DEVELOPMENT OF A TRANSLATIONAL, TISSUE-ENGINEERED APPROACH TO REPLACEMENT OF MYOCARDIAL INFARCT SCAR TISSUE

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DEVELOPMENT OF A TRANSLATIONAL, TISSUE-ENGINEERED APPROACH TO REPLACEMENT OF MYOCARDIAL INFARCT SCAR TISSUE

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Jason Bradley Schulte
December 2014

Accepted by:
Dr. Agneta Simionescu, Committee Chair
Dr. Dan Simionescu
Dr. Martine LaBerge
Dr. Richard Visconti
Dr. Christopher Wright
ABSTRACT

Myocardial infarction (MI) affects nearly 600,000 individuals each year, and the resulting damage initiates a pathophysiological progression towards congestive heart failure (CHF). A shortage of donor organs precludes heart transplantation as a practical solution, and neither surgical intervention nor stem cell therapy have yielded consistent and sufficiently positive results in clinical investigation.

To prevent CHF, prospective therapies must target the cause of the maladaptive cardiac remodeling which precedes it—the nonfunctional, dyskinetic infarct scar—and aim to replace it with functional cardiac muscle. Tissue engineering holds promise for the development of novel therapies to either halt or reverse post-MI cardiac remodeling. However, early efforts to implant thick (> 100 µm), functional, tissue-engineered grafts have failed in animal models because such grafts lack the vascularization, among other features, necessary for long term survival. Therefore, the primary goal of this research was to develop a tissue-engineering approach to fulfill the need for thick, fully vascularized, and functional myocardial grafts to replace infarct scar tissue and prevent or reverse development of CHF in affected patients.

First, myocardial flap scaffolds based on decellularized (DCELLed) porcine left-ventricular myocardium and its associated coronary arteries and veins were generated and characterized. All scaffolds displayed a fully intact and patent vasculature to the level of capillaries, were devoid of cellular proteins, contained only residual DNA, retained collagen, elastin, and basal laminar components, exhibited excellent mechanical properties, and were compatible towards seeded cells.

To evaluate the host response to the scaffold material in a xenogeneic scenario, scaffold samples were implanted subdermally in rats. Long-term, macrophages at the implant site were
observed to shift in polarization from an inflammatory to a more constructive, remodeling phenotype.

In a subsequent series of studies, these scaffolds were seeded with human adipose derived stem cells (hADSC) and exposed to stimuli reflective of the cardiac environment using a custom-designed platform. Seeded constructs were subjected to various combinations of mechanical, electrical, and pharmacological stimuli with the objective of directing the differentiation of hADSC into a cardiomyocyte phenotype en route to resembling functional myocardial tissue.

Finally, the feasibility of infarct scar surgical replacement with myocardial scaffold-based grafts was evaluated in a porcine model. A surgical anastomosis of the scaffold’s vessels to the host’s vasculature demonstrated complete perfusion of the scaffold. Hemostasis was achieved with no major bleeding events, and no complications were encountered.

It is expected that this research will have a positive impact upon efforts to treat a growing population of patients suffering from complications of MI. It will not only enable clinicians to prevent or reverse the progression towards CHF—saving lives and improving the quality of life for recipients of this therapy—but also contribute to the advancement of the cardiac tissue engineering field.
DEDICATION

For my family – past, present, and future.
ACKNOWLEDGMENTS

The author would like to acknowledge the incredible support and guidance he received from his advisors, Drs. Agneta and Dan Simionescu. Their mentorship has shaped him both as a scientist and a person. For their time and indispensable advice, he would like to thank committee members Christopher C. Wright, M.D., of the Greenville Health System, Greenville, SC; Richard P. Visconti, Ph.D., of the Medical University of South Carolina, Charleston, SC; and Martine LaBerge, Ph.D., of Clemson University, Clemson, SC. He is grateful for the friendship and assistance of all Biocompatibility and Tissue Regeneration Laboratories (BTRL) members, past and present, including: Jeremy Mercuri, Ph.D, George Fercana, Ph.D., James Chow, Ph.D., Lee Sierad, Ph.D, Richard Pascal, Grace Dion, Allison Kennamer, Theresa Hafner, Katy Jaeggli, Natasha Topoluk, Chris DeBorde, and Alex Bina. He would like to extend a special thanks to all those who contributed directly to this project: Joshua Biggs, Robert Marti, Laura McCallum, Katelyn Rye, Mike Jaeggli, and Barry C. Starcher, Ph.D. Finally, the author would like to thank the Clemson University Department of Bioengineering and its staff, including: Maria Torres, Leigh Humphries, Sherri Morrison, Maranda Arnold, Michelle Kirby, Mellissa McCullough, and Tammy Rothell. Funding for this research was furnished by the National Institutes of Health through several grants: R21EB009835 and 2P20GM103444-06 (to AS) and R01 HL093399 (to DTS).
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CHAPTER ONE: REVIEW OF LITERATURE

1.1 Introduction and Background:

1.1.1 Etiology and Pathology:

Hypertensive, hypercholesterolemic individuals often develop coronary artery disease (CAD), which is characterized by atherosclerosis in one or both of the coronary arteries. Atherosclerotic lesions in these arteries, especially the left anterior descending (LAD) coronary artery, are associated with a high risk for occurrence of adverse cardiac events, including unstable angina, acute MI, and sudden death from cardiac failure.\[1\] These lesions, or atheromatous plaques (atheromas), are suspected to develop according to the established inflammatory mechanisms of atherosclerosis, but typically do not grow large enough to achieve any appreciable stenosis of the vessel. Rather, the mechanism responsible for triggering cardiac events is thrombosis or subsequent thromboembolism within the coronary arteries or their branching vessels.\[1\]

In the pathogenesis of MI, thrombus formation occurs following the rupture of the endothelium and fibrous cap which overlay the atheroma. The atheroma’s lipid core is left exposed and free to contact and activate platelets, initiating blood clot formation.\[1\] The resulting thrombus occludes the coronary circulation either at the site of the lesion or embolizes to some distal point, and all cells nourished by the occluded vessel are subjected to severe ischemic insult if they are located distal to the blockage. Hypoxia and lack of sufficient nutrient and waste exchange resulting from ischemia cause necrosis of the affected cells, and the injured area, or necrotic core, becomes inflamed.\[2\]
Following the inflammatory response, the tissue is remodeled into a dynamic, but functionally inferior, scar composed of fibrous matrix proteins (i.e. collagen type I and III) and myofibroblasts (Figure 1.1).[^3] This fibrotic response is heavily favored over any competing regenerative response (e.g. restoration of native structure and function of the tissue) because the former is a much more rapid process, and there is a strong impetus to repair (e.g. restore tissue integrity) injury in a vital organ such as the heart as quickly as possible.[^4] This remedy for the injury is analogous to sealing a hole in a ship’s hull with duct tape instead of welding a new piece of metal in place of the void—it is a haphazard attempt at repair.

![Figure 1.1: Schematic of the arterial stenosis and subsequent blockage that causes acute myocardial infarction (MI). The area of affected tissue is also shown. The cells in this area succumb to necrosis and the tissue is remodelled into a fibrous scar.](image)

In the first 2 days post-MI, activated macrophages contribute to increased expression of transforming growth factor-beta (TGF-β), which is thought to drive the phenotypic shift of interstitial cardiac fibroblasts into myofibroblasts. These myofibroblasts, in conjunction with increased levels of angiotensin II and TGF-β, mediate transcription of collagen type I and III mRNAs at the site of the infarct.[^3]

[^3]: Reference (pages 4-5)
[^4]: Reference (pages 6-7)
The resulting fibrosis is not localized to the necrotic core or tissue immediately adjacent to it, but is frequently observed to affect sites remote to the infarct. In an infarct of the left ventricular anterior wall, for example, areas of increased fibrillar collagen deposition can appear intraseptally, in the septal endocardium, in the right ventricle, and in the visceral pericardium. This effect is most likely an indirect result of the damage caused by the infarct and is mediated by physiologically-driven changes in cell morphology, and therefore, changes in the expression of fibrillar matrix proteins.

Following MI, the body will attempt to maintain homeostasis despite the infarct-induced death of cardiomyocytes (CM) and accompanying loss of their energetic contribution to the stroke volume (volume of blood pumped during systole) of the left ventricle. This means the collective demand of the body’s cells for blood will not deviate from pre-infarct levels, and, consequently, the remaining healthy myocardium must work harder to maintain the original cardiac output. As a result, the heart undergoes changes at both the cellular and organ level in order to normalize stroke volume.

The noncontractile infarct scar is a weak point in the ventricular wall that acts as a stress concentration during ventricular systole. Thus, ventricular pressure without counteracting force evolved from wall tension in that area causes stretching and thinning of the infarct and an increase in the radius of the chamber. Pursuant to the Law of Laplace, which can be used to model the relationships between pressure, volume, and wall geometry in the left ventricle, an increase in radius helps to normalize wall tension and stroke volume initially. With time, however, this increased radius diminishes the preload (and ejection fraction) the ventricle is able to generate, and other mechanisms of remodeling such as
increased CM contraction force and wall thinning are enlisted to help augment preload generation.

Increased CM contraction force is managed through cellular hypertrophy. Via mechanotransduction, CM experience chronically increased mechanical stresses and synthesize additional muscle filaments (actin, myosin, and titin) to handle the larger workload. Since the ventricle increases in volume, the myocardium is under volume overload, and it has been established that volume overload produces eccentric CM hypertrophy.[6] Specifically, CM add muscle filaments in series rather than in parallel (as in pressure overload scenarios) so that CM lengthen. The aggregate of this lengthening is an increasing ventricular radius, which circles back to create the original problem of reduced preload.

Initially, this hypertrophic response is effective in maintaining cardiac output, but the long term effects are deleterious. Since these cells are surrounded by an extracellular matrix (ECM) of collagen, there is an attendant increase in the synthesis of fibrous ECM components to support the hypertrophic cells.[7, 8] This fibrosis leads to decreased compliance, poor diastolic filling, and chronic excessive wall stress. There is also evidence that chronic hypertrophy involves shedding of integrins—the transmembrane proteins responsible for myocyte-ECM adhesion—into the adjacent ECM, potentially causing disordered mechanotransduction and diminished transfer of myocyte contraction force to the ECM.[9] Additionally, activation of matrix metalloproteases (MMP) under chronic volume overload can lead to degradation of the collagen network (the perimysium) and cause increased elasticity and muscle fiber slippage.[10] A possible manifestation of this
slippage is the expansion of the ventricle and thinning of the ventricular wall, which leads to global enlargement of the heart, weakening of the ventricular walls, and dangerously reduced cardiac output.\(^{[11]}\) Together, these constitute the hallmark symptoms of dilated cardiomyopathy (DCM) and congestive heart failure (CHF), a fatal condition which can result in the years immediately following an MI, especially if the infarct is left untreated beyond reperfusion.\(^{[7, 12]}\)

The hearts of patients with CHF consistently display such characteristic ventricular wall distension and aneurysmal dilation, which result in the development of widespread edema (including pulmonary edema) and severe exercise intolerance. CHF is the final stage of a degenerative cascade of events which begins with MI and the scar tissue it produces.\(^{[7, 12]}\) Therapies which seek to halt progression toward CHF must necessarily target the prevention of infarct scar formation or its replacement with functional myocardium.

### 1.2 Treatment:

#### 1.2.1 Clinical Therapeutic Options:

Orthotropic, or whole organ replacement, is currently the only curative therapy for myocardial damage, namely prevention of left ventricular dysfunction and remodeling, CHF or subsequent MI.\(^{[13, 14]}\) Transplantation is limited by a severe shortage of donor hearts, as fewer than 2,500 transplant procedures are performed annually, and over 3,000 patients perennially remain on the waiting list.\(^{[15, 16]}\) The unmet need is not fully conveyed by these figures, however, if one considers the annual number of patients who suffer a first
MI (roughly 600,000). This would seem to suggest the existence of a vastly underserved population of patients who could benefit from transplantation, but are either ineligible, or deem it futile to request a place on the waiting list.

The inflammatory response following MI occurs rapidly. It is estimated that necrosis of CM begins 20-30 minutes after the initial blockage of the coronary vasculature. The current gold standard in clinical care post-MI is percutaneous coronary intervention (PCI), a transcatheter procedure wherein an angioplasty balloon is used to dilate the vessel lumen immediately adjacent to the site of thrombotic occlusion, thus restoring flow to the downstream tissues. One complication limiting the efficacy of PCI is ischemia-reperfusion injury, an inflammatory condition created by sudden reversal of a hypoxic state wherein microvascular endothelial cells become activated and produce reactive oxygen species. Currently, chest pain centers and cardiac care units have set a benchmark door-to-balloon time (elapsed time between patient admission to completion of PCI) of 90 minutes in order to limit the extent of damage produced by MI. While minimizing door-to-balloon time has shown a direct correlation with reduced infarct scar size, it is difficult to imagine achieving reperfusion of the ischemic tissue before CM necrosis sets in. This means patients are inevitably faced with the development of an infarct scar and its complications unless response and transport times dramatically increase or an at-hand/pre-hospital admission treatment is developed.

An alternative to PCI is CABG, a surgical procedure which involves using an autograft (either a length of the saphenous vein or mammary artery) to reroute blood flow around the thrombus (thus, bypassing it) that blocks the coronary circulation and restoring
blood supply to the ischemic myocardium (Figure 1.2). CABG is used frequently in an interventional capacity among patients with angina caused by coronary artery disease. It can be indicated as a treatment for aborting and mitigating damaged caused by MI, but is associated with increased morbidity (i.e. stroke, brain dysfunction) as the immediacy of its performance following MI increases (although this correlation could be derived from general instability of the patient prior to surgery).\cite{22,23} The overall efficacy of CABG as assessed by long-term (10 year) mortality rate is favorable, with rates of only 26 – 32% reported in several clinical trial.\cite{22-25} PCI, including percutaneous transluminal coronary angioplasty (PTCA) performed with either balloons or stents is associated with slightly higher mortality rate, but presents a less invasive alternative with comparable relief of symptoms.\cite{26,27}

Figure 1.2: Graphic illustration showing the points of anastomosis (attachment) of the vascular graft in coronary artery bypass grafting surgery (CABG).

The therapies discussed above are only marginally effective in limiting the size of the infarct scar, and focus solely on myocardial revascularization. By the time these
treatments are implemented, they are not completely preventive in regards to scar formation and thus do not avert the greatest threat that MI presents to patients – the pathological progression towards CHF. These shortcomings in treatment of MI-induced damage have prompted the development of surgical EHTniques aimed at restoring ventricular function subsequent to remodeling and onset of DCM.

The first such attempt was the ill-fated construction of a muscular sling using the latissimus dorsi muscle in hopes that its contraction would augment that of the dysfunctional left ventricle in heart failure patients. Another approach hypothesized that reducing the size of dilated LV would somewhat restore cardiac efficiency by increasing ejection fraction and stroke volume. This is known by several names: endoventricular circular patch plasty (EVCPP), surgical ventricular reconstruction, or the Dor procedure (after the surgeon who pioneered the EHThnique). The procedure entails incising the aneurysmal area, which is coincident with the area of the infarct scar, and threading a purse-string suture at the peri-infarct border along the endocardial wall. The circular suture pattern is then tightened, effectively reigning in the aneurysm and reducing the chamber diameter. EVCPP is typically performed simultaneously with CABG, and a recent clinical trial was conducted to elucidate the added benefit, if any, of performing EVCPP with CABG. Upon completion of the Surgical Treatment for Ischemic Heart Failure (STITCH) trial, Jones et al. concluded that EVCPP did significantly reduce end-systolic volume, but this effect was not associated with a decrease in the frequency of postoperative hospitalization or death from cardiac causes (the primary endpoint of the trial)
when compared to CABG performed alone.\[30\] These results suggest that EVCPP is ineffective as a treatment for DCM and in forestalling development of CHF.

A final approach to treatment of MI complications, specifically CHF, is the use of left-ventricular assist devices (LVAD). LVADs are implantable medical devices which mechanically pump blood from the left ventricle into the aorta, essentially replacing the muscular function of the ventricle with an artificial pump. Originally developed as a bridge-to-transplant therapy for end-stage heart failure patients on the transplant waiting list, they were ultimately explored as a destination therapy for transplant-ineligible heart failure patients.\[31\] The Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure (REMATCH) trial of 2001 placed 129 end-stage (New York Heart Association class IV) heart failure patients into either an optimum medical management group or an LVAD-treated group. Results demonstrated a 48% decrease in mortality among the LVAD-group when compared with the medical management group. One and two year survival rates were 52% and 23%, respectively, in the device group.\[31\] Despite this high mortality rate, it must be noted that these patients were in an advanced stage of deterioration, and if the devices could drastically increase survival at such a point, their beneficial effects might be significantly enhanced if implanted in patients with less advanced heart failure. The potential of LVADs is tempered by concerns of hemocompatibility, infection, and durability, and both the cumbersome nature of the devices and daily attention required for their operation still represent limitations to quality of life.\[31, 32\]
1.2.2 Experimental Clinical Techniques:

When it is understood that the key to limiting the impact of MI lies in restoring the lost functionality of necrotic myocardium, the goal then becomes to replace the resultant fibrous infarct scar with functional tissue.

The marriage of advancements in the fields of stem cell biology and percutaneous (catheter-based) device engineering have made it possible to imagine that pluripotent stem cells could be transplanted into infarcted myocardium in order to facilitate a regenerative response within the scar tissue. This approach, termed cellular cardiomyoplasty, uses in situ injection of autologous and potentially therapeutic cells through intracoronary or endoventricular catheter introduction (Figure 1.3). A litany of stem and progenitor cells have been proposed for use with this method, including skeletal myoblasts, bone marrow stem cells, peripheral blood stem cells, vascular endothelial cells, mesenchymal stem cells (MSC), adipose-derived stem cells (ADSC), umbilical cord cells, induced pluripotent stem cells (iPSCs), endothelial progenitor cells, cardiac stem cells, and embryonic stem cells (ESC). For a more in-depth discussion and analysis of studies and clinical trials involving cellular cardiomyoplasty performed using these various cell types, the reader is referred to several comprehensive reviews of the subject. The therapeutic goals and potential effects associated with this method, as outlined by Chachques, et al. are to reduce the size of infarct scars, improve myocardial viability, limit global ventricular dilation, improve ventricular wall compliance and diastolic function, and provide advantageous paracrine effects.
**Figure 1.3:** Graphical representation of the various methods used to deliver potentially therapeutic cells via cellular cardiomypoplasty. Intramyocardial, transendocardial, intravenous, and intracoronary routes of administration are shown.

Despite indications of success in several clinical trials, controversy lingers over the precise mechanisms of action, most appropriate cell sources, and timing of cell transplantation following MI. Furthermore, apparent limitations with transplanted cells, such as cell “regurgitation” from the injection site, poor cell engraftment and integration with host tissue, extensive apoptosis, and absent or limited differentiation into desired cell types, must be addressed. One critical factor in the success of *in situ* cellular cardiomypoplasty is the inhospitable microenvironment into which these cells are implanted. Depending upon the timing of the procedure, the cells are either thrust into a strong, local inflammatory response capable of inducing apoptosis (early post-MI) or into a poorly vascularized and dense matrix of collagen (late post-MI). It would be perhaps overly optimistic to expect a high rate of cell attachment and viability in these environments, much less proper signaling, integration, or differentiation. Intracoronary or intramyocardial injection of stem cells is thus clearly limited by the lack of a stable cell
delivery method, and so in situ cellular cardiomyoplasty, at least at the moment, does not “stick”

1.2.3 Rationale for Tissue Engineering:

Just as it has in many pathologies where organ transplant is considered the gold standard in clinical practice, tissue engineering has gained popularity as a potential therapy for regenerating functional myocardium. The strategy of combining cells, engineered matrices (or scaffolds), and growth factors in order to produce an implantable graft presents clinicians with a much needed alternative to using donor organs or autografts in the treatment of such conditions, mainly to better serve demand for organ or tissue replacement.

Tissue engineering is a relatively young field, and myocardial tissue engineering is younger still. As such, implantation studies of EHT are limited. The first ever clinical trial (MAGNUM-trial) involving an EHT construct was begun in 2003, and used autologous bone marrow cells (BMC) seeded onto a 3-D collagen type I scaffold. The construct was implanted over the epicardial surface of the post-infarct scar in a group of 10 patients while they were undergoing CABG. Follow-up at 10 months indicated prevention of cardiac remodeling and thickening of the scar (via cell ingrowth) with functional, healthy tissue. There were no indications of immune or inflammatory reactions, no arrhythmias, and the EHT appeared to integrate well with the host tissue.

It is important to note that this particular EHT construct was not intended to be functional, meaning it was neither contractile nor vascularized. Its mechanism of action
was one of providing “passive” support, and the collagen matrix most likely acted as a stable microenvironment from which the BMC could secrete paracrine signals to the healthy cells in the peri-infarct tissue. Regardless of the mechanism at work in this graft, its success demonstrates the need for a scaffold in which to house transplanted cells and offers a possible explanation for the shortcomings of cellular cardiomyoplasty. In addition, it highlights the need for tissue engineering approaches in order to successfully replace infarct scar and regenerate functional myocardium.

1.3 Design Requirements for Engineered Heart Tissue:

Desired characteristics of an engineered heart tissue (EHT) grafts are the attenuation of ventricular wall remodeling or dilation, development of systolic force, possession of sufficient compliance to withstand diastolic loading, electrical coupling of the CM in the graft with those of the host, and the avoidance of arrhythmias, inflammation, encapsulation, or calcification.\textsuperscript{[39, 42, 43]}

If biomimicry, “remuscularization” (or restored functionality), and full replacement of a transmural (spanning the full thickness of the ventricular wall) infarct scar are assumed to be the goals of a particular EHT strategy, an attempt must be made to reproduce characteristics of the native myocardium as closely as possible. In addition, the full or partial resection of the infarct scar may be necessary for full integration and improvement in function of infarcted hearts.\textsuperscript{[42, 44]}

The average thickness of the ventricular wall is 1.5 cm, and the cell density of native myocardium is estimated at $2 \times 10^8$ cells/cm$^3$.\textsuperscript{[45]} In the ventricles, this figure encompasses
4 predominant cell types, each with their own specific function, and organized in such a way as to provide the optimum function of the tissue.\cite{39, 45} These cell types (CM, cardiac fibroblasts (CF), vascular smooth muscle cells (VSMC), and endothelial cells (EC)) appear in defined and consistent ratios within healthy myocardium.\cite{46} In addition, the myocardium is very metabolically demanding, and is thus highly vascularized with an estimated capillary density of 2,500 capillaries/mm\textsuperscript{2}.\cite{47} This latter point illustrates the need for an EHT graft which is highly vascularized, as the function and survival of a graft with poor blood supply could be limited.

The ECM of the myocardium is also of great importance, as it largely influences the orientation of the cells, the extent of their electrical coupling, and the mechanical properties of the tissue.\cite{46} Developing a scaffold with both porosity and mechanical properties comparable to native ECM is a significant challenge.

An aspect often overlooked in tissue engineering efforts, the method of surgical implantation used in delivering EHT grafts, could be a major determinant of both their success and feasibility. The current trend in implant delivery is toward trans-catheter EHThnikes, which are minimally invasive and thus reduce the risk of surgical complications.\cite{48} Since these advantages appeal to surgeons, the question then becomes one of how to design an EHT graft which is compatible with trans-catheter delivery.

Each of these design considerations impose their own particular range of caveats and limitations upon the potential success of a given approach to generating EHT. These limitations will be discussed in detail in subsequent sections of this review.
1.4 Current Strategies for Producing Engineered Heart Tissue:

Despite its short lifetime, tissue engineering and the application of its principles to the treatment of post-MI damage and CHF have generated great interest. An assortment of innovative tissue engineering strategies have been developed by a wide range of research groups.\(^{[39, 40, 42, 43]}\) These strategies include (a) the classical approach of using a synthetic biomaterial to generate a scaffold which is then functionalized via repopulation with appropriate cell types; (b) the same approach used above, with the exception that the synthetic biomaterial is substituted for natural materials in creating the scaffold; (c) again, the same approach as above, but using a combination synthetic and natural biomaterial scaffold instead; (d) and an approach of constructing “scaffold free” cell sheets, or stacked monolayers of cells (Figure 1.4).

**Figure 1.4:** Various concepts in generating engineered heart tissue (EHT): (I) natural or synthetic material for cell repopulation; (II) cells seeded on soluble collagen and extracellular matrix (ECM) proteins; (III) assembly of cell monolayer "sandwiches."
1.4.1 Synthetic Biomaterials:

Synthetic biomatrices have been created using polymers with established properties of biocompatibility and biodegradability, such as polylactic acid (PLA), polyglycolic acid (PGA), and formulations of their copolymer, polylactic-co-glycolic acid (PLGA).\cite{39, 42, 43} In addition, other elastomeric polymers, including polycaprolactone (PCL), poly(glycerol-sebacate) (PGS), poly(trimethylene carbonate-co-lactide) (PTMCLA), and polyurethane (PU), have been employed to fabricate scaffolds for EHT.\cite{39}

A variety of different materials processing approaches have been implemented to create scaffolds with defined structures similar to the native ECM. This control over the geometrical and chemical characteristics of scaffolds is an advantage inherent to the use of synthetic materials. Scaffolds with defined porosity, alignment, and surface chemistry can be generated through such methods as salt (particulate) leaching, laser sintering, and electrospinning.\cite{39, 43, 49}

Lesman et al. seeded highly porous PLA/PLGA grafts generated via particulate leaching with a combination of human embryonic stem cell-derived CM, human umbilical vein EC, and human embryonic fibroblasts which were subsequently implanted into a nude rat model for 2 weeks. The grafts appeared to successfully attach to the recipient myocardium and showed evidence of neovasculature formation. Some grafts, however, were encapsulated by fibrous tissue, indicating poor integration with the host tissue.\cite{49}

In another study, Ke et al. implanted a PGA patch seeded with undifferentiated mouse ESC into a rat model of MI. After evaluation of cardiac function at 8 weeks following implantation, nearly twice the number of animals in the group which received
the graft survived in comparison with the sham control group.\textsuperscript{[50]} Similarly, BMC-seeded PCL patches were applied to the infarcted hearts of rats, and the authors reported a reduction in left ventricular remodeling, preservation of left ventricular systolic function, and deEHTtion of myosin heavy chain proteins.\textsuperscript{[51]} The latter seem to indicate possible transdifferentiation of BMC into cardiac cell types. Lack of ability to induce the differentiation of stem cells grown on polymer grafts has been cited in the criticism of such grafts.\textsuperscript{[42]}

Owing to its elastomeric characteristics, PGS has been investigated as potential scaffolding by several groups.\textsuperscript{[5, 52-54]} Its versatility and ability to be machined into precise, repeatable accordion like structures provided it with anisotropic mechanical properties similar to those of the right ventricular myocardium.\textsuperscript{[55]} It was reported to induce cell alignment\textsuperscript{[54, 55]} and to support the viability and vascularization of a cell-seeded scaffold when implanted in a rat infarct model [52]. When it was compared via an implantation study to scaffolds composed of either poly(ethylene terephthalate)/dimer fatty acid (PED) and PED reinforced with titanium oxide (PED-TiO\textsubscript{2}), the PGS scaffold adhered to the host myocardium while undergoing degradation. The PED did not remain attached to the host tissue, and the PED-TiO\textsubscript{2} contributed to increased chamber dilation and infarct scar size.\textsuperscript{[5]} Thus, the application of PGS and similar thermoplastic elastomers cast doubt on the contention that synthetic materials cannot adequately reproduce the physiological mechanical properties of the myocardium.\textsuperscript{[42]}
1.4.2 Natural Biomaterials:

The use of naturally occurring materials to generate scaffolds for EHT has involved collagen, collagen gels (e.g. Matrigel), GAGs, fibrin, hyaluronic acid, and alginate. Collagen is the most widely used natural material in efforts to produce EHT, and can be found in dry sheet, gel, or preserved in its native form as the predominant component of the cardiac ECM. Ergo, this section will focus predominantly on material formulations which employ collagen as the main component. For a discussion of the other materials mentioned above, the interested reader is directed to an extensive review of materials, both natural and synthetic, with potential applications to myocardial tissue engineering.

The work of Zimmermann et al. over the last decade has focused on development of an EHT construct using neonatal CM and an extracellular matrix consisting of collagen type I and Matrigel. Implantation of this construct in an infarcted rat model has shown that it is able to electrically couple with the host myocardium, contract for up to 8 weeks in vivo, contribute to newly formed myocardium, and that it is associated with wall thickening in the infarcted area. One disadvantage to this approach is the use of Matrigel, the safety of which is questionable since it is composed of ECM extracted from mouse tumors. The group addressed this issue with the claim that they had developed both serum-free and Matrigel-free EHT constructs.

Additional studies have confirmed beneficial results such as graft-host myocyte electrical coupling, neovascularization or angiogenesis, and increased ventricular wall...
thickness in EHT grafts generated using collagen along with either neonatal rat CM, mouse ESC, or bone marrow-derived MSC.[60-62]

A particularly unique method of generating a collagenous scaffold is that of decellularization (DCELL), or the removal of all cell constituents and cell membrane components which could be potentially immunogenic if implanted as an allo- or xenograft. DCELL can employ one or both of two mechanisms, chemical and physical agitation, to render a donor tissue completely free of epitopes which might trigger an immune response from a graft recipient.[56] Following DCELL, the remaining tissue is composed of structural ECM proteins (i.e. collagens, elastin, and GAGs). These proteins elicit little to no immune response upon implantation due to the extensive evolutionary conservation of the genes which code for them.[56] Notwithstanding the tremendous advantages offered by a scaffold with physiologically accurate concentrations, distributions, and organization of natural ECM proteins, the option to create it from readily available xenogeneic tissue sources only enhances the appeal of this method.[63, 64] Perhaps the most attractive feature of DCELL, however, is the ability to preserve whole, intact vascular networks along with the native ECM. This provides the means to perfuse the scaffold with media, and could even enable direct surgical attachment of such a perfused graft to the host vasculature upon implantation.[63, 64]

This method has been applied to tissue engineering efforts in regeneration or replacement of the kidneys, esophagus, heart valves, urethra, bladder, skeletal muscle, peripheral nerves, and, most recently, the myocardium.[63, 65-72] Effective DCELL procedures should not be so harsh that they nonspecifically degrade everything within the
treated tissue. Rather, they must strive to remove all unwanted components, while leaving
the desired components unaltered in both conformation and quantity. For example, some
DCELL methods have used the action of enzymes to speed the process by catalyzing
protein cleavage.\textsuperscript{73, 74} One such enzyme is the protease, trypsin, which readily degrades
elastin and fibrillin via cleavage of peptide bonds following positively charged amino acid
residues.\textsuperscript{7, 75}

This type of nonspecific action can destroy components of the native ECM which
are actually important to maintain, especially if a subsequent attempt to repopulate a
DCELLed scaffold is to be carried out successfully. Such components include basal
laminar proteins and proteoglycans to which seeded cells would bind (e.g. collagen type
IV and laminin).\textsuperscript{7, 76} Evaluation of several DCELL methods has shown they are effective
in accomplishing this goal, and seeded cells both adhere to and proliferate on the resulting
scaffolds.\textsuperscript{63, 77} These studies provide proof of principle for the DCELL approach, and
their authors also suggest it can be used as a technique for whole organ replacement.\textsuperscript{63, 72,
77} However, due to the finite number of requisite cells which can be generated via \textit{in vitro}
culture and differentiation of autologous stem cells, efforts to repopulate an entire
DCELLed organ (e.g. the heart) at the appropriate cell density are over-ambitious and
unlikely to succeed.\textsuperscript{78} This approach becomes more feasible when applied to the
generation of smaller grafts, such as those of the dimensions needed for replacement of
infarct scars.
1.4.3 Combination Biomaterials:

A few groups have sought to produce scaffolds which are composites of both synthetic and natural materials in an attempt to pool their respective advantages. Stankus et al. created electrospun scaffolds composed of type I collagen and poly(ester urethane)urea, which they reported to improve cell adhesion despite having deficient mechanical properties.\(^79\) In a similar approach, neonatal CM seeded on collagen type I-coated, electrospun PCL nanofibers exhibited enhanced attachment. The cells integrated into a nanofibrous mesh which could be stretched to provide a passive load and allow the beating cells to contract at natural frequency. This represents an advantageous method for mimicking the ECM and for providing mechanical stimuli.\(^80\) Another group investigated the \textit{in vitro} performance of a dilinoleic acid (fatty acid dimer)-conjugated poly(ethylene terephthalate) elastomer with mouse and human ESC-derived CM. They reported favorable results but have not generated any \textit{in vivo} data up to this point.\(^81\)

In yet another variation of this approach, Krupnick et al. delivered BMC to the infarct scars of rat hearts after seeding the cells onto a non-woven PLA mesh reinforced with poly(tetrafluoroethylene) (PTFE). The mesh was coated with the cells, which were suspended in a mixture of Matrigel and collagen type I and IV before the addition of collagenase and subsequent implantation. The grafts appeared to integrate well with the host tissue, displaying no signs of arrhythmogenesis or inflammation and demonstrating possible differentiation toward cardiac lineage by staining positive for myosin.\(^82\)
1.4.4 “Scaffold-Free” Cell Sheet Engineering:

This method for generating EHT was first conceived by Yamada et al. following their development of temperature-responsive culture dishes composed of poly(N-isopropylacrylamide) (PIPAAm). These unique culture surfaces alternate between being hydrophobic and hydrophilic within a small range of temperatures (i.e. they are hydrophobic at optimum culture conditions (37°C) and hydrophilic at room temperature (<32°C)). The researchers realized this afforded them the capability to grow entire monolayers of cells and then readily detach them simply by altering the ambient temperature of their PIPAAm culture vessels. A critical advantage provided by this method was the elimination of the need for proteases to detach cells from culture vessels, thus sparing the cell-synthesized ECM from any alteration or degradation.

