa Clara, CA). The cells were then analyzed through flow cytometry (BD Acurri C6 Plus, BD biosciences, San Jose, CA). To access the effect of transwell culture on cell survival, live/dead staining was done according to manufacturer protocol (Thermo Fisher). Cells were imaged using fluorescent microscopy.

4.2.7. Differentiation of iPSC using dissolved GSK3/Wnt Inhibitors

By adapting previously published protocols(64), AFSC-iPSC were differentiated into cardiac cells by temporally inhibiting the GSK3 and Wnt signaling pathways (Fig 4A). Upon reaching approximately 70% confluency, undifferentiated colonies were dissociated in collagenase type 2 (Worthington Biochemical Corp., Lakewood, NJ) for 5 min then manually dislocated from the feeder layer, dispersed
into single cell suspension, then plated as a monolayer of cells onto Matrigel (BD Biosciences) at 260,000 cells/cm². Cells were expanded 3 days in mTeSR1 media (STEMCELL Technologies, Cambridge, MA). Media was then changed to RPMI 1640 (Thermo Fisher) and the GSK3 inhibitor, CHIR99021, was added at a concentration of 6µM, representing differentiation Day 0. After 24 hours, media was replaced with fresh RPMI 1640. At day 3 the Wnt inhibitor, IWP2, was added to RPMI/B27 without insulin at a concentration of 2.5µM. At day 7, insulin and ascorbic acid were added to the RPMI 1640 media. The occurrence of beating colonies was monitored through phase contrast microscopy after day 7.

**4.2.8. Differentiation of iPSC using pSi-released inhibitors**

To test the effectiveness of inhibitor release from loaded pSi, dissociated iPSC were plated at 260,000 cells/cm² onto Matrigel coated 12-well polyethylene terephthalate ThinCert cell culture inserts (Greiner Bio-One, Monroe, NC) with 0.4µm pore size.
Based on calculated loading efficiencies, approximately 1.5mg CHIR99021 loaded pSi and 4.0mg IWP2 loaded PLGA-pSi were suspended in 24ml of RPMI 1640. At day 0 of differentiation, 1ml of particle suspension was added to each seeded transwell and an additional 1ml RPMI 1640 surrounded the insert. Every 48 hours, 1ml of fresh RPMI1640 replaced the media surrounding the insert. After day 8, insulin and ascorbic acid were added to the RPMI 1640 and 1ml of media was added each to the insert and surrounding.

**4.2.9. Flow Cytometry**

Cells were detached at day 12 into suspension with Accutase (ThermoFisher) and stained with a fluorescently conjugated antibody for cardiac troponin T (BD Biosciences) at a dilution of 1:100. BD Acurri C6 Plus software (BD Biosciences) was
used for all flow cytometry data collection. FlowJo software (Tree Star, Inc., Ashland, OR) was used for data analysis.

**4.2.10. Gene Expression Analysis**

Gene expression was quantified using qRT-PCR. To assess the upregulation of early and late stage cardiac markers, cell samples were collected at 0, 1, 5, 10, and 20 days after the start of differentiation. At each time point, RNA samples were collected using an RNA collection kit following manufacturer protocol (Affymetrix, Santa Clara, CA). mRNA samples were then reverse transcribed to DNA using a cDNA kit following manufacture protocol (Applied Biosystems, Foster City, CA). The resulting DNA samples were analyzed with quantitative real-time polymerase chain reaction (qRT-PCR) using a proprietary assay system following manufacturer protocol (Affymetrix). All samples were assayed for expression of Oct4, Isl1, Nkx2.5, cTnT, connexin 43, myosin heavy chain, and GAPDH as a house keeping gene using DNA primers (Thermo Fisher). The expression of each gene was first normalized to the level of GAPDH. Relative fold changes were determined by calculating ΔΔCt comparing to Day (-3) or Day 0. Biological triplicates for each group were assessed, and results were reported as mean ± standard deviation.

**4.2.11. Immunofluorescence**

Cell cultures were fixed in 4% paraformaldehyde (Alfa Aesar, Ward Hill, MA) at 4°C for 20 minutes. Fixed cells were permeated with Triton X100 (Sigma-Aldrich) for 5min at room temperature. Next, cells were incubated with specific antibodies
(Abcam, Cambridge, UK) for cardiac markers myosin heavy chain and connexin 43, then in DyLight-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Cells were imaged using an epifluorescence microscope (DMI 6000B, Leica Microsystems, Wetzlar, DE).

4.2.12. Calcium handling and membrane voltage potential

The calcium handling of spontaneously contracting cells was measured by imaging calcium-sensitive dye Indo-1 AM (Thermo Fisher) with an epifluorescence microscope (Olympus, Center Valley, PA) and photomultiplier tube detection system and software (Ion Optix, Westwood, MA). Cells were washed with PBS at 37°C, replaced with Tyrode's buffer containing 2µl of 2mM Indo-1 AM, and allowed to incubate 30min at room temperature. The cells were then washed three times with fresh Tyrode's buffer and analyzed using the Ion Optix system. Detection wavelengths were recorded at 405nm and 485nm. Membrane voltage potential utilized the same procedure as calcium measurements but with voltage sensitive dye Di-8-ANEPPS (Thermo). Detection wavelengths were recorded at 560nm and 620nm.

4.2.13. Peak shortening percentage analysis

Cells were recorded upon spontaneous contraction from day 20-30 using phase contrast time-lapse video microscopy (Nikon Eclipse TE300, Melville, NY). Images were taken every 100ms with a CoolSNAP HQ2 CCD camera (Photometrics, Tuscon AZ) and analyzed with ImageJ. Minimum representative
rectangles were used to outline individual contracting cells and the lengths of the smallest and largest rectangles were used to calculate the peak shortening percentage.

4.2.14. Statistics

Statistical analyses were done in SigmaPlot (Systat Software Inc., San Jose Ca). Data were compared using one-way analysis of variance (ANOVA) followed by Tukey's test, p<0.05 was considered significant. Results were presented as mean ± standard deviation with number of samples/trials indicated in captions.
4.3. Results

4.3.1. pSi characterization and GSK3 and Wnt inhibitor release from pSi

The PLGA-pSi particles measured 8.24±3.25µm in diameter and uncoated pSi 265nm per dynamic light scattering. Zeta potentials for pSi and PLGA-pSi were -13.1±0.42 and -39.6±1.42, respectively (Figure 4.1F). CHIR99021 and IWP2 were loaded into silica particles by direct absorption from a 600ug/ml solution. HPLC data showed a burst release profile with 81.1±5.3% of CHIR99021 released within the first 24 hours (Figure 4.1G). After the initial 24 hours a CHIR99021 continued to be released through day 7 with nearly 100% cumulative release at 144 hours.