Okano et al. then expanded on this approach, growing monolayers of neonatal rat CM and stacking them on each other to generate constructs of spontaneously aggregating and beating CM in vitro. The thickness of these constructs was limited to 3-6 layers, or a thickness of about 100 μm, as a consequence of their lack of vascularization. In an attempt to overcome this problem, they devised a “polysurgery approach” wherein these cell sheet stacks were successively implanted on top of one another through the performance of recurrent surgical procedures. Each individual thin stack became vascularized prior to implantation of the next thin stack, producing a thicker tissue construct.

Other cell types besides CM have been employed with this method. In particular, cell sheets consisting of undifferentiated ADSC were implanted into a sub-acute rat model.
of infarct. Upon explantation, these constructs contained differentiated CM and newly formed blood vessels. They also appeared to limit the extent of ventricular wall distension.\textsuperscript{[85]} The major disadvantage of this approach is the limited ability to induce vascularization within the constructs \textit{in vitro}. Lack of blood supply restricts the thickness of the constructs to the maximal diffusion range of oxygen and other nutrients within tissue, a distance estimated to be about 200 µm.\textsuperscript{[63]} Consequently, limited thickness translates to extremely low mechanical properties and the creation of constructs which are not easy to handle or implant without fear of ripping them apart.\textsuperscript{[39, 42]}

These disadvantages were addressed by first attempting to reinforce the constructs with layers of collagen gel. This resulted in improved cardiac function and integration with the host myocardium, but it must be noted that collagen gel is not ideal for this application since it has a lower modulus and greater compliance than other forms of collagen.\textsuperscript{[39, 86]} In a more direct approach to the problem, Murry et al. employed coculture of CM, fibroblasts, and EC to generate prevascularized cardiac patches which could actively contract and electrically pace. More importantly, the prevascularized patches demonstrated 10-fold greater cell engraftment upon implantation than did patches comprised of CM alone.\textsuperscript{[87]}

1.5 Limitations, Challenges, and Barriers in Myocardial Tissue Engineering:

1.5.1 Effects of Scaffold on Engineered Heart Tissue Viability and Function:

A major determinant of success in producing EHT is the ability to seed appropriate cell types onto a scaffold and subsequently maintain their viability, promote their integration with host tissue, and, ideally, induce their proliferation. To that end, the logical
place to begin improving EHT design efforts lies in optimizing the scaffold for cell attachment.\cite{7, 46, 56} Matching the characteristics of native ECM, rather than native tissue, is the desired outcome in those EHT strategies which attempt to repopulate a scaffold with relevant cell types. The rationale for this is that cells will respond favorably (i.e. attach and assume normal morphology) to a scaffold which bares similarities to the structure which they naturally bind—the ECM.\cite{7}

Mimicking the ECM requires that native proteins and ground substance, or elements which mimic both their structure and surface chemistry/bioactivity, are present within the scaffold. Reproducing native ECM structure (i.e. porosity, composition, and interconnection of the individual constituents) is of great importance, since it will align the mechanical properties of the scaffold with those of the physiological ECM.\cite{7, 46, 56} Given that the ECM’s role in relaying external mechanical stimuli to cells has been well established, creating a scaffold with similar mechanical properties ensures that seeded cells are relayed the same mechanical signals as they would be if they were residing within healthy myocardium. Via the process of mechano-transduction, these mechanical signals are translated into chemical signals which mediate intracellular pathways, and are capable of producing changes in levels of gene expression.\cite{46, 56} Collectively, this process has an immense impact upon cell fate (i.e. differentiation and morphology) and survival\cite{88} (Figure 1.5). Furthermore, recent findings have suggested that the ECM is significantly involved with conduction of the electrical signals responsible for synchronous contraction of the myocardium.\cite{89, 90}
Interaction of cells with the ECM can induce apoptosis, migration, and/or differentiation. Matrix characteristics such as porosity, architecture, compliance, and surface chemistry mediate these influences.

Having outlined the cell-ECM relationship, some regard must be given to the connections that make the cell-ECM interface possible. In the myocardium, CM attach to the ECM through integrins and a specific protein complex, which bind laminin and perlecan (constituents of the basal lamina), respectively.\textsuperscript{91} Thus, when attempting to create a scaffold which is conducive to cell attachment, it is necessary to consider incorporating these macromolecules, or functional substitutes thereof, within the material. Results from a recent study, however, indicate that functional analogs of ECM proteins, such as RGD, performed poorly in inducing CM attachment when compared to the holoprotein (i.e. laminin or collagen type I).\textsuperscript{92}

The difficulty of the scaffold creation process is only compounded when the aim of repopulating it with cells is considered, as their addition will undoubtedly lead to changes in the properties of the resulting EHT graft. Assuming repopulation is successful, the
concern then becomes the graft’s ability to match the mechanical properties (Young’s modulus between 0.2 – 0.5 kPa) of the healthy host tissue upon implantation.\textsuperscript{[43]} Matching the mechanical properties of the host tissue should be conducive to receipt of the proper signals by the cells in the graft, and result in its successful integration. If, however, there is compliance mismatch between the graft and the host tissue, micromotion-induced encapsulation will occur, and the graft will fail.\textsuperscript{[5]}

\textit{1.5.2 Vascularization and Growth Promoting Factors:}

The success of an EHT graft is critically dependent upon the extent to which it is vascularized. Cells, particularly CM, cannot survive without access to nutrients or a mechanism to eliminate wastes and accomplish gas exchange.\textsuperscript{[2]} As mentioned previously, the dimensions of EHT constructs without a preformed and functional vascular network are limited to the observed diffusional range of solutes and gasses \textit{in vivo} – about 200 µm.\textsuperscript{[63]} In order to produce grafts with the larger dimensions necessary for replacing an infarct scar, strategies to overcome this limitation must be developed. The creation of a vascular network within EHT constructs must provide the means to nurture the cells in both the \textit{in vitro} and \textit{in vivo} environments. Methods for accomplishing this include the use of continuously perfusable vascular channels, or even blood vessels, to introduce culture media (\textit{in vitro}) and blood (\textit{in vivo}) into the EHT construct.\textsuperscript{[63, 93]} These same channels could then be surgically anastomosed to the host vasculature upon implantation, providing a means of direct perfusion of the graft for enhanced integration, function, and survival.\textsuperscript{[64, 70]}

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Other modes of EHT graft vascularization could involve direct incorporation of vascular endothelial growth factor (VEGF) into the scaffold to induce angiogenesis and neovascularization by either or both of host and graft EC. In conjunction with this, endothelial progenitor cells (EPC) might be seeded on the graft in hopes that they will also participate in the neovascularization process by differentiating into EC, recruiting other stem/progenitor cells, or secreting angiogenic paracrine signals.\[78]\n
### 1.5.3 Cell Sourcing and Expansion:

For EHT grafts to avoid immune rejection, the cell types incorporated in them must necessarily be autologous. Thus, in a clinical scenario employing EHT as the therapy, the cells used to generate the EHT graft will be the patient’s own cells.\[42, 78]\ The possibility of simply using terminally differentiated CM from myocardial biopsies is precluded by the fact that these cells are essentially senescent, and display little potential for division or turnover beyond birth.\[43, 78, 88]\ The focus then shifts to identifying suitable stem or progenitor cell sources which can differentiate into the necessary cell types (CM, EC, and VSMC; adult CF are sufficiently proliferative and can be readily harvested). Furthermore, generating grafts of clinically relevant size will require significant populations of phenotypically pure cells to be grown \textit{in vitro}, which will be difficult to achieve using clonal expansion culture techniques.\[78]\ At the very least, this will pose additional challenges by increasing the lead time for producing EHT, as well as costs.

An extensive discussion of pertinent cell sources will not be presented here, but can be found in several reviews.\[39, 42]\ Briefly, ESC have been investigated for use in tissue
engineered grafts and EHT. ESC are extremely advantageous in that they can differentiate into any cell type in the body, although effectively directing their differentiation, especially toward cardiac lineages, can be difficult. That aside, use of ESC in research that has clinical applications as its endpoint is irresponsible and shows lack of forethought. ESC can be immunologically rejected, are perpetually mired in ethical debates over the origin of their cell lines, and the Food and Drug Administration (FDA) is unlikely to approve their use due to concerns over their ability to form teratomas. More practical sources of pluripotent autologous stem cells include ADSC, MSC, EPC, and BMC. Several groups have published in vitro culture methods for directing these cell types toward CM and EC phenotypes, as well as methods for increasing proliferation and expansion toward the goal of producing necessary cell numbers for EHT graft seeding.

1.5.4 Bioreactors for Seeding and Preconditioning:

Since 3-D tissue constructs have so many unique properties that 2-D monolayer cultures lack, they demand a unique form of cell culture. In the case of EHT grafts, the goal of culture is to produce functional myocardial constructs by imparting them with characteristics specific to myocardium, namely electrophysiological and mechanical properties. The ideal system for accomplishing this goal is a bioreactor that is specifically tailored to simulate the physiological environment and stimuli experienced by native myocardium.

Typically, a bioreactor serves several purposes, including the facilitation of uniform seeding of a scaffold via dynamic seeding conditions (i.e. perfusion and gentle, cyclical
stretch/compression); the enhancement of diffusion of gasses, solvents, and mass transfer, in general; simulation of physiological stimuli and external forces (e.g. fluid flow, shear stress, compression and tension, blood pressure, and electrical impulses; and maintenance of conditions to replicate the in vivo environment, such as temperature, pH, electrolyte, ion, and metabolite concentrations, and oxygen content.\textsuperscript{[99]}

There is a great deal of evidence to indicate that dynamic seeding, as well as sustained dynamic culture conditions, can aid in producing EHT grafts with a high cell density and enhanced cell survival. The use of perfusion, however, seems to be the consensus method for achieving the best results, as illustrated by the findings of Radisic et al. that use of a perfusion bioreactor increased cell density of EHT grafts to near physiological cell density, allowed for the generation of thicker (~ 5 mm) grafts, and facilitated mass transfer.\textsuperscript{[93, 100]}

If a bioreactor is rationalized from a purely engineering design standpoint, it would be described as a system with both inputs and outputs. In this case, the desired output is the production of a functional myocardial tissue substitute with CM that are electrically connected, conduct impulses, and respond to these impulses by producing a certain systolic force.\textsuperscript{[101]} With this constraint established, the task of the bioengineer then becomes to identify the most appropriate inputs which will produce the desired output. These inputs are the stimuli applied to the EHT construct within the bioreactor, and should strive to not only approximate, but exceed, physiological levels. In this way, a factor of safety is built into the EHT graft, and it should theoretically be able to handle the most elevated conditions of heart rate, blood pressure, and external stresses which could conceivably
occur in vivo. Examples of such exaggerated conditions might be a pulsatile flow rate which mimics a heart rate of 200 bpm or a media inlet pressure of 200 mmHg. The same principle should be applied to the repetitive electrical impulses and cyclical tensile stretching or compression that the graft is exposed to in order to precondition, or “train”, it to become a functional graft of cardiac muscle. The next section of this review will focus on the mechanics of myocardium on both the cellular and tissue level to further expound on the role of dynamic stimuli in myocardial tissue engineering strategies.

1.5.5 Applications of Cardiomyocyte and Myocardial Extracellular Matrix Mechanics:

The state of the art in tissue engineering assumes the premise of biomimicry, or the ability of an engineered tissue construct to mimic the tissue which it is intended to replace, as central to its overriding goal: the replacement or regeneration of diseased or dysfunctional tissues.\cite{102, 103} This mandates that EHT not only appear analogous to healthy, native tissue, but also replicate the function of this tissue. There are many specific tissue types for which tissue-engineering therapies hold promise, but the development of some present a considerably more difficult challenge than others. Mimicking the structure and function of bone or skin, for example, is less daunting than the prospect of recreating muscle and nervous tissue.\cite{102, 103} Simply put, as the complexity of the tissue (in terms of structure and function) increases, the difficulty of reengineering it follows suit.

One aspect of particular importance in determining how closely EHT mimics its target tissue is mechanics. A thorough characterization of EHT must be performed in order to determine its intrinsic mechanical properties, which may then be compared to those of
the native tissue as a means to predict EHT’s ability to perform the mechanical function of its analogue.\textsuperscript{[74]} As is evident from the development of the biphasic and multiphasic theories, these mechanical properties cannot be fully described without a prior understanding of the tissue architecture, its components (i.e. cells, extracellular matrix, and interstitial fluid), and their respective contributions to the overall mechanism(s) underlying the mechanical behavior of the tissue.\textsuperscript{[104]}

This divergent approach to analyzing tissue mechanics may prove particularly useful in the development of tissue-engineered myocardium. Myocardium, or cardiac muscle, is unique among tissues in that it plays an active mechanical role, synchronously contracting to pump blood into both the pulmonary and arterial circulation.\textsuperscript{[78, 103]} Like other types of muscle, it stands apart because it is motile and active, performing actual mechanical work as opposed to providing passive support or facilitating transfer of mechanical forces (e.g. bone, membranes, cartilage, ligaments, tendons).\textsuperscript{[105]} The component which imparts it with this dynamic character are the cardiac myocytes (CM), which collectively shorten (contract) to “pull” on their collagenous extracellular matrix (ECM) and compress their respective chamber walls, reducing the chamber volume (thus, increasing chamber pressure) and forcing blood out.\textsuperscript{[105, 106]}

Since CMs play such an important role in the function of myocardium, it is desirable to develop a working knowledge of their mechanical behavior and characteristics, especially if the aim is to reproduce myocardial tissue. Study of their cytoskeleton, intercellular junctions, extracellular matrix linkages and the interaction of these various mechanical components is warranted to gain a holistic understanding of the biomechanics
of myocardium. These investigations could also aid in the completion of specific milestones in developing engineered myocardium, namely the efficient in vitro culture and differentiation of adult stem cells or cardiac progenitors into phenotypically accurate adult CMs.

1.5.5.1 The Cardiomyocyte Cytoskeleton:

Owing to their highly sophisticated mechanical function, CMs have developed one of the most intricate cytoskeletons found among all cell types. In addition to an abundance of aligned actin and myosin filaments (the actinomyosin complex), the CM cytoskeleton consists of titin and a network of microtubules.\textsuperscript{107} The actinomyosin complex and titin constitute the bulk of the structure known as the sarcomere, repetitive units of which make up the length of myofilaments and occupy most of the cytoplasmic volume of the CM. The combined action of sarcomeres is responsible for the macroscopic contraction of CMs.\textsuperscript{106, 107} Following stimulation by an influx of Ca\textsuperscript{2+} ions, sarcomeres shorten via the sliding filament mechanism (modeled by cross-bridge theory), and return to their original length upon cessation of contraction with help from the spring-like recoil of highly elastic titin molecules.\textsuperscript{104, 106, 107}

Microtubules emanate from the microtubule organizing center and extend to the periphery of the middle portion of CMs. Consistent with the tensegrity hypothesis of cellular mechanics, they may act to bear compressive loads by buckling and unbuckling during CM contraction.\textsuperscript{108-110} In addition, there is evidence indicating that they are mechanically coupled to other elements of the cytoskeleton, helping to transduce both
intra- and extracellular forces throughout the cell and drive the overall intracellular response to loading. Evidence in the literature increasingly suggests that cytoskeletal proteins assume an invaluable role in mechanosensory and mechanotransduction, thus mediating cellular responses in normal development and in pathophysiological conditions (e.g. dilated and ischemic cardiomyopathy, pressure and volume overload hypertrophy). \[111-122\]

1.5.5.2 Cytoskeletal-Extracellular Matrix Linkages:

As crucial as the mechanical components of the cytoskeleton are to the functional role of CMs, the points where these elements attach and integrate with the ECM are critical in determining how myocardial tissue, and the heart as a whole, function. \[3, 7, 123\] These linkages ultimately mediate the transfer of external forces (e.g. blood pressure driven by metabolic and physiological demand) imposed on the myocardium through the myocardial ECM into the CMs. This pathway can also be reversed, as CM contraction imparts force on the ECM via these same linkages, manifesting as the contraction that is the pumping action of the heart. \[3, 11\]

These “linkages” are actually a complex of several cytosolic, transmembrane, and extracellular proteins, and are localized to specific areas on the periphery of each CM, known as costameres. \[107\] Costameres are found at Z-disks between sarcomeres, and effectively form mechanical “tethers” which anchor the Z-discs to ECM proteins. These tethers consist of a chain of proteins, beginning with talins, vinculins, dystrophins, and syntrophins in the immediately submembranous cytosol connecting to transmembrane
integrins and dystroglycan complexes, which, in turn, are bound to laminins of the ECM.\textsuperscript{107, 124} The tethers form a mechanical link between the actinomyosin complex and fibrillar collagens (type IV and I), the primary load-bearing components of the CM cytoskeleton and myocardial ECM, respectively.\textsuperscript{3, 107} Although this chain contains many links so as to make the connection seem almost indirect, it serves as the primary means of relaying mechanical signals between the two components of the tissue.

1.5.5.3 Cell-Cell Linkages:

Individual myocytes can transmit mechanical energy to and from their surrounding ECM, but it is the summation of this individual force over billions of myocytes which accounts for the muscular beating of the heart. In addition to interacting with the ECM, CMs accomplish this by working in concert with one another. They are connected at their longitudinal borders (intercalated disks) by adherens junctions, or cadherin/catenin complexes, which link the opposing ends of actin filaments.\textsuperscript{107} As actin filaments from opposing cells pull against each other, cadherins act as mechanosensors and reinforce the junctions. This facilitates the bidirectional transfer of cytoskeletal tensile stress between cells in a manner which compounds the forces generated by sarcomeric shortening in individual myocytes.\textsuperscript{107}

In both this section and the previous section, linkages in the directions of both the minor and major axes of the CM were discussed. Taken together, these linkages allows the CM to sense and transmit mechanical stimuli with directional specificity.\textsuperscript{107} This
property means that CMs can integrate this information and act on it in a way which gives the most desirable biological response to changes in their mechanical environment.

1.5.5.4 Using Models to Define Effective Mechanical Stimuli in Cell Differentiation:

Gaining an understanding of intra- and intercellular mechanics of the CM, as well as the mechanics of CMs’ interactions with their surrounding ECM, is important in studying pathophysiological and pathological conditions of the myocardium. Since it has been well established that CMs and their ECM interact through the phenomenon of mechanotransduction, and that this process elicits a biological response from the CMs, it is of interest to be able to predict the nature and extent of this response to a given mechanical stimulus. \[107, 123\] This is accomplished through the application of mathematical models which accurately characterize the constituents of the tissue, and, given a mathematically defined set of inputs, can output a prediction of how the tissue will respond.

Within the field of myocardial tissue engineering, for example, the goal is to develop an EHT construct with the ability to generate systolic force and use it to effectively replace the lost functionality of diseased myocardium. A critical component of such EHT is donor-immunocompatible cells, specifically CMs. Considering the criteria of immunocompatibility and physiological cell density (2 x 10^8 cells/cm^3), the possibility of using allo- or xenogeneic cells is precluded, and a large number of CMs is necessary in order to properly populate EHT. \[40, 41, 45\] Consequently, the cell source of choice becomes an adult stem or progenitor cell population with cardiac lineage potential, and the approach
becomes to culture, maximize proliferation, and differentiate these cells into a population with high phenotypic similarity to human adult CMs.\textsuperscript{[41]}

The current body of work suggests that directed differentiation of these cells into CM requires the application of a mechanical stimulus to the cells in culture. Groups have shown that periods of both static and dynamic stretch of monolayer and 3D \textit{in vitro} cultures induce changes in CM and stem cells, alike.\textsuperscript{[125-128]} The question, then, is no longer: do stem cells respond to mechanical stimulation by exhibiting evidence of differentiation toward a cardiac phenotype, but becomes: what specific mechanical stimuli, delivered in what specific way, will result in the generation an accurate adult CM phenotype from cardiac precursors?

This begs a host of other questions about how forces imparted upon a culture substrate are transmitted to the cells being cultured on the substrate: How much of the original mechanical stimulus is transmitted to the cell (i.e. is it degraded, amplified, or altered)? How does it propagate through the cell, and what type of force (i.e. compression or tension, magnitude, frequency) corresponds with what biological response (i.e. are there thresholds for a particular response)? The answers to such questions can only be arrived at through the use of a model. To further complicate things, the nature of these questions requires that the mechanical behavior of the cells and the mechanical behavior of the matrix be separated, and that a model for each component be generated.\textsuperscript{[123]}
Several \textit{in vitro} culture systems have been designed to expose cells within them to mechanical stimuli of varying nature.\cite{105, 125-128} These stimuli can come in the form of uniaxial or biaxial strain, be either static or dynamic with disparate strain rates, strain percentages, duration of strain, and frequencies. Cells can also be cultured on substrates of either natural or synthetic materials with different stiffness, dimensionality (2D or 3D), or degrees of anisotropy.\cite{123, 129}

So the question remains, given the existing evidence in the field as it relates to mechanical modeling of CM and myocardial ECM, which set of conditions will promote the most successful differentiation of cardiac progenitors into a mature CM phenotype? One must begin by defining the desired biological response and then must work backwards to determine the appropriate mechanical inputs. The functions of key mediators in the biological response to mechanotransduction, such as mechanoreceptors, signaling molecules, and target effector proteins are involved in signaling pathways initiated by the receipt of mechanical stimuli.\cite{107} If thresholds can be determined for triggering the initiating events in the pathways of choice, a model (e.g. the tensegrity model) can be used to determine how stresses are transferred along cytoskeletal elements to specific organelles or structures where pathway initiation occurs. Working back up the mechanical ladder, the extracellular loads required to precipitate these intracellular stresses can be determined, and the macroscopic loading of the EHT necessary for generation of these extracellular loads can, in turn, be determined using a mechanical model of the ECM.
1.5.6 Surgical Considerations:

With a lack of intrinsic regenerative capability in the myocardium, the MI-induced necrotic core will be completely remodeled into fibrous tissue between 2-6 weeks post-MI.\cite{11, 39, 49} The timing of therapy delivery in infarct treatment is an important and, as yet, unanswered question. A graft implanted in the acute phase could be sabotaged by the hostile milieu of inflammatory cytokines associated with CM necrosis, while a graft implanted in the chronic, post-scar formation phase could be impeded from integrating with healthy tissue by the scar.\cite{130} In order to implant a construct immediately after MI, it would need to be ready before the event, in an “off the shelf” fashion. This possibility is precluded by complications that arise due to the need for autologous cell sourcing and \textit{in vitro} cell proliferation, seeding, and preconditioning in a bioreactor. Such a process could take several weeks, if not months, to complete and could not benefit the patient immediately unless the MI was predicted.\cite{39, 42, 43} Consequently, patients are typically treated with PCI to abort MI and allowed to heal over the next 6 weeks before any surgical intervention is considered.\cite{18} While the infarct scar does form during this time, the extent of remote fibrosis and global remodeling is usually minimal.\cite{11, 18} The significance of this treatment approach is that surgeons will be presented with a decision when implanting EHT grafts: do they resect the infarct scar and then implant, or do they attach the graft directly to either the endocardium or epicardium (depending on the surgical access used; see discussion below)?

Whether or not grafting engineered heart tissue (EHT) directly to the fibrotic scar inhibits proper adherence and perfusion of the graft with the host tissue through an
inflammatory response is unclear. A number of studies have reported positive outcomes when implanting EHT on top of infarct scars created in animal models.\textsuperscript{[50, 51, 58]} When EHT constructs are not placed directly on the epicardium over the scar, but rather at the endocardial surface (by means of EVCPP)\textsuperscript{[131]} or in a depression made by resection of the scar tissue, solid engraftment has been repeatedly achieved.\textsuperscript{[44, 82, 132]} These opposing implantation methods will need to be compared for efficacy by performing both with a single type of EHT, then perhaps the difference, if one exists, can be elucidated.

An additional uncertainty that pertains to surgical methodology is the nature of the surgical access used. Will EHT grafts be large and require attachment to the epicardium, so as to demand a full sternotomy? Or will surgeons be able to insert a moderately sized EHT graft through a relatively small intercostal opening? Better yet, what if there is the possibility that an EHT graft might be small enough to be implanted percutaneously via catheter? And finally, how will an anastomosis of the vessels used to perfuse potential EHT grafts affect the choice of surgical approach?

In order to answer these questions, the first step which must be taken is determining an estimate of the required size of an EHT graft. Given that a typical MI sufficient to cause heart failure leads to the death of about 50 g of myocardium, it can be estimated that the minimal dimensions required in an EHT graft for replacing a transmural infarct should be approximately 2.6 cm x 2.6 cm x 1.5 cm for a square geometry or 2.9 cm (diameter) x 1.5 cm (height) for a cylindrical geometry.\textsuperscript{[39, 78, 133, 134]} If attempting to adapt EHT grafts to a catheter-based delivery, the access pathway of choice would be through the thoracic aorta and the aortic valve into the left ventricle because this approach offers the largest
anatomical dimensions (i.e. the largest vessel lumens). The largest catheter size approved for use by the FDA in a transluminal aortic approach was 25 on the French scale, and the particular application was aortic heart valve replacement.\textsuperscript{[135]} This catheter size corresponds to a diameter of about 0.833 cm, which is much too small to accommodate a graft of the aforementioned dimensions loaded in almost any imaginable configuration. For EHT to one day be implemented using a trans-catheter approach, the graft dimensions will need to be decreased, or a method must be devised to sufficiently compress a construct containing live cells without damaging them. Until such time, it appears that the surgical implantation method will be through some sort of “cut-down” access.\textsuperscript{[136]}

1.5.7 Myocardial Tissue Engineering in the Diseased Patient:

The vast majority of patients who could benefit from a clinically feasible and cost-effective EHT have some form of cardiovascular disease (CVD).\textsuperscript{[43]} In fact, CVD encompasses high blood pressure (HBP), coronary heart disease (CHD) and its associated complications (MI and angina pectoralis), CHF, and congenital cardiovascular defects.\textsuperscript{[17]} The American Heart Association estimates that approximately 90\% of patients with CHD have been exposed to at least one of the following risk factors: hypercholesterolemia, hypertension, cigarette smoking and diabetes mellitus (DM). Data show that the incidence of CVD in women with DM and a healthy weight was 54.8\%, but rose to 78.8\% among obese women. Among diabetic men with a healthy weight, the prevalence of CVD was 78.6\%, whereas it climbed to 86.9\% among obese men.\textsuperscript{[17]} Additionally, end-stage renal
disease and acute renal failure (ARF) are comorbidities of CVD. Of first-time diagnoses of ARF, a combined 12.3% were associated with a hospitalization for either CHF or MI.\(^{[17]}\)

These data serve to underscore the point that researchers developing EHT should rid themselves of the illusion that potential candidates for their therapy will be a tabula rasa of health. Rather, they should prepare EHT constructs to withstand a hostile, diseased environment. In the MAGNUM-trial discussed earlier, for example, 11 of the 20 participants were hypertensive, 13 were hypercholesterolemic, 7 were diabetic, and 10 were smokers.\(^{[41]}\)

In terms of inflammatory processes, a few of the most threatening conditions to the long-term viability of EHT are atherosclerosis, graft arterial disease (GAD), and arteriolosclerosis. Atherosclerosis is the most well-known of these diseases, as it is implicated in having a causal role in both CHD and peripheral artery disease. The characteristic atheromas or lesions common to atherosclerosis present the potential for tissue calcification.\(^{[1, 17, 137]}\) GAD is distinguished from athero- and arteriosclerosis by characteristic toto-circumferential intimal thickening, and has been observed to occur predominantly in implanted allografts, which contain immunogenic cell constituents.\(^{[138]}\) EHT constructs, however, should ideally consist of nonimmunogenic ECM proteins seeded with autologous cells, rendering them free from concerns about GAD or transplant vasculopathy.\(^{[138]}\)

Finally, arteriolosclerosis, or the thickening of the intimal layer in arterioles due to hyperplasia or hyaline matrix deposition, could occur in EHT.\(^{[137]}\) EHT grafts implanted in hypertensive or diabetic patients would be at an increased risk of contracting
arteriolosclerosis because of the causal role of both hypertension and diabetes in development of arteriolosclerosis.\textsuperscript{137}

Both atherosclerosis and arteriolosclerosis could have a major impact upon the viability of EHT constructs, because such constructs will most likely contain a preformed or preserved vascular network that will stenose or occlude if these diseases are present, leading to ischemia and necrosis of the cells in the construct.

1.6 Summary and Conclusions:

It is difficult to make conjectures about the ultimate success or failure of EHT constructs. Given the requirements placed upon them and the mechanisms or processes that might be at work \textit{in vivo}, producing an EHT construct that remains viable for some time will be a challenging task. In the future, prevascularized scaffolds should be designed which allow for perfusion seeding and culture \textit{in vitro}, as well as the possibility of surgical anastomosis at implantation for improved graft function and survival. Scaffolds should possess a composition and architecture which mimic the native ECM in order to elicit favorable host responses; facilitate cell attachment and infiltration; and to maintain desired morphology, phenotype, and function. In designing EHT grafts and methods to produce them, researchers should not lose sight of the limitations presented by the necessity of autologous cell sourcing, and should investigate \textit{in vitro} culture methods to optimize clonal expansion of cell lines. Nor should they overlook the potential diseased and hostile microenvironment that awaits their EHT graft when they are applying stimuli to precondition their EHT constructs. Lastly, investigators should devote some thought to the
surgical approaches for implantation of EHT and consider the desires of surgeons themselves, as they will be the end-users of potential EHT grafts. If researchers working to develop EHT can overcome the inherent challenges associated with it, they can alleviate the devastation CVD causes so many patients and their families throughout the western world.

1.7 References:


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CHAPTER TWO: PROJECT MOTIVATION, SPECIFIC AIMS, AND SIGNIFICANCE

2.1 Introduction and Clinical Relevance:

Myocardial infarction (MI), the principal complication associated with cardiovascular disease, affects over 1,000,000 individuals annually. Incidences of acute MI are increasingly survivable, however, the associated transient ischemia causes tissue damage, scar formation, and initiates a degenerative progression toward congestive heart failure (CHF) in more than one fourth of affected patients. Consequently, the prevalence of CHF is currently estimated at 5.7 million afflicted, and total expenditures for CHF treatment are expected to increase from 44.6 billion dollars in 2015 to 97 billion dollars by the year 2030. It is clear that CHF represents a vast economic burden and should be regarded as a significant public health concern.

CHF develops as a result of global cardiac remodeling (i.e. left ventricular dilation and wall thinning) that occurs in response to the death of cardiomyocytes (CM) during MI. These CM are not regenerated, but are instead replaced by largely non-contractile, collagenous scar tissue. Mechanically speaking, the scar is a poor substitute for functional cardiac muscle, as it disrupts the continuity of the contractile syncytium of the myocardium. Persistence of the scar over time leads to maladaptive, compensatory CM hypertrophy, fibrosis, contractile dysfunction, and dilated cardiomyopathy en route to CHF. To prevent CHF, prospective therapies must aim to replace the infarct scar with functional cardiac muscle.
2.2 Current Standards of Care and Limitations:

Coronary artery bypass grafting, percutaneous coronary intervention, ventricular reconstruction, and left ventricular assist devices either focus solely on revascularization or do not adequately restore functionality to infarct-damaged tissue.\textsuperscript{[15]} Heart transplantation falls short as a solution, as demand for donor hearts far outpaces supply.\textsuperscript{[16, 17]} Success of stem cell therapy for MI in the form of intramyocardial (transendocardial) or intracoronary delivery of stem cells has been anecdotal. Several clinical trials have yielded findings that suggest marginal improvement in cardiac function, but none have shown any sustained improvement beyond 18 months. This is most likely due to poor cell engraftment and integration with host tissue, extensive apoptosis, and absence of cell differentiation.\textsuperscript{[18-20]}

Tissue engineering approaches may address the need for effective cell delivery by providing a stable environment for regenerative cells in the infarcted area.\textsuperscript{[2, 7, 21, 22]} Early efforts to generate a biomimetic, functional, tissue-engineered construct have shown promise with thinner grafts (<1 mm),\textsuperscript{[23, 24]} but thicker grafts (7 mm to 15 mm) are necessitated because the infarct scar is nearly transmural in many cases. Long-term survival and functionality of thicker, more clinically relevant-sized grafts implanted in animal models, however, has been hindered by insufficient vascularization within the grafts themselves, as well as lack of perfusion by the host’s blood supply.\textsuperscript{[21]} In order to replace the infarcted area with a functional graft of such thickness and sustain its constituent cells, vasculature is necessary to transport nutrients and wastes further than diffusion alone can
As of yet, no tissue engineering approach exists which has addressed and overcome these limitations.

The overarching goal of this project is to develop a biomimetic, acellular, and vascularized graft for replacement of infarct scar tissue. The graft is to be in the form of a “vascularized flap” to allow for surgical manipulation and implantation via anastomosis (similar to CABG) of the graft’s major vessels to the recipient vasculature.

It is hypothesized that this vascularized-flap graft, which will consist of a DCELLeled myocardial ECM scaffold derived from the left ventricular wall of porcine hearts and relevant cardiac cell types derived from autologous adult stem cells, will integrate with the host cardiac conduction system and produce systolic force upon implantation. In doing so, it will replace the lost functionality of cardiac muscle damaged by MI and avert (or possibly reverse) the remodeling which precipitates CHF. Our approach, as envisioned in a clinical scenario, is depicted in Figure 2.1.
Figure 2.1: **Overview of proposed translational approach and clinical scenario:** Patient suffers a myocardial infarction, has subcutaneous adipose tissue harvested, and adipose stem cells are isolated from the tissue; patient receives optimal medical management during the rest of the process until graft implantation. Cells are cultured with the aim of growing large numbers for later repopulation by reseeding of the myocardial scaffold generated from xenogeneic sources (i.e. a pig). The seeded construct is placed into a bioreactor wherein it is exposed to stimuli which mimic the cardiac environment in order to differentiate stem cells and condition the construct into a functional graft. The graft, consisting of autologous cells and nonimmunogenic scaffold, is implanted in the patient as a functional replacement for infarct scar tissue.

2.3 **Specific Aims:**

Four related and overlapping aims have been established toward achieving the overall goal of this research, and they are directed at understanding: 1) how to generate a DCELLed scaffold with the necessary features to overcome existing limitations of tissue engineering approaches; 2) the role and fate of DCELLed scaffolds in cardiac tissue engineering; 3) bioreactors, stem cells, and strategies for producing functional grafts; and 4) desired outcomes and benefits of infarct scar replacement with functional grafts.

**Aim 1 (Chapter 3): To develop and characterize a decellularized, vascularized scaffold for use in myocardial tissue engineering**

**Hypothesis:** *Acellular left ventricular porcine myocardium will serve as an ideal supporting material for tissue engineered myocardial grafts due to its low immunogenicity, potential as an excellent “niche” for inducing differentiation of stem cells toward cardiac phenotypes, and its network of preserved, patent vascular channels.*
**Approach:** Scaffolds will be generated using a combination of reagents, including detergents, chelators, bases, and enzymes to remove all cellular proteins from porcine myocardium. In doing so, scaffolds should: 1) have all potentially immunogenic proteins and molecules removed, 2) retain the biochemical structure, composition, architecture, and relative quantities of all ECM proteins (e.g. collagens type I, III, and IV, elastin, fibronectin, and laminin), and 3) provide a means of nourishing reseeded regenerative cells (i.e. an inherent vasculature).

**Innovation:** In addition to the properties listed above, the myocardial scaffold will preserve the native vasculature of the xenogeneic tissue and its connection to the coronary artery and cardiac vein to allow for convenient surgical manipulation and implantation. This will be in the mode of a “free flap transfer” with anastomoses of the above vessels to specific locations, providing a strategy for graft implantation that can be adopted quickly in the clinic.

**Aim 2 (Chapter 4): To characterize the host response to scaffolds and optimize their in vivo degradation rate**

**Hypothesis:** Scaffolds which resist in vivo degradation for longer than 8 weeks will provide implanted cells sufficient stability and time to integrate with host tissue, improving functional outcomes. Treatment with a specialized cross-linking agent will mitigate the severity of the immune response to implanted scaffolds.

**Approach:** As shown in previous studies, treatment of scaffolds with penta-galloyl glucose (PGG), a plant-derived antioxidant and matrix-binding polyphenol, will protect scaffolds from calcification, mitigate immune rejection, and prevent excessive matrix
metalloprotease-driven degradation. Myocardial scaffolds will be treated with two different concentrations of PGG and implanted subdermally in rats. At 4, 8, and 12 weeks, scaffolds will be explanted and examined histologically and immunohistochemically to characterize the host response, as well as the extent of degradation; results will be compared to non PGG-treated scaffolds.

**Innovation:** Tissue engineers who employ scaffolds as a significant component of their prospective therapy have struggled with questions surrounding the fate of scaffolds once implanted (i.e. how long should they persist, should they be remodeled, and how long should remodeling take in order to assure the optimum therapeutic benefit?). Our approach aims to answer these questions, determine the in vivo degradation rate of the scaffold, and to elucidate the precise nature of the host response to the scaffold (i.e. inflammatory vs. remodeling) including delineation of the source(s) of any stem cells that may be involved.

**Aim 3:** To condition cell-seeded myocardial constructs in vitro into functional grafts

**Hypothesis:** Exposure to conditions mimicking the physiological environment of the myocardium will induce stem cell differentiation and maturation of cell-seeded constructs into functional myocardial grafts.

**Approach:** A bioreactor will accommodate constructs seeded with hADSCs, fibroblasts, and endothelial progenitor cells. It will allow for precise control of physical and biochemical conditions (pressures, electrical stimulation, and media compositions). Constructs will be exposed to these conditions for 4 weeks to direct differentiation of stem cells toward electrically integrated and synchronously contracting cardiomyocytes. Resultant grafts will be examined for evidence of physiologic maturation using histology,
gene and protein expression, and by measuring excitation thresholds, calcium transients, fractional shortening, and force generation; results will be compared to unstimulated control constructs.

**Innovation:** Using bioreactors to deliver the stimuli necessary to differentiate and condition stem or progenitor cells into cardiac cells helps produce more functional myocardial grafts. The precise application of these stimuli in terms of what combination of stimuli to use, how to coordinate them, and the magnitude and frequency of their application will be determined by the studies outlined in this aim.

**Aim 4: To develop an animal model of infarction and demonstrate feasibility of surgical replacement of infarct scar with tissue-engineered constructs**

**Hypothesis:** A porcine model of infarction and surgical approach to construct implantation must be developed and optimized.

**Approach:** Coronary artery ligation in pigs will be used as an infarct model, and electrocardiography (EKG), echocardiography (ECG), blood chemistry, and histology will ascertain induction of MI. For implantation, the scaffold’s major vessels will be anastomosed to the host vasculature, and the flap-like portion of the scaffold will be affixed to the exposed viable myocardium with fibrin glue and sutures. Two rounds of implantation will be conducted: the first to evaluate the degree of hemostasis and vascular integrity, and the second to evaluate the subacute host response. For the former, pigs with healthy hearts will be implanted with a scaffold and observed for 1 hr. For the latter, pigs at 4 weeks post-MI will undergo infarct scar resection and replacement with a scaffold and
be allowed to survive for 1 week. EKG, ECG, histology, immunohistochemistry, and electrophysiology methods will be used to evaluate the responses.

**Innovation:** This aim will demonstrate the feasibility of the proposed surgical implantation method while allowing for identification of challenges and aspects of the procedure in need of revision. This will set the stage for a larger, full-scale study of the efficacy of tissue-engineered myocardial grafts in the future.

2.4 **Project Significance:**

Building upon recent advances in stem cell biology and tissue engineering, the proposed research will develop a meaningful therapy and further it toward application in the clinic. We expect the outcomes of this translational approach to have a positive impact upon efforts to treat a growing population of patients suffering from complications of coronary heart disease. Also, it could represent a significant step toward better understanding of the complex interaction between cells and extracellular signals in the cardiac environment. This research will not only enable clinicians to prevent or reverse the progression towards CHF—undoubtedly saving lives and improving the quality of life for recipients of this therapy—but also contribute to the advancement of the cardiac tissue engineering field.

2.5 **References:**


CHAPTER THREE: DEVELOPMENT AND CHARACTERIZATION OF AN ACELLULAR MYOCARDIAL SCAFFOLD ENRICHED WITH PATENT VASCULAR NETWORKS AND BIOCOMPATIBLE CELL NICHES

3.1 Introduction:

The prevailing paradigm in the field of tissue engineering advocates the combined use of cells and scaffolds for therapeutic application to the target tissue. Among the biomaterial scaffolds under investigation, none have recently gained as much attention as acellular tissue matrices. These DCELLed extracellular matrix (ECM) scaffolds have been successfully generated by subjecting tissues from a number of sources to various combinations of chemical, enzymatic, and physical agitation, breaking up and removing cells and cellular proteins in the process.\textsuperscript{[1, 2]} The remaining ECM acts as an agreeable “niche” for reseeded cells, complete with the appropriate binding sites, mechanical properties, and architecture necessary to induce formation of a tissue-engineered construct that is physiologically comparable to native tissue. The function of ECM proteins does not differ among species, and thus their structure has been highly conserved over the course of evolution. As such, these proteins should not perturb the host immune system when implanted in an allogeneic or even xenogeneic capacity.\textsuperscript{[3]} Another major advantage offered by DCELL is that the resulting scaffold is left with an intact vascular network, a design which nature has perfected and would be painstaking, if not impossible, to duplicate from the bottom-up. This vascular network can be used to sustain cells seeded onto DCELLed scaffolds once the resulting constructs are implanted.
Porcine hearts are also approximate in size to human hearts, which is important considering that hearts with different ventricular volumes will experience different pressures, and thus, have different wall stresses and amounts of constituent collagen to bear those stresses.\cite{4} In terms of mechanics, this means that scaffolds generated with porcine hearts are macromechanically equipped to withstand forces in the human cardiac environment, as well as micromechanically amenable to attachment and proper mechanotransduction of seeded cells.

Whole heart and myocardial patch DCELL has been demonstrated by a number of groups.\cite{5-11} Typically, remnants of the DCELL process are composed of extracellular matrix (ECM) proteins native to the myocardium and the native vascular network. Few studies, however, have attempted a comprehensive, parallel analysis of both the vascular and myocardial matrices focusing on DCELL efficacy, elastic moduli and burst pressures of the intrinsic coronary arteries, patency of capillary perfusion, exact amounts of collagen and elastin, and identification of important basement membrane proteins, among others. In our view, complete and efficient DCELL of such complex tissues appeared problematic and required a comparative analysis of several DCELL methods.

An optimal DCELL technique for this application should fulfill the following criteria: 1) remove all potentially immunogenic proteins and molecules, rendering the implant biocompatible; 2) preserve the biochemical structure, composition, architecture, and relative quantities of all matrix proteins including basement membrane, thus generating an agreeable biological and biomechanical “niche” for reseeded cells; 3) provide a means of nourishing reseeded regenerative cells by preserving the natural
vasculature; and 4) allow for convenient surgical manipulation, implantation, and reconnection to the host vasculature.

To this end, we DCELLed porcine myocardium and its constituent vasculature using four different procedures comprising combinations of sodium dodecyl sulfate (SDS) and sodium hydroxide (NaOH) in a comparative study of their ability to remove cells and cell remnants and preserve the features of the native ECM. The scaffold was uniquely shaped as a “flap” so that the native vasculature could be used in a three-fold manner: first, to facilitate DCELL by perfusion, second, to serve as a conduit for recellularization with appropriate cells, and third, to allow for surgical manipulation and implantation via anastomosis of the graft’s vessels with the recipient vasculature (similar to coronary bypass surgery). We systematically evaluated both the myocardial and coronary arterial ECM and investigated their ability to support the attachment and survival of relevant cardiac cell types.

3.2 Methods and Materials:

3.2.1 Decellularization of Porcine Myocardium:

Whole hearts were obtained from healthy, adult pigs at a local abattoir. Immediately following harvest, 50 mM ethylenediaminetetraacetic acid (EDTA) in warm phosphate buffered saline (PBS) was injected into the left coronary artery branch at the aortic trunk to prevent clotting in the vasculature, and the hearts were transported on ice for immediate processing. A 1 cm thick, 6-8 cm wide, and 12 cm long “flap” of myocardium was excised from the left-anterior ventricular wall, with care not to sever the
coronary artery or cardiac vein, and these vessels were cannulated with barbed Luer connectors (Cole-Parmer) secured with zip-ties in order to facilitate perfusion (Figure 3.1). Larger, open-ended vessels severed during excision of the flap were closed with 4-0 silk suture (Ethicon). The flaps were then subjected to combined perfusion and immersion of solutions in a continuous-flow DCELL system. The system comprised a series of fluid reservoirs and a multi-channel peristaltic pump (Masterflex, Cole-Parmer) which circulated 2 L of solution through and around each scaffold. The inflow reservoir was elevated 90 cm above the cannulated ends of vessels, generating a hydrostatic pressure of about 80 mmHg and a flow rate of about 200 ml/min (largely dependent on tissue resistance). A solution of 30 mM EDTA in PBS (pH=7.5) was perfused for 12 h to clear any remaining clotted blood from the vasculature, followed by 1% SDS and 0.1 M NaOH solutions in sequence and in various durations for 10-15 days, while solutions were changed every 2 days. The four separate DCELL treatment groups (n=4 scaffolds per group) were as follows: Group 1: 2 days SDS followed by 8 days NaOH, Group 2: 5 days SDS followed by 5 days NaOH, Group 3: 10 days SDS followed by 2 days NaOH, Group 4: 15 days SDS. DCELL efficacy was gauged by monitoring changes in scaffold color and mass. Following completion of the DCELL process, scaffolds were immersed and rinsed overnight in 70% ethanol and 3 changes (one overnight) of PBS on an orbital shaker. For post-SDS DCELL studies, Group 4 scaffolds (n=3) were further subjected to treatments of either 24 h perfusion with DNAse/RNAse (Worthington Biochemical) solution (720 mUnits/mL each) in PBS containing 5 mM magnesium chloride at pH 7.5, perfusion of 0.1 M NaOH for 2 hours, or 3 days perfusion of PBS.
Figure 3.1: A pedicled “flap” of A) native myocardium excised from the left ventricular wall of the porcine heart and a representative image of the decellularized (Decelled) scaffolds resulting from perfusion decellularization. The cannulae used to connect the vasculature to the perfusion system are shown secured with orange zip ties. Red arrows indicate the open end of the coronary artery prior to cannulation (Native) and the inlet to the coronary artery after cannulation (Decelled). Similarly, blue arrows indicate the same
features for the cardiac vein. Arrows also indicate the direction of anterograde blood and decellularization solution flow. Areas designated by circles indicate origins of specimens of decellularized coronary arterial extracellular matrix (CA ECM) and decellularized myocardial extracellular matrix (MYO ECM) used in histological, immunohistochemical, DNA, biochemical, mechanical, and cell-seeding analyses. Time-course plot B) of the mean recorded masses of scaffolds exposed to 4 different decellularization methods. Results of a C) PicoGreen® quantitative assay for DNA are shown which compare the DNA content of both decellularized MYO ECM and CA ECM samples generated with various decellularization treatments to that of native myocardium and native coronary arteries, respectively. DNA content of D) MYO ECM samples from scaffolds generated with the Group 4 decellularization treatment and subsequent exposure to various reagents as measured by PicoGreen® assay. DNA content of Group 4 and native myocardium represented in D are carried over from C for comparison (*: indicates statistical significance from all other groups, p<0.0001). Images of DAPI-stained sections E) from MYO ECM and CA ECM of decellularized porcine myocardial scaffolds from Group 4 and Group 3, showing residual nucleic acids (blue, white arrows; 10X, bar=100 μm). Images of H&E-stained sections F) from MYO ECM and CA ECM of scaffolds from Group 4 and Group 3, showing residual “pools” (black arrows) of nucleic acids (blue; 10X, bar=100 μm).

3.2.2 Histology and Immunohistochemistry:

Samples of myocardial ECM (“MYO” in Figure 3.1A) and coronary arterial ECM (“CA” in Figure 3.1A) were examined by histological staining with Hematoxylin and Eosin (H&E), Gomori’s trichrome, Movat’s pentachrome, and Verhoeff-Van Gieson’s stains. Immunohistochemical (IHC) analysis was performed on formalin-fixed and paraffin-embedded sections using heat-mediated antigen retrieval (10 mM citric acid buffer at 95-100 °C for 10-15 minutes) followed by incubation with antibodies to collagen IV (2 μg/mL, Abcam), laminin (4 μg/mL, Abcam), actin (2 μg/mL, Abcam), cardiac myosin heavy chain (4 μg/mL, Abcam), or fibronectin (4 μg/mL, BD
Biosciences) and application of Vectastain DAB kit (Vector Labs) reagents for detection. For pore size determination, images (n=25) of H&E-stained sections of MYO ECM from each group were analyzed using ImageJ software (NIH) to determine the mean diameter of pores in both longitudinal (n=210) and transverse (cross-sectional) (n=278) directions.

3.2.3 DNA, Collagen and Elastin Quantification:

DNA content of MYO ECM (n=8) and CA ECM (n=10) and samples of similarly located native tissues (myocardium: n=8, coronary artery: n=10) was measured using a Quant-IT PicoGreen® kit (Invitrogen). Lyophilized samples of MYO ECM (n=8), CA ECM (n=8), native myocardium (n=8), and native coronary artery (n=8) were analyzed for desmosine content by radioimmunoassay (RIA) and for hydroxyproline content by amino acid analysis as previously reported, and collagen and elastin content were calculated as previously described.

3.2.4 Mechanical Properties:

Rectangular 2.5 x 1 cm specimens of DCELLed porcine MYO ECM (n=4) were either left with the epicardium attached or had the epicardium removed (n=4). Similar samples (n=4) of native porcine myocardium with and without the epicardium were prepared for comparison. Specimens were secured to a frame with 10 N load cell (MTS Systems), submerged in PBS at 37 °C, and preconditioned for 10 cycles between 0-15% strain at a rate of 3.0 mm/min. After 2 minutes rest, they were subjected to 3 cycles to 40% strain at 3.0 mm/min. Tangential slopes of each stress-strain curve between 35-40%
strain were averaged and taken as the Young’s modulus. Samples of native porcine coronary arteries (n=8) and DCELLed CA ECM (n=8) isolated from DCELLed porcine myocardial flap scaffolds were subjected to burst pressure measurements as previously described.\textsuperscript{[15]}

3.2.5 Evaluation of Scaffold Vasculature:

A native porcine heart (obtained as above) was injected with 50 mM EDTA in warm PBS at the left coronary arterial branch from the aortic trunk to clear the vasculature of any clots. The left coronary artery and superior portion of the cardiac vein were cannulated with barbed Luer connectors (Cole Parmer). A cannulated, acellular flap scaffold was embedded in concentrated (126 mg/mL) Knox gelatin to seal any open-ended vascular channels created during the excision portion of the DCELL process. Red and blue-pigmented polymethyl methacrylate (PMMA) from Batson’s #17 Anatomical Corrosion Kit (Polysciences Inc.) were sequentially injected into the vasculature of both the native heart and scaffold. Red PMMA was infused anteriogrady through the coronary arterial inlet, and blue PMMA retrogradely through the cardiac vein inlet. After polymerization, a cast of the vasculature was obtained by tissue maceration. Vascular casts were imaged on a dissection microscope and ImageJ software was used to measure the diameters of blood vessels.

For injection and imaging of fluorescent dyes, fluorescein isothiocyanate (FITC)–dextran and rhodamine B isothiocyanate (RITC)–dextran (Sigma) were simultaneously injected into the scaffold vasculature (FITC-dextran anteriogradely through the arterial
inlet and RITC-dextran retrogradely through the venous inlet). The scaffolds were immediately imaged macroscopically using an in vivo imaging system (IVIS® Lumina XR, Caliper Life Sciences). The system generated images of photon density on a greyscale which were artificially colored using the accompanying Living Image® software (Caliper Life Sciences).

Carbon black spherical particles of 2-12 μm in diameter (Sigma) were passivated via overnight incubation in 100% fetal bovine serum (FBS, Atlanta Biologicals), washed 2X in PBS, suspended in PBS at a concentration of 2x10^6 particles/mL, and injected into the vascular inlets of a Group 4 scaffold. Movat’s pentachrome stain was used to examine 5 μm-thick sections of MYO ECM distal (at the apical end of the scaffold) to the inlet cannulae.

3.2.6 Cell Seeding and Analysis:

Cylindrical, 5 mm-diameter punch biopsies (n=4) of MYO ECM were taken from DCELLeled porcine myocardial scaffolds from Group 4, sterilized in 0.1 % peracetic acid (Sigma) in PBS for 1 h, washed 3X in sterile PBS, and lyophilized. For initial cytotoxicity studies, approximately 5x10^6 rat dermal fibroblasts (Cell Applications) at passage 11 were seeded onto the dry scaffold samples and cultured statically for either 7 or 14 days. At each time point, seeded scaffold samples were stained for viability using a Live/Dead kit (Invitrogen).

For seeding of neonatal rat cardiomyocytes (Lonza), 1 mm³ samples of MYO ECM from Group 4 scaffolds (n=3) were sterilized with peracetic acid and washed as
described above, seeded with $2 \times 10^6$ cells at passage 2, and cultured statically for 7 days. Samples were analyzed by immunofluorescence with primary antibodies to connexin43 (2 μg/mL, Abcam), actin (2 μg/mL, Abcam), cardiac myosin heavy chain (4 μg/mL, Abcam), and α-sarcomeric actinin (2 μg/mL, Abcam), secondary antibodies AlexaFluor 488 and 594 (4 μg/mL, Invitrogen), and 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain.

3.2.7 Statistical Analysis:

Statistical analysis was performed using JMP software (SAS Institute Inc.). One-way analysis of variance (ANOVA) and Fisher’s least significant difference method for post-hoc comparison of mean values (with $\alpha=0.05$) were used. All quantitative data are represented as mean ± standard deviation. Error bars represent ± standard error.

3.3 Results

3.3.1 Removal of Cellular Components:

Scaffold color and mass loss were indicative of effective muscle cell removal, as scaffolds initially of red-brown color became progressively paler throughout the DCELL process before becoming white and translucent (Figure 3.1) and wet masses decreased with time. Notably, NaOH treated scaffolds displayed a higher rate of mass removal than SDS treated scaffolds (Figure 3.1B). Upon switching from SDS to NaOH as called for by each treatment, the mass removal rate increased by more than a factor of three. The treatment for Group 4 (SDS alone) removed 49.6±1.4% of the original tissue mass, while
the other three treatments, which all incorporated varying lengths of NaOH exposure, resulted in an average $58.8\pm1.3\%$ removal of the initial mass ($p=0.0006$). These results indicate that NaOH is a more rapid and more efficient DCELL agent as compared to SDS.

Histological analysis of MYO and CA ECM (Figure 3.2A) illustrated removal of cell nuclei and cytoplasmic proteins from native tissues, leaving voids in the resulting scaffold. IHC analysis confirmed complete removal of actin and cardiac myosin heavy chain muscle proteins from both tissues (Figure 3.2B). Data in Figure 3.6 also reflect effective DCELL, as the disparity between the weight percentages of matrix proteins in DCELLed and native tissues was caused by removal of cell proteins.

Quantification of DNA showed that all DCELL treatments which employed NaOH (Groups 1-3) cleared 95-98% of DNA from myocardial and arterial tissues. However, DNA content was not significantly different in Groups 1-3. The treatment in Group 4 (SDS alone) reduced DNA content by 33.2% in the myocardium and only 27.5% in the coronary arteries (Figure 3.1C). H&E- and DAPI-stained sections of Group 4 scaffolds showed positive staining for nucleic acids which had “pooled” in sub-epicardial and sub-intimal areas of the MYO and CA ECM, respectively, while Group 3 scaffolds were completely devoid of nucleic acids (Figure 3.1E and F). Quantification of DNA in MYO ECM from Group 4 scaffolds perfused with additional solutions, described as post-SDS procedures, including DNAse/RNAse or NaOH, resulted in further reduction of DNA content (Figure 3.1D).
Figure 3.2: Histological images of A) native porcine myocardium and decellularized (Decelled) porcine myocardial ECM (MYO) stained with H&E and sectioned in the longitudinal and transverse (cross-sectional) directions with respect to cardiomyocytes, depicting the cylindrical voids left in their absence (40X, bar=20 μm); H&E-stained native porcine coronary artery and decellularized porcine coronary arterial ECM (CA; 10X, bar=100 μm). Decellularized sections are representative of all four treatment groups. Cell nuclei (blue), cytoplasmic proteins (dark pink), and collagen (light pink) are shown. B) Immunohistochemical (IHC) staining of native and decellularized (Decelled) porcine myocardium (MYO) for actin and cardiac myosin heavy chain (cardiac MHC; 40X, bar=20 μm). IHC of native and decellularized porcine coronary artery (CA) for actin (10X, bar=100 μm). Decellularized sections are representative of all four treatment groups. IHC positive staining = brown. Inserts = IHC negative controls. L = lumen and black arrows indicate the internal elastic lamina.
Figure 3.3: Histology of native porcine myocardium and decellularized (Decelled) porcine myocardial ECM (MYO) from Group 4 scaffolds (40X, bar=20 μm) and native porcine coronary artery and decellularized porcine coronary arterial ECM (CA) from Group 4 scaffolds (10X, bar=100 μm) stained with Movat’s pentachrome, which shows nuclei (dark red), collagen (yellow-orange), elastin fibers (maroon), and cytoplasmic proteins (red). Native porcine myocardium and decellularized porcine myocardial ECM from Group 4 scaffolds (40X, bar=20 μm) and native porcine coronary artery and decellularized porcine coronary arterial ECM from Group 4 scaffolds (20X, bar=50 μm) stained with Gomori’s trichrome, which shows nuclei (dark purple), collagen (blue-green), elastic fibers (purple), and cytoplasmic proteins (red). Native porcine myocardium and decellularized porcine myocardial ECM with epicardium from Group 4 scaffolds (20X, bar=50 μm) and native porcine coronary artery and decellularized porcine coronary arterial ECM from Group 4 scaffolds (10X, bar=100 μm) stained with Verhoeff-Van Gieson’s (VVG) stain for visualization of nuclei (grey/black), elastin fibers (black), and collagen (pink). Images of decellularized sections for all stains are representative of all four treatment groups. L = lumen and black arrows indicate the internal elastic lamina.
3.3.2 Preservation of Matrix Architecture and Mechanical Integrity:

H&E-stained histological sections cut in the longitudinal and transverse planes of DCELLed MYO ECM reflected retention of the native myocardial structure and organization (Figure 3.2A). Taken together, images from these two planes illustrated the cylindrical voids left within scaffolds following removal of cardiomyocytes. Quantitative analysis of the pores in these images showed they had mean dimensions of about 20 x 40 μm among all treatment groups (Table 1). Histological sections of DCELLed CA ECM displayed elliptical and oblong pores vacated by smooth muscle cells and fibroblasts, intact internal elastic lamellae, and little delamination of the vessel tunics (intima, media, adventitia; Figures 3.2, 3.3, 3.4, 3.5). Uniaxial tensile testing of DCELLed MYO ECM samples showed that scaffolds from all groups had a higher elastic modulus than that of native tissue (p<0.0001). Group 1 scaffolds (with epicardium attached) had a significantly greater modulus than scaffolds from other groups (p=0.0011). Separation of the epicardium from MYO ECM samples was not found to have a significant effect upon the modulus (Figure 3.6C).

Tubular segments of DCELLed CA ECM from all groups exhibited burst pressures which were not significantly different from those of native coronary arteries and exceeded 2000 mmHg (Figure 3.6D).
**Figure 3.4:** Immunohistochemistry (IHC) of native porcine myocardium and decellularized porcine myocardial ECM, showing the degree of preservation of collagen IV (Coll IV), laminin, and fibronectin in Group 2, Group 3, and Group 4 (40X, bar=20 μm). IHC positive staining = brown. Inserts = IHC negative controls.
**Figure 3.5:** Immunohistochemistry (IHC) of native porcine coronary artery and decellularized porcine coronary arterial ECM, showing the degree of preservation of collagen IV (Coll IV), laminin, and fibronectin in Group 2, Group 3, and Group 4 (20X, bar=50 μm). IHC positive staining = brown. Inserts = IHC negative controls.

**Table 2. Immunohistochemical Staining of Basement Membrane Proteins and Fibronectin**

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<td>Fibronectin</td>
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<td>+</td>
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**Grade:**

+++ Intense and highly localized
++ Diffuse, but less localized
+ Visible, but faint, sporadic, and/or nonlocalized
- Not visible
3.3.3 Preservation of ECM Proteins and Basal Lamina:

Gomori’s trichrome, Movat’s pentachrome and Verhoeff-Van Gieson’s-stained sections illustrated the preservation of collagen and elastin following DCELL in both MYO ECM and CA ECM (Figure 3.3). Notably, elastin was diffuse in the CA ECM (Figure 3.3) but was also observed in the epicardium and microvascular channels within the MYO ECM (Figure 3.7). These results were consistent among scaffolds from all treatment groups and indicate excellent preservation of collagen and elastin. To evaluate preservation of basement membrane components, we performed IHC for collagen IV, laminin, and fibronectin. These were all present within native tissues and localized to the peri-cellular interstitium (Figure 3.4 and 3.5). IHC of sections from Group 4 confirmed excellent retention of collagen IV, laminin, and fibronectin in both the MYO and CA ECM following DCELL (Figures 3.4 and 3.5, Table 2). Scaffolds from groups exposed to NaOH (Groups 1, 2, and 3), however, displayed a trend of increasingly diminished staining of these proteins as length of NaOH exposure increased (Figures 3.4 and 3.5, Table 2). IHC of sections from Group 1 scaffolds displayed sparse labeling of collagen IV in comparison to other groups and little to no labeling of either laminin or fibronectin. Images of these sections are not shown due to their similarity to images of sections from Group 2 scaffolds.

Quantification of collagen and elastin content using total hydroxyproline and desmosine analysis showed that MYO and CA ECM were composed mainly of collagen and elastin, with slight differences among the various groups (Figure 3.6). Elastin content was significantly different only between Group 2 and Group 4 in the MYO ECM.
(p=0.0005; **Figure 3.6A**) and between Group 2 and Group 3 in the CA ECM (p=0.0432; **Figure 3.6B**). Both types of matrices from all DCELLead treatment groups had mean collagen and elastin weight percentages which were significantly different from those of native tissue (p<0.0001; **Figure 3.6A and B**). The ratio of mean collagen weight percentage to mean elastin weight percentage for native myocardium was 19.5. This ratio increased significantly in samples of DCELLead MYO ECM from all treatment groups, and, among treatment groups, was only significantly different between Groups 1 and 4 (p=0.0457, Table 3). This same ratio was 4.3 in native coronary arteries, and it was not significantly different from that of CA ECM in any of the groups (Table 3).

**Figure 3.6**: Biochemical quantification of collagen and elastin content of A) decellularized myocardial ECM samples generated with four different decellularization treatments as compared to that of native myocardium.
Collagen and elastin content of B) decellularized coronary arterial ECM samples generated with four different decellularization treatments as compared to that of native coronary arteries (*: indicates statistical significance from all other groups, p<0.0001; **: indicates significance from all groups except Group 3, p<0.0001; ***: p=0.002; #: p=0.0419; ##: p=0.0432; ###: p=0.0009).  C) Young’s elastic moduli of decellularized myocardial ECM samples generated with four different decellularization treatments as compared to that of native myocardium (*: indicates statistical significance from all other groups, p<0.0001; **: indicates statistical significance from all other groups in “without epicardium” group, p=0.0011). Groups of samples with and without the epicardium attached were tested.  D) Burst pressures of decellularized coronary arterial ECM segments generated with four different decellularization treatments as compared to that of native coronary arteries.

<table>
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<tr>
<th>Table 3. Ratio of Collagen Weight Percentage to Elastin Weight Percentage</th>
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<tr>
<td>Myocardial ECM</td>
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<td>Group 1</td>
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<td>Group 4</td>
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<td>Native</td>
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*: significant from all other groups (p = 0.0007)  
**: significant from Group 1 (p = 0.0457)

3.3.4 Preservation of Vascular Integrity and Patency:

Simultaneous injection of FITC- and RITC-conjugated dextran into arterial and venous vascular inlets showed extensive perfusion of scaffolds with fluorophores (Figure 3.7A and B). An overlaid image of both emission spectra demonstrated that the fluorophores had actually mixed within the same vessels (Figure 3.7C), and an overlaid
image of the fluorescent channel with a white-light photograph of the scaffold (Figure 3.7D) illustrated localization of fluorophores to the scaffold vasculature. Corrosion casts of a scaffold from Group 4 demonstrated retention of structure and patency of both macro- and microvasculature following DCELL. Intact capillaries 5-8 μm in diameter were observed in casts of native myocardium as well as DCELLed scaffolds (Figure 3.7). Carbon microspheres injected through the vascular inlets of a scaffold from Group 4 were observed to accumulate within capillaries in histological sections of distal portions of the scaffold (Figure 3.7M and N). Microvasculature was also routinely observed in histological sections, and was illustrated by staining of internal elastic lamina or laminin via VVG and IHC, respectively (Figure 3.7K and L).
Figure 3.7: Top Left Panel: Macroscopic images of a decellularized porcine myocardial scaffold from Group 4 following injection with A) fluorescein isothiocyanate (FITC, red) through the arterial inlet and B) rhodamine isothiocyanate (RITC, blue) through the venous inlet. C) Merged image of images A and B showing colocalization of fluorescent dyes within arteries and veins. D) Merged image of image C and a white-light image of the scaffold displaying fluorescent dyes confined to the vasculature. Bottom Left Panel: Corrosion casts of the vasculature of E, F) native left-ventricular porcine myocardium and a G, H) decellularized porcine myocardial scaffold from Group 4, showing preservation of both G) macro- and H) microvasculature following the decellularization process. A 500 μm-diameter needle was included in the frame as a scale reference (black bar in E-H=500 μm). Right Panel: Representative images across all decellularization treatments of I) H&E- and J) VVG-stained sections of decellularized vasculature from scaffolds showing the coronary artery (CA) and adjacent cardiac vein (CV; 2.5X, bar=500 μm). Micrographs of K) VVG staining and L) IHC for laminin in sections of microvasculature from Group 4 scaffolds showing preservation of elastin (black) and laminin (brown) around vessels, respectively (40X, bar=20 μm). Micrographs M, N) of decellularized myocardial scaffold vasculature from Group 4 following injection of 2-12 μm-diameter, carbon particles via the vascular inlets. Sections were from sites distal (apical portions of scaffold) to inlets, and black carbon particles are shown localized to small vessels (M: 10X, bar=100 μm; N: 20X, bar=50 μm).