The addition of the PLGA outer layer eliminates the initial burst release, typical of most delivery systems. When IWP2 was encapsulated within the porous silica particles and coated with 5wt% PLGA 50:50, the results showed a cumulative release of 27.8±7.2% within the first 48 hours (Figure 4.1G). The particles were shown to have 40.2±9.1% cumulative release at the end of 72 hours. After the 144 hours, IWP2 was shown to have a cumulative release of 56.4±7.2%.
Figure 4.1 - Porous silica particle characterization (A) Schematic showing formulation of CHIR99021 and IWP2 loaded pSi. Gray areas represent porous silica and blue area represents PLGA coatings (B) TEM image of a single porous silica particle (scale 20nm) (C) SEM image of pSi (scale 2μm) (D) SEM image of PLGA-pSi (scale 10μm) (E) Confocal image of FITC-labeled pSi within PLGA coating (scale 10μm) (F) Zeta potential and particle diameters (G) Cumulative release profiles of CHIR99021 and IWP2 (H) Small molecule loading efficiencies into pSi and PLGA-pSi
4.3.2. Cellular toxicity with pSi exposure

Forward scattering of annexin V labeled cells measured by flow cytometry showed that 27.3±2.1% of cells internalized uncoated pSi and 3.61±1.4% internalized PLGA-pSi after 3 days in culture (Figure 4.2A). Uncoated pSi and PLGA-pSi were tested for cellular toxicity through an annexin V and propidium iodide assay (Figure 4.2B). After 3 days, annexin V staining of iPSC exposed to pSi loaded with both GSK3 and Wnt inhibitors (12.5±1.9%) showed no significant difference compared to the cells alone (10.1±3.3%), suggesting no increase in apoptosis. Propidium iodide staining also showed no significant difference between iPSC exposed to pSi loaded with both inhibitors (3.1±1.6%) compared to cells alone (4.1±2.5%) suggesting no increase in cell necrosis.
Figure 4.2 - iPSC Toxicity to Particles (A) Internalization of pSi and PLGA-pSi in AFSC-iPSC after 72 hours (B) Apoptosis and necrosis of AFSC-iPSC after 72 hours

4.3.3. Cardiac gene expression in pSi differentiation comparable to dissolved inhibitors

Live/Dead staining showed greater than 90% live cells in both 2D monolayer culture and transwell culture with no significant difference after 3 days in culture.
Figure 4.3C). The morphology of differentiating cells in transwells at day 0 showed denser cell localization and less cell spreading compared to 2D monolayer differentiation. During the first 5 days of differentiation, the 2D monolayer differentiation had a phase-dark layer of fibroblastic cells below a layer of spherical phase-bright cells, while a phase-bright cell layer was absent in transwell culture. At time points greater than 10 days, both groups exhibited a clustered morphology with elongated web-like sheets.
Spontaneous beating occurred in all groups at approximately 18-25 days of differentiation. Localized beating colonies were observed in all groups and were not synchronously paced.

cTnT expression showed 30.3±1.9% differentiation efficiency in pSi differentiated cells at day 15. Differentiation efficiency was greater in both 2D and transwell dissolved inhibitor groups with 45.6±3.5% and 39.3±5.3%, respectively (Fig 3B).
4.3.4. pSi released inhibitor differentiation expressed markers of cardiac differentiation

Cardiac differentiation was monitored through gene expression of brachyury, early stage cardiac markers (nkx2.5 and isl-1), and later stage cardiac markers (cTnT, β-MHC, Cx43). Pluripotent stem cell marker OCT4 was also monitored.
throughout the differentiation in order to quantify remaining pluripotency in the cell population.

Brachyury expression was measured 24 hours after the introduction of CHIR99021 in both conditions. The relative expression compared to day (-3) of differentiation showed that all groups had little to no brachyury expression at the start of differentiation, but after 24 hours all groups showed significant upregulation. The 2D monolayer differentiation and transwell dissolved inhibitors had a fold change increase of 236.3±6.7, where the transwell with inhibitor loaded particles showed a significant fold change increase of 99.3±27.1 (Figure 4.4B).

When compared to day (-3), OCT4 expression was shown to be downregulated over the course of differentiation in all groups (}
Figure 4.4C). At day 10 and 20, OCT4 fold expression in all conditions was below 0.06±0.01 and 0.007±0.006, respectively.

Early stage cardiac markers, Nkx2.5 and Isl-1, were both upregulated throughout the differentiation compared to day 0.
At each of the time points, Nkx2.5 expression was lower in the transwell groups compared to the 2D monolayer group. When compared to the other time points within groups, day 10 showed to have significant upregulation of
Cardiac troponin T was shown to be upregulated as early as day 5 in the transwell group with free inhibitors when compared to day 0 (Figure 4.4C).
However significant upregulation of cTnT was only shown in the pSi group at day 20 of differentiation. β-MHC is upregulated at day 10 in the 2D monolayer group, where later time points showed β-MHC significantly upregulated across all groups (Fig 4G). At day 30, the transwell group with pSi had the highest expression of β-MHC. Gap junction protein connexin 43 expression was not significantly different compared to day 0 at each time point in all groups (Fig 4H).
4.3.5. Early cardiac maturation and arrangement

Immunofluorescence staining showed expression of late stage cardiac markers $\beta$-MHC and connexin 43 in pSi differentiation at day 30 (
Cardiac cells were shown to have random arrangement with myosin heavy chain expressed throughout the cytoskeletons. There were no noticeable sarcomeres or banding patterns. Similar random expression of connexin 43 was shown as well with extracellular localization.

**Figure 4.5** - Confocal Immunofluorescence of pSi Differentiated AFSC-iPSC (A)

Actinin (red) Cx43 (green) DAPI (blue) (Scale 20µm)

4.3.6. Electrophysiological activity and peak shortening of pSi differentiated cells

Both control and experimental groups had spontaneously beating cells at day 18-21. Calcium handling measurements showed pSi differentiated cells spontaneously beat at a frequency of 3.0±0.6Hz.
Figure 4.6A). Differentiation with dissolved inhibitors resulted in cells with a beating frequency of 1.4±0.8Hz (...

Figure 4.6A). Contractile cell peak length shortening was not significantly different between dissolved inhibitor and pSi differentiation groups at 5.0±1.2% and 4.8±1.0%, respectively.
Figure 4.6 – T races of Spontaneously Beating Cardiac Cells (A) Calcium (Indo-1) and Voltage (Di-8-ANEPPS) sensitive dye traces for pSi and 2D differentiation at day 30 (B) Averaged peak length shortening percentages at day 30 (C) Image of beating cardiac cells within transwell (scale 20µm)

4.4. Discussion

In this study we have shown that GSK3/Wnt inhibitors released from pSi can be used to differentiate iPSC into cardiac cells. Previous studies showed highly efficient cardiac differentiation by temporal exposure to dissolved GSK3/Wnt inhibitors (63, 170). Here we showed that controlling the release of inhibitors by pSi encapsulation could produce spontaneously contracting cardiac cells, minimizing handling interactions.
iPSC did not exhibit significant cellular apoptosis or necrosis when exposed to pSi compared to the control. Previous studies have shown that internalization of pSi can induce cell programed death(186, 187). Therefore the preservation of iPSC cellular activity was likely due to minimal particle internalization. Both pSi formulations were internalized in iPSC but did not show a significant difference in apoptosis and necrosis compared to controls. This was likely due to the high metabolic nature of iPSC and lack of cellular waste exchange for 72 hours inciting innate apoptosis.

Inhibitors were able to direct cardiac differentiation after release from pSi. The release profiles from pSi were not binary and therefore had a sustained release past day 1 and 5 for GSK3 and Wnt inhibition, respectively. Temporal regulation of these inhibitors has been previously shown to be crucial for efficient cardiac differentiation, but a sustained release produces cardiac cells with less efficiency(188). Our data supports these findings as we showed an average pSi differentiation efficiency of 30.3%. Calcium handling and membrane potentials show contractility of pSi differentiated cells similar to that of direct inhibitor differentiation. A lower rate of spontaneous contraction in pSi differentiated cells may result from the delayed cardiac development compared to dissolved inhibitors.

Although plating density was the same in all groups, the differentiating cellular morphologies were different. Previous studies have shown that single iPSC can sense micro and nanoscale topographies altering their pluripotency(189, 190). The rounded and clustered appearance of cells within the transwells was likely due
to differences in substrate topography and may have promoted maintenance of pluripotency in a population of cells. After 10 days from the start of differentiation, the morphology between the two groups became more similar with beating colonies forming in localized areas. In terms of cardiac maturation, the morphology of differentiated cardiac cells did not show a clear pattern of cellular alignment. β-MHC and connexin 43 were present in differentiated cells but lacked sarcomeric pattering and organization. Continued culture and mechanical/chemical conditioning may be required for maturation(191, 192).

Genetic expression of cardiac differentiation markers in pSi differentiation showed comparable results to direct inhibitor differentiation. The release of CHIR99021 from uncoated pSi was sufficient in transitioning pluripotent iPSC into the mesendoderm lineage, and further Wnt inhibition by IWP2 loaded pSi upregulated late stage cardiac markers. Peak expressions of β-MHC and connexin 43 were later in pSi differentiated cells compared to control. While the cumulative dosage of pSi released inhibitors was similar to that of direct addition of inhibitors, 6uM CHIR99021 at day 0 and 2.5uM IWP2 at day 3, the non-binary and sustained release may have delayed cardiac maturation. Alterations to the pSi release kinetics could be altered to further increase cardiac efficacy. The downregulation of OCT4 within all groups of differentiation is important from a translational perspective, signifying reduced risk of pluripotent cells able to form teratomas(193).

A major limitation of this study is the significantly lower differentiation efficiency compared to that of fibroblast derived iPSC. Previous studies have shown
differentiation efficiency variability based on cell line\(194, 195\). This may result from innate differences in gene expression or epigenetic traits. AFSC derived iPSC have previously shown to have a lower differentiation efficiency compared to fibroblast derived iPSC lines\(184\). Therefore pSi AFSC-iPSC differentiation was not expected to increase differentiation efficiency compared to dissolved inhibitor control. The pSi platform used on a different cell line may produce greater differentiation efficiencies with a less heterogeneous population of resulting cells. Optimization of release parameters can also help to direct a more efficient cardiac differentiation. This could be done by altering the degradation kinetics of the particles in terms of polymer ratio and coating thickness. This would ensure the proper dosage of inhibitors per area of cell. Another limitation to this study is the diffusion of waste products. Transwell culture allowed for the isolation of iPSC and pSi, but elimination of cellular waste was limited to simple diffusion. Further development of this platform would benefit from a bioreactor ensuring sufficient exchange of nutrients.

4.5. Conclusion

This study shows that small molecule inhibitors temporarily released from pSi can direct cardiac differentiation of iPSC. The dual release of GSK3 and Wnt inhibitors from pSi is a simple and effective way to generate spontaneously beating cardiac cells. This self-directing cardiac differentiation platform has potential in future cardiac tissue engineering applications.
Electrospun patch functionalized with nanoparticles allows for spatiotemporal release of VEGF and PDGF-BB promoting in vivo neovascularization.

Abstract: The use of nanomaterials as carriers for the delivery of growth factors has been applied to a multitude of applications in the field of tissue engineering. Growth factors delivered with tailored spatiotemporal characteristics are leveraged to induce cellular signaling which can have downstream remodeling effects. However, issues of toxicity, stability, and systemic effects of these platforms have yet to be fully understood, especially for cardiovascular applications. In this study, we proposed a delivery system composed by poly(DL-lactide-co-glycolide) acid (PGLA) and porous silica nanoparticles (pSi) to deliver vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). The tight
spatiotemporal release of these two proteins has been proved to promote neovascularization and could be easily applied to ischemic tissue. In order to avoid tissue toxicity, augmenting the localized release, and stability of the platform we conjugated them to and electrospun (ES) gelatin patch patches.

When compared to freely disperse particles, ES gelatin patches were shown in vitro cultured with neonatal cardiac cells to have significantly less internalization of PLGA-pSi (2.0±1.3%) particles compared to free PLGA-pSi (21.5±6.1) or pSi (28.7±2.5) groups.