3.3.5 Cytocompatibility:

Samples of MYO ECM from scaffolds exhibited excellent cytocompatibility to fibroblasts after 7 and 14 days in culture (Figure 3.8A). Rat neonatal cardiomyocyte-seeded samples labeled for connexin43, actin, cardiac myosin heavy chain (MHC), α-sarcomeric actinin, and DAPI appeared viable and positive for these markers. Although no tests of functionality were performed, cells appeared to interconnect and self-organize on the scaffolds (Figure 3.8B).
**Figure 3.8:** A) Images of decellularized porcine myocardial ECM from Group 4 scaffolds seeded with rat dermal fibroblasts (FB) and cultured for either 7 (top) or 14 (bottom) days. Cells were labeled with Live/Dead reagents at each time point. Live cells were labeled with calcein AM (green) and dead cell nuclei were stained with ethidium homodimer (EthD-1, red; 20X, bar=50 μm). B) Immunofluorescence (IF) images of decellularized porcine myocardial ECM from Group 4 scaffolds seeded with neonatal rat cardiomyocytes (CM) and cultured for 7 days. Scaffolds were stained for α-sarcomeric actinin, cardiac myosin heavy chain (MHC), actin, connexin43 and DAPI (40X, bar=20 μm).

### 3.4 Discussion

#### 3.4.1 Evaluation of Decellularization Efficiency:

In this study, we have taken a systematic and thorough approach to developing a naturally vascularized, acellular myocardial flap, addressing the key features and characteristics of the myocardial matrix, the major arteries and veins, and the intrinsic microvasculature. Several groups have successfully DCELLed myocardium in either whole or excised portions of both rat and pig hearts using a wide range of chemical
agents and physical methods.\textsuperscript{[5-11, 16]} Many reported on characteristics (e.g. acellularity, biochemical composition, vascularity, thickness) of the resulting scaffolds and their applicability to tissue engineering efforts. However, with regard to optimization of a DCELL method, these studies vary in the comprehensiveness of their analysis.

Our choice of chemical agents for DCELL was predicated upon previously published results from studies which used SDS \textsuperscript{[2, 6, 8, 11]} and upon our previous experience with NaOH as a DCELL agent. The latter has long been used in “hot alkali” methods for elastin isolation (indicating its suitability for preserving elastin),\textsuperscript{[13]} and we previously observed extensive removal of DNA and collagen from carotid arteries treated with 0.1 M NaOH at 37°C.\textsuperscript{[15]} In several pilot studies, we used this same concentration of NaOH to decellularize myocardium at room temperature, and did not observe any noticeable degradation of the collagenous matrix components (data not shown). We surmised that the lower temperature reduced protein degradation kinetics so as to remove cellular proteins without causing extensive damage to the collagenous ECM. Thus, it was of interest to investigate the combinatorial effect of SDS’s ability to solubilize plasma membranes and denature proteins with NaOH’s affinity for solubilizing protein and degrading nucleic acids.\textsuperscript{[2]}

3.4.2 Removal of Cellular Material:

Despite its reputation as an effective DCELL reagent,\textsuperscript{[1, 2, 8, 11, 17]} it has been suggested by some that SDS may leave cellular remnants when used in DCELL of MYO ECM. However, there is scant evidence furnished by the authors in support of this...
contention.\textsuperscript{[9]} Our own histology and IHC results demonstrated that every DCELL treatment we investigated, including that which used solely SDS following EDTA (Group 4), was effective in disrupting cell and nuclear membranes and in completely removing intracellular proteins from both the MYO ECM and CA ECM. Measurements of the dimensions of pores within our DCELLed scaffolds are consistent with those previously reported.\textsuperscript{[7]} DNA quantification was carried out to provide an additional measure of acellularity, and with the exception of the Group 4 treatment, all DCELL methods left only small amounts of residual DNA. Although quantitative assays, as well as H&E- and DAPI-stained sections, confirmed that Group 4 scaffolds contained visible amounts of residual DNA (Figure 3.1), none of the corresponding histological stains or IHC showed muscle proteins or other apparent cellular remnants (Figure 3.2). Furthermore, the nucleic acids in these sections were not observed in small, concentrated areas (which would be indicative of intact nuclei), but rather seemed faint and diffuse, occasionally “pooling” within pores of the matrix. This was especially evident in areas adjacent to denser portions of the matrix, such as the epicardium, adventitia, and internal elastic lamina, where resistance to diffusion of large molecules is potentially greater.

In replicating a similar SDS-based protocol developed by Ott et al., Akhyari and colleagues showed only a 43\% reduction of native DNA content.\textsuperscript{[11, 16]} Weymann et al. DCELLled an intact porcine heart using perfusion of 4\% SDS over 12 h, concluded with perfusion of PBS for 24 h, and reported an 82\% reduction in DNA content.\textsuperscript{[8]} While SDS was effective in lysing the nuclear membranes, it likely did not interact with this newly extra-nucleated, or free DNA. This lack of interaction is explainable, given that SDS is a
strongly anionic molecule and DNA is negatively charged along its phosphate backbones. NaOH, by contrast, is exceedingly effective in breaking down DNA, as it causes both denaturation[18] and hydrolysis of the molecule.[19]

To reduce content of residual DNA within Group 4 scaffolds, we prepared a new set of scaffolds using the Group 4 treatment and compared the effects of three prospective post-SDS “washing procedures” upon levels of residual DNA. The DNAse/RNAse and NaOH (2 hours) treatments were effective in removing some residual DNA, but did not achieve the very low levels of residual DNA observed in groups with significant exposure to NaOH (2 days or more). Despite this, we demonstrated that minor additions to our Group 4 treatment could result in further removal of DNA to acceptable limits.

3.4.3 Preservation of Basal Lamina Components:

Basement membrane proteins contain important cell binding moieties, and any alteration of their conformation by chemical or physical means during DCELL could render them nonfunctional.[16, 20] Laminin is a vital link in the chain which binds the cardiomyocyte cytoskeleton to the collagenous ECM components, thereby facilitating transfer of mechanical forces and stimuli between the two.[21, 22] It has multiple domains which allow it to simultaneously bind integrins, proteoglycans, and collagen IV, making laminin the all-purpose “glue” of the basement membrane.[4, 23] Attachment to laminin has also been shown to be necessary for cardiomyocyte survival in vitro, indicating its ability to mediate important cell signaling pathways.[24, 25] Collagen IV assembles into an open network overlaying the predominant structural ECM proteins.[18] It serves as the
linkage between fibrillar collagens and both laminin and fibronectin. Fibronectin acts as an important intermediary between cardiomyocytes and the basal lamina, binding integrins, proteoglycans, and collagens.[23] Although these are minor components within the ECM, they will likely play a major role in the attachment, mechano-transduction, differentiation, maintenance of phenotype, and ultimately, functionality of reseeded cells.

In our studies, DCELL treatments which employed NaOH altered and/or removed basement membrane proteins. While collagen IV and fibronectin appeared less affected by this exposure, laminin was aggressively degraded by NaOH (full degradation at 48 h exposure to 0.1 M NaOH). Scaffolds from Group 4 (SDS alone) retained all basement membrane proteins we attempted to identify by IHC.

3.4.4 Preservation of Collagen and Elastin:

Quantitative analysis showed that collagen and elastin were the predominant components of both myocardial and coronary matrices within scaffolds from all groups. Aside from studies which reported Western blotting results[16] and qualitative or semi-quantitative results,[5-10] to our knowledge, no other attempts were made to quantify these important matrix proteins. Elastin plays an important mechanical role in normal cardiac tissue, as it facilitates passive recoil of the matrix following muscle contraction and distension of the collagenous components within both the MYO and CA ECM.[23, 26] Akhyari et al. reported that DCELL with a similar method reduced elastin content relative to native tissues,[16] but our data show that elastin content is largely preserved, particularly within the CA ECM.
3.4.5 Evaluation of Mechanical Properties:

Overall, the elastic moduli of MYO ECM and CA ECM did not differ significantly from native tissues, which suggests that DCELL procedures assessed in this study did not alter their mechanical properties. The elastic moduli we report here (0.4-0.9 MPa) for DCELLed MYO ECM is somewhat lower than that reported by other groups, but this does not indicate that our scaffolds are by any means mechanically deficient. This disparity can be attributed in part to differences in mechanical testing methods (e.g. strain rate, modulus calculation method, preconditioning, preloading, and cross-sectional area measurement techniques for calculation of engineering stress). Our mechanical testing procedure was most comparable to that described by Eitan et al. in terms of preconditioning and strain rate, but it was not clear as to which specific portion of the stress-strain curve was used for calculation of their reported elastic modulus.\[10\] Our myocardial matrix is also considerably less stiff than another acellular porcine MYO ECM generated with an SDS-based protocol, which had a reported modulus of 5.219 MPa (strain rate was 6.0 mm/min – twice the rate used in the current study).\[6\]

Importantly, both the above DCELL protocols employed trypsin extensively (Sarig et al.: 0.05% (w/v) for 96 hr and Wang et al.: 0.01% for 2.5 weeks).\[7,9\] Trypsin is a serine protease known to partially degrade elastin, an observation confirmed by our own work with this enzyme (data not shown). Acellular myocardial matrix generated by the first trypsin protocol\[9\] was reported to be completely devoid of elastin, and while recently published results from the second group confirm the presence of elastin histologically, no quantitative data was provided.\[17\]
3.4.6 Vascular Integrity and Patency:

Corrosion casts of scaffolds demonstrated that our DCELL procedure leaves the inherent vasculature intact to the extent of preserving the patency of microvasculature and capillaries. Simultaneously anterio- and retrogradely injected fluorescent dyes were able to move freely through the scaffolds’ vascular tree and mix with one another. In addition, carbon microspheres appeared within distal microvascular channels following their injection into the scaffold’s main vascular inlets. These observations confirm that the microvasculature and capillary beds linking the arterial and venous circulation are left intact by our DCELL process. Using a similar perfusion SDS-based protocol, Ott et al. presented evidence of vascular preservation with corrosion casting results, but also by heterotopically connecting the vasculature of their whole, DCELLeed rat heart to the aorta of a recipient rat and observing perfusion of the scaffold with host blood.[11]

Microvasculature is absent in trypsin-exposed scaffolds, possibly complicating subsequent efforts to maintain viability of reseeded cells and limiting feasibility of reperfusion or graft integration upon implantation.[9, 16]

3.4.7 Cytocompatibility and Cardiomyocyte Cell Seeding:

Several groups have already established DCELLeed MYO ECM as an acceptable substrate for supporting reseeded cells.[5, 6, 9-11, 16] Relevant cardiac cell types seeded onto samples of DCELLeed MYO ECM from scaffolds in Group 4 attached, maintained a cardiac phenotype, and remained viable for up to 14 days in static culture. These data support the cytocompatibility of our scaffolds and their suitability for maintaining
cardiomyocyte cell phenotype, although more definitive studies must be conducted to substantiate the latter claim.

3.4.8 Analysis of Optimal Decellularization:

The acellular myocardium is an outstanding matrix scaffold with promising tissue engineering applications for in vitro testing, as well as for in vivo regeneration. Our results suggest that a rational design and selection of DCELL methods for myocardium needs to take into account several criteria which serve the intended final application. These criteria were used to comment on some of the positive and negative effects of each of the DCELL reagents we investigated, including EDTA (Table 4).

Our comparative study shows that scaffolds DCELLed with SDS alone exhibit excellent preservation of major and minor ECM components but are not fully DNA-free despite extended perfusion with SDS, which removed all detectable cell proteins. Post-SDS treatments with nucleases may reduce DNA content further and thus provide
acceptable scaffolds for implantation. NaOH, on the other hand, is very effective at removing all traces of DNA but also removes some of the basement membrane components. Currently, there is no definitive evidence to support residual exogenous nucleic acid content as being a critical determinant of the success or failure of implanted scaffolds or constructs.\[27] Producing a DNA-free scaffold is neither the sole, nor necessarily the most important, criterion for determining whether a scaffold is optimal, and it must be weighed against other criteria in judging a scaffold’s suitability for tissue engineering applications. There is evidence, however, that basement membrane proteins play an important role in cell survival, attachment, and interactions with the ECM.\[16, 23-25]

Thus, in selecting a scaffold for regenerative medicine applications, we lend more credence to preservation of native ECM characteristics than we do to absolute removal of DNA. For this reason, our future work will be conducted using scaffolds from Group 4 which have been treated to further remove residual DNA. Other scaffolds we generated are also suitable materials for tissue engineering. Some may see merit in using them, especially since their lower DNA content may assuage fears of immunogenicity or other unforeseen negative effects. Ongoing studies focus on cell-seeding and preconditioning of constructs made from our scaffolds, and on implantation studies to assess biocompatibility and feasibility of implantation techniques.

3.5 Conclusions:

To the best of our knowledge, this is the first study which has carried out a comprehensive, parallel analysis of both the vascular and myocardial matrices in a
myocardial flap containing intact coronary arteries, veins, and associated microvasculature. DCELL of such complex tissues was challenging and required a comparative analysis of detergent versus alkaline DCELL methods. Our scaffolds are porous, retain collagen and elastin content and structure, exhibit excellent mechanical properties, and are cytocompatible. Moreover, scaffolds retain laminin, fibronectin, and collagen IV. We’ve also preserved the vasculature’s integrity and patency down to the capillary scale, as shown by several assays. Together with our demonstration of cytocompatibility and support of cardiomyocyte survival, these results indicate that our scaffold is ideally suited for repopulation with relevant cardiac cell types as a tissue-engineered construct for myocardial repair. Given its composition, intact vasculature tree, mechanical properties, and geometry, we believe constructs produced from this scaffold have the potential to functionally integrate with healthy host myocardium and to be nourished by direct anastomotic connection with the host’s own vasculature. We also believe such constructs may be useful as physiologically accurate models for in vitro studies of cardiac physiology and pathology.

3.6 Updates to These Methods:

It is noteworthy that our process of tissue preparation and DCELL changed from those depicted in Figure 3.9 since these studies were conducted. To make preparation of the tissue for DCELL less labor-intensive, we elected to decellularize the entire porcine heart via retrograde perfusion of the aorta to generate scaffolds for use in future studies (Figure 3.10C). We designed a new vessel to accommodate an entire porcine heart using
SolidWorks software, and machined three of these containers with the help of Clemson University Machining and Technical Services (Figure 3.10B and D). The critical point of this method involved preventing the outflow of solutions from the heart at too high of a rate. It was necessary to maintain pressure within the left ventricle so that the main path travelled by solutions was into the coronary arteries, through the microvasculature, and out through the veins. As such, the pulmonary veins at the posterior of the heart needed to be closed to prevent solutions from preferentially flowing rapidly through the left ventricle, into the left atrium, and out of these severed veins. We sealed the veins with suture or a plugged cannula secured with zip-ties (in the case of large openings).

Upon achieving complete DCELL of the organ, regions of interest were dissected and used for experiments in Aims 2, 3, and 4. The reagents used and the sequence in which they were used did not change, however, and characteristics of scaffolds generated with this new method were identical to those presented here (data not shown).

3.7 References:


CHAPTER FOUR: EVALUATION OF THE HOST RESPONSE TO IMPLANTED MYOCARDIAL SCAFFOLDS

4.1 Introduction:

In myocardial tissue engineering, the optimal therapeutic approach is one of delivering a graft containing functional, autologous CM that will integrate electrically and mechanically with existing healthy myocardium. Delivery of a sufficient number of new CM to replace CM lost in an MI requires a thick graft, nourished by an organized vasculature. The optimal scaffold must not only support and nourish the replacement CM, it must also resist significant degradation by the host until these CM can integrate with native CM and until the graft’s vasculature can form connections with native vasculature of the host. The important questions then become 1) how long do these processes take, and 2) what is an acceptable degree of degradation?

Since our scaffold is composed of ECM, which is normally in a constant state of turnover via degradation and synthesis, it is expected that some degree of remodeling will occur in our implanted grafts. Over time, host CM, endothelial cells, vascular smooth muscle cells, and especially macrophages and cardiac fibroblasts will likely infiltrate the myocardial grafts and degrade the scaffold ECM while replacing it with endogenous, fully remodeled ECM that incorporates the delivered cells into the tissue. Groups who have implanted ECM or other biologic scaffold-based grafts in the myocardium have observed cell and vascular integration as early as 4 days and consistently at 4 weeks and beyond.[1-6] Despite the rapidity with which integration can occur, scaffolds should not be considered
dispensable before the 3-week period encompassing the acute and subacute host responses has elapsed.\cite{7, 8} Furthermore, in light of the thickness of the grafts and the potentially inhospitable environment into which they will be placed, the scaffolds should remain largely intact for up to 8 weeks to ensure that their constituent cells have ample time to integrate with host tissue and vascular supply.

To learn more about the possible host response to our scaffolds, we implanted them in an in vivo model that represented a very active immune site, so that if the material was immunogenic, we would be certain to observe a response. The model, subdermal implantation in juvenile rats, does so because the integumentary system boasts a strong presence of immune cells, including Langerhans cells and dendritic cells, and because the adaptive immune system in these juvenile animals is sufficiently developed and, overall, vigorous.\cite{9}

### 4.2 Biocompatibility Study 1:

#### 4.2.1 Methods:

**4.2.1.1 In Vitro Cytotoxicity Assessment:**

The indirect cytotoxicity of scaffolds was evaluated according to methods adapted from ISO standard 10993-5. Possible extractables were eluted from high density polyethylene (HDPE; negative control) and scaffolds via incubation of (n=6), 5 x 5 mm samples of each material in culture media (DMEM with 10% FBS) at 37 °C for 7 days. NIH 3T3 fibroblasts were then seeded into two 24-well plates at 50,000 cells/well (n=3 wells/condition) and cultured for 3 days in standard media prior to being cultured with
extraction media containing possible cytotoxic eluates for 7 days. Upon 7 days of culture with extraction media, cell viability was examined using a tetrazolium-based MTS assay (Promega) and a Live/Dead® assay (Invitrogen). In each assay, measurements collected from the cells exposed to extracts were compared to those of cells cultured in either negative (normal media) or positive (70% ethanol) conditions to quantify the extent of cytotoxicity.

4.2.1.2 Scaffold Sample Preparation for Implantation:

DCELLed scaffolds were prepared according to the original method of dissection and DCELL as described in Chapter 3. Following DCELL, a 10 mm-diameter biopsy punch was used to cut pieces of scaffold, and the resulting cylinders were cut in half to create the final samples used for implantation. Prior to implantation, the samples were sterilized by immersion and gentle agitation in 0.1% (w/v) peracetic acid in PBS for 1 h, followed by rinsing in 4 changes of sterile PBS. Peracetic acid has been demonstrated to be an effective antibacterial and antimicrobial agent by means of oxidation, and has been used successfully in our lab as a non-degradative wet sterilization method.[10-12]

4.2.1.3 Subdermal Implantation Procedure:

In accordance with an approved animal use protocol, 16 juvenile Sprague-Dawley rats were anesthetized with buprenorphine at 0.03-0.05 mg/kg and acepromazine at 0.5 mg/kg administered subcutaneously. A small incision was made in the center of the dorsal area of each rat about 2 cm inferior to the scapulae, and, using blunt dissection, two pockets
were created between the dermis and fascia lateral to the incision (one pocket in each direction). A scaffold sample was placed into each pocket, and the incision was closed using staples. Rats were allowed to recover and were housed individually for the remainder of the study.

4.2.1.4 Explantation and Histological Evaluation:

At both 4 and 8 weeks post implantation, \( n=8 \) rats were euthanized via CO\(_2\) gas (Euthanex system) followed by bilateral pneumothorax. Scaffold samples and small amounts of their adjacent host tissues were excised and fixed in 10\% neutral buffered formalin and processed for histological examination. Paraffin-embedded samples were sectioned at 5 \( \mu \)m and stained by H&E and immunohistochemistry (IHC) for CD68, a marker for monocytes/macrophages, and CD8, a marker for cytotoxic T-lymphocytes.

4.2.2 Results:

4.2.2.1 In Vitro Cytotoxicity Assessment:

Preliminary testing of the scaffolds revealed no evidence that they contained cytotoxic leachables. Two methods, Live/Dead staining (Figure 4.1A) and an MTS assay (Figure 4.1B) were used to measure cytotoxicity. Results were reported as comparisons to measured values from fibroblasts grown in normal cell culture media. Neither assay demonstrated a significant difference in viability between cells exposed to extract media from scaffolds and cells grown with normal media. The assays were proven to be effective.
in measuring cytotoxicity by the inclusion of both a positive and negative control in the experimental design. The positive control resulted in a great increase in cell death, and the negative control saw no significant deviation from the normal control in the Live/Dead assay. Rather unexpectedly, cells from the negative control displayed a significant reduction (40%) in cell viability compared to normal cells as measured by MTS assay, although this reduction was not to the extent observed with the positive control (60%).
**Figure 4.1: In vitro cytotoxicity assessment:** Live/Dead® A) and MTS® B) assays for cytotoxic effects of myocardial scaffolds. In each assay, the reported value for each group is relative to the measured value for NIH 3T3 fibroblasts under standard culture conditions (i.e. a value of 1 in A and 100% in B; *: indicates statistical significance from control, p<0.0001 in A and B; **: indicates statistical significance from control, p=0.0005 in B).

4.2.2.2 Explantation and Histological Evaluation:

DCELLed myocardial scaffold samples implanted in rats were almost completely degraded at 8 weeks post-implantation. In a few cases (2 of 8 rats) at this time point, it was not possible to macroscopically identify any remaining scaffold upon explantation. H&E staining, as well as IHC for CD8 and CD68, revealed the presence of large populations of immune cells, including macrophages and lymphocytes, inside the scaffolds and at the implant-host tissue interface. This was consistent in histological sections of explants from both time points, seemingly indicative of a chronic inflammatory response (Figure 4.2). It was also noted that a significant number of cells in the IHC sections did not stain positively for either marker, indicating that they were not macrophages, monocytes, or cytotoxic T-lymphocytes.
Figure 4.2: Histological analysis of explanted scaffolds from Biocompatibility Study 1: Representative images of H&E staining of decellularized myocardial scaffolds following subdermal implantation in juvenile rats for 4 weeks show heavy infiltration of leukocytes within and around the scaffolds (S); dashed black line denotes scaffold-host tissue interface. Representative images of IHC for CD8 and CD68 show T-cells and monocytes and/or macrophages, respectively, are present within the scaffolds. Representative images of H&E staining of explanted scaffolds at 8 weeks post-implantation show persistent and, in some cases, increased presence of leukocytes within and around scaffolds in comparison to scaffolds explanted at 4 weeks. The amount of identifiable scaffold in each sample explanted at 8 weeks was also greatly diminished with respect to that of samples explanted at 4 weeks (in some cases scaffolds were not identifiable at all).
4.2.3 Discussion:

In our experience with implanted scaffolds, we had not observed an inflammatory response of this magnitude. Furthermore, as discussed previously in this document, the expected response to an acellular scaffold should not involve an aggressive or protracted immune response. We hypothesized that this response could have been due to insufficient rinsing of the scaffold prior to implantation (instead of perfusing wash solutions through the scaffold as was done with DCELL solutions, scaffolds were simply immersed in wash solutions with agitation on an orbital shaker), and therefore to the presence of residual SDS from DCELL or peracetic acid from sterilization methods.

To test for residual SDS, we retroactively performed an assay to quantify the SDS concentration (described in further detail in methods section for Biocompatibility Study 2) in lysates of leftover, unimplanted scaffolds. It was found that the SDS concentration in the pre-implanted scaffolds was $2.14 \pm 1.17 \mu g/cm^3$ tissue. In searching for some context as to whether this concentration was sufficient to induce cytotoxicity, we came across a study which focused upon determining whether SDS used to decellularize pulmonary valves could be removed from the tissues, and correlated residual SDS concentrations with cytotoxicity and metabolic activity as measured by lactate dehydrogenase (LDH) and MTS assays, respectively. The authors found that after 5 wash cycles with PBS, they could reduce the SDS concentration to under 50 µg/mL, and that this concentration did not result in any cytotoxicity or reduction in metabolic activity. Yet another study cited the threshold toxicity concentration (defined as a 10% decrease in cell viability) of SDS as 0.047 mM, or roughly 13.6 µg/mL (in this case, specific gravity dictates 1 mL=1 cm³). Given that
the SDS concentration within our scaffolds was nearly 25-fold and 6-fold less these figures, respectively, we ruled out residual SDS as the cause of any heightened immune response.

Similarly, previous studies conducted by our lab involving peracetic acid sterilization of implanted scaffolds did not result in any negative outcomes associated with immune or inflammatory responses.\textsuperscript{[11]} In these cases, simple repetitive washing of the scaffolds with PBS on an orbital shaker (identical to the method used here) was sufficient to remove peracetic acid.

In the absence of any other obvious explanation for the response we observed, it was worth delving deeper into the results of similar studies, both internal and external to our lab. Previous scaffolds we implanted were not only generated from vastly different tissues (thus, of different structure and composition), but were also either fixed with PGG or seeded with ADSC, both of which have been shown to mitigate the immune response.\textsuperscript{[13, 14, 18]}

Our conventional thinking about host responses to implants has been informed by observations of responses to non-biological, and thus, less bioactive materials. It is narrow-minded to assume that the host response to a material composed of ECM proteins should proceed in the same manner. Instead, the host uses the only real means at its disposal to respond to the presence of this material – leukocytes (e.g. lymphocytes, monocytes, and macrophages). Together, these cells dictate the nature of the response to the material and direct its remodeling through mechanisms traditionally associated with inflammation.\textsuperscript{[19, 20]}

One could argue that fibroblasts also play a role in the host response to biologic scaffolds because they are capable of both synthesizing and degrading (via secretion of
matrix metalloproteases; MMPs) ECM components.\textsuperscript{[21]} Their synthesis of MMPs, however, is largely mediated by signaling from TNF-\(\alpha\) and TGF-\(\beta1\) – cytokines produced by leukocytes, so it is unlikely that fibroblasts could direct the remodeling or degradation of a biological scaffold on their own.\textsuperscript{[22]}

In addition, there is further evidence in the literature to suggest that what we viewed as an aggressive inflammatory response was simply a normal response to a non-crosslinked biologic scaffold. While our previous studies with PGG-treated scaffolds showed limited involvement of macrophages in the host response, several studies conducted by the Badylak group demonstrated a prominent role of various subsets of macrophages, neutrophils, and monocytes in the response to implantation of non-chemically modified scaffolds.\textsuperscript{[20, 23-25]}

It has been established that macrophages are possessed of significant plasticity, changing, or “polarizing”, to fit the conditions of the local environment. While there are multiple phenotypes (M1, M2a, M2b, and M2c), polarization generally refers to the polar extremes of either an M1 (classically activated, proinflammatory) or M2 (alternatively activated, immunomodulatory and remodeling) phenotype.\textsuperscript{[23, 24, 26]} Enough is known about these two distinct populations that they can be identified by certain markers: inducible nitric oxide synthase (iNOS), CD80, and CCR7 for M1, and CD206, CD163, and Fizz1/Ym for M2.\textsuperscript{[23, 27]} In general, it has been observed that desirable outcomes with implanted biological scaffolds (i.e. remodeling into appropriate, functional tissue as opposed to scar tissue formation) correlate with a shift from the M1 to the M2 polarity as the host response
With this in mind, it became of interest to identify the types of macrophages present within the implant site at different time points.

An additional observation from this first study was that not all cells which had infiltrated the scaffolds stained positively for markers of monocytes, macrophages, and lymphocytes. This is not surprising given that we have previously found that other types of scaffolds (DCELLed porcine carotid arteries and DCELLed porcine pericardium) implanted in this same model attracted cell infiltrates that stained positive for vimentin, prolyl-4-hydroxylase, and alpha-smooth muscle actin in addition to macrophages.[13, 14]

In light of the questions surrounding the outcomes of this study, we planned a subsequent study to investigate means to forestall complete degradation of the scaffold and to more closely examine the identities, functions, and origins of cells at the implant site in order to gain a better understanding of the host response and the ultimate implant fate.

4.3 Aims for Biocompatibility Study 2:

In the eventual clinical adaptation of this approach, we believe that extending the in vivo lifespan of the scaffold so as to partly preserve it up to 12 weeks or beyond will lead to more favorable outcomes with respect to myocardial graft integration and incorporation of constituent cells. To test if this was possible, we employed penta-galloyl glucose (PGG), an ECM protein-binding, plant-derived polyphenol, as a cross-linking agent to impart the scaffolds with greater resistance to degradation by the host. Indeed, previous studies conducted by our laboratory demonstrated that treatment of scaffolds with PGG protected them not only from excessive matrix metalloprotease-driven degradation,
but also prevented calcification while mitigating the severity of the immune response (Figure 4.3).\textsuperscript{15}

\textbf{Figure 4.3: Effects of PGG as assessed by histology and cytokine array:} Histology and IHC performed on decellularized carotid artery scaffolds following subdermal implantation in rats for 4 weeks. Scaffolds treated with PGG show decreased susceptibility to calcification and deposition of osteogenic proteins in comparison to scaffolds not treated with PGG (nontreated), as reflected by alizarin red staining for calcium (Azn Red), and IHC for osteopontin (OPN) and alkaline phosphatase (ALP). PGG-treated scaffolds also displayed immune-modulatory characteristics, as fewer CD68 positive cells were identified within them than in nontreated scaffolds (as assessed by IHC), and less TNF-α was observed in PGG-treated scaffolds in a cytokine array (graph).
We expected that myocardial scaffolds treated with PGG would demonstrate improved resistance to degradation over non-treated scaffolds, and the degradation profile of PGG-treated scaffolds would be sufficiently gradual to allow regenerative cells delivered within them in the future to integrate with host cells and provide functional benefits. If at least a portion of the scaffold remained at 8 weeks post-implantation, this expected outcome was considered met.

This study was also intended to analyze the host biological response to the implanted myocardial scaffold, specifically in terms of macrophage polarization. The results allowed us to determine whether this biomaterial elicited a classical inflammatory response or a more constructive, remodeling response. Given PGG’s purported anti-inflammatory properties, we expected to observe a significant reduction of inflammatory cells within and around the implant site of PGG-treated scaffolds as compared to non-treated scaffolds. Similarly, we expected to observe a greater ratio of M2 to M1 macrophages and greater recruitment of stem cells, indicative of a more regenerative response, in the PGG-treated scaffolds.

Cellular infiltration, vascularity, and collagen content were analyzed in order to evaluate the structure of the tissue that was formed as a result of the host response. We also intended to determine the phenotype of the previously unidentified cells by staining for common markers of fibroblasts, smooth muscle cells and mesenchymal and hematopoietic stem cells, which could be recruited to the site in order to remodel the scaffold.
4.4 Biocompatibility Study 2:

4.4.1 Methods:

4.4.1.1 Quality Control Characterization of Scaffolds: DNA Quantification, SDS Quantification, and alpha-1, 3-galactose Screening:

4.4.1.1.1 DNA Quantification:

Picogreen and Oligreen assays for double-stranded DNA (dsDNA) and single-stranded (ssDNA), respectively, were carried out on (n=6) samples from randomly selected areas of the DCELLed scaffolds used in this implantation study. Briefly, samples were completely digested in 1 mL of papain digestion buffer and run against a standard curve generated from known concentrations of a supplied DNA standard in order to determine the DNA content of each sample.

4.4.1.1.2 SDS Quantification:

In addition to acellularity, it was also important to demonstrate the removal of SDS from scaffolds following DCELL. Residual SDS in an implanted scaffold could be cytotoxic to the immediate host cells and elicit an aggressive inflammatory response if its concentration was sufficiently high and/or persistent. The same digested scaffold samples (n=6) described above in DNA quantification methods were also used in this assay. The assay was derived from a previously published method and designed around a phase separation principle.[28] It involved an aqueous phase comprised of slightly alkaline PBS (pH=8.0) and toluidine blue dye, as well as a chloroform organic phase, and the two phases were combined in a single test tube. A small volume (5-10 µL) of digested scaffold was
added to the aqueous phase, the two phases were mixed by inversion, and the mixture was incubated at room temperature. The critical mechanism of the assay was the binding of toluidine blue to SDS. This complex was able to traverse the interface between the two phases due to the amphiphilic nature of SDS. The SDS-toluidine blue complex then collected in the chloroform phase, and a volume of this phase was pipetted into a 96-well plate for spectrophotometric analysis of absorption at 600 nm. The optical density of each sample of unknown SDS concentration was compared against a standard curve of samples of known SDS concentration, and the concentration of unknown samples was extrapolated (Figure 4.5B).

4.4.1.1.3 Screening for alpha-1, 3-galactose:

The alpha-1, 3-galactose, or alpha-gal, carbohydrate is found in all non-primate mammals, and therefore triggers an aggressive immune response when introduced into humans. This is particularly concerning given that porcine tissue contains this antigen, which must be removed from scaffolds intended for xenogeneic implantation in humans, lest an immune response lead to implant rejection and failure.\cite{29} Since alpha-gal is normally occurring in rats, its presence in the scaffold would not be detrimental to this particular implantation study. However, with implantation in humans as a future goal for this scaffold material, it was prudent to demonstrate the removal of alpha-gal from the tissue as a result of the DCELL process.

Removal of alpha-gal was demonstrated using standard immunohistochemistry techniques. Instead of a primary IgG or IgM antibody, biotinylated isolectin B4 generated
in the griffonia simplicifolia shrub was used to label alpha-gal. Avidin/biotinylated enzyme complex (ABC; Vector Labs.) with horseradish peroxidase was applied to tissue sections, followed by the enzyme substrate, 3, 3’-diaminobenzidine (DAB). As a result of this detection system, brown color was developed wherever alpha-gal was located.

4.4.1.2 Scaffold Preparation and PGG fixation:

Scaffolds were prepared by the method of whole heart DCELL and individual samples were prepared as previously described. Scaffolds were exposed to differing concentrations of PGG (Omnichem Ajinomoto, Belgium), 0.15% and 0.3% w/v in 50 mM phosphate buffer, pH=5.5, containing 20% isopropanol for 24 h, or were left untreated as a control. Scaffolds were then rinsed with PBS and sterilized for implantation as described above in the first implantation study.

4.4.1.3 Subdermal Implantation in Rats:

Forty-five male, juvenile Sprague-Dawley rats were randomly assigned between three groups: 1) non PGG-treated (NT), 2) 0.15% PGG-treated, 3) or 0.3% PGG-treated (n=5 rats/group/time point). The corresponding scaffolds were subdermally implanted into each rat. Anesthesia, surgical procedure, implantation (2 scaffolds/rat), closure, and recovery proceeded in the same manner as previously described.
4.4.1.4 Explantation and Semi-quantitative Histological Analysis:

At 4, 8, and 12 weeks post-implantation, animals were euthanized as previously described, and the scaffolds and immediate surrounding tissue were excised and examined histologically to characterize the host response, as well as the rate and extent of degradation. Following standard formalin-fixation and paraffin embedding, 5 µm-thick sections were prepared and stained via H&E and Masson’s trichrome methods. Sections were imaged using an inverted Zeiss Axiovert microscope and accompanying AxioVision software. Micrographs were obtained of high powered fields (400X magnification) at the scaffold/host tissue interface (10 images/section; n=30 images/condition). Characteristics of the local host response, such as cellularity (cell infiltration), foreign body/multinucleated giant cell proliferation, vascularity, and collagen content were analyzed semiquantitatively using ImageJ software (NIH) to quantify these components of interest in the field of view) (Figure 4.7).

Macrophage polarization was assessed by immunofluorescence double-staining of formalin-fixed, paraffin-embedded, 5 µm-thick section. Sections were deparaffinized and heat mediated antigen retrieval with 10 mM citric acid at pH=6.0 was performed. Primary antibodies for known markers of M1 and M2 macrophages, C-C chemokine receptor type 7 (CCR7, Cell Applications; 1:250 dilution) and CD206 (Santa Cruz Biotechnology; 10 µg/mL), respectively, were applied and sections incubated overnight at 4 °C. Nuclei were counter-stained with DAPI. Sections were imaged using an inverted Zeiss Axiovert microscope equipped with mercury lamp, and accompanying AxioVision software. Micrographs were obtained of high powered fields (400X magnification) at both the
interior of the scaffold (3 images/condition) and the scaffold-host tissue interface (3 images/condition). ImageJ software was once again used to process the images and count numbers of positively-stained cells for each marker (Figure 4.10). Resulting data was used to compare the response in different groups and time points.

4.4.1.5 Statistical Analysis:

Wherever possible, analysis of variance (ANOVA) was performed to identify differences in population means and to test for significant effects of overall factors (i.e. time point and treatment). Post-hoc comparisons between means of individual conditions (treatment and time point) were performed using Fisher’s least significant difference test (LSD). In all tests, a p-value of 0.05 or less had to be calculated to attain statistical significance.

4.4.2 Results:

4.4.2.1 DNA Quantification:

PicoGreen and OligGreen assays of pre-implanted scaffold lysates displayed approximately 92% and 84% reductions in dsDNA and ssDNA from native levels, respectively (Figure 4.4, p<0.0001 in both cases).
**Figure 4.4: Screening of myocardial scaffolds for DNA content:** Results of PicoGreen® and OliGreen® quantitative assays for double-stranded (dsDNA) and single-stranded (ssDNA) DNA, respectively, in samples of native porcine myocardium and myocardial scaffolds prior to implantation (*: indicates statistical significance from native, p<0.0001).

**4.4.2.2 SDS Quantification:**

As shown in Figure 4.5A, the residual SDS content of scaffolds at different steps of the washing process following complete removal of cells was determined. SDS concentration is reduced significantly to innocuous levels following the first wash step and remains stable for the duration of the washing procedure. While the measured value in the final step in Figure 4.5A is very near zero, these were not the data from the samples used in the actual study; they only serve as a demonstration that SDS can be removed from scaffolds. SDS concentration within scaffolds used for Biocompatibility Study 2 was
found to be 2.402 +/- 0.867 µg/cm³ tissue, which was not significantly different from the value measured for Biocompatibility Study 1.

**Figure 4.5: Screening of myocardial scaffolds for SDS content** A) Results of a quantitative assay for SDS content in samples of myocardial scaffolds from each of the stages of rinsing following decellularization with SDS. The rinsing process is reflected by moving from left to right on the ordinate (ddi H2O = distilled,
deionized water, EtOH = ethanol). B) Image of the well plate used in the SDS assay, depicting toluidine blue bound to SDS from the organic phase of test reactions. Upper wells are from scaffold samples, with each successive rinsing step represented from left to right. Lower wells are samples of known SDS concentration, which increased from right to left, used to generate a standard curve.

4.4.2.3 Screening for alpha-1, 3-galactose:

Examination of histological sections of pre-implanted scaffolds stained for the presence of alpha-gal showed that the antigen had been completely removed from the tissue during DCELL (Figure 4.6). This was the case in both the arterial and myocardial matrices of scaffolds, although only samples of the myocardial matrix were used in this study. Sections of explanted scaffolds stained for this antigen displayed diffuse positive staining, indicating that endogenous alpha-gal had been deposited by the infiltrating rat cells (data not shown).

Figure 4.6 (below): Screening of myocardial scaffolds for alpha-1, 3-galactose: A) Micrographs of native A-D) and decellularized E-H) porcine myocardium and constituent coronary artery stained via immunohistochemistry (IHC) for evidence of the alpha-gal epitope, showing its removal as a result of the decellularization process. IHC positive staining = brown; A, B, E, F 10X, bar=100µm; C and D 40X, bar=20µm; G and H 20X, bar=50µm.
4.4.2.4 Semi-quantitative histological analysis of cellularity, foreign body giant cell proliferation, vascularity, and collagen content:

H&E and Masson’s trichrome staining of histological sections of explanted scaffolds provided ideal images for analysis of these characteristics using ImageJ software (Figure 4.7). Individual components of interest were readily identifiable to the naked eye, as well as to the software.

Figure 4.7: Histological evaluation of explanted scaffolds from Biocompatibility Study 2: Micrographs of explanted scaffolds stained with hematoxylin and eosin (H&E) A and B) and Masson’s trichrome C and D). The former show collagen and cytoplasm (pink), nuclei (navy blue), and red blood cells (bright red), while the latter show collagen (royal blue), multinucleated foreign body giant cells (FBGCs) and cytoplasm (red), and nuclei (maroon). ImageJ was used in conjunction with H&E images to count nuclei and blood
vessels (black arrows in B), and with Masson’s images to count FBGCs (black circles in C) and measure the area of collagen within the field of view (All images 40X).

4.4.2.5 Cell infiltration, Figure 4.8A:

ANOVA identified a significant effect of group. PGG-treated groups were not significantly different from one another, but both had significantly fewer infiltrating cells than the NT control (0.15%, 0.3% < NT; p<0.0001). ANOVA also identified a significant effect of time point. Significantly less cell infiltration was observed at 12 weeks than at 4 or 8 weeks, and cell infiltration was not significantly different between 4 and 8 weeks (12 < 4, 8; p<0.0001).

4.4.2.6 Vascularity, Figure 4.8B:

ANOVA identified a significant effect of group. 0.3% PGG-treated and NT groups were not significantly different from one another, but both had significantly more vasculature than the 0.15% PGG group (0.15% < 0.3%, NT; p<0.0001). The amount of vasculature between PGG-treated groups was not significantly different until 12 weeks, with a significant uptick in the 0.3% PGG group. ANOVA also identified a significant effect of time point. Significantly less vasculature was observed at 12 weeks than at 4 or 8 weeks (although this effect does not hold for the 0.3% PGG group, which had significantly more vascularity than the other groups at this time point). Vascularity was not significantly different between 4 and 8 weeks (12 < 4, 8; p=0.0012).
Figure 4.8: Semi-quantitative analysis of histological images of explanted scaffolds from Biocompatibility Study 2: A) Number of nuclei as counted by ImageJ per high powered field (HPF), or each micrograph taken at 40X (400X total) magnification; images were those described in Figure 4.7. Scaffolds were treated with either 0.15% PGG or 0.3% PGG, or not treated (NT) with PGG at all prior to implantation. Scaffolds in each of the groups were explanted at either 4 weeks (4 Wk), 8 weeks (8 Wk), or 12 weeks (12 Wk) post-implantation; #: indicates statistical significance from all other groups except one another and 0.15% PGG, 4 Wk, p<0.0001; *: indicates statistical significance between specified groups, p=0.0008. ANOVA indicated there was a statistically significant effect of treatment; cell infiltration in 0.15% PGG and 0.3% PGG < NT, p<0.0001. ANOVA indicated there was a statistically significant effect of time point; cell infiltration in 12 Wk < 4 Wk and 8 Wk, p<0.0001. B)
Number of blood vessels as counted manually per HPF; images were those described in Figure 4.7. Explanted scaffolds were grouped according to the same conditions as described in A; #: indicates statistical significance from all other groups except one another and 0.3% PGG, 4Wk and 12 Wk, p<0.0001; *: indicates statistical significance between specified groups, p=0.0006; **: indicates statistical significance between specified groups, p<0.0001. ANOVA indicated there was a statistically significant effect of treatment; vascularity in 0.15% PGG < 0.3% PGG and NT, p<0.0001. ANOVA indicated there was a statistically significant effect of time point; vascularity in 12 Wk < 4 Wk and 8 Wk, p=0.0012.

4.4.2.7 FBGC count, Figure 4.9A:

ANOVA did not identify a significant effect of group (p=0.1725). There were no significant differences between groups within the same time point. 0.3% PGG and 0.15% PGG groups were the closest to being significantly different from one another (p=0.1507). ANOVA identified a significant effect of time point. Fewer FBGC were observed at each successive time point in every group (4 > 8 > 12; p < 0.0001). While there appeared to be a trend of decreasing FBGC presence in the NT group, none of the means at different time points within this group were significantly different. In contrast, both of the PGG-treated groups experienced a significant decrease in FBGC presence between 4 weeks and 12 weeks.

4.4.2.8 Collagen Content, Figure 4.9B:

ANOVA identified a significant effect of group. Scaffolds from PGG-treated groups contained significantly more collagen than the NT group, and the 0.15% group contained significantly more collagen than the 0.3% group (0.15% > 0.3% > NT;
p<0.0001). ANOVA also identified a significant effect of time point. A significant increase in collagen content was observed at each successive time point in every group (4 < 8 < 12; p < 0.0001).

**Figure 4.9:** Semi-quantitative analysis of histological images of explanted scaffolds from Biocompatiblity Study 2: A) Number of foreign body giant cells (FBGC) as counted manually per high powered field (HPF), or each micrograph taken at 40X (400X total) magnification; images were those described in Figure 4.7. Scaffolds were treated with either 0.15% PGG or 0.3% PGG, or not treated (NT) with PGG at all prior to implantation. Scaffolds in each of the groups were explanted at either 4 weeks (4 Wk), 8 weeks (8 Wk), or 12 weeks (12 Wk) post-implantation; *: indicates statistical
significant between specified groups, p≤0.0214 in all cases. ANOVA indicated there was no statistically significant effect of treatment, p=0.1725. ANOVA indicated there was a statistically significant effect of time point; FBGC number in 12 Wk > 8 Wk > 4 Wk; p < 0.0001. B) Collagen content per HPF as measured by ImageJ; images were those described in Figure 4.7. Explanted scaffolds were grouped according to the same conditions as described in A; *: indicates statistical significance between specified groups, p≤0.0481 in all cases (not all significant differences are shown). ANOVA indicated there was a statistically significant effect of treatment; collagen content in 0.15% PGG > 0.3% PGG > NT, p<0.0001. ANOVA indicated there was a statistically significant effect of time point; collagen content in 4 Wk < 8 Wk < 12 Wk, p<0.0001.

Figure 4.10: Evaluation of explanted scaffolds from Biocompatibility Study 2 by immunofluorescence:
Micrographs of explanted scaffolds stained with antibodies to CD206 (green) and CCR7 (red); nuclei were
counterstained with DAPI (blue). ImageJ software was used to count cells that stained positively for each protein, and data were analyzed to characterize the nature of the host response to scaffolds.

4.4.2.9 Semi-quantitative histological analysis of macrophage polarization:

The ratio of cells which stained positively for CD206 to cells that stained positively for CCR7 in areas within the scaffold is shown in Figure 4.11A. Initially, at 4 weeks after implantation the macrophage population overwhelmingly favored polarization toward an M1 phenotype, but the ratio reverted significantly back in favor of M2 macrophages as implantation time increased. The ratio overwhelmingly favored M2 macrophages at later time points in both PGG-treated groups, but did not change significantly over time in the NT group (although it did favor M2 macrophages and did show a trend of increasing M2 polarization). ANOVA to test for overall effects was not possible because small sample size did not provide sufficient degrees of freedom.

The ratio of cells which stained positively for CD206 to cells that stained positively for CCR7 in areas immediately surrounding the scaffold is shown in Figure 4.11B. At 4 weeks post-implantation, the NT group already showed that much of the macrophage population consisted of M2 macrophages, while macrophages in the PGG-treated groups tended be split between the two phenotypes or slightly favored the M1 phenotype. Over time, just as they did at the interior of the scaffold, the macrophages in the PGG-treated groups shifted drastically toward an M2 phenotype. In the NT group, the ratio remained in favor of M2 but decreased significantly at the 8 and 12 week time points. ANOVA to test for overall effects was not possible because small sample size did not provide sufficient degrees of freedom.
The percentage of total macrophages (CD206+ and CCR7+ cells) which stained positively for CD206 within the scaffold is shown in Figure 4.12A. There was an overall trend of increasing M2 percentage with time regardless of treatment with PGG. The NT group had a slightly greater percentage of M2 macrophages than the PGG-treated groups at 4 weeks, but this was only weakly significant (p<0.0845). As seen in Figure 4.12B, this same trend applied to cells at the scaffold-host tissue interface. In fact, in NT scaffolds M2 macrophages predominated throughout all time points, whereas PGG-treated scaffolds displayed a dramatic M1 to M2 shift in between 4 and 8 weeks post-implantation.

Figure 4.11 (below): **Semi-quantitative analysis of immunofluorescence images of explanted scaffolds from Biocompatibility Study 2**: A) Ratio of CD206+ (M2) macrophages to CCR7 (M1) macrophages at the interior of scaffolds per high powered field (HPF), or each micrograph taken at 40X (400X total) magnification, as counted by ImageJ; images were those described in Figure 4.10. Scaffolds were treated with either 0.15% PGG or 0.3% PGG, or not treated (NT) with PGG at all prior to implantation. Scaffolds in each of the groups were explanted at either 4 weeks (4 Wk), 8 weeks (8 Wk), or 12 weeks (12 Wk) post-implantation; *: indicates statistical significance from all other groups, p<0.0003; **: indicates statistical significance from all other groups except for 0.15% PGG at 8 weeks, p<0.0001; #: indicates statistical significance from other groups at same time point, p<0.0029. B) Same ratio as described above, but determined from cells observed in tissues immediately surrounding the scaffolds; images were those described in Figure 4.10. Explanted scaffolds were grouped according to the same conditions as described in A; *: indicates statistical significance from all other groups except for 0.15% PGG at 8 and 12 weeks, p<0.0001; **: indicates statistical significance from all other groups except for 0.15% PGG at 12 weeks, 0.3% PGG at 12 weeks, and NT at 4 weeks, p=0.0002; #: indicates statistical significance from other groups at same time point, p<0.0029.
A. CD206+ (M2) Macrophages at Scaffold Interior

B. CD206+ (M2) Macrophages at Scaffold-Host Tissue Interface
Figure 4.12: Semi-quantitative analysis of immunofluorescence images of explanted scaffolds from Biocompatibility Study 2: A) Percentage of total macrophages positive for CD206+ (M2 macrophage marker) in each image of the interior of scaffolds as counted by ImageJ; the remainder were positive for CCR7 (M1 macrophage marker). Images were high powered fields (HPF), or micrographs taken at 40X (400X total) magnification; images were those described in Figure 4.10. Scaffolds were treated with either 0.15% PGG or 0.3% PGG, or not treated (NT) with PGG at all prior to implantation. Scaffolds in each of the groups were explanted at either 4 weeks (4 Wk), 8 weeks (8 Wk), or 12 weeks (12 Wk) post-implantation; $: indicates weak statistical significance from other groups at same time point, p<0.0845; #: indicates statistical significance from other groups at same time point, p<0.0061; *: indicates statistical significance between specified groups, p=0.0164; **: indicates statistical significance between specified groups, p=0.0168. B) Same percentage as described above, but determined from cells observed in tissues immediately surrounding the scaffolds; images were those described in Figure 4.10. Explanted scaffolds were grouped according to the same conditions as described in A; #: indicates statistical significance from other groups at same time point, p<0.0001; ##: indicates statistical significance between specified groups, p<0.0352; *: indicates statistical significance between specified groups, p<0.04; **: indicates statistical significance between specified groups, p=0.0003.

4.4.3 Discussion:

4.4.3.1 General Observations:

In summary, PGG treatment decreased cell infiltration as compared to NT scaffolds, and cell infiltration decreased over time among all treatment groups. Vascularity appeared to correlate almost exactly with this trend. FBGC presence decreased significantly over time in the PGG-treated groups, and displayed a trend of doing so in the NT group. Collagen content significantly increased over time in all groups, and PGG
treatment significantly increased the cumulative amount of collagen formed at the implant site with respect to the NT group.

It was clear that PGG-treated scaffolds degraded less over time, and that treatment of scaffolds with higher concentrations of PGG exaggerated this effect. This was not only evident macroscopically at explantation, but also microscopically in sections of the NT scaffold, as scaffold remnants were considerably more difficult to identify in that group than in the PGG-treated groups. Scaffold remnants were readily identifiable in PGG groups, even at 12 weeks after implantation. This was not surprising given that our group previously showed PGG treatment of collagenous scaffolds decreased the activity of MMPs when in implanted scaffolds, and demonstrated PGG treatment reduced the susceptibility of collagen and elastin to collagenase and elastase degradation.[11, 18]

Despite the trend of decreasing vascularization in scaffolds over time, closer histological inspection of all groups at 12 weeks post-implantation seemed to indicate a great number of vessels within and around the implant site. Numbers of vessels in these areas also seemed to be increased relative to the distal, surrounding dermis, although we collected no quantitative data in support of this observation. Along with an angiogenic effect, the implant site was also rich with collagen. We did not stain for collagen type III or prolyl-4-hydroxylase, so we cannot claim this was newly synthesized collagen. There was, however, a noticeable, localized difference in shades of blue and pink staining in Masson’s and H&E stains, respectively. This seemed to suggest a difference in the structure, and therefore, in both type and age of these areas of collagen.
From previous studies conducted in our lab, we know PGG slows the degradation of scaffolds, presumably through the physical action of crosslinking ECM proteins; but also by modulating the activity of immune cells. The observation that neovascularization and angiogenesis in the implant area was reduced with PGG treatment seemed to correlate with the observed reduction in the number of infiltrating leukocytes, as they likely arrived at the site via the vasculature.

FBGC presence was not significantly different between the groups, but it was interesting to see their numbers decline significantly over time, especially in the PGG treated groups. There was also a slightly greater number of FBGC in the 0.3% PGG group than in the 0.15% PGG group (although not significant). Taken together with the late increase in vascularity observed in the 0.3% PGG group, this might suggest that fixation with a greater concentration of PGG leads to a more chronic inflammatory response, or at least a delay in the host’s ability to respond to the presence of the implant.

Increased collagen content with PGG treatment could be reflective of either better, more organized remodeling of the implant or the formation of dense, collagenous scar tissue at the site. Formation of collagenous tissue could also have less to do with scarring and be more the product of appropriate remodeling for the implant site given its proximity to the dermis, which is mostly collagenous. Alternatively, higher collagen content in the PGG-treated groups, especially earlier on, could be the result of scaffolds’ increased resistance to degradation. Thus, the collagen being measured in these groups may not be newly synthesized endogenous collagen, but simply stubborn remnants of the scaffold. This theory is contradicted by the fact that the group treated with a greater concentration
of PGG did not have the greatest collagen content, as one would assume the higher concentration would cause more crosslinking and impart greater resistance to degradation. In addition, most of the scaffold area in histological sections was highly cellularized, so the image analysis method used to measure collagen content would not have recognized these areas of scaffolds as collagenous.

4.4.3.2 Macrophage Polarization:

Macrophages in both the scaffold interior and at the scaffold-host tissue interface displayed a shift in polarization from the M1 phenotype to the M2 phenotype. This effect was less pronounced in the NT group, however, as the population seemed to remain roughly split between the two phenotypes over the course of the study. This shift occurred primarily between 4 and 8 weeks following implantation. After 8 weeks, the macrophage population remained predominantly of the M2 phenotype.

It is difficult to say how these M2 macrophages arrived at the scaffold interior. There were very few M2 macrophages at 4 weeks in the PGG groups’ interface, but their number drastically increased at 8 weeks in the scaffold interior. There were also relatively high numbers of M2 macrophages at 4 weeks in the NT group’s interface, but no drastic increase in M2 macrophages at 8 or 12 weeks in the scaffold interior. Taken together, these observations seemed to indicate that M2 macrophages arrived at the scaffold interior via the many newly formed vessels in the area. However, if one considers that both vascularity and cell infiltration were decreasing over time in all groups, it suggests that M2 macrophages did not simply arrive at the scaffold interior as M2 macrophages, but either
arrived as monocytes and differentiated into M2 macrophages, or existing M1 macrophages in the area shifted to an M2 phenotype. Again, given the decreases in both cellularity and vascularity over time, the latter seemed the more likely scenario.

Several studies investigating the response to implanted biological scaffolds – with or without a cellular component – have associated adverse outcomes (chronic inflammation, loss of implant functionality, and scar tissue formation) with a macrophage population of predominantly M1 phenotype. Conversely, responses that involved macrophage populations shifting from M1 phenotype to predominantly M2 phenotype were correlated with positive, constructive responses (recruitment of stem cells, formation of functional tissue).[20]

While the results of this study indicated that a shift from M1 to M2 phenotype occurred in macrophages at the implant site, it was difficult to make conclusions about the overall outcome based upon the tissue which was formed. Because of the implants’ subdermal location, the highly vascularized collagenous tissue they were transformed into could be appropriate in such a site. On the other hand, it was not clear that this tissue was not granulation or scar tissue. The resultant tissue will need to be studied in more detail to determine the exact matrix constituents, their source, and the identities of cells before conclusions can be made regarding its fitness for the implant site.

There is evidence that chemical cross-linking of biologic scaffolds (via carbodiimides or glutaraldehyde) limit the ability of macrophages to respond to and remodel the material, leading to a pro-inflammatory, M1-dominated response.[20, 24] One study which focused on carbodiimide-crosslinked scaffolds noted an M2 to M1 shift between 2
and 4 weeks post-implantation, and a persistence of this condition out to 16 weeks.[24] Our results with PGG-crosslinked scaffolds in this study contradicted these findings, demonstrating an M1 to M2 shift after 4 weeks of implantation. We have no particular insight as to the cause of this disparity, other than the differing crosslinking mechanisms of the two chemicals, PGG and carbodiimide, must have caused different changes in structure of the scaffolds, leading to differences in the way cells responded to the material.

Additionally, we have shown previously that PGG reduced the infiltration of macrophages into scaffolds composed primarily of collagen when those scaffolds were implanted in the same model used here.[18] This result would seem to support the above claims that chemical crosslinking inhibits macrophage response, but the explanted scaffolds in this case were stained only against CD68, a pan-macrophage marker (not specific for polarization), so we could not comment as to whether this resulted in an M1-dominated macrophage response. Interestingly, PGG treatment did reduce cellular infiltration of scaffolds in the current study, but this did not result in a predominantly M1 response, as the above study involving crosslinking predicted.

Limitations of this study included a lack of staining to identify specific phenotypes of non-monocytes, non-macrophages, and non-lymphocytes; a lack of methods to assess whether endogenous matrix was synthesized and, if so, what specific matrix components were produced. This study also would have benefitted from the use of another method to measure M1 and M2 macrophage presence, such as qRT-PCR to determine gene expression of iNOS (M1) and arginase (M2).
4.4.4 Conclusions:

Studying the host response to biologic scaffolds is as much a matter of curiosity as it is practicality. By virtue of their composition, these materials cannot be “inert”, as the conventional definition of biocompatibility would have us believe is a requisite for safety upon implantation. Instead, they are “bioactive” and can be degraded or transformed to serve whatever purpose the host determines is most beneficial. This response is mediated by cells of the immune system, which normally participate in inflammation and wound healing. Because these cells are traditionally associated with these processes, and because these processes commonly have a negative connotation, it is our first instinct to perceive the responses to biological scaffolds in a negative manner. But, when we understand that these cells constitute the only available apparatus for remodeling or repurposing an implanted material, we can focus on discovering the mechanisms at work behind the immune cell-mediated host response and developing a better understanding of the characteristics of the remodeled tissue. This will serve to better inform our thought processes and opinions on the safety and effectiveness of therapies, particularly tissue engineering therapies, which are predicated upon the use of biological scaffolds.

The studies discussed above were designed to examine the host response to the myocardial scaffolds whose generation was described in Chapter 3. We aimed to gain insight into how and why the scaffold would be remodeled, and to apply this to knowledge to our overall translational approach. In particular, we wanted to know how long the scaffold would persist before it was degraded, and understand what type of local
microenvironment would be presented to cells that might eventually be delivered to damaged hearts using the scaffold as a vehicle.

Our results allowed us to answer these questions in a general sense. We learned that fixation with PGG can extend the lifetime of scaffolds up to, and possible beyond, 12 weeks – presumably a sufficiently long period for delivered cells to integrate with host tissue in future implantations of recellularized scaffolds. We also learned that while the response to this scaffold does involve some elements of the classical inflammatory response (FBGCs), over time the environment becomes less pro-inflammatory as evidenced by the predominance of M2 macrophages. Lastly, although we were unable to thoroughly describe the functionality, or “appropriateness”, of the tissue ultimately formed, it was clear that this scaffold encouraged angiogenesis, which is a very desirable quality for our purposes in generating functional, vascularized muscle.

Moving forward, we will be able to use either PGG-treated or non-treated scaffolds in these therapies, depending upon the desired degradation profile. The response to scaffolds may also prove to be drastically different at the eventual target site, the heart, and when there is an autologous cellular presence within the scaffolds, as would be the case with myocardial grafts.\[30\]

4.5 References:


5.1 Introduction:

In its infancy, Vacanti and Langer described tissue engineering as an interdisciplinary effort to create “functional substitutes for damaged tissue”.[1] As the field grew, more groups continued to develop scaffolds and attempted to repopulate, or “seed”, them with cell types relevant to the tissue being replaced.[1-3] It was soon recognized that, regardless of the source or type of cells used, static seeding and culture methods were insufficient to produce organized, highly populated constructs with well-differentiated and functional cells, and the importance of incorporating extracellular factors such as mechanical, electrical, and biochemical stimuli was realized. In order to mimic the structure and function of native tissue more closely, tissue engineers began seeking a means to allow their constructs to mature, or be “conditioned”, by these extracellular stimuli, and they developed bioreactors for this purpose. Bioreactors are specially designed, self-contained systems which provide for the adjustment and control of specific conditions (e.g. pH, oxygen concentration, nutrient supply, mechanical forces, and electrical impulses) to simulate the dynamic surroundings and processes which support the functions of native tissue.[2, 4]

As critical as delivering the correct stimuli to cells may be, choosing a source of stem cells with the appropriate characteristics may be even more important. Large
quantities of cells are necessary to produce recellularized tissue-engineered grafts, so these cells must be either abundant at the source or proliferative to the extent necessary to repopulate scaffolds at the cell density of native myocardium (200 million cells/cm$^3$).\cite{5} These cells must also be compatible with the recipient’s immune system and be of the proper phenotypes (i.e. CM, cardiac fibroblasts, vascular smooth muscle cells, and endothelial cells) which comprise the myocardium (or possessed of the ability to differentiate into them). In view of these criteria, the cell source of choice should be autologous cells (i.e. from the recipient patient) which are clonally expanded, differentiated ex vivo, and implanted as a component of a tissue-engineered therapy.

Recently, the approach of using IPSC generated from fibroblasts, BM-MSC, or other terminally differentiated adult cells has been popularized. This approach can be used in myocardial tissue engineering strategies as an autologous therapy if the somatic cells used to generate IPSC are harvested from the patient for whom the tissue-engineered graft is intended. This method uses transfection of either retroviral vectors or episomes containing plasmid DNA or other vectors to first dedifferentiate the somatic cells into IPSC.\cite{6}

These cells can then be differentiated into a CM phenotype followed by specialized low attachment/suspension culture techniques to generate embryoid bodies and subsequent treatment with WNT signaling molecules.\cite{7, 8} There are also protocols that use the principle of induced pluripotency, but differentiate somatic cells in to CM in a more direct process, without an intermediate step wherein IPSCs are generated.\cite{9}
A major disadvantage of using iPSCs is that concerns remain over their stability once dedifferentiated, specifically whether the epigenetic reprogramming strategies, either retroviral or molecular, might cause unintended changes in expression or regulation of other genes. This could lead to undesirable consequences once the pluripotent cells are differentiated and implanted, such as tumor formation.\textsuperscript{[10-12]} While they may seem highly conjectural, these concerns could derail progress of any therapies based on iPSCs toward clinical translation.

After consideration of available cell sources and their respective pros and cons, we identified ADSCs as the most ideal fit in view of the criteria presented above. These cells are highly abundant within subcutaneous fat, with about $100-300 \times 10^6$ cells/100-500 g of adipose tissue. More importantly, harvest of this tissue is accomplished through a minimally invasive liposuction procedure, which can generate between 100 mL to 3 L of lipoaspirate per procedure. Processing of lipoaspirate to isolate ADSC is also a well-established process that can be automated and even performed in the operating room.\textsuperscript{[13, 14]}

The ability of ADSC to differentiate into CM, EC, and VSMC lineages has not been widely demonstrated. However, it has been well-established that they can become osteoblasts, chondrocytes, and adipose cells. Given this demonstrated multipotency, it’s not far-fetched to believe they can differentiate into CM, EC, or VSMC if subjected to the right conditions. Additionally, in vitro cultures of ADSC have shown a reduction in major histocompatibility complex expression with progressive passages, possibly suggesting they could be used allogeneically.\textsuperscript{[14]} This mode of use would need to be proven safe in separate clinical trials, as it is possible that expression of these complexes is only suppressed in
vitro, and could return once the cells are transplanted into a new host. Furthermore, a comprehensive post-hoc analysis of their effectiveness in an array of clinical trials for MI treatment was recently conducted, and many of the results appeared promising.\textsuperscript{[15]}

The studies described in the next few sections were aimed at evaluating the potential of select stimuli that mimic the cardiac environment to cause differentiation of ADSC toward a CM phenotype. They were designed as part of the overall project aims, and in accordance with the tissue engineering paradigm of using bioreactors to apply stimuli to scaffolds seeded with stem cells. As will be detailed in the introduction to each study, evidence from the literature was also used to construct detailed aspects of these experiments and the methods and systems used within.

\textbf{5.2 Pilot Study: Differentiation of hADSC Cultured Statically on Decellularized Myocardial Scaffolds:}

\textbf{5.2.1 Introduction:}

There is little evidence in literature of a robust protocol for differentiation of hADSC or MSC into CM. Successful studies are anecdotal, especially in human cells, and the efficiency of differentiation protocols is low (≈50\% of original cells were differentiated at best).\textsuperscript{[16, 17]} Some studies demonstrated that varying the surface chemistry of the substrate for cell growth, such as coating with laminin, can have a positive effect.\textsuperscript{[16]} Others have shown that varying surface topography of the substrate can also have a cardiomyogenic effect. In addition, effects of both these variables on differentiation have been enhanced by the concurrent application of 5-aza-2-deoxycytidine (AZT), a DNA-
demethylator. In brief, AZT inhibits the action of DNA methyltransferase enzyme, leaving portions of the genome unmethylated and therefore more accessible to binding by transcription factors that may be upregulated as a result of signaling cascades activated by changes to the substrate or the application of other stimuli.

These studies supported the idea that a possible mechanism for inducing cell differentiation was simply the characteristics of the substrate (i.e. structure and composition), so we were interested in studying the effect of our DCELLed myocardial scaffold, which was shown to preserve proteins that mediate cellular attachment (e.g. laminin, collagen IV, and fibronectin) and therefore play a role in mechanotransduction. By virtue of this characteristic, they may have the potential to affect differentiation of cells grown on scaffolds, even in the absence of dynamic mechanical stimuli.

5.2.2 Methods:

5.2.2.1 Scaffold preparation, cell seeding, AZT stimulation, and cell culture:

Human adipose-derived stem cells (hADSC) were seeded onto DCELLed, sterile myocardial scaffolds (prepared by whole heart DCELL and peracetic acid sterilization as previously described) at 3x10^6 cells/scaffold and cultured for 7 days. In addition, hADSCs were seeded in standard, 6-well, tissue-culture plastic plates at 50,000 cells/well and cultured during the same period; this group served as a negative control for the effect of the scaffold. At this point, scaffolds and well plates were either treated with 9 µM AZT for 24 h or left untreated (n=1 for scaffolds; n=6 for well plates). Cells in all conditions were then cultured for an additional 3 weeks before being processed for analysis via
histology, immunofluorescence and IHC, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), Western blotting, and ELISA to evaluate gene and protein expression of the CM markers alpha-actinin, connexin 43, troponin T, troponin I, beta myosin heavy chain, and desmin.