Internalization was positively correlated to late stage apoptosis resulting in PLGA-pSi and pSi groups with over 15% of cells apoptotic compared to 4.1±0.5% in the ES gelatin group after 3 days in culture. Release of growth factors from the ES gelatin patch was shown to be sustained and with the sequential release of VEGF and PDGF over time based on different PLGA encapsulation formulations. Through fluorescence microscopy, particles crosslinked to ES gelatin were shown to be uniformly dispersed with VEGF loaded particles 4.3±1.5µm and PDGF loaded particles 8.2±0.8µm in diameter. When implanted subcutaneously, ES gelatin patches were shown to have greater neovascularization than controls evidenced by increased impression of α-SMA and CD31 after 21 days. qRT-PCR results support increased angiogenesis by upregulation of VEGFA, VEGFR2, Vwf and COL3A1 exhibited a synergistic effect with the release of VEGF and PDGF after 21 days in vivo. The results of this study proved that PLGA-pSi particles crosslinked to ES
gelatin reduced cellular toxicity and could be tailored to have a dual release of growth factors promoting neovascularization.

5.1. Introduction

In recent years nanomedicine has attracted much attention in terms of drug delivery applications. The unique physical and chemical properties of nanoparticles and various methods used to formulate delivery vehicles can aid in overcoming pharmacological barriers of free drugs or compounds, i.e. bioactivity, solubility, targeting and circulating half-life (66, 196, 197). However, because of this wide range of nano-based delivery systems, there are no universal assessments of nanotoxicity (198).

The application of nano carriers in tissue engineering has numerous applications in delivering biochemical cues promoting implant integration and remodeling at the cellular level. Different cells respond differently to nanoparticles and selecting the right particle formulation and application becomes crucial in obtaining the desired cellular affect while minimizing collateral toxicity. In cardiac tissue engineering the preservation of native cardiac cells is extremely important due to their unique function and terminally differentiated state. Conditions such as a myocardial ischemia and subsequent infarction can lead to necrosed areas of heart tissue due the absence of blood supply. Repair or prevention of infarcted areas with strategies aimed at neovascularization can greatly increase prognosis. Neovascularization can be activated by the spatio-temporal exposure of VEGF and
PDGF-BB(73, 77, 199). It has been shown that release of VEGF is an important regulator for the activation of angiogenesis(200) and subsequent PDGF-BB exposure is essential for continued vascular maturation(199). Direct injection of growth factors is limited by their circulation half-life(201, 202). Therefore sustained dual delivery of these growth factors is important in tissue engineering applications.

The applications of porous silicon or silica have been studied for a number of years and range in numerous fields of study(203-205). In terms of drug delivery models, porous silica particles (pSi) have many unique properties such as high loading efficiency, tunability, availability of surface modifications and ease of synthesis(178). Further modifications to pSi release kinetics can be done by polymer encapsulations. For numerous years, poly(DL-lactide-co-glycolide) acid (PLGA) has been utilized to encapsulate drugs and other particles because of its biocompatibility and tunable degradation(206, 207). Delivery of biochemical cues can further be controlled from nano delivery platforms by conjugated particles to a biomaterial substrate(208). This ensures localized delivery to targeted area as well as mitigates free particle internalization.

This study investigated the angiogenic potential of an electrospun (ES) gelatin patch with cross linked PLGA-pSi loaded with VEGF and PDGF-BB. Freely dispersed pSi and PLGA-pSi were directly compared to ES gelatin conjugated PLGA-pSi for internalization and toxicity of neonatal rat cardiac cells in vitro. VEGF and PDGF-BB were both loaded into pSi and coated with different formulations of PLGA in order to have a sequential and sustained release. ES gelatin patches were
implemented into a subcutaneous BALB/c mouse model and explanted patches were
assessed through immunohistology and qRT-PCR.

5.2. Materials & Methods

5.2.1. Neonatal cardiac myocytes extraction

Cardiac myocytes were isolated from 2-3 day old rat hearts as previously published(209). Briefly, extracted hearts were washed, minced and suspended in
dissociation solution consisting of 0.32mg/ml collagenase type II and pancreatin
dissolved in 116mM NaCl, 20mM HEPES, 1mM Na₂HPO₄, 5.5mM glucose, 5.4 mM
KCl, and 0.8mM MgSO₄. Tissues in suspension were placed on an orbital shaker for
5min at 37°C, and supernatant was replaced with fresh dissociation solution for an
additional 30min. The supernatant was then collected in 5ml horse serum,
centrifuged and resuspended in 5ml horse serum. Fresh dissociation solution was
added to the original, undigested tissue and repeated an additional 2-3 times Cells
from all digestions were pooled together and resuspended in plating media (DMEM
with 10% horse serum, 5% FBS, 1% penicillin/streptomycin). Cells were then
plated onto a 3mg/ml gelatin coated surface at 10⁵ cells/cm². Media was changed to
maintenance media (DMEM with 5% horse serum, 1% FBS, 1%
penicillin/streptomycin) after 24 hours and changed every 2 days.
5.2.2. Porous silica particle fabrication

pSi particles were fabricated using a modified tannic acid template and Stoher method as previously published (210). Briefly, 272mg of tannic acid were dissolved in 50ml ethanol, 25ml ammonium hydroxide were added and allowed to stir for 1min. 300ul of tetraethyl orthosilicate (TEOS) were added dropwise and allowed to react for 3 hours with constant stirring. The particle suspension centrifuged, washed and re-dispersed in 40ml of 1:1 ethanol:water. Washes were repeated until washing solution was clear. After the final ethanol:water wash, the supernatant was removed and replaced with 15ml DI water then filtered through a 40µm mesh. Water was separated by centrifugation and particles were re-suspended in 1ml isopropanol. The solution was sonicated at 20AMP for 1min and allowed to dry in a vacuum oven at 60°C and -36Pa until dry. Fluorescein isothiocyanate (FITC) labeled pSi were fabricated by adding a 1mg of FITC (Sigma-Aldrich) dissolved in 58.33 µl APTES (Sigma-Aldrich) to the above TEOS solution.