5.2.2.2 Isolation of mRNA and protein:

Cells on scaffolds and culture plates were harvested and processed to isolate mRNA and cellular protein using Trizol™ reagent (Invitrogen). For cell on scaffolds, scaffold samples (n=1) were first flash frozen in liquid nitrogen, fractured into small pieces using a Biopulverizer (BioSpec, Bartlesville, OK) and then processed according to the Trizol isolation process for mRNA and protein.

For cells on well plates, culture media was aspirated and wells (n=3) were rinsed with warm, sterile PBS. One mL of Trizol reagent was added to each well and the wells were incubated for 5 min at room temperature. A cell scraper (BD Biosciences) was used to scrape cells free of the culture surface, and the solution was collected in a sterile, nuclease-free tube. To lyse cells, the solution was aspirated and forcefully passed through a 26-guage needle several times. The sample was processed according to the Trizol isolation process for mRNA and protein.

5.2.2.3 Histology, immunofluorescence, and IHC staining:

Following standard formalin-fixation and paraffin embedding, 5 μm-thick sections were prepared and stained with H&E.
Formalin-fixed, paraffin-embedded, 5 µm-thick sections were deparaffinized and heat mediated antigen retrieval with 10 mM citric acid at pH=6.0 was performed. Primary antibodies for alpha-actinin, troponin T, connexin 43, and desmin (Abcam; 4 µg/mL) were added to sections and incubated overnight at 4 °C. At this point, sections were labeled with the correct secondary antibody for the application (i.e fluorescently conjugated for IF or biotinylated for IHC). Sections were counterstained with dilute (1:1) hematoxylin in distilled, deionized water (IHC) or with DAPI (IF).

5.2.2.4 Western blotting:

In order to normalize the amount of protein in samples used for Western blotting, a bicinchoninic acid (BCA) assay (Pierce) was used to quantify protein concentration in the samples isolated as described above. To prepare samples for electrophoresis prior to blotting, the sample volume necessary to obtain 20 µg of protein was calculated, and diluted in reducing buffer containing 1 µL β-mercaptoethanol (βME)/20 µL. Electrophoresis and transfer were run at standard conditions. Blots were labeled with primary antibodies to desmin, troponin T, and connexin 43 (Abcam, 1 µg/mL, 1 µg/mL, and 0.128 µg/mL) and detected using secondary antibody and other reagents supplied in a BM Chemiluminescence Western Blotting Kit (Roche). Blots were imaged on a BioRad ChemiDoc system and bands were analyzed for relative intensity (densitometry) and molecular weight using the accompanying Image Lab software.
5.2.2.5 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The concentration of isolated mRNA was measured using a NanoDrop 2000 benchtop spectrophotometer (Thermo Scientific), and a total of 1 µg of mRNA was reverse-transcribed using M-MLV reverse-transcriptase enzyme and other reagents supplied in an Ambion RT kit (Invitrogen). Resultant complimentary DNA (cDNA) and SYBR Green Master Mix (Invitrogen) were used to prepare reactions for qRT-PCR. Reverse transcription and PCR reactions were run on a RotorGene cycler (Qiagen). Relative expression of markers was calculated using the $2^{\Delta\Delta Ct}$ method.[20]

5.2.2.6 Enzyme-linked immunosorbent assay (ELISA):

ELISA for troponin I was performed on samples of protein isolated as described above. Samples were prepared to contain 10 µg protein, and were run using reagents and instructions supplied by a cardiac Troponin I ELISA kit (Calbiotech). The blue color evolved by addition of the supplied detection reagent was measured spectrophotometrically at 450 nm. Sample concentrations of troponin I were calculated according to the relationship between known concentration and OD established by the supplied standard curve samples.

5.2.3 Results:

5.2.3.1 Histology, immunofluorescence, and IHC staining:

H&E staining of seeded scaffolds showed the presence of hADSCs, mainly concentrated several layers-thick on the outer surface, although some pockets of cells were
identified within the scaffolds (Figure 5.1 middle). Immunostaining for the above markers on scaffolds revealed cellular expression of alpha-actinin, connexin 43, and desmin (Figure 5.1 top and middle). Correspondent staining of hADSC grown on culture plastic appeared less intense (Figure 5.1 bottom). Upon inspection of these images, AZT treatment did not appear to affect the expression of the proteins in cells on scaffolds, but seemed to cause increased expression of CX43 and troponin T in cells grown on culture plastic. Sections were imaged using an inverted Zeiss Axiovert microscope equipped with mercury lamp, and accompanying AxioVision software.
Figure 5.1. Analysis of Differentiation of hADSC Cultured Statically on Decellularized Myocardial Scaffolds by IHC and IF: At top, Images of IHC-stained sections of scaffolds seeded with hADSCs and cultured for 4 weeks show expression of the indicated proteins. At 7 days, cell-seeded scaffolds and culture plates were either treated with 5-aza-2-deoxycytidine (+AZT) or not (-AZT). At middle, IF images of the same scaffolds show positively stained sections (green) counterstained with DAPI (blue) and H&E-stained sections display tissue morphology of seeded scaffolds. At bottom, IF images of cells on culture plastic stained in the same manner. IHC positive staining=brown; all images 40X, bar=20µm.

5.2.3.2 Western blotting:

When assayed for relative expression of the same markers by Western blotting, however, intensity of the bands produced by cells on culture plastic was greater than that of cells on scaffolds (Figure 5.2). This relationship held regardless of treatment with AZT. Treatment with AZT significantly increased protein expression of troponin T and desmin in cells grown on culture plastic, and there was a trend suggesting the same effect in cells grown on scaffolds, although low sample numbers did not permit analysis for statistical significance.
Figure 5.2: Analysis of Differentiation of hADSC Cultured Statically on Decellularized Myocardial Scaffolds by Western blotting: Western blotting and densitometry calculations of the resulting bands quantify relative expression of specific proteins in hADSCs seeded and cultured statically on scaffolds and hADSC cultured with standard methods on cell culture plastic. Cells in each condition were also either treated with 5-aza-2-deoxycytidine (+AZT) or not (–AZT); *: indicates statistical significance from corresponding –AZT, p=0.0471.

5.2.3.3 qRT-PCR:

Gene expression as measured by qRT-PCR showed that CX43 and troponin T were slightly upregulated in hADSCs on scaffolds as compared to hADSCs grown on culture plastic, while desmin and beta myosin heavy chain were both downregulated, all in the absence of treatment with AZT (Figure 5.3B). In fact, treatment with AZT did not appear
to increase expression of any of the markers of interest when directly comparing cells grown on the two different substrates. When the effect of AZT was studied within cells grown on the same substrate, AZT significantly increased expression of troponin T and βMHC, and showed a trend of increased expression of desmin in cells on culture plastic (Figure 5.3A). AZT treatment also showed a trend of increased expression of desmin and βMHC in cells grown on scaffolds. It did not significantly decrease expression of the other markers in either case.
A. Relative Gene Expression of CM Markers in hADSCs on Scaffolds or Culture Plastic when Treated with AZT

- CX43
- TnT
- Desmin
- βMHC

Fold Change wrt Non AZT-treated

B. Relative Gene Expression of Cardiac Markers in hADSCs on Scaffolds Versus hADSCs on Culture Plastic

- CX43
- TnT
- βMHC
- Desmin

Fold Change in Expression wrt to Cells on Culture Plastic
Figure 5.3: Analysis of Differentiation of hADSC Cultured Statically on Decellularized Myocardial Scaffolds by qRT-PCR: A) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) performed on RNA isolated from hADSC-seeded and cultured statically on scaffolds and hADSC cultured with standard methods on cell culture plastic. Cells in each condition were also either treated with AZT or not treated. Expression is reported as the fold change in expression of each gene in each group relative to expression in its non AZT-treated counterpart (represented with a value of 1); *: indicates statistical significance from non AZT-treated control, p=0.002; **: indicates statistical significance from non AZT-treated control, p=0.029. B) qRT-PCR performed on RNA isolated from the same conditions as above. Expression is reported as the fold change in expression of each gene in the scaffold group relative to expression in the culture plastic group (represented with a value of 1). Cells in each condition were also either treated with AZT or not; *: indicates statistical significance from culture plastic group, p=0.045; **: indicates statistical significance from culture plastic group, p=0.0314; #: indicates statistical significance from culture plastic group, p=0.039; ##: indicates statistical significance from culture plastic group, p=0.0573.

5.2.3.4 Enzyme-linked immunosorbent assay (ELISA):

ELISA for troponin I found none of this marker was present in cells grown on culture plastic regardless of treatment with AZT. In contrast, cells grown on scaffolds were found to express troponin I, and treatment with AZT appeared to enhance expression of this marker, although statistical significance of this effect could not be established (Figure 5.4).
**Figure 5.4: Analysis of Differentiation of hADSC Cultured Static on Decellularized Myocardial Scaffolds by ELISA**

Enzyme-linked immunosorbent assay (ELISA) was used to quantify expression of troponin I in protein isolated from hADSCs seeded and cultured statically on scaffolds (S) or on cell culture plastic (P). Cells in each condition were also either treated with 5-aza-2-deoxycytidine (+AZT) or not (−AZT); hADSCs cultured with standard methods on cell culture plastic (P) did not contain measurable amounts of the protein.

**5.2.4 Discussion:**

The markers studied here all play critical roles in the functionality of CM (as discussed in sections 1.5.5.1 and 1.5.5.2). CX43 is a gap junction protein through which calcium ions are transported in order to facilitate propagation of an excitation stimulus. This makes coordinated muscular contraction of the myocardium possible.\[^{21,22}\] Troponin
T and I are components of the troponin complex, which forms part of the larger tropomyosin complex. The troponin complex is found near the heads on myosin filaments and normally impedes their binding to actin filaments unless the complex is bound by a calcium ion.\[^{23}\] Thus, this complex represents an important intermediary in the mechanism of sliding filament muscle contraction. Desmin is an important intermediate filament protein which links the contractile apparati of CM with one another at Z-disks and to other components of the cell, including the nucleus and mitochondria. Collectively, this maintains the structural and mechanical integrity of CM during contraction, and aids in both force transmission and longitudinal load bearing.\[^{24, 25}\] It may also play a role in connecting the sarcomere to the ECM within desmosomes, thus forming a mechanical linkage between the ECM and sarcomere useful in mechanotransduction. Similar to desmin, alpha-actinin plays a mechanical role by anchoring the actin filaments in CM to the Z-disc.\[^{26}\]

In light of their functions, it is clear to see why we chose these proteins as the focus of this initial differentiation experiment. Since we were studying the effect of the substrate on the differentiation of bound cells, it was incumbent upon us to select markers with an established role in the attachment and mechanotransduction pathways of CM.

When cultured on scaffolds, hADSCs expressed cardiac genes and proteins regardless of treatment with AZT (which was expected to increase marker expression), implying that the myocardial scaffold might possess some intrinsic characteristics that cause this expression. These characteristics likely include the composition (collagen, elastin, fibronectin, basement membrane proteins) and architecture (pore size, ECM
organization), which together provide signals to hADSC through mechanotransduction that may upregulate the gene and protein expression of these markers.\textsuperscript{[27]}

There was also some evidence that treatment of cells grown on either substrate with AZT might have increased expression of these markers, but more supporting data must be collected before this claim can be made. When deciding whether to include AZT as part of a differentiation protocol, some consideration must be given to nonspecific and widespread deleterious changes the chemical could produce in the genome and its structure. AZT has been commonly studied as an anti-cancer treatment, mainly because it has the ability to induce cytotoxicity by causing DNA damage (i.e. strand breaks)\textsuperscript{[28]} Dosages and concentrations used in these studies were on par with those used here, so some of these effects could be imparted on hADSCs seeded on scaffolds. These effects, and consequently the use of AZT, may be undesirable outside of the context of cancer treatment or other more suitable applications.

It was noteworthy that hADSCs grown on culture plastic (i.e. “undifferentiated” cells) expressed CX43, troponin T, and desmin in at least a minimal level. In retrospect, we found that some groups who have carried out extensive analysis of the expression profile of hADSCs found that CX43 is normally expressed in these cells. Since they have been theorized to be of a perivascular phenotype, they may employ CX43 in intercellular signaling with endothelial and smooth muscle cells as part of their normal function.\textsuperscript{[13]}

It would be more beneficial in the context of an experiment such as this, which is focused on differentiation of cells, to measure a marker that is not constitutively expressed in the initial, undifferentiated phenotype. However, given the importance of CX43 to CM
function, it will still be critical to demonstrate its expression in the differentiated cell population. As such, we will continue to measure it in future differentiation experiments with the aim of demonstrating the development of a CM phenotype.

That said, in order to posit differentiation of these cells in experiments where dynamic stimuli will be applied, increased expression of these markers must be observed with respect to unstimulated control cells, as well as expression of other cardiac markers in the stimulated cells with little to no expression in the control cells (i.e. differential expression suggesting a change in phenotype). This effect was best illustrated in this study by ELISA for troponin I, which was only present in cells grown on scaffolds. In addition, there was some indication that culture of hADSCs on scaffolds resulted in increased expression of a few of these markers with respect to cells grown on culture plastic, as seen in the IF staining. Aside from those specific results, this study largely demonstrated the opposite outcome, specifically Western blots which showed greater expression of CX43, troponin T, and desmin in cells cultured on plastic as opposed to scaffolds. It’s possible that this result was skewed by the presence of solubilized matrix proteins in samples of seeded scaffolds. This extracellular protein could have contributed to the total 20 µg of protein which is normally run in all samples to normalize them so they may be quantitatively compared. Consequently, the protein samples from the seeded scaffolds might have contained a smaller proportion of cellular protein compared to those samples isolated from cells grown on tissue culture plastic. So, while samples from both groups were found to contain the proteins of interest, samples from the cells on culture plastic might not have contained more of those proteins if there had been a correction for the
extraneous extracellular proteins. A control will need to be devised to account for the presence of these proteins in future experiments wherein the undifferentiated control group consists of cells grown on culture plastic.

5.2.5 Conclusion:

Use of myocardial scaffolds as a substrate for hADSC growth precipitated expression of cardiac genes and proteins regardless of treatment with AZT (which was expected to increase marker expression). Although evidence of increased expression of these markers in cells on scaffolds with respect to cells on culture plastic was less abundant, the structure and composition of myocardial scaffolds could be an inductive mechanism for cardiomyogenic differentiation of hADSCs.

This study focused only on the effect of the DCELLeed myocardial scaffold as a substrate in differentiating hADSCs into CM. Its findings, while of interest, neglected variables such as electrical and mechanical stimuli which we believe are necessary to achieve full differentiation of stem cells into a CM phenotype. The system described in the next section of this chapter was designed with the idea of incorporating such factors in mind.

5.3 hADSC Differentiation with Flexcell System:

5.3.1 Introduction:

To date, many groups have recognized the need for reproducing the physiological stimuli observed in the cardiac macro- and microenvironments in order to generate more
biomimetic constructs or grafts. Eschenhagen and colleagues demonstrated the beneficial effects of the application of mechanical strain on engineered heart tissue, noting increased CM alignment, sarcoplasmic protein organization, and concomitant enhancement of contractile force generation in the stretched constructs.[29] The effects of electrical stimulation have been studied and reviewed extensively by Vunjak-Novakovic’s group, among others, who have shown that culturing myocardial tissue-engineered constructs in the presence of electrical fields with defined parameters can encourage development of electrical connectivity and excitability of CM, as evidenced by increased expression of connexin 43, lowered excitation thresholds, and increased stimulus capture rates.[30, 31] Few groups, however, have taken these two most defining stimuli of the cardiac environment and applied them simultaneously to cell-seeded constructs.[32] Even fewer have recognized the natural interplay between them in the excitation-contraction coupling of the myocardium and attempted to mimic this within a bioreactor by carefully synchronizing timing, duration, and characteristics of electrical impulses and mechanical forces.[33-35]

Wang et al. recently investigated the effects of coordinated electrical and mechanical stimuli on DCELLed myocardium scaffolds reseeded with rat mesenchymal stem cells. They observed positive immunostaining for the cardiac markers myosin heavy chain, sarcomeric α-actinin, cardiac troponin T, connexin43, and N-cadherin but did not analyze gene and protein expression of the cells within their constructs following culture and stimulation. Most importantly, they did not observe contractile behavior in their constructs, nor did they find any direct evidence of electrical activity or connectivity between the constituent cells.[35] It is likely this is due to their use of too few seeded cells;
the roughly 833,000 cells/cm³ density of their constructs did not allow for the necessary proximity of cells to produce intercellular connectivity.

As discussed in previous sections, cells within a tissue-engineered graft should be functional (contractile and able to integrate with the host cardiac conduction system) upon implantation. Otherwise, the graft does not help alleviate the mechanical dysfunction which leads to remodeling and CHF. Thus, characterizing changes in stem and progenitor cells which have been exposed to physiological stimuli in bioreactors becomes a key milestone in developing feasible approaches to engineering new myocardium with therapeutic potential.

5.3.2 Methods:

5.3.2.1 System Design:

This platform was designed to integrate the mechanical and electrical stimulation of cells seeded on DCELLed myocardial scaffolds, and to allow for precise control of the characteristics of these two stimuli. Design inputs included: 1) means of applying a uniaxial tensile force of up to 140 mmHg to a cell-seeded construct, with the ability to control characteristics of the waveform 2) means of applying at least 2 V electrical stimulus to seeded constructs, with the ability to control other characteristics of the waveform 3) means to coordinate the timing of delivery of these two stimuli to the construct, and in such a manner so as to allow for changing how the two waveforms overlap in successive experiments 4) allow for conventional culture of seeded constructs in a standard incubator with standard media, and facilitate convenient changing of media every 2-3 days 5)
accommodate a sufficient number of experimental replicates to achieve statistical significance among experimental groups in measured endpoints.

A commercially available system sold by Flexcell International Corp. served as the basic component around which this platform was designed (Figure 5.5). The system was sold under the name FX-5000™ Compression System, and was comprised of a main controller housing with pressure regulators, valves, and transducers used to precisely control the flow of compressed air into a baseplate upon which four 6-well plates were attached (Figure 5.5B and C). The bases of the 6-well plates were hermetically sealed against rubber gaskets on the baseplate by means of telescoping bolts that clamped the well plates to the baseplate (Figure 5.5E).

The system functioned by pumping compressed air into the space beneath the baseplate and the well plates above, forcing the membrane at the bottom of each well upward (Figure 5.6F). The result was that the membrane moved upward like an inflating balloon when air was pumped into the baseplate and returned downward to a flat position when air pressure was released. This movement was very similar to the motion of the left ventricular free wall during diastole and systole, and was used to simulate just that. The accompanying Flexcell software was used to create a pressure waveform modeled after the left ventricular pressure waveform observed during the cardiac cycle (Figure 5.6A). A pressure transducer was used to measure the real-time pressure applied to the silicone membrane, and the data was fed to a custom-written LabView program. This program was designed to deliver an electrical pulse which was triggered when the pressure reading reached a specific value input by the user (Figure 5.6A and C). In other words, the LabView
program was used to tightly control the relative temporal delivery of both mechanical and electrical stimuli to samples contained within the well plates. This fact allowed us to simulate the excitation-contraction behavior of cardiac muscle in an effort to expose hADSCs to proper physiological stimuli and induce their differentiation into CM.
**Figure 5.5: Design of Flexcell system:** A) Schematic illustration of main components of the Flexcell system. B) An incubator containing the modified Flexcell® system comprised of tubing, baseplate, and 6-well plates with silicone membranes pumps air into the baseplate shown in C, where E) telescoping bolts are used to seal the edges of F) modified 6-well plates against a rubber gasket. D) Flexcell controller (2 bidirectional, software-controlled valves inside) and data acquisition (DAQ) modules, which collectively enable all measurement and output of physical signals in the system, are shown.
**Figure 5.6: Detailed operation of Flexcell system:** 

A) Screenshot of display from computer used to control the Flexcell system. The window at back left is the Flexcell software, running the programmed regimen and displaying pressure waveform data collected by the Flexcell controller’s pressure transducers. The window at front right is the LabView program, displaying pressure waveform data from a pressure transducer which was tapped into the positive pressure line running to the Flexcell baseplate. This data was acquired by one data acquisition (DAQ) module and fed into the LabView program; this program triggered an electrical pulse, output by a second DAQ module, every time the pressure fell within a user-defined range (in this case, at the rising edge of each pressure wave).  

A, C) This trigger is illustrated by two virtual LEDs in the LabView display (dashed yellow ovals), which indicate the status of the pulse by lighting up with an “ON” or “OFF”.  

B, D) Auxiliary setup for plates receiving only electrical stimuli; these plates could not be kept in the same incubator as those receiving mechanical stimuli because said incubator was set at a higher temperature to offset the cooling effect of running compressed air through the baseplate.  

D) The LabView program responsible for delivering the electrical stimulus to electrical only plates; the characteristics of the pulse (amplitude, frequency, duration) were controlled to match those of the electrical pulse produce by the program in A and C.

E,F) Pressure from air pumped into the baseplate (blue arrows in F) with 6-well plate sealed atop it causes upward deflection of the silicone membrane in each well. Scaffolds are shown in F with horizontal ends fixed in place at the sides of each well. When the membrane deflects upward under pressure, a resultant uniaxial tensile force (black arrows with yellow outline in E and F) is applied to the scaffold.  

F) Heat map illustration of the potential field and graph of electrical potential vs. position generated from a Comsol™ model based upon the well and electrode dimensions and the applied electrical pulse. The region of interest (R.O.I.), or the area of scaffold where the effects of mechanical stimuli and electrical stimuli will both be applied to seeded cells, is indicated with a dashed box.
5.3.2.2 Modified Flexcell Well Plate Design:

We modified Flexcell Bioflex® 6-well plates by attaching customized, plastic well inserts to the lid of each plate using a combination of cyanoacrylate glue and silicon sealant in order to provide a watertight seal (Figure 5.7A, B, F, H). The inserts were designed to accommodate a 1-2 mm thick, 3.5 cm x 2 cm rectangular scaffold sheet that had been seeded with cells. Inserts held the sheets stretched directly above the silicone membrane by providing fixation points for the edges of each sheet at the periphery of the well on opposing sides (Figure 5.7E and Figure 5.6F). Once stretched across the diameter of the well, each sheet was clamped between the bottom of the insert and a ring which fastened to the insert via a snap hook mechanism (Figure 5.7E). This ensured that as the membrane deflected upward under pressure, the seeded scaffolds were subjected to a uniaxial tensile force (Figure 5.6E). A lock-in-groove feature was included between the rings and inserts to prevent slippage of the sheets over time due to cyclical application of tensile force (Figure 5.7C and D).

The inserts also incorporated fixtures for two cylindrical, 3 mm-diameter, carbon electrodes which were positioned parallel to each other but perpendicular to the direction of the tensile force (Figure 5.7F, G, and H). The electrodes (one positive, one negative) were wired to electrical adapters through a network of wires attached to the lid of the plate (Figure 5.7G). The wiring and inserts were both attached to the undersides of the plate lids to facilitate media changes in the wells.

Plates were connected to the electrical signal output from the LabView program, and the pulses delivered to them resulted in the development of an electrical field within
each well, the direction of which was parallel to that of the tensile force applied to the scaffolds (Figure 5.6F). Scaffolds lay directly below the electrodes such that cells seeded on them were exposed to the electrical field.
Figure 5.7: Design of Flexcell plates and well inserts:  A) CAD rendering of a well insert and the B) 3D-printed ABS plastic part produced from the same SolidWorks file.  C) Protrusions from the bottom of the well inserts were designed to lock with grooves on the accompanying locking ring shown in D.  E) Scaffold sheets were secured near the bottoms of wells at both ends by stretching the sheets across the well inserts and then placing a locking ring over them.  Sheets were clamped between the two parts and held securely by means of the lock-in-groove feature described above; compression was maintained by 4 snap-hooks (black arrows) that retained the locking ring in close apposition.  F) Fully-assembled lid of a Flexcell plate used for groups receiving electrical stimulation, with well inserts attached by super glue and silicone sealant.  G) Carbon electrodes (black rods) were used to create an electrical potential within each well, and are shown connected to larger wires (yellow and green) via thin platinum wire (black arrows).  The ends of the electrodes fit inside hollowed-out channels, allowing them to slide up and down with the motion of the scaffold as it was mechanically stretched.  For groups that did not receive mechanical stimulus, electrodes were not attached and plates were not wired.  H) Each circuit, consisting of a positive and negative electrode, was wired to a single connector (black part at left) that conducted electrical impulses produced by a data acquisition module.

5.3.2.3 Experimental Setup:

We surmised there were 4 major stimuli that determined the nature and degree of hADSC differentiation into CM: mechanical stimulation (M), electrical stimulation (E), substrate properties (S; scaffold vs. no scaffold/silicone membrane only), and pharmacological stimuli (P; AZT-treated).  We used the Flexcell system’s ability to accommodate many samples (n=24) to our advantage in achieving this aim – to discover which of these stimuli had the greatest impact on hADSC differentiation, and to determine if there was any synergistic effect between them in this respect.  For the first experimental run using the Flexcell system, 4 different conditions were tested: 1) M+E+S, 2) M+S; 3)
M+S+P; 4) E+S (referred to hereafter as E+M, M, M+AZT, and E, respectively). We used one, 6-well plate per condition (n=6), and hADSC were seeded onto each scaffold via injection through a small-diameter needle at various points in the area between the two electrodes. Upon completion of 3 weeks of culture under the conditions listed above, the scaffolds were collected in order to characterize the extent of hADSC differentiation into CM.

At the conclusion of culture in this system, sections of seeded scaffolds from each well were divided up and processed for various methods of evaluation: 1) fixation in 4% paraformaldehyde for histology and immunofluorescence/IHC; 2) isolation of protein and RNA via homogenization and Trizol® reagent for Western blotting and qRT-PCR, respectively; 3) preservation in cell culture media for tests of cell/tissue functionality and viability, including Live/Dead assay and live cell calcium imaging using Fluo-4.

Statistical analysis techniques, including multivariate analysis of variance (ANOVA) and post-hoc contrast analysis of means via Fisher’s least significant difference test, were used to determine main effects and interactions of the 4 stimuli tested on the measured outcomes.

5.3.2.4 Preparation of Scaffolds:

Scaffolds were prepared by first dissecting 2.0 x 3.8 cm rectangular segments of DCELLeD myocardial scaffold generated via whole heart DCELL. Then, 1-2 mm thick layers of these segments were sheared off using a scalpel to generate scaffold sheets. Sheets were washed 3X (1 h, 3 h, 1 h) before being sterilized with 0.1% peracetic acid in
PBS at pH 7.4 (2X; 30 min, 1 h). Sheets were then washed with sterile PBS 3X (1 h, 3 h, 1 h) in preparation for cell seeding.

5.3.2.5 Cell Seeding and AZT stimulation:

Each sheet was incubated overnight at 37 °C in DMEM with 10% FBS. Immediately prior to seeding, the excess media was removed from the sheets and they were incubated another 2 h at 37 °C in an attempt to slightly dehydrate the scaffolds. The rationale was that cell solution would be more likely to penetrate a slightly dehydrated scaffold than one that was swollen with media, increasing the effective density of seeded cells. We used a cell culture scale-up apparatus, the CellSTACK® (Corning), to produce sufficient numbers of cells for this study. Once the sheets were secured to the inserts, a suspension of hADSCs was injected at several random points in the center of each sheet, and a total of 3.1 x 10^6 cells were seeded onto each sheet. The remainder of the cell suspension solution was drop-seeded onto the upper surface of each sheet, and the seeded scaffold sheets were placed in wells with no media and incubated for 4 h at 37 °C to allow cells to attach to the sheet. About 6.5 mL of media was placed in each well and the sheets were cultured statically for another 20 hours. At this point, scaffolds in the M+P+S (M+AZT) group were treated with 9 µM AZT and cultured for an additional 24 h, while the other groups were only statically cultured during this time.
5.3.2.6 Construct Attachment and Stimulation Parameters:

Seeded myocardial scaffolds were placed across the space between the two extensions of each well insert, and clamped into place using the rings as previously described (Figure 5.7E).

In its final configuration, a 3.6 cm² rectangular area of hADSC-seeded myocardial scaffold received user-controlled, synchronized, and cyclic mechanical and electrical stimulation (Figure 5.6F). This area would serve as the region of interest (R.O.I.) for all methods performed to assess differentiation. These stimuli were adjusted to mimic those of the native cardiac environment as closely as possible, with a cyclic pressure waveform (0-120-0 mmHg amplitude in 350 msec with 450 msec static at 0 mmHg, 1.17 Hz or ~70 BPM) and a 5V (2.78 V/cm), 20 msec-width, square-wave electrical pulse applied at the rising edge of every pressure wave.

Prior to stimulating scaffold sheets with the above protocol, we thought it prudent to allow the newly seeded cells sufficient time to attach to the scaffold, and to ramp-up the mechanical stimulus in a stepwise manner so the cells could acclimate to the stress. This was accomplished by applying waveforms similar in period and frequency to the one described above, but with amplitudes of 40 mmHg and 80 mmHg for 24 h each. The results of this analysis will determine which stimulus or combination of stimuli should be used to differentiate seeded cells most effectively in the bioreactor phase of this aim.
5.3.2.7 Isolation of mRNA and protein:

An approximate 1 cm$^2$ area of each scaffold was cut from the R. O. I. and mRNA and protein were isolated as previously described.

5.3.2.8 Histology, immunofluorescence, and IHC staining:

An approximate 0.5 cm$^2$ area of each scaffold was cut from the R. O. I. and fixed in 10% neutral buffered formalin. These sample were histologically process and stained via IHC for alpha-actinin (Abcam; 4 µg/mL) as previously described. Sections were counterstained with dilute (1:1) hematoxylin in distilled, deionized water. Sections were imaged using an inverted Zeiss Axiovert microscope and accompanying AxioVision software.

An approximate 0.5 cm$^2$ area of each scaffold was cut from the R. O. I. and fixed in 4% paraformaldehyde for 2 hours at 37 ºC. Following adequate fixation, samples were rinse 3X in PBS and stored in PBS. For IF staining, samples were cut into thin (<1 mm wide) strips and placed into wells of 96-well plates, where they could be subjected to heat mediated antigen retrieval and the various steps of the IF staining process. They were incubated overnight at 4 ºC with primary antibodies to actin and CX43 (Abcam; 4 µg/mL), and nuclei were counterstained with DAPI. Samples were imaged using an inverted Zeiss Axiovert microscope equipped with mercury lamp and accompanying AxioVision software.
5.3.2.9 Live/Dead Assay:

An approximate 0.5 cm² area of each scaffold was cut from the R.O.I. and placed in wells of a 24-well plate which contained complete culture medium (DMEM with 10% FBS and 1% antibiotic/antimycotic) until they could be assayed. When ready, the samples were cut into thin strips as described above, rinsed 2X with warm PBS, submerged in a Live/Dead working solution containing calcein AM and ethidium homodimer-1, and incubated for 20 min at 37 °C. Samples were imaged using an inverted Zeiss Axiovert microscope equipped with mercury lamp and accompanying AxioVision software.

5.3.2.10 Calcium Staining Assay:

An approximate 0.5 cm² area of each scaffold was cut from the R.O.I. and placed in wells of a 24-well plate which contained complete culture medium (DMEM with 10% FBS and 1% antibiotic/antimycotic) until they could be assayed. When ready, the samples were cut into thin strips as described above using sterile surgical instruments and then placed back into their respective wells containing media. An equal volume of 2X Fluo-4 Direct™ calcium reagent loading solution (Life Technologies) was added to the well and the plate was incubated at 37 ºC for 45 min. Samples were imaged using an inverted Zeiss Axiovert microscope equipped with mercury lamp and accompanying AxioVision software.
5.3.3 Results:

5.3.3.1 Isolation of mRNA and protein:

Appreciable amounts of mRNA and protein were obtained from the scaffolds, indicating a significant cellular presence on all of the scaffolds following the 3 week culture and stimulation period (data not shown). There were sufficient amounts of each to perform the qRT-PCR and Western blotting for all markers of interest (up to 10), however those assays were not carried out prior to publication of this document.

5.3.3.2 Live/Dead Assay:

Examination of Live/Dead staining of scaffolds revealed that all groups contained live cells along with some dead cells (Figure 5.8). Some cells were observed to have nuclei which stained red, indicating they were dead, while their cytoplasm concurrently stained green, indicating they were alive. Importantly, cells displayed an elongated morphology, and appeared to align with one another in a direction parallel to that of both the applied tensile stress and electrical field. More cells were visible in the E and E+M groups than in the other groups. It was also difficult to find cells spread evenly throughout the scaffold. They were not visible on every strip that was prepared and stained, and it appeared they were mostly confined to the surface of scaffolds.
Figure 5.8: Analysis of hADSC differentiation with Flexcell system:

Images of cells contained within scaffold sheets exposed to the indicated stimuli and stained with Live/Dead™ assay reagents (calcein AM/live (green) and ethidium homodimer-1/dead (red)). Stimuli were part of dynamic differentiation regimens within the Flexcell system. Small areas of the sheets were cut into thin (< 1 mm wide) segments and stained with these reagents; second image from top 10X, bar=100µm; all other images 2.5X, bar=500µm.
5.3.3.3 Calcium Staining Assay:

Fluo-4 Direct™ Calcium reagent stained intracellular Ca²⁺ within all groups (Figure 5.9). The same elongated morphology and consistent orientation along directions of applied tensile force and electrical field observed with Live/Dead staining were observed in this assay. Small nodules or pockets of calcium could be discerned throughout the entirety of the cells’ length, with clear voids which were presumably the locations of nuclei. It was unclear if the staining extended into the cytoplasm or was concentrated at the cell membrane. Similar to the Live/Dead assay, more cells were observed in the E and E+M groups than in the other two groups.