5.2.3. Loading of PDGF-BB and VEGF into pSi

Fabricated pSi (~4mg) were loaded with 10µg/ml growth factor solution consisting of PDGF-BB or VEGF suspended in PBS. Particles were well dispersed into the loading solution placed on a rotating mixer at 37°C for 20min. The suspension was then centrifuged and the supernatant stored at -30°C for loading efficiency analysis. Loaded pSi were placed in -80°C for 1 hour and lyophilized overnight.
5.2.4. Fabrication of PLGA-pSi

Loaded pSi were encapsulated with PLGA through a solid-in-Oil-in water emulsion as previously published (178, 211). Briefly, PDGF-BB loaded pSi were coated with 10wt% 75:25 PLGA and VEGF loaded pSi coated with 5wt% 50:50 PLGA. The water solution consisted of 40ml of 2.5% polyvinyl alcohol. Growth factor pSi were suspended in PLGA dissolved in 1ml dichloromethane (DCM) to create the solid in oil suspension. 2ml of the water phase was added to the solid-oil suspension and homogenized for 5min. The homogenized solution was added to the remaining water phase dropwise and allowed to stir for 6 hours. Particles were then separated by centrifugation and supernatant reserved for encapsulation efficiency analysis. Particles were washed with 10ml DI water and separated again through centrifugation. Resulting particles were then frozen and lyophilized and stored at -20°C until later use. In order to distinguish the two PLGA-pSi formulations by confocal microscopy, PLGA-pSi loaded with VEGF and PDGF-BB were fluorescently labeled with FITC and tetramethylrhodamine (TRITC) (Thermo Fisher), respectively. This was done by adding either fluorescent marker to PLGA in DCM solution at a concentration of 1mg/ml.

5.2.5. ES gelatin patch with conjugated pSi fabrication

Fabrication of electrospun gelatin patch was done as previously published (212). Briefly, porcine and bovine skin gelatins (Sigma Aldrich) were dissolved in a 10% (w/w) hexafluoro-2-propanol (HFIP) (Sigma Aldrich) solution overnight at room temperature with constant stirring. Gelatin solution was loaded
into a syringe fitted with a 1.0mm diameter needle. Electrospinning parameters
applied to the solution were 18kV with a flow rate of 2 ml/h. The resulting patch
was placed in a vacuum desiccator to allow for residual solvent evaporation. PLGA-
pSi were conjugated to the surface of the patch by 1-ethyl-3-(3-
dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS). This
further crosslinked the ES gelatin patch as well as bonded the PLGA-pSi. The patch
was soaked in a solution of ethanol:water (9:1, v/v) with 75nM EDC (Sigma Aldrich),
30mM NHS (Sigma Aldrich) and 30 mg of PLGA-pSi particles for 24 hours at 4°C.
Patches were washed thoroughly with PBS, frozen at -30°C for 4 hours and
lyophilized overnight.

5.2.6. Characterization of ES gelatin patch with conjugated particles

ES gelatin patches were visualized by SEM and confocal microscopy.
Conjugated particles distribution and size were analyzed with ImageJ.

Lyophilized ES gelatin patches were evaluated for swelling within a 7 day
period. Dry weight ($W_d$) of lyophilized ES gelatin patches were recorded and then
submerged into PBS. At specified time points, hydrated samples were removed and
weighed ($W_h$). Percent swelling was calculated using the following equation:

$$\text{Swelling (\%)} = \frac{(W_h - W_d)}{W_d} \times 100$$

Equation 5-1 - Swelling Percentage of ES Gelatin
5.2.7. Neonatal cardiac myocytes culture on ES gelatin-pSi scaffold in vitro

ES gelatin-pSi scaffolds were cut into 0.25cm² squares and sterilized under ultraviolet light for 1 hour. Neonatal cardiac myocytes were dissociated from tissue culture wells by adding 0.5ml TrypLE Express (Thermo Fisher) for 5min at 37°C and neutralized with maintenance media. Cells were collected after centrifugation and resuspended at 10 million cells per ml. 20ul of cell solution was added to the top of each ES gelatin-pSi scaffold and incubated at 37°C for 30min to allow for cellular attachment. Maintenance media was then added to submerge each scaffold and cultured at 37°C until desired time point.

5.2.8. pSi internalization and cardiac cell toxicity

In order to visualize pSi internalization in neonatal cardiac myocytes, scanning electron (SEM) and confocal microscopy were utilized. SEM (FEI Quanta 400 ESEM FEG; FEI, Hillsboro, OR, USA) samples were coated with 3nm Pt/Pb and analyzed at 7kV.

Confocal microscopy was utilized to image FITC labeled pSi and PLGA-pSi. Cells were fixed in a 4% paraformaldehyde solution (Sigma-Aldrich) for 20min at 4°C and then washed 3 times with PBS. Cells were permeabilized with a 0.5% Triton X-100 (Sigma-Aldrich) in PBS solution for 15min. DAPI and phalloidin staining were done according to manufacturer’s protocol (Thermo Fisher).

To determine neonatal cardiomyocyte toxicity as a result of material interactions, cells were cultured for 3 days exposed to pSi alone and ES gelatin with
conjugated pSi. Cells were analyzed through AnnexinV/Dead Cell Apoptosis kit (ThermoFisher) at days 1 and 3 compared to the control at day 0. Cells were analyzed by flow cytometry (BD Accuri C6, BD Biosciences).

**5.2.9. *in vitro* release of PDGF-BB/VEGF**

Loading efficiency was calculated based on the concentration of growth factor remaining in initial loading solution and PLGA encapsulation solution. Remaining growth factor concentrations for PDGF-BB and VEGF were determined through enzyme linked enzyme-linked immunosorbent assay (ELISA) (R&D Systems).

Release profiles were determined by dispersing 6.0mg PLGA-pSi in 1.0ml of PBS. ES gelatin scaffolds were cut into 0.25cm² squares and submerged into 1.0ml PBS. Samples were incubated at 37°C with gentle agitation until predetermined time points. At each designated time point, suspensions were centrifuged for 5min at 4000rpm, supernatant collected and particles re-dispersed in 1ml fresh PBS. Obtained samples were quantified by ELISA. Supernatants were collected up to 21 days.