Figure 5.9 (below): Analysis of hADSC differentiation with Flexcell system: Fluo-4 Direct™ Calcium reagent shows binding to intracellular calcium (green) of cells contained within scaffold sheets exposed to the indicated stimuli. Stimuli were part of dynamic differentiation regimens within the Flexcell system. Small areas of the sheets were cut into thin (< 1 mm wide) segments and stained with this reagent; left column 10X, bar=100µm; right column 40X, bar=20µm.
5.3.3.4 Histology, immunofluorescence, and IHC:

IF staining for actin and CX43 showed positive staining only in the E group. Actin staining was definitive, but CX43 staining (green dots) was less intense and could have been background autofluorescence (Figure 5.10A, C, and E). Portions of scaffolds from all groups were stained in the same manner, but an area of positively staining cells for either marker could not be found in these groups, even though some sporadic nuclei were identified. It was noted that the area of cells depicted as staining positively here was found on a very thin piece of tissue. Again, cell morphology was elongated, cells were oriented in the same direction, and some appeared in close enough proximity to allow for contact with one another.

Figure 5.10 (below): Analysis of hADSC differentiation with Flexcell system: A, C, E) Immunofluorescence staining of cells contained within scaffold sheets exposed only to an electrical stimulus. Cells showed positive staining for actin (red), but staining for connexin 43 (green) was less definitive. DAPI (blue) was used as a nuclear counterstain. Groups exposed to other stimuli from the Flexcell system are not shown because positive staining for either marker was not present. Small areas of the scaffold sheets were cut into thin (< 1 mm wide) segments and stained; A 20X, bar=50µm; C and E 40X, bar=20µm. B, D, F) Histological sections of cells contained within scaffold sheets exposed only to an electrical stimulus shown stained by immunohistochemistry (IHC) for alpha-actinin. Groups exposed to other stimuli from the Flexcell system are not shown because of difficulties encountered in obtaining histological sections containing cells. IHC positive staining = brown; B 10X, bar=100µm; D and F 40X, bar=20µm.
Sections from the E group stained positively for the presence of alpha-actinin as demonstrated by IHC (Figure 5.10B, D, and F). These sections were known to contain cells because previous H&E staining of serial sections had shown the presence of cells.
(data not shown). We could not determine whether cells from other groups expressed this marker because we were unable to obtain sections from them that contained any cells. In contrast to IF, IHC allowed for examination of how cells fit into scaffold interstices. Many of them appeared to align in the predominant direction of collagen fibers (Figure 5.10F). One dense group of cells looked as if it was ensconced in a network of newly synthesized, or otherwise heavily remodeled, matrix; this area of matrix appeared very different from others (Figure 5.10B and D).

5.3.4 Discussion:

Results of this experiment were not the complete battery they were intended to be; there was no quantitative data on protein or gene expression because Western blotting and qRT-PCR were not completed prior to publication of this document. Despite this, amounts of mRNA and protein isolated from scaffolds will be sufficient for these methods to be completed in the future, and they will allow more substantive conclusions to be made.

Live/Dead staining and calcium staining confirmed the presence of live cells on scaffolds from all groups. Calcium staining was much more diffuse, but it could be coincidental given that cell density on scaffolds was very heterogeneous, and portions of the R.O.I. used for calcium staining might have contained more cells than portions used for Live/Dead staining. The particular appearance of calcium in these cells was intriguing because of how diffuse it appeared, spread throughout the cells. The fact that the images shown here were taken from specimens with significant depth, as opposed to histological sections with only 5 µm of depth, is advantageous because close inspection of high
powered images revealed some nodules in the plane of focus while others (apparently still within the same cell) were slightly out of focus. This could be taken to mean that calcium was dispersed through the entire volume of the cell cytoplasm, as one would expect in a CM. Regardless of this observation, staining of calcium stores alone was far from definitive evidence that these cells had acquired a CM phenotype. This is because calcium is rather ubiquitous, and is involved in processes (e.g. second messengers in signal transduction and enzyme cofactors) not necessarily specific to CMs. It is regularly stored within mitochondria and the endoplasmic reticulum (the sarcoplasmic reticulum in muscle cells). It would have been helpful to have performed the same staining on both a positive and an undifferentiated control as references for how intracellular calcium deposits should appear in those situations (unfortunately, there is also a lack of other studies which have used Fluo-4 to label calcium in these instances). Another more definitive test would involve stimulating the cells with agonists to calcium uptake, such as heptanol and carbachol, and monitoring the response. Similarly, the provision of an electrical stimulus would provoke a spike in intracellular calcium concentration in a CM-like cell.

5.3.4.1 Histology, immunofluorescence, and IHC:

Although positive staining for actin was observed, this particular antibody was not specific to cardiac muscle alpha actin, so other isoforms could be labeled here. The signal did not localize to bands, as is sometimes seen in staining of CM. CX43 staining was sparse, with widely dispersed dots that might be construed as positive staining (CX43 normally appears as dots at intercellular junctions). Alpha-actinin staining of cells was
similar to what we observed with the static differentiation study – positive staining along the body of the cells, but no distinctive banding from concentration at Z-disks as in CMs. Since we identified cells in all groups with Live/Dead and calcium staining, we were somewhat surprised that we could not do the same with other cardiac specific markers such as those discussed above. We did observe some nuclei (as stained by DAPI) in these groups, but no accompanying staining for the markers of interest. This would seem to suggest that these few cells simply did not express these markers. While that may well have been the reality, one conflicting observation was that positively stained cells shown in Figure 5.10 were found on a very thin piece of scaffold (probably only 0.25-0.5 mm wide) relative to other pieces (0.5-1.0 mm wide). By virtue of being thin, the solutions used in the IF protocol may have come into closer proximity with the cells, enhancing their action and resulting in more intense staining. In contrast, thicker pieces of scaffold may have created a physical barrier to the entry of these solutions, resulting in poor staining.

Our purpose behind employing the proceeding method, by which pieces of scaffold were stained without paraffin embedding, dealt with the drawbacks we encountered with the method used to obtain IHC results. With paraffin-embedded and sectioned samples, we found it difficult to obtain sections with any cells. The cells were contained in pockets so dispersed that many of our 5 µm sections failed to capture any. With the exception of the E only group, we could not obtain sections with cells from any of the other groups, even when we staggered our sections 50 µm apart between every 2 or 3 serial sections. Since our R.O.I. was relatively small – 3.6 cm² – and we had to use portions of it for other assays, we could not afford to be inefficient with our samples. We decided that the best
approach to immunostaining was to use small portions of scaffold for each antibody, and to use IF to stain the samples without embedding. This enabled us to stain more of the sample by avoiding the wasted sections associated with paraffin or cryo-embedding. Not insignificantly, this strategy also maximized our chances of identifying all pockets of cells that might stain for our markers of interest.

5.3.4.2 Design Iterations and Challenges:

The development of this system was not without its challenges, and, as a result, went through several iterations before taking on a final form. In several tests of the system following this experiment, it was observed that the pH of the media from wells run on the Flexcell baseplate was slightly alkaline, as the phenol red indicator shifted from an orange-red to a red-purple color. When measured with a pH meter following several days of culture within the system, media from these wells had a pH of around 7.9. Since we were using normal DMEM media buffered by bicarbonate and ambient CO₂, we surmised that the local CO₂ concentration within the wells was somehow too low. A plausible explanation for this was our use of house compressed air, consisting of room temperature air with an atmospheric concentration of CO₂ (∼0.035%), as the source of air which was pumped through the Flexcell baseplate.

This was problematic because silicone is a gas-permeable material, so the compressed air with a much lower CO₂ concentration was permeating the membrane and reducing the local CO₂ concentration from its desired level of 5%. In turn, this caused the bicarbonate buffer system equilibrium to shift toward alkalinity. Additionally, the lower
temperature of the compressed air was found to reduce the temperature of the culture media from 37 °C to as low as 31 °C. This, too, could have shifted the effective range of the buffer but, more importantly, may have also reduced enzyme activity and metabolic processes within the cells.

A simple solution would have been the addition of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to the media since its buffering mechanism is not dependent upon CO₂, but it was found that HEPES has been shown to produce free radicals when an electrical potential in excess of 0.8 V is applied to the molecule. This precluded its use in our system, as we were using much greater electrical potentials than this figure and free radicals can produce oxidative damage in cells, possibly impacting their viability or their responses to the experimental treatments.

We therefore elected to change the source of the air pumped through the Flexcell to air from the incubator so that it possessed the correct CO₂ concentration and temperature. However, we found that since this air was also heavily humidified, it presented a risk of damage to the vacuum pump and the Flexcell pump in our system. It then became necessary to remove the water vapor from the incubated air by means of an inline dryer. This dryer used a silica desiccant that became saturated and required changing every 8-12 hours. Once implemented, it was found that this system normalized the pH of the media to around 7.5, but the temperature of the media did not recover to 37 °C as expected. To correct this, the ambient temperature of the incubator was iteratively increased and the temperature of the media was measured until a temperature setting was determined which produced the desired media temperature of 37 °C.
After these changes were made, the system functioned as intended and the culture conditions were all holding steady at their proper levels. But, over the course of three experiments, the Flexcell controller proved to be unreliable, suffering hardware malfunctions in the midst of two of them. This portion of the system was critical to the experiment, and its frequent downtime severely hindered progress in this study. The redesigned system is depicted in Figure 5.11.
Figure 5.11: Modification of Flexcell system: A) Schematic illustration of main components of the second generation Flexcell system. In order to better regulate temperature and pH, the system was modified to circulate air from within the incubator through the Flexcell controller and baseplate; the functions of all other components remained unchanged. B) An incubator containing tubing, baseplate, and a compressed air
storage tank; the tank and as much tubing as possible were kept inside the incubator to reduce condensation of water vapor from the humidified incubator air. C) An in-line dryer filled with silica gel dessicant was used to dehumidify air drawn from the incubator by a D) vacuum pump. This was done to protect the vacuum pump and Flexcell controller against possible damage from the buildup of condensate.

5.3.4.3 Cell Seeding Challenges:

The challenges presented by insufficient cell density to obtaining and evaluating results in this experiment prompted us to change our cell-seeding methods in a subsequent experiment. This new experiment also incorporated the changes to the system design enumerated above. We hypothesized that seeding lyophilized (freeze-dried) scaffolds would encourage more cell infiltration because the cell suspension would be drawn into the porous, dehydrated scaffold in the same manner that a dry sponge draws in water. To test this, scaffolds sheets were prepared and sterilized via the same methods as the previous experiment. Sheets were then placed in sterile petri dishes and lyophilized by first freezing at -80 °C, followed by application of continuous vacuum at 1.6 mBar.

We used a cell culture scale-up apparatus, the CellSTACK® (Corning), to produce sufficient numbers of cells for this study. Each lyophilized sheet was injected with 25 µL of cell suspension at a concentration of 3.8 x 10^6 cells/mL at 20 evenly spaced points by means of a custom-designed and 3D-printed array of needle holes. The array was positioned over the center of each sheet and a 26 gauge needle was passed through each of the holes successively until a uniform grid of points was injected. Following injection, each sheet was then placed into a petri dish containing 11 mL of cells suspended in media at a concentration of 4.9x10^5 cells/mL. Sheets were incubated in this cell suspension
overnight until they had rehydrated and swelled to their original volume. Based upon the surface area of each sheet and the concentration of the surrounding cell solution, each sheet was coated at a cell surface density of at least $5.6 \times 10^3$ cells/cm$^2$.

These seeded scaffolds were mounted on Flexcell plates and run on the redesigned Flexcell system for 3 weeks. Subsequent Live/Dead staining and mRNA and protein isolation indicated that there were no cells on any of the scaffolds, even in the E only group scaffolds cultured in an incubator with normal settings. After some careful consideration of possible causes, it was determined that injected cells did not remain inside scaffolds. Our understanding of this was that the lyophilized scaffolds initially repelled media until their outer surface could become hydrated. Because of this, the injected media was expelled from the interior of the scaffold and the cells along with it. Since scaffolds were immediately submerged in cell suspension following injection, there was not sufficient time for serum proteins in the culture media to adsorb to their surfaces. This meant cells were deprived of any chance to attach to the scaffold, and were washed away once the scaffolds were placed in cell suspension. Apparently, the scaffolds also failed to attract any cells whilst bathing in the cell suspension.

### 5.3.4.4 Perspectives on Dynamic Stimuli in Cardiomyogenic Differentiation:

There have been a number of studies whose results support the positive effects of dynamic mechanical and electrical stimuli, as well as AZT, on the differentiation of various populations of stem or progenitor cells into CM-like cells.[18, 35, 38-42] Our platform incorporates many of these strategies into one comprehensive system that should produce
similar results. Some of these results should include increased gene and protein expression of most or all of the following: alpha-actinin, troponin-T, CX43, desmin, N-cadherin, β-myosin heavy chain, cardiac alpha myosin heavy chain 6, cardiac alpha actin, cardiac myosin light chain-2α, titin, GATA4, Nkx2.5, and Islet-1. Cells should also display responsiveness to electrical stimuli, measurable via multi-electrode arrays (MEA) and calcium transients with the Fluo-4 assay.

Even though many of these markers were not measured in this experiment, histological methods showed that cells in all but one group demonstrated poor expression of the markers which were measured. It is still unclear as to whether poor cell seeding, ineffective assays and methods, or undesirable environmental conditions were the cause. Low cell density, in particular, was cited by another group who conducted a similar study as the reason why the cells they were attempting to differentiate did not become electrically excitable.\[35\] Cell density, of course, is a function of the cell seeding methods.

Poor outcomes were likely a result of all three factors, but with more efficient cell seeding strategies and tighter control of the culture conditions, along with improved reliability of the Flexcell system, this platform should produce results that allow us to optimize differentiation of hADSCs and subsequent maturation of grafts produced with them. Finally, with the addition of qRT-PCR and Western blotting results, it is almost certain that a clearer perspective will emerge as to the success or failure of this initial experiment.
5.3.5 Conclusions:

Through several design iterations, we successfully engineered a system with the desired features outlined in the previous sections of this chapter. It is capable of delivering mechanical and electrical stimuli in a coordinated manner to cell-seeded myocardial scaffolds. Based upon the initial results of experiments with this platform, it would be advisable to revise cell seeding methods so as to deliver as many cells to the scaffolds as is practical, and to make certain cells are retained within the scaffolds immediately following the seeding process. Without an abundance of cells in the scaffolds, their differentiation may be adversely affected, and even evaluation of the effects of the experiment may become too challenging.

In the future, this system will also serve as an efficient platform for testing the effects of seeding other cell types (e.g. cardiac fibroblasts, smooth muscle cells, or endothelial cells), in addition to hADSC, on the differentiation of the latter into CM and the overall development of the construct into a functional tissue. We can also test the effects of changing the coordination of the mechanical and electrical stimuli. For example, the electrical impulse can be alternatively delivered at the peak of the pressure waveform, simulating an excitation followed by a compression of sorts (the relaxation of applied tensile force). It might be determined that this method is more effective at producing differentiation and contractility in hADSC than delivering the stimulus just prior to the beginning of the pressure waveform.

Other changes that might improve the operation of the system include improving the reliability of the Flexcell control unit, which malfunctioned during two separate
experiments involving the platform. Implementing continuous temperature and pH monitoring instruments in some of the Flexcell plates could also be a valuable modification.

5.3.6 References:


6.1 Introduction:

As discussed in Chapter Two, one of our aims was to evaluate the feasibility of surgically implanting a tissue engineered myocardial graft in the manner we have previously described (Figure 6.1). The success of our approach in addressing this problem hinges on the ability to connect the graft’s vasculature with that of the recipient. Otherwise, the cells delivered within the graft will likely succumb to ischemia and die.\[1\] The need for a vascular supply to the graft was driven by the need for the graft to be thick. Without sufficient thickness, the graft would be largely ineffective in preventing or reversing ventricular remodeling. But, a thick graft introduces the complication of diffusional limitations, so a vasculature becomes necessary to transport nutrients and wastes.

We devised this animal study to demonstrate that tissue-engineered myocardial grafts (autologous stem cell-seeded myocardial scaffolds) could potentially be used to revitalize infarcted myocardium by replacing the scar tissue. The goal was to show that the scaffold could be connected to a patient’s vasculature through anastomosis of its arterial and venous conduits to the aorta and pulmonary artery, respectively; and that an infarct scar could be resected and replaced with a portion of the scaffold while this vascular connection was maintained (Figure 6.1F and G).

In this study, we strove to provide proof of concept for the clinical use of this novel scaffold. We focused on the scaffold (without seeded cells) to both simplify and avoid
waste, as the implanted scaffolds did not need to contain cells in order to provide the information we were seeking. Three sub-studies are proposed which, if successful, will provide sufficient supporting data for an NIH grant submission (Figure 6.2). More specifically, the studies aim to prove: 1) that we can generate an infarct model in the pigs, 2) that we can secure the scaffold in place, and 3) that the scaffold will integrate into the host tissue. Perhaps most importantly, we wanted to show that all of this was possible without causing harm to the pigs.

Figure 6.1: Overview of an animal model to test clinical implementation of myocardial grafts: This depiction is representative of the therapy as it is intended to be used in human subjects. A) Intended recipient
with infarcted heart has subcutaneous adipose tissue harvested and **B)** adipose stem cells are isolated from the tissue. Cells are cultured with the aim of growing large numbers for later repopulation by reseeding **C)** of the myocardial scaffold **D)** generated from xenogeneic sources (i.e. a pig). **E)** The seeded construct is placed into a bioreactor wherein it is exposed to stimuli which mimic the cardiac environment in order to differentiate stem cells and condition the construct into a functional graft. **F)** In the operating room, the surgeon trims the graft to fit the size of the infarct, resects the infarcted tissue, **G)** attaches the flap area of the graft over the resection site, and anastomotically connects the graft’s arterial and venous vessels to the aorta and the pulmonary artery, respectively.
Figure 6.2: Overview of preliminary animal study to test clinical implementation of myocardial grafts:

This flow chart illustrates the different groups in the feasibility study. The expected outcomes and methods to assess them are indicated for each group. In addition, critical decision points are identified wherein specific outcomes from each group must be observed in order to proceed to the next group. Procedures in each successive group are more difficult, and thus present more risks.

Pigs were chosen for this study because their cardiovascular anatomy, physiology, and pathologies are very similar to human aspects. Also, pig models of MI are well established and widely accepted for testing such procedures.[2-4] Smaller animals such as mice, rats, and rabbits (the originally proposed subjects for this study) were avoided because they have smaller anatomical features that would present undue technical challenges during surgery and recovery that may have directly resulted in poor outcomes.

This is a feasibility pilot study that will form the basis for a larger, more extensive study. We estimated the number of samples required for each assay to reach statistical significance once studies are completed at n=3. For proposed studies, the estimated need is 12 pigs, which will be equally divided between 3 groups (n=3 per group, 3x3=9). Since survival rate for this type of surgery is estimated at about 70%, we added 3 more pigs to the study (1 pig/group). For pigs to be of a sufficient size to avoid surgical complications stemming from the size of anatomical features, we expected they would need to weigh upwards of 35 kg; pigs were divided into three groups as described above.

After quantitative data sets are obtained, analyses of variance for a completely random design will be done and means will be compared using Fisher's protected least significant difference test (LSD).
We hypothesized that our novel scaffold would allow for efficient integration into myocardium, and, by virtue of its direct connection to the host vasculature, it would rapidly become perfused and nourished by host blood, without significant post-op bleeding. We expected to see positive scaffold integration and adequate perfusion after surgical connection to the host vasculature.

Confirmation of our hypothesis will serve as the basis for a larger study of significant clinical relevance, where autologous ADSC will be seeded onto our scaffold, and, after maturation in vitro, the tissue construct will be implanted into post-infarcted hearts to help with regeneration and healing (Figure 6.1). From this research we will also gain valuable insight into surgical techniques and procedures necessary for proper implantation of such constructs, which will help avoid complications and limit discomfort, distress, or loss of animals in future studies.

6.2 Pilot Study: In Vitro Test of Surgical Attachment:

6.2.1 Introduction:

Initially, we had planned to conduct these studies in smaller animals to minimize the costs of surgery and husbandry. We wanted to avoid animals that were extremely small, such as rats and mice, so settled on using rabbits. Studies using rabbit models of infarction are prevalent, but few have used the animals to investigate complicated surgical techniques that might prove necessary with these studies.\textsuperscript{[5-8]} Consequently, we were concerned that the size of the rabbits’ anatomical features in and of itself would cause surgical complications that would convolute the outcomes of the study.
6.2.2 Methods:

6.2.2.1 Porcine Heart Model:

To test the feasibility of the above methods in an in vitro, non-surgical model, an adult porcine heart obtained from a local slaughterhouse was used as a stand-in for a living heart that received a myocardial scaffold-graft (Figure 6.3A). The left ventricular free wall was cut out of a whole, DCELLeled porcine heart (Figure 6.3B) and further trimmed to fit a defect (simulating a resected scar) created in a fresh heart (Figure 6.3C, D, and E). The defect was not transmural, reflecting a scenario where the surgeon spares a layer of viable myocardium adjacent the endocardium when resecting the infarct scar. The coronary artery and cardiac vein of the scaffold were anastomosed to the ascending aorta and the coronary sinus, respectively (Figure 6.3F and G). The void was coated with a layer of Tisseel™ fibrin glue (Baxter), and the flap portion of the scaffold was pressed into the void (Figure 6.3H and I). A running 4-0 prolene suture was placed at the periphery of the scaffold and weaved back and forth between the scaffold and the native heart in an attempt to better seal the severed vessels of the scaffold (Figure 6.4A, B, and C).

The opening of the aortic anastomosis was accessed through the aorta and a syringe with attached Luer connector was used to cannulate the arterial conduit. The syringe, which was loaded with water colored with red food dye, was depressed and the solution was forced into the scaffold vasculature.

Figure 6.3 (below): In vitro test of scaffold implantation method, Part 1: A) Illustration of the graft implantation method; in the operating room, the surgeon trims the graft to fit the size of the infarct, resects the infarcted tissue, attaches the flap area of the graft over the resection site, and anastomotically connects
the graft’s arterial and venous vessels to the aorta and the pulmonary artery, respectively. **B-I)** Demonstration of graft preparation and implantation in the laboratory. 

**B)** The entire left ventricular free wall was cut away from a whole, decellularized heart and **C)** further trimmed to fit the size of a **E)** simulated defect created by removal of an imaginary infarct in a **D)** fresh porcine heart. Anastomoses were made between **F)** the scaffold’s coronary artery and the heart’s aorta, and between **G)** the scaffold’s cardiac vein and the heart’s coronary sinus. **H)** Tisseal™ fibrin glue was applied to the defect, and the flap portion was attached securely with fibrin glue and sutures.
6.2.2.2 Rabbit Heart Model:

We performed the simulated implantation of the scaffold in a fresh rabbit heart (Animal Technologies; Tyler, TX) in the same manner as described above (Figure 6.5). The only difference in the two methods was that a much smaller portion of scaffold had to
be trimmed, and the portion needed to contain a vascular unit (an artery and vein that connect to one another through a capillary network, thereby supplying a piece of tissue with a functional vascular supply) (Figure 6.5A and B). This vascular unit was tested for integrity by suturing open vessels and injecting water through both the arterial and venous inlet to verify there were no leaks.

6.2.3 Results:

6.2.3.1 Porcine Heart Model:

Injection of red dye into the arterial anastomosis resulted in its migration through the arterial vasculature and into the venous vasculature (Figure 6.4D, E, and F). Veins were bulging with pressure, and after injection of more than 10 mL of dye solution, the whole flap portion began to bulge out from the native heart. (Figure 6.4D and E). No leaks were observed in the major vessels throughout the flap portion of the scaffold, but the venous anastomosis leaked slightly (Figure 6.4E).
Figure 6.4: In vitro test of scaffold implantation method, Part 2: A-C) Myocardial scaffold shown securely attached to the left ventricle of a fresh porcine heart, and with anastomoses of the coronary and venous conduits made to the fresh heart. D-F) Images of the scaffold following injection of water colored with red food dye through the aortic anastomosis. D, E) The scaffold shown bulging outward from the ventricular wall following dye injection. E, F) The scaffold’s vessels were clearly perfused with the dye, and apparent bulging of the cardiac vein demonstrated intact connections between the arterial and venous vascular channels of the scaffold.
6.2.3.2 *Rabbit Heart Model:*

Anastomoses of the major vessels were made, but it was clear during the procedure that they would not be functional. It was not possible to find the opening of either anastomosis in order to inject fluid, so the functionality of the graft was not determined (Figure 6.5C).
Figure 6.5: In vitro test of scaffold implantation method. Part 3: A) Myocardial scaffold shown adjacent to a fresh rabbit heart, and an outline of an attempt to identify a functional vascular unit (an efferent artery and an afferent vein) to patch a B) defect created in the fresh heart. B) Vascular unit after being cut from the myocardial scaffold and C, D) anastomoses of the coronary and venous conduits made to the fresh heart. C, D) The lower part of the vascular unit was secured to the defect in the fresh heart using fibrin glue.

6.2.4 Discussion:

Migration of dye from the arterial to the venous circulation in the porcine model demonstrated that the scaffold possessed intact arterioles, venules and capillaries. The scaffold’s bulging veins showed that the anastomoses and, to some extent, the interface between the scaffold and the native heart held pressure. Bulging of the scaffold indicated some leakage was building up underneath the flap portion, but also that the seals around the edge of the flap were intact. Leakage of the venous anastomosis was due to surgical inexperience. This anastomosis was certainly the more challenging of the two because the vein was flimsy and not as amenable to handling and suturing.

The rabbit model proved to be more technically challenging, and would probably demand the use of microsurgical techniques in order to be done properly.

6.2.5 Conclusions:

The porcine model proved to be feasible, but the rabbit model seemed as if it would present unnecessary complications due solely to its smaller anatomical features. The difficulty of surgical procedures and stresses on animals will only become heightened with each successive study described below. In view of this, we decided to move forward with
studies which used pigs as the recipients of scaffolds, and to abandon the idea of using a rabbit model.

6.3 Group 1: Acute Implantation Testing:

6.3.1 Methods:

6.3.1.1 Modification of Myocardial Scaffold with Vascular Scaffolds for Anastomosis:

To facilitate attachment of the myocardial scaffold’s coronary arterial inlet and venous outlet to the pig vasculature, 6-10 cm lengths of DCELLed porcine carotid arteries, or vascular scaffolds, were anastomotically attached to both of those vessels. This was necessary to ensure that the surgeon had enough length of conduit with which to work, as the graft’s own vessels may not have been long enough to reach the desired points of anastomosis on the aorta and pulmonary artery.

6.3.1.1.1 Vascular Scaffold Preparation

Fresh, 6–12 cm long, porcine carotid arteries with internal diameter tapering from about 8 mm to 3 mm were obtained from young animals via Animal Technologies (Tyler, TX). The arteries were DCELLed in a bioprocessing system according to a recently published protocol developed in our lab. The system, shown below, used pressure vessels and a peristaltic pump to generate translumenal pressure (50-80 mmHg) in arteries mounted inside two custom-designed housings. The combined action of several reagents and this pressure facilitated completely uniform DCELL of the arteries. Filling and drainage ports enabled timely and convenient changing of solutions.
Perfusion bioprocessor for decellularization of vascular scaffolds: Porcine carotid arteries were mounted inside the clear housings, and a system of tubing and pressure heads was used to continuously pump a series of reagents through the vessels until the cells were removed from the tissue.

6.3.1.1.2 Cleaning and Preparation

Once received, arteries were kept on ice and cleaned of excess fat. Luer lock fasteners were zip-tied to each end of the arteries for eventual mounting in the bioprocessing system. To test for leaks, one luer lock was closed off with a luer cap while the luer on the opposite end was fastened onto a syringe filled with saline. The arteries were pressurized and monitored for any leaks. If a leak was observed, the hole was sutured shut using 3-0 Ethibond Excel braided polyester suture. The arteries were once again checked for leaks before proceeding with the remainder of the process.
6.3.1.1.3 Tissue Decellularization and Sterilization

Samples were first treated overnight in 30 mM EDTA, 0.02% sodium azide (NaN₃) (pH to ~7) on an orbital shaker at 3.5 rpm at 4°C. The following day, the arteries were removed from the EDTA solution and mounted in the bioprocessing system. From this point on, the DCELL process continued inside of the bioprocessing system. The steps for DCELL and sterilization are listed below.

1. Mounted the arteries in the perfusion bioreactor.
2. Filled the bioreactor with 30 mM EDTA, 0.02% NaN₃ solution and left overnight.
3. Rinsed bioreactor with ddH₂O.
4. Introduced 1% SDS solution into the reservoir and ran with pressure for 12 days; changed solution at 6 days.
5. Rinsed with ddH₂O overnight with pressure.
6. Rinsed 3X with 1 L of 70% ethanol (EtOH).
7. Rinsed with ddH₂O for 30 min with pressure.
8. Drained water, added 1400 mL of 0.1 M NaOH into system, incubated for 2 hr with pressure.
9. After 2 hr, rinsed with ddH₂O.
10. Added 1400 mL PBS, circulated overnight with pressure.
11. Treated scaffolds with DNase/RNAse solution (720 mUnits/mL DNase, 720 mUnits/mL RNAse, 5 mM MgCl₂, in 1x PBS, pH=7.5 with 1% v/v antibiotic/antimycotic) for 96 hours at room temperature.
12. Added 1400 mL PBS, circulated 1 hr with pressure.

The system was moved into a sterile hood to ensure sterility

13. Rinsed with ddH₂O.
14. Rinsed 2X with 1400 mL of sterile PBS; circulated the last 500 mL for 20 min with pressure.
15. Placed in 1400 mL 0.1% w/v peracetic acid, circulated the last 500 mL for 2 hr with pressure at room temperature.

Pigs (n=3) were used for testing and optimizing the surgical implantation of the myocardial scaffold in healthy hearts and observed for 30 min - 1 h (non-survival surgery). Pigs were preanesthetized by an intramuscular injection of ketamine hydrochloride (20 mg/kg) and xylazine hydrochloride (2 mg/kg). The animals were positioned supine, and a 22-gauge indwelling needle was inserted in the central vein of the auricle for continuous i.v. anesthetic injection. The animals were intubated with an endotracheal cannula (6
French) and then connected to an artificial respirator with a stroke volume of 200–300 cm$^3$/stroke and frequency of 20/min. Propofol (6 mg/kg/h) and vecuronium bromide (0.05 mg/kg/h) was continuously infused using a syringe pump. Pigs were prepped for aseptic surgery using nolvasan scrub and sterile saline rinse in triplicate. Surgery was performed using aseptic technique.

The animals were fixed in a recumbent position so that the left thorax was exposed, and a left thoracotomy was performed as follows: the outer layer of skin and muscles between the third and fourth ribs were dissected. The distance between the third and fourth ribs was widened with a rib spreader to allow a direct view of the left auricle LAD coronary artery. The pericardium was dissected along the LAD from the upper part of the left auricle (approximately 6 cm) to expose the myocardium. The myocardial scaffold was attached to the descending aorta via its arterial vascular scaffold conduit and to the pulmonary aorta via its venous vascular scaffold conduit. An area of about 10 cm$^2$ was shaven off the lower left anterior ventricle and the scaffold glued onto the shaven area with fibrin glue, followed by placement of 6-8 sutures to secure the scaffold. The scaffold was allowed to stay in place for 1 hour to allow blood to clot around the implant and also test for blood leakage. After 1 hour, the pigs were humanely euthanized via intravenous injection of FatalPlus (1 mL/10 lb; supplying 390 mg/mL sodium pentobarbital) followed by performance of a bilateral pneumothorax for euthanasia verification, and hearts and scaffolds were collected for analysis via histology.
6.4 Group 2: Induction of Myocardial Infarct:

Pigs (n=3) will undergo induction of MI by left anterior descending (LAD) coronary artery ligation and be allowed to survive for 4 weeks. Electrocardiography (EKG), echocardiography (ECG), blood chemistry and histology will ascertain induction of MI at 4 weeks (survival surgery). EKG and ECG will be performed prior to surgery in order to establish baseline levels for these signals. Pigs will be prepped for aseptic surgery using nolvasan scrub and sterile saline rinse in triplicate. Surgery will be performed using aseptic technique. Hearts will be exposed by left thoracotomy as described above. The pericardium will be dissected along the LAD from the upper part of the left auricle (approximately 6 cm) to expose the myocardium around the LAD. The LAD on the proximal side, below the left auricle from the myocardium, will be exfoliated for approximately 1 cm. A lidocaine hydrochloride jelly will be applied to anesthetize the area. First, a complete ligation of the LAD will be made immediately distal to the second diagonal branch using a 2-0 suture. Approximately 20 min later, an ameroid constrictor (3.5 mm internal diameter) will be fastened around the coronary artery just distal to the left circumflex artery branch using sutures. The ameroid constrictor is designed to slowly (within 3-5 days) ligate the artery. To enhance the effect of the ameroid constrictor, 2 additional suture strings will be loosely rounded at the site of the ameroid so that these strings will be located between (below) the ameroid constrictor and arterial wall. This dual ligation approach has been shown to improve survivability of the procedure by reducing the likelihood of fatal arrhythmia and to produce a more pathologically relevant model with
respect to models generated using a single ligation point. Following ligation, the fascia and intercostal space will be closed, buprenorphine (0.04 mg/kg) and prophylactic antibiotics (ceftiofur; 5.0 mg/kg) administered i.m., and pigs allowed to recover.