**5.2.10. *in vivo* ES scaffold implantation**

In accordance with approved protocol by the Institutional Animal Care and Use Committee (IUCUC) at Houston Methodist Research Institute and NIH guidelines for the care and use of laboratory animals, BALB/c mice (10 weeks old; weighing 21-23g) were used to assess the angiogenic potential of ES gelatin-PLGA-pSi patches
by subcutaneous implantation. Briefly, mice were anaesthetized and a 3 x 3 cm² area of skin was shaved and sterilized with ethanol. Midline skin incisions were made to prepared area and two 0.64 cm² patches were implanted into the area. A total of 12 mice were used for each time point (7, 14 and 21 days). At each time mice were euthanized by CO₂ inhalation and implants were retrieved for further analysis.

5.2.11. Histological evaluation

Explanted ES gelatin scaffolds (n=3 per experimental group) were sectioned and mounted with Cytoseal XYL (Thermo Fisher) mounting medium. Sections were stained for α-smooth muscle actinin, CD31 and DAPI according to manufacturer’s protocol (Abcam) and analyzed with a histology microscope (ECLIPSE Ci-E, Nikon).

5.2.12. qRT-PCR

Functionalized electrospun patches loaded with empty vector (Blank), PDGF-BB alone (PDGF), VEGF alone (VEGF), or both growth factors (Dual) were implanted in the subcutaneous mouse model. Implants were retrieved at 7, 14, and 21 days. RNA was extracted by using RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentration and purity were measured by NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA was reverse transcribed from 1µg of total RNA with iScript retrotranscription kit (Bio-Rad Laboratories, Hercules, CA). Amplifications were carried on using TaqMan® Fast Advanced Master Mix on a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA). The
following target probes (Applied Biosystems) were applied to evaluate the expression of local neovascularization.

1. Vegfa, vascular endothelial growth factor A, Mm01281449_m1
2. Kdr/Vegfr2, kinase insert domain protein receptor, Mm01222421_m1
3. Vwf, Von Willebrand factor homolog, Mm00550376_m1
4. Col3a1, collagen, type III, alpha 1, Mm01254476_m1

The expression of each gene was first normalized to the level of Gapdh (glyceraldehyde-3-phosphate dehydrogenase, Mm99999915_g1). Relative fold changes were determined by taking implants which were loaded with empty vectors and collected at 7 days as reference. Biological triplicates for each group were assessed, and results were reported as mean ± standard deviation.

5.2.13. Statistics

Statistical analyses on quantitative results were done using one way ANOVA. p<0.05 was considered significant. Results are present with ± standard deviation and number of samples/trials indicated in captions.

5.3. Results

5.3.1. Neonatal cardiac cell particle internalization and toxicity

Qualitative analysis of particles internalized by neonatal cardiac cells was visualized by SEM and confocal microscopy. SEM images of PLGA-pSi and pSi groups
Figure 5.1A and

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Figure 5.1B) showed clear localization of particles on top and below cellular membranes, while cells attached to ES gelatin patches revealed minimal particle internalization.

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Figure 5.1B) showed clear localization of particles on top and below cellular membranes, while cells attached to ES gelatin patches revealed minimal particle internalization.
Figure 5.1C and

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Figure 5.1F). Confocal microscopy supported SEM images showing the localization of FITC labeled particles only within cell cytoskeletons of PLGA-pSi and pSi groups.
**Figure 5.1D and**

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Flow cytometry quantified significantly greater internalization of free PLGA-pSi (21.5±6.1) and pSi (28.7±2.5) compared to ES gelatin-PLGA-pSi scaffolds (2.0±1.3) (Fig 1G).

Apoptosis and necrosis quantified through flow cytometry revealed no significance between groups tested on day 0 with 30min exposure to particles.
Figure 5.2A). Days 1 and 3 revealed significant differences in total apoptosis when compared to untreated control. Day 1 revealed that there was significantly higher late stage apoptosis signified by the double positive cell population of Annexin V and propidium iodide. Where PLGA-pSi and pSi groups showed 17.6±6.6% and 14.7±4.2%, respectively, ES gelatin patches had 3.0±1.2% late apoptosis.
Figure 5.2B). After 3 days of particle exposure, both PLGA-pSi and pSi had internalization comparable to the control, but ES gelatin group was significantly lower (4.1±0.5%).
Figure 5.1 - Neonatal cardiomyocyte particle internalization. (A-C) SEM images of cells internalizing PLGA-pSi, pSi and PLGA-pSi on ES gelatin scaffolds. (D-F) Confocal microscopy images showing internalization of PLGA-pSi, pSi and PLGA-pSi on ES gelatin scaffold. (G) 24 hour internalization of particles quantified by flow cytometry.
Figure 5.2 - Apoptosis resulting from particle exposure. (A) Annexin V and propidium iodide staining of neonatal cardiac cells while exposed to particles for 3 days. (B) Total apoptosis percentages over time compared to untreated control group (n=3, p≤0.05 is significant)
5.3.2. Characterization of PLGA-pSi and release of VEGF and PDGF-BB

Averaged particle diameter of 5% 50:50 PLGA-pSi particles was measured to be 4.3±1.5μm where 10% 75:25 PLGA-pSi produced a larger particle at 8.2±0.8μm (Figure 5.3A). The loaded PLGA-pSi formulations showed a sustained release of VEGF and PDGF-BB over the course of 21 days. 5% 50:50 VEGF PLGA-pSi and 10% 75:25 PDGF-BB PLGA-pSi showed after 15 days to have a cumulative release of 91.2±12.4% and 66.2±1.6%, respectively (Figure 5.3B). After particles were conjugated to ES gelatin patches, the cumulative release of VEGF and PDGF-BB was lower than unconjugated particle release after 21 days. The conjugation of PLGA-pSi to ES gelatin patches was not shown to have an impact on the swelling ratio of the material after 7 days (Figure 5.3C). Confocal microscopy revealed that even distribution of both formulation of PLGA-pSi (Figure 5.3D, Figure 5.3E and Figure 5.3F). SEM image supported even PLGA-pSi distribution on the surface of the ES gelatin patch and clear distinction of different particle formulations evidenced by their size (Figure 5.3G).
Figure 5.3 - Electrospun gelatin-PLGA-pSi characterization and growth factor release. (A) Average diameter of PLGA-pSi formulations (B) Release profiles of VEGF and PDGF-BB loaded into PLGA-pSi (C) Swelling ratios of ES gelatin patches over time. (D-G) Confocal and scanning electron microscopy showing morphology and distribution of particles on ES gelatin patches.
5.3.3. Vasculogenic potential in vivo