Blood will be collected (described below) immediately before and after the surgical procedure and troponin (TN), lactate dehydrogenase (LDH), creatine phosphokinase (CPK) levels measured. After 4 weeks, EKG and (ECG) will be performed under anesthesia. For ECG, pigs will be scanned in a parasternal short-axis view with 2D and M-mode imaging and a broadband high-frequency (12-MHz) transducer. Left ventricular internal diastolic diameter (LVIDd), end diastolic volume (LVEDV), internal systolic diameter (LVIDs), and end systolic volume (LVESV) will be calculated and the left ventricle ejection fraction (LVEF) will be calculated. ST segment elevation and abnormal Q waves on EKG will be indicative of MI. Pigs will be euthanized under anesthesia as described above. Hearts will be excised and analyzed for additional signs of infarction.

6.5 Group 3: Short-term Implantation Testing:

Pigs (n=3) will undergo induction of MI as previously described in Group 2 and be allowed to survive for 4 weeks before implantation of the myocardial scaffold; following scaffold implantation, pigs will be assessed for evidence of scaffold integration at 2 and 4 weeks post implantation (multiple survival surgery). At 4 weeks post-infarction, scaffolds will be implanted as described in Group 1, with the exception that the area to be cut away from the left ventricular wall will be the infarct scar. After ensuring hemostasis, the fascia and intercostal space will be closed, buprenorphine (0.04 mg/kg) and prophylactic
antibiotics (ceftiofur; 5.0 mg/kg) administered i.m., and pigs allowed to recover. At both 2 and 4 weeks, EKG and ECG will be performed as previously described, and pigs will be euthanized after 4 weeks under anesthesia as described above. Hearts will be excised and analyzed for evidence of scaffold integration.

Subdermal adipose tissue (post-mortem) and blood (pre-op/ante mortem and post-op) will be collected for isolation of ADSCs and circulating endothelial progenitor cells, respectively, which will be used for subsequent in vitro applications and future in vivo studies (blood collected will primarily be used for assays germane to results of the studies). Each collection procedure is described below.

6.5.1 Blood collection: [10]

While anesthetized, pigs will be laid laterally and the subcutaneous abdominal vein will be located. The area will be swabbed with 70% ethanol to disinfect, and thumb pressure will be applied on the proximal end of the vessel to occlude blood flow. A 21 G or 19 G needle, depending on the animal size, will be introduced into the engorged vessel. Approximately 50 mL of blood will be collected, and hemostasis will be achieved by application of thumb pressure for about 5 min. Blood collection will be carried out before and after the procedure described in Group 2, and also just prior to euthanasia in all groups in order to isolate circulating endothelial progenitor cells.
6.5.2 Fat collection:

Subcutaneous fat (10 g per pig) will be harvested from the dorsal abdominal and inguinal areas of the pigs immediately following euthanasia. We will collect fat from these areas and compare stem cell counts. After euthanasia, the subdermal adipose tissue is accessed through a 15 cm longitudinal incision in the abdominal or inguinal area. After tissue identification, 10 ml of saline solution containing 1:1,000,000 dilution of epinephrine are added to minimize blood loss and after 3 minutes, a subdermal adipose tissue (5-10 cm diameter) is collected for isolation of ADSCs.

6.5.3 Post-operative Recovery and Monitoring:

Immediately post-op, the respiratory rate, reflexes, mucus membrane color, movement, and appetite of pigs will be monitored for 15-30 minutes, or until ambulatory. We will also be alert to redness, swelling or oozing at the surgical site; changes in appetite, attitude/activity, and temperature will be monitored every 6-12 hr for the first 48 hr, and daily thereafter.

Pigs will be housed singly during post-op recovery and may be housed in groups as determined by the attending veterinarian (AV). If possible, single housing pen construction should allow for social interaction, as pigs are social animals.

Other measures taken to monitor animal health include: 1) Appetite: documented daily. 2) Hydration: documented by skin test daily. 3) Weight: measured 2 times per week for the first 2 weeks, then weekly for 2 weeks. After 1 month every 2 weeks or at the
discretion of the AV. 4) Behavior will be assessed daily and considered together with other
criteria over 3-4 days period. 5) Surgery site monitored daily for proper healing for first
week, every other day thereafter.

6.6 Results:

6.6.1 Group 1:

To date, 1 pig from this group underwent a surgical procedure. The anastomoses
of vascular scaffolds to the coronary artery and cardiac vein were carried out on a back
table in the operating room just prior to the surgery. The integrity of the anastomoses was
tested by inserting a syringe loaded with saline into the end of each vascular scaffold and
injecting the liquid. No leaks were observed (Figure 6.7A-C).

After opening the thoracic cavity of the animal and exposing the heart, the vascular
scaffold connecting the venous circulation was successfully anastomosed to the pulmonary
artery. When blood was allowed to flow through this connection, no leakage was observed,
and blood spread slowly through the venous circulation (Figure 6.7D and E). This
connection was clamped while the arterial anastomosis was made to an area of the
descending aorta just inferior to the aortic arch (Figure 6.7H). Blood was allowed to flow
through this connection, and flowed at a much greater volume and velocity. Leakage was
observed from several arterial branches of the LAD CA that fed the septum, as these had
been severed when the left ventricle was cut away from the rest of the heart. These leaks
were quickly closed with small metal clamps, and hemostasis was achieved shortly
thereafter.
At the surgeon’s discretion, the procedure was halted at this point. There were no complications which precipitated this decision – it was simply determined that the selected approach of a left thoracotomy did not provide the correct angle for sufficient access to the anterior left ventricle in order to proceed with resection and scaffold attachment.

Following anastomosis, blood was allowed to flow freely through the scaffold. There was a clear change in its color, as it went from an opaque white to a reddish-pink (Figure 6.7G and I). Blood was observed to perfuse the arteries and veins, even down to vessels of a size barely discernable to the naked eye (Figure 6.7J). The scaffold was left in this state and observed for a period of about 30 minutes, during which time no purpling of the blood was noticed. Palpation of the vascular scaffolds and the LAD CA also indicated good pressure during this time, although a pulse was not detected in this manner.
Figure 6.7: **Acute implantation testing of myocardial scaffold in a porcine model:**

A) A vascular scaffold is shown as it was anastomosed to the cardiac vein of the myocardial scaffold.

B) Once the vascular scaffolds were attached, sterile saline was injected through them to test the integrity of the anastomoses.

C) Myocardial scaffold shown with vascular scaffolds anastomosed to the coronary artery and cardiac vein to allow for extensibility and surgical flexibility.

D) Initial incision for left thoracotomy was
made and electrocautery equipment and retractor were used to cut down and open the thoracic cavity. **E)** Anastomosis of the venous conduit to the pulmonary artery; shown clamped, but, when opened, low pressure and low velocity flow of blood into the scaffold was observed. **F)** Myocardial scaffold shown as it would lay on the heart once fully implanted; in clinical application, scaffold would be trimmed down significantly from the size shown here. **H)** After both conduits had been anastomosed to their respective vessels (arterial to the descending aorta (DA) and venous to the pulmonary artery (PA)), **G)** the scaffold’s vessels were clearly perfused with blood, especially when compared to an **I)** unimplanted scaffold. **J)** Endocardial side of the myocardial scaffold, where perfusion of the scaffold’s microvasculature is visible.

### 6.6.2 Histological Assessment of Scaffold Perfusion:

Histological sections obtained from both the arterial vascular scaffold conduit and the myocardial scaffold showed extensive perfusion of the entire construct with host blood (**Figure 6.8**). Vessels of wide-ranging diameters were perfused, and inspection of high magnification micrographs revealed perfusion of vessels as small as 6-8 µm in diameter (**Figure 6.8B, D and F**). A comparison of the blood within the vascular scaffold and that within the smaller vessels in the myocardial scaffold demonstrated differences in its structure. The former showed clumps of loosely coagulated erythrocytes, while the latter appeared to be solid, compact fibrin with no identifiable erythrocytes (**Figure 6.8G and H**). Additionally, erythrocytes and leukocytes (unclear if they were neutrophils or monocytes) were observed to attach to the vascular scaffold’s lumen (**Figure 6.8C and E**).

**Figure 6.8 (below): Histological evaluation of acute implantation of myocardial scaffold:** **A, C, E, G)** Micrographs of a circumferential section of the arterial vascular scaffold conduit, increasing in magnification downward. **B, D, F, H)** Micrographs of a section through the myocardial scaffold from a region about 1.5
cv distal to the scaffold LAD CA, increasing in magnification downward. A) Vascular scaffold shown with erythrocytes in lumen. B, D) Myocardial scaffold showing extent of perfusion throughout a wide range of different-sized vessels. C, E) Erythrocytes and leukocytes shown adhering to the lumenal wall. F) High magnification micrograph of myocardial scaffold identifies perfusion of vessels on the scale of capillaries. G) Magnification of erythrocytes in vascular scaffold lumen distinguishes individual cells, whereas magnification of blood in a more distal artery shows dense, fully formed thrombus. A, B) 2.5X, bar=500µm; C, D) 10X, bar=100µm; E-H) 40X, bar=20µm.
6.7 Discussion:

It was not clear to what extent or how rapidly coagulation set in, but judging from color alone, blood flow in the scaffold seemed to remain unobstructed for about 15-20 min. Instead of darkening slightly as thrombus does, the blood remained a scarlet red. The lack of purple coloration (indicating deoxygenation) also suggested that the direction of its flow was from the arterial into the venous circulation. In addition to having the proper directionality, this meant that blood must have flown through what remained of the capillary beds. This confirmed the results of several in vitro methods we had previously used to demonstrate that the scaffold vasculature was patent and intact down to this level (see Chapter Three).

Histological evaluation of the scaffolds agreed with these observations, as vessels of similar size to capillaries were perfused with blood. As was expected, thrombus formation appeared to have begun first within smaller vessels and propagated proximally through larger vessels. This was supported by the observation that smaller vessels contained only solid, coagulated plasma, while the lumen of the much larger vascular scaffold contained identifiable erythrocytes that did not appear significantly coagulated.

It was also noted that we did not observe any dispersion of erythrocytes outside of vessels and into the rest of the scaffold matrix, which would be indicative of leaks. This suggested that all vessels within the scaffold retained their integrity post-DCELL. This inference applies to capillaries as well, which is intriguing since a common question with the DCELL approach is whether the capillary conduits are left intact once endothelial cells are removed. Had capillary conduits not remained intact, we would expect to see many
erythrocytes scattered throughout the scaffold because these cells would no longer be
contained once they reached that level of the vasculature.

Since this scaffold and its vascular channels consisted of bare collagen and other
ECM components, it was our expectation that coagulation and eventual occlusion of the
vasculature would occur.\textsuperscript{[11, 12]} We noted conditions that seemed to suggest arterial to
venous blood flow for 15-20 min, implying patency of microvasculature during this time.
While this was encouraging, future grafts implanted in this manner will require
endothelialization, heparinization, or both to avoid the formation of significant
thrombus.\textsuperscript{[11]}

To correct problems with accessibility to the ventricle, it was decided that future
surgeries would proceed through a median sternotomy approach. This approach involves
cutting down the midline of the sternum by using a saw, and can be more traumatic than a
left thoracotomy. Despite this, it is important to use an approach that allows unencumbered
access to the entire anterior surface of the heart so that an area of the ventricle can be
resected and replaced with the scaffold in future experiments.

Subsequent procedures should consider using longer lengths of vascular conduit so
as to reach the right atrium and avoid crossing the conduits over one another as seen in
\textbf{Figure 6.7H}. The right atrium was specified above, rather than the pulmonary artery,
because moving the point of venous anastomosis from the former to the latter would
provide a more favorable pressure gradient for blood flow through the scaffold. In contrast
to pulmonary arterial pressures, which can range from 30-50 mmHg, right atrial pressures
range from 5-12 mmHg. This would provide a greater pressure differential between arterial
conduit inflow pressure (120 mmHg) and the retrograde resistance at the venous conduit, increasing flow velocity through arterioles and capillaries and avoiding potentially hypertensive conditions in these vessels. Capillary pressure, which largely influences the extent of nutrient and waste exchange between blood and interstitial fluid, would be normalized by this change and it is therefore highly recommended in future surgical procedures.

It was also apparent that open branches in the scaffold should be ligated or sealed prior to the surgical procedure. This will be done by injecting a colored solution (e.g. sterile DMEM) through the vascular grafts and noting the position of any leaks before sealing them. In doing so, hemostasis may be achieved more quickly.

Overall, we noted there were no complications with the pig during surgery, and blood loss was limited to 250 mL or less. By our estimates (70 mL blood/kg in a 37 kg pig), this volume was equal to about 10% of the circulating blood volume, well below the maximum allowable blood draw of 15% in a single procedure.\[13\]

This was not the first instance of a successful procedure of this kind. Other groups have attempted to employ this approach to solve the problem of providing a vascular supply to tissue-engineered grafts.\[1, 14\] There is also precedent established for a few of the key implantation techniques we intend to use, including the resection of significant portions of the infarct scar to enable attachment of the device within the void created, and the anastomosis of the vessels supplying the graft to the patient’s vasculature.\[14\] For the former, the reasoning is that attachment of the graft adjacent to any healthy, functional tissue will allow for greater electrical and vascular integration
with the host than would its attachment directly over nonfunctional, less vascularized infarct scar tissue (discussed in section 1.5.6). Robinson et al. demonstrated the feasibility of scar resection prior to device implantation without compromising the ventricular wall’s integrity under systolic pressures.\cite{Robinson15} Ott et al. successfully anastomosed the aorta of a whole, DCELLeed rat heart to the abdominal aorta of another rat, and showed that the acellular organ had an intact and patent vasculature that conducted the host’s blood adequately without bursting or leaking.\cite{Ott14}

As discussed above, it will not be sufficient to merely connect grafts to the vasculature in the future. Measures must be taken to prevent thrombus formation, otherwise the functionality of such grafts will be compromised. Furthermore, vascular integration at the interface of host tissue and the graft may be necessary to ensure sufficient vascular supply, especially to peripheral portions of the graft.

**6.8 Conclusions:**

Given the functionality of the anastomoses and how well the surgery was tolerated by the animal, we considered this initial procedure a success. We will continue with these studies as planned in order to establish the feasibility of future implantations of scaffolds seeded with cells. To correct problems with accessibility to the ventricle, it was decided that future surgeries would proceed through a median sternotomy approach. It may also be necessary to place pigs on heart-lung bypass in order to perform resection of the ventricle. Moving the point of anastomosis of the venous conduit from the pulmonary artery to right atrium may prove more beneficial to the overall vascular flow.
within the scaffold. It was also apparent that open branches in the scaffold should be ligated or sealed prior to surgery in future procedures.

6.9 References:

CHAPTER SEVEN: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

7.1 Summary of Project Development:

Based upon data furnished by the American Heart Association, an estimated 1.43 million individuals are currently at risk of developing CHF as a result of tissue damage caused by MI. Costs to treat CHF represent a significant burden to our society; currently, they stand at $44.6 billion but are predicted to double by 2030. Patients with CHF perennially account for 6% of all Medicare beneficiaries, and are some of the most expensive to treat. In fact, among the top 5% of patients in terms of overall expenditures, patients with CHF comprise a full third of the population\(^\text{[1]}\). These facts and figures clearly outline why developing practical and effective therapies to treat these patients is a worthwhile cause.

After gaining an understanding of the pathological link between MI and CHF and undertaking a critical evaluation of the current standards of care, limitations in our ability to treat this population of patients were identified. These limitations represented an opportunity for innovation, so we set out to develop a novel, translational approach to treatment of the underlying causes of this condition. We relied on a knowledge of the advantages offered by tissue engineering to inform our unique approach. After studying some of the successes and failures by others in this application, we crafted an approach that demanded the development of a tissue-engineered graft that was thick, fully vascularized,
and functional (contained cells that mimicked the function of healthy myocardium) to replace infarct scar tissue and prevent or reverse development of CHF in affected patients.

7.2 Progress toward Achievement of Specific Aims:

To accomplish the overall objective of this project, we established 4 specific, yet interrelated aims and directed our efforts in toward achieving them. These aims were described at length in Chapter Two but are presented in brief below:

**Aim 1** (Chapter 3): To develop and characterize a decellularized, vascularized scaffold for use in myocardial tissue engineering

**Hypothesis:** Acellular left ventricular porcine myocardium will serve as an ideal supporting material for tissue engineered myocardial grafts due to its low immunogenicity, potential as an excellent “niche” for inducing differentiation of stem cells toward cardiac phenotypes, and its network of preserved, patent vascular channels.

**Aim 2** (Chapter 4): To characterize the host response to scaffolds and optimize their in vivo degradation rate

**Hypothesis:** Scaffolds which resist in vivo degradation for longer than 8 weeks will provide implanted cells sufficient stability and time to integrate with host tissue, improving functional outcomes. Treatment with a specialized cross-linking agent will mitigate the severity of the immune response to implanted scaffolds.

**Aim 3** (Chapter 5): To condition cell-seeded myocardial constructs in vitro into functional grafts
**Hypothesis:** Exposure to conditions mimicking the physiological environment of the myocardium will induce stem cell differentiation and maturation of constructs into functional myocardial grafts.

**Aim 4 (Chapter 6):** To develop an animal model of infarction and demonstrate feasibility of surgical replacement of infarct scar with tissue-engineered constructs.

**Hypothesis:** A porcine model of infarction and surgical approach to construct implantation must be developed and optimized.

Our progress toward completing these aims, as well as our conclusions in regards to their attendant hypotheses, have been discussed at length in each of the indicated corresponding chapters, but a summary of the major conclusions is as follows:

7.2.1 **Aim 1 (Chapter 3):** To Develop and characterize a decellularized, vascularized scaffold for use in myocardial tissue engineering:

1) We evaluated both the myocardial and arterial matrix of our scaffolds, and found they are porous, retain collagen and elastin content and structure, exhibit excellent mechanical properties, and are cytocompatible. Moreover, scaffolds retain laminin, fibronectin, and collagen IV. We’ve also preserved the vasculature’s integrity and patency down to the capillary scale.

2) Cytocompatibility and support of CM survival indicate that our scaffold is ideally suited for repopulation with relevant cardiac cell types as a tissue-engineered construct for myocardial repair.
3) Given its composition, intact vasculature tree, mechanical properties, and geometry, we believe constructs produced from this scaffold have the potential to functionally integrate with healthy host myocardium and to be nourished by direct anastomotic connection with the host’s own vasculature.

4) Constructs resulting from recellularization of this scaffold may also be useful as physiologically accurate models for in vitro studies of cardiac physiology and pathology.

7.2.2 **Aim 2 (Chapter 4): To characterize the host response to scaffolds and optimize their in vivo degradation rate:**

1) The host response in a xenogeneic implantation study was directed predominantly by cells commonly associated with immune functions (i.e. of leukocytic lineages). The scaffold is “bioactive”, as opposed “inert”, with a heavy infiltration of host cells.

2) Treatment of scaffolds with PGG can extend their lifetime up to, and possibly beyond, 12 weeks – presumably a sufficiently long period for delivered cells to integrate with host tissue in future implantations of recellularized scaffolds.

3) The host response to scaffolds does involve some elements of the classical inflammatory response (FBGCs), but over time the environment becomes less pro-inflammatory as evidenced by the predominance of M2 macrophages.

4) It was clear that this scaffold encouraged angiogenesis, which is a very desirable quality for our overall approach in generating functional, vascularized muscle.
5) We were unable to thoroughly describe the functionality, or “appropriateness”, of the tissue ultimately formed by host remodeling of the scaffold, as this is dependent upon the implantation site and the intended function of the implant.

7.2.3 Aim 3 (Chapter 5): To condition cell-seeded myocardial constructs in vitro into functional grafts:

1) Use of myocardial scaffolds as a substrate for hADSC growth precipitated expression of cardiac genes and proteins regardless of treatment with AZT.

2) Evidence of increased CM marker expression in hADSC grown on scaffold vs. standard culture plastic was not abundant, but what little there was could be explained by an inductive role of the structure and composition of myocardial scaffolds in cardiomyogenic differentiation of hADSCs.

3) The addition of variables such as electrical and mechanical stimuli may be necessary to achieve full differentiation of hADSCs into a CM phenotype.

4) We successfully engineered a system capable of delivering mechanical and electrical stimuli in a coordinated manner to cell-seeded myocardial scaffolds.

5) Early indications are that this system will serve as an efficient platform for testing the effects of these stimuli on the differentiation of hADSC into CM.

6) Flexcell International Corporation makes poorly designed and unreliable products.
7.2.4 **Aim 4 (Chapter 6): To develop an animal model of infarction and demonstrate feasibility of surgical replacement of infarct scar with tissue-engineered constructs:**

1) The rabbit model seemed to present unnecessary complications due solely to its smaller anatomical features, and was abandoned.

2) To correct problems with accessibility to the ventricle, it was decided that future surgeries would proceed through a median sternotomy approach.

3) It was also apparent that open branches in the scaffold should be ligated or sealed prior to the surgical procedure. This will be done by injecting sterile DMEM through the vascular grafts and noting the position of any leaks before sealing them. This will ensure that hemostasis is achieved more quickly.

4) The anastomoses were functional and hemostasis without significant blood loss. The surgery was tolerated well by the animal; we considered this initial procedure a success and surgeries will continue as planned.

7.2.5 **Perspectives on Progress Made and Comments on Potential Continued Research:**

The studies conducted and reported on here have fulfilled the objectives of Aim 1 and made significant progress toward completion of the other aims. Continued study of the host response may lead to better understanding of outcomes of future experiments wherein cells, autologous or otherwise, are delivered within scaffolds.

Development of the Flexcell platform carried with it significant technical challenges, but as it is constituted now, the system should become a valuable tool in working toward the completion of Aim 3. Along those lines, further experimentation with
the system may demonstrate that differentiation of hASDSs using only dynamic stimuli is a less effective or practical approach than using human IPSC, which are now being shown to be highly amenable to cardiomyogenic differentiation.\cite{2-4} In the context of our translational approach, it is still the opinion of the author that hADSCs represent the most practical option for reasons already delineated above. However, it may be advisable to focus on using this platform as a tool to “condition” cells that already display a number of CM traits to become more functional, rather than using it to affect fully de novo differentiation.

Initial progress toward Aim 4 is both promising and exciting, and significant thought and planning should be put into how future implantation studies are conducted, as their results have the potential to make or break the translational potential of this therapy.

Regardless of future directions, it is expected that this research will have a positive impact upon efforts to treat a growing population of patients suffering from complications of MI. It will not only enable clinicians to prevent or reverse the progression towards CHF—saving lives and improving the quality of life for recipients of this therapy—but also contribute to the advancement of the cardiac tissue engineering field.

### 7.3 Recommendations for Future Work:

#### 7.3.1 Aim 2:

Moving forward, we will be able to use either PGG-treated or non-treated scaffolds in this translational approach, depending upon the desired degradation profile. The response to scaffolds may also prove to be drastically different at the eventual target site,
the heart, and when there is an autologous cellular presence within the scaffolds, as would be the case with myocardial grafts. It is advised that future implantation studies examine the response under these conditions.

As for things that can still be studied in samples from both Biocompatibility Studies, a number of IHC and IF stainings can be done to look for certain factors of interest. Sections could be stained for peroxisome proliferator-activated receptors (PPAR), whose downregulation has been implicated in causing a phenotypic shift from M2 to M1, in order to provide further confirmation of polarization and gain insight into its possible mechanistic progression. Stem cells that may be recruited to the vicinity of the implant can be identified by staining for CD34 or CD29, mesenchymal stem cell markers. Their specific populations of origin may be determined by staining for the hematopoietic stem cell, adipose-derived mesenchymal stem cell, and bone marrow-derived mesenchymal stem cell markers CD144, CD146, and CD106, respectively. Other cell types such as fibroblasts and smooth muscle cells can be identified by staining against HSP47 and vimentin, and α-smooth muscle actin, respectively. Lastly, one can look for evidence of newly secreted collagen within the scaffolds by staining for collagen type III and prolyl-4-hydroxylase.

7.3.2 Aim 3:

Cell seeding of scaffolds is absolutely critical. The platform can function perfectly and provide excellent conditions for cell differentiation, but that is meaningless without the presence of cells on the scaffold. Even with too few cells on the scaffolds their
differentiation may be adversely affected, and evaluation of the effects of the experiment may become too challenging because of a lack of cell density. Pilot experiments should be conducted which directly compare the efficacy of different cell seeding methods.

Future experiments could investigate the co-culture of cardiac fibroblast seeded along with hADSCs onto scaffolds and their effect when combined with the dynamic conditions provided by the platform. The effects of changing the coordination of the mechanical and electrical stimuli could also be tested. For example, the electrical impulse can be alternatively delivered at the peak of the pressure waveform, simulating an excitation followed by a compression of sorts (the relaxation of applied tensile force). It might be determined that this method is more effective at producing differentiation and contractility in hADSC than delivering the stimulus just prior to the beginning of the pressure waveform.

The following is a suggested study once initial studies with the Flexcell system have been used to determine which specific stimuli are useful in cell differentiation. This information can then be applied in concert with the use of a perfusion bioreactor described herein.

7.3.2.1 In Vitro Conditioning of Cell-Seeded Constructs in a Bioreactor

7.3.2.1.1 Bioreactor Design:

The bioreactor will be designed using SolidWorks software, and machined by Clemson University Machining and Technical Services. We have extensive experience in this area[^8], and the process will be completed using formal methods of design. Based upon
the results of the Flexcell studies, some of the design features described below may be changed or excluded, but the design we envision includes the capability to deliver all stimuli that will be tested in the Flexcell studies. Design features and outputs are as follows (Figure 7.1): (1) a main housing with 6 chambers (one construct per chamber) which can be filled with chemically defined cell culture media, (2) silicone membranes to which each construct is fixed via suturing to extensions of lid that insert until nearly flush with bottom of each chamber. The membranes will operate on the same principle as the Flexcell system, cyclically rising and falling in response to increased or decreased air pressure (driven by a compressed air source and controlled by LabView) in the chambers below them – this imparts cyclic tensile and compressive forces on the constructs (Figure 7.1 bottom); (3) insulated electrical leads extending from a LabView-controlled circuit which will be attached to electrodes as in the Flexcell system, providing paced electrical stimulation similar to the cardiac conduction system; the scale of the housing and chambers will be such that the entirety of constructs will lie between the electrodes, exposing all resident cells to the developed electrical field, (4) dynamic fluid circuit, digital peristaltic pumps, and pinch valves to control fluid pressure and simulate pulsatile blood flow using circulating culture media. Computational fluid dynamics (CFD) analysis will be conducted on the pathway of fluid flow through the bioreactor. CFD analysis software (SolidWorks) will provide measurements of wall shear stress at the coronary artery inlet. These measurements will be informative regarding possible responses of endothelial cells seeded on the scaffold vascular lumen. Additional design considerations will include sterility,
visibility, continuous monitoring of pressures, and ability to fit inside a standard cell culture incubator (Figure 7.1 bottom).

Figure 7.1: Conceptual drawing of possible perfusion bioreactor: At top, schematic of bioreactor components and their layout. Components are as follows: (1) main housing with 6 chambers (2) compliance chamber/pressure head (3, 4) media reservoirs with attached micron-scale air filters (6) pinch valve for pulsatile flow (7, 8) media routing manifolds (9) data acquisition module and electrical stimulus generator (10) media outflow lines. At bottom, CAD software-generated drawings (Solidworks™) of the main housing and its features (isometric, lateral, and top views of the housing, respectively, from top to bottom). Blue arrows show path and action of air used to generate positive pressure and deflect silicone membrane (horizontal black line) upward. Red arrows indicate the path of media flow in and out of the housing. A seeded construct (SC) is shown occupying its designated position in one of the chambers with electrodes (E) located on both sides and just above it. The flow of media in and out of the seeded construct is also denoted by red arrows.
7.3.2.1.2 Scaffold Preparation:

DCELLed scaffolds (n=6) prepared as previously described will be trimmed to 3 cm x 3 cm at the distal or apical portion of the left ventricular wall so that the intrinsic vasculature and afferent coronary artery are left attached; the resulting “flap” scaffolds will be sterilized, rinsed, and serum neutralized.

7.3.2.1.3 Cell seeding, incorporation of constructs into bioreactor, and conditioning:

Results of the Flexcell studies will determine which, if any, cell types (e.g. fibroblasts) will be seeded in addition to hADSC. We will begin the seeding process by injecting hADSC suspended in media at $5 \times 10^6$ cells/mL through the coronary vasculature of the scaffolds described above. The intent of seeding cells in this manner is to reendothelialize the vasculature. There is evidence that hADSC can differentiate into an endothelial cell phenotype under conditions of applied fluid shear stress, and we believe that once attached to the lumens of vasculature, they will be differentiated into an endothelial phenotype (hADSC-EC) by virtue of their exposure to the flow of media through the bioreactor and constructs. Prior to injection, the circumflex artery and the more proximal diagonal branches of the coronary artery will be ligated with sutures, and the edges of the scaffold, including the edge extending to the right ventricular wall and the ventricular septum, will be tightly clamped to prevent any initial leakage of the perfused cell suspension through severed vessels. The seeded scaffolds will then be laid flat and cultured statically in a petri dish for 6 h to allow cells to attach securely to the vessel lumens. At this point, the scaffolds will be turned over and laid on their opposite surface,
and the above cell suspension perfusion process will be repeated. The reasoning behind this is to ensure a more uniform coverage of the lumen, as cells will settle out of suspension during static culture, thus covering only up to ½ of the lumen surface in each perfusion step. Following another 6 h of static culture, scaffolds will be placed within their respective housings in the bioreactor and the arterial and venous cannulas will be connected to inlets and outlets, respectively. The scaffolds will be seeded with a combination hADSC (70%; to be differentiated into CM; hADSC-CM) and human CF (30%; should they be found in Phase 1 to be beneficial) cell suspension via injection at 15-20 points throughout the scaffolds’ interstitium and also on the epicardial surface by drop-wise pipetting. At this point, the bioreactor will be sealed and will continuously circulate culture media (DMEM with 10% fetal bovine serum and 1% antibiotic/antimycotic) through the vasculature as well as throughout the chambers (immersion-perfusion). We will adjust various stimuli and conditions within the bioreactor to approximate normal physiological conditions of the myocardium (fluid pressure = 120/80 mmHg, fluid shear = 12 dynes, fluid viscosity = 3-4 cP (using dextran), electrical stimulus = 1.25 Hz, 2-4 V/cm, mechanical force = 2 N tension, 20% strain). With these conditions set, the system will be placed inside an incubator at 37 °C and maintained in this state for 4 weeks to induce formation of inter-CM and CM-fibroblast electrical connections, growth of a confluent endothelial layer on vessel lumens, development of global, synchronous contraction, and overall construct maturation. Culture media will be changed every 4 days under sterile conditions.
7.3.2.1.4 Cell Culture:

Each of the cell types listed above will be cultured individually in cell-specific media and subcultured to attain sufficient numbers to seed the scaffolds (n=6) at about ¼ of the correct physiological cell density (5 x 10^7 cells/cm³)\(^{[10]}\) of the native human myocardium (roughly 0.35 billion cells/scaffold). We will use a cell culture scale-up apparatus, the CellISTACK® (Corning®), to produce sufficient numbers of cells for this study. To account for differing proliferation rates among cell types, the proportions of each cell type seeded will be altered from their physiological levels (i.e. CF – 60% of total cell number in myocardium, CM – 25 %, and EC – 5%)\(^{[11, 12]}\) to levels which we estimate will yield approximately correct ratios once conditioning and maturation is complete (i.e. CF – 30% of total seeded cell number, hADSC-CM – 67.5%, hADSC-EC – 2.5%). Should we find that treatment with AZT enhances differentiation of hADSC into CM in the Flexcell study, we will add AZT into the culture media in the bioreactor. For hADSC targeted for EC differentiation, we will use endothelial cell growth supplement in cultures prior to seeding and treat them with AZT or another DNA-demethylating reagent shown to induce hADSC to EC differentiation, BIX-01294 (Sigma), just prior to harvest for seeding.\(^{[13]}\)

7.3.2.1.5 Characterization of conditioned constructs:

After 4 weeks of culture within the bioreactor, we will evaluate constructs for cell density and distribution, maintenance of cell viability, and adoption of cell and tissue morphology, mechanical properties, and electrical stimulus propagation comparable to native myocardium. Constructs will be cut into sections and processed for various methods
of evaluation: 1) fixation in 4% paraformaldehyde for histology and immunofluorescence/IHC; 2) extraction of protein and DNA via Trizol® reagent and homogenization for western blotting and RT-PCR, respectively; 3) preservation in cell culture media for tests of cell/tissue viability and functionality, including micro-electrode array (MEA) probing and patch clamping in both the presence and absence of agonists, calcium transient imaging using Fluo-4, and measurement of the force of any contraction produced by cells using a custom-designed force transducer. We will also prepare samples of recellularized arteries for inspection via scanning electron microscopy to quantify confluency and study features of the newly differentiated endothelium. These cells’ ability to take up oxidized LDL will also be evaluated as a test of their functionality. We will evaluate the mechanical properties (modulus of elasticity and burst pressure, respectively) of