Gross observation of ES gelatin explants showed significant differences in observable neovascularization. After 7 days *in vivo* no group had observable vascularization (Figure 5.4A, Figure 5.4B and Figure 5.4C). Observable red capillary like areas surrounding the patch area were only detectable by gross analysis in ES gelatin patch containing particles loading both PDGF-BB and VEGF (Figure 5.4F and Figure 5.4I). Immunohistological analysis of explanted ES gelatin patches showed significantly higher expression of neovasculogenic markers, alpha smooth muscle actinin (α-SMA) and CD31, after 21 days *in vivo* (Figure 5.5). Control ES gelatin patches revealed slight α–SMA expression within the patch after 21 days (Figure 5.5C). In the ES gelatin patch releasing PDGF-BB and VEGF there was greater α–SMA and CD31 expression at 7, 14, and 21 days compared to the control (Figure 5.5D, Figure 5.5E and Figure 5.5F). At 14 and 21 days of the growth factor releasing patches, the co-localization of α–SMA and CD31 was observed, signaling neovascularization.
Figure 5.4 - Gross observations of ES gelatin explants up to 21 days *in vitro*. ES gelatin containing conjugated PDGF-BB-PLGA-pSi (A, D and G), VEGF-PLGA-pSi (B, E and H) and both particles were evaluated and compared to control patches.
Figure 5.5 - *In vivo* immunohistological sections of implanted ES gelatin patches up to 21 days. Sections were stained for α-SMA (green) and CD31 (red). Dotted lines represent border of ES gelatin patches. (scale bar, 20µm)

(B,C) Quantification of α-SMA and CD31 (n=3, p≤0.05 is significant)

5.3.4. Genetic expression of vascularization markers

We assessed and evaluated local neovascularization after the implantation of four different groups of electrospun patches which were loaded with empty vector (Blank), PDGF-BB alone (PDGF), VEGF alone (VEGF), or both growth factors (Dual) at 7, 14, and 21 days on molecular level. Expression of Vegfa (Figure 5.6A) and its receptor Vegfr2 (Figure 5.6B), endothelial marker Vwf (Figure 5.6C), and Col3a1 (Figure 5.6D), which is enriched in connective tissue that supports blood vessel walls, were determined. Implantation of blank electrospun patches didn’t affect the
expression of any gene and their expression stayed unchanged throughout the process. Implantation of patches which released PDGF-BB alone led to a gradual increase in expression of all four genes between 7, 14, and 21 days, while patches that released VEGF alone increased the expression of Vegfr2, Vwf, and Col3a1, but not Vegfa. Though release of individual growth factor by the patches elevated vascular marker gene expression, the most notable augmentation was observed in the dual-release group. The differences between individual versus dual release group were most significant after 21 days implantation. Taken together, the spatio-temporal delivery of both growth factors demonstrated the most dramatic facilitation of genes implemented in local neovascularization.

Figure 5.6 - Expression of genetic markers for neovascularization from explanted ES gelatin patches. (A)Vegfa (B)Vegfr2 (C) Vwf (D) Col3a1
5.4. Discussion

The application of drug delivery in tissue engineering strategies has been widely researched for a number of reparative strategies (66, 213, 214). Currently there are no standardized regulations for nanoparticle toxicity and assessment due to the myriad of cell types and particle formulations (215), but previous studies have shown a strong correlation between cellular toxicity and particle internalization (216-218). The limit of diffusion also hinders many tissue engineering strategies leading to implant failure over time caused by inadequate nutrient transfer past 200µm (219, 220). Ongoing research into prevascularized (221, 222) and angiogenic (223, 224) scaffolds have highlighted the need for implantable biomaterials supporting cellular growth and proliferation as well as recruit native remodeling cascades (225). In this study we have shown an electrospun gelatin platform containing PLGA-pSi delivering angiogenic growth factors reduced pSi internalization, toxicity and promoted neovascularization.

Biomaterials for tissue engineering needs to provide both structural support as well as promote integration into native tissue. Natural and synthetic scaffolds alone have shown positive results in tissue structural support, but often times do not promote native tissue integration. The key components to tissue integration involve cellular migration in attachment, however these migrated cells will not survive without a linked vasculature network. The promotion of angiogenesis has been linked to a number of signaling molecules and growth factors. However, targeting this angiogenesis into an implanted material is challenging without a localized release that can signal vasculogenic cells to
the material. Drug releasing materials are a solution to this targeting of an implanted biomaterial, but other factors arise such as drug stability, dosage and timing. By encapsulating drugs within delivery vehicles such as nanoparticles, the stability of the drug is preserved and delivery dosage and timing can be tuned based on the encapsulating material(178). Also, the cellular trafficking of these molecules is crucial to gain the desired cellular response. Growth factors meant to affect an extracellular receptor will likely have an undesired cellular response if internalized rather than exposed to the surface of the cell(226, 227). Therefore, internalization of loaded particles is not always a desired effect, particularly in the case of angiogenesis promoted by growth factors. By conjugating drug loaded particles to the surface of the implanted biomaterial, the spatiotemporal delivery kinetics are localized at the scaffold and the surface of cells, which in turn improves the efficacy of the delivery system(228).

In this study we have shown an electrospun gelatin platform containing PLGA-pSi delivering angiogenic growth factors reduced cardiac cell internalization, toxicity and promoted neovascularization. Ideally the vehicle for drug or compound delivery in tissue engineering applications would be biocompatible and have a localized payload(229). Particle internalization has been shown to have a negative effect on cellular signaling and activate apoptotic pathways(230). Intracellular trafficking and sorting in different intracellular regions of an individual cell can be dependent on the cell type as well as internalized particle formulation(231). The results we presented on particle internalization of both PLGA-pSi and pSi alone showed to have negative effects on apoptotic signaling pathways. This is likely due to alterations in cellular signaling resulting from the uptake of foreign materials(232). The size of the
particle plays a role in the particle internalization concentration. As shown in

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Figure 5.1, greater numbers of smaller pSi particles are able to be internalized by a single cell compared to the larger PLGA-pSi. However since the goal for this study was to deliver growth factors that have extracellular signaling effects, particle internalization was not ideal. Therefore, by conjugating PLGA-pSi to ES gelatin patches cells were shown to be less able to internalize particles and therefore payloads of growth factor were hydrolytically controlled rather than enzymatic
digestion from cells. This trend was conferred when monitoring the early and late stage apoptosis in neonatal cardiac cells. Initial total apoptosis was not significant in all groups and may have resulted from inherent apoptotic signaling due to incomplete cellular attachment. After 3 days of exposure to pSi alone, total neonatal cardiac cell population was significantly decreased compared to other groups. This may have influenced the total apoptosis percentage in that a significant population of cells have already lysed and were fragmented. However particles crosslinked to ES gelatin patches proved to decrease total apoptosis and have the highest percentage of viable cells.

The sustained and sequential release of VEGF and PDGF-BB was tailored based on previously published work (77, 199). By choosing a 5% 50:50 PLGA coating for VEGF loaded pSi, the coating was engineered to be thinner and more susceptible to hydrolytic degradation compared to the 10% 75:25 PLGA coating for PDGF-BB loaded pSi. These two polymer coating formulations were translated in the average particles diameters (Figure 5.3A) and release profiles (Figure 5.3B). VEGF was designed to be released first promoting early stages of neovascularization, where the later and sustained release of PDGF-BB was aimed to promote endothelial recruitment and in turn vascular growth and maturation. The conjugation of PLGA-pSi to ES gelatin patches did alter the release profiles of PDGF-BB and VEGF in vitro. Both growth factors were shown to have a more sustained release over time released for ES gelatin patches compared to release for particles. This longer sustained release proves to be beneficial in the neovascularization process in vivo due to continued vessel maturation in tissue remodeling. The localization of
particles to the patch area also minimized nonspecific distribution of growth factors limiting any significant systemic affects in vivo.

Conjugation of PLGA-pSi to ES gelatin patches did not change the bioactivity of the growth factors in terms of promoting in vivo vascularization. The swelling ratio of patches with conjugated PLGA-pSi did not change as well supporting clinical patch applications. Gross observations on explanted patches proved to have a synergistic vascularization effect greater than either growth factor alone. Immunohistological results supported greater vascularization in dual released ES gelatin patches evidenced by the co-localization of α-SMA and CD31 at later time points. Lumen formations are larger and more frequent in the 21 day time points compared to 14 days suggesting that the timing of growth factor release was sufficient to promote sustained vessel maturation. Gene expression analysis over time also supports the synergistic effect of PDGF-BB and VEGF with significantly greater expression of both early and late angiogenesis at 21 days. This suggests that neovascularization remodeling was ongoing in the implant area.

In general size-dependent cytotoxicity has been accepted that smaller particles are more toxic due to their increased specific surface area (233, 234). The results of this study did not show significant differences in cytotoxicity of the smaller pSi vs the larger PLGA-pSi. This may have been a result of pSi aggregation therefore decreasing the specific surface area of the particles as a collective. SEM images did reveal some areas of pSi internalization where aggregates were present. Another limitation of this study is that the measure of particle internalization at the
single cell level was a qualitative assessment. The exact number of particles internalized per single cell was undoubtedly variable. Therefore the effective dosage of particles internalized by a single cell leading to apoptotic signaling pathways was not determined. However the correlation between particle internalization with cardiac cell apoptosis was sufficient to support the opposite where decreased particle internalization decreased cellular apoptosis. Our platform of PLGA-pSi particles bound to an ES gelatin substrate supports this deduction.

The promotion of neovascularization after myocardial ischemia has been shown to have greater outcomes in terms of cardiac output and function. This has been shown to be driven by the cellular recruitment of vasculogenic progenitors and cells (235-237). Our platform utilizing the localized and temporal release of angiogenic growth factors could be beneficial as a cardiac patch after ischemia proving to have little toxicity to cardiac cells as well as promote angiogenesis.

5.5. Conclusion

This study has shown that PLGA-pSi within neonatal cardiac cells can have apoptotic signaling effects when internalized. PLGA-pSi conjugated to ES gelatin showed less cellular internalization and toxicity compared to all groups tested. As a platform, alterations in patch characteristics and particle formulation can further tune delivery of different payloads allowing for a wide range of clinical applications.
Conclusions and Future Directions

The overall objective of this thesis was to investigate the potential of a self-contained cardiac patch using reprogrammed amniotic fluid-derived stem cells and for congenital heart defect repair. To that end, we demonstrated the following: 1) AFSC could be reprogrammed by mRNA transfection and subsequently differentiated into functional cardiac cells by small molecule inhibition of the GSK3/Wnt signaling pathways, 2) Small molecule inhibitors of the GSK3/Wnt signaling pathway could be loaded into porous silica particles and released temporally in vitro to direct iPSC-AFSC into functional cardiac cells, and 3) pSi crosslinked to electrospun gelatin patches were shown to decrease cellular toxicity in neonatal cardiac cells and when loaded with VEGF and PDGF-BB shown to promote neovascularization in vivo.

Moving forward, there are a number of areas that require further investigation. These include the following:
The differentiation efficiency of AFSC-iPSC to cardiac cells was not as efficient as published for fibroblast-derived iPSC. Does the timing or concentration of small molecule inhibition need to alteration in order to result in a more efficient differentiation?

AFSC-iPSC differentiated into cardiomyocytes were classified as functional but immature cardiac cells. Can different chemical or physical conditioning be used to promote further maturation?

The efficiency of cardiac differentiation from pSi was lower than that of differentiation with dissolved inhibitors. Can the release platform be optimized to increase the differentiation efficiency? Does this differentiation efficiency change with a different iPSC cell line?

Can the pSi differentiation platform be translated into the electrospun gelatin platform?

Can the angiogenesis promoting ES gelatin patch promote cardiopreservation in an MI model?

Future studies inquiring into these questions can be used to support the findings in the presented research and further push clinical translation. The differentiation of fully functional cardiac cells from reprogrammed amniotic fluid derived stem cells is a significant finding in the field of congenital heart defect tissue engineering. This source of cells is renewable, easily obtained, and has been proven to be created through safe and efficient means. The current findings go one step further in showing the potential in creating a clinically translatable, self-contained and differentiating cardiac patch. If further studies prove successful, this cardiac
patch platform will generate autologous cardiac tissue all while the baby is still
developing. Clinically, this technology is capable of improving the lives of countless
children. The translation of this platform may also be expanded to other cardiac
disorders such as myocardial ischemia, thus having the potential to improve the
lives of countless individuals.
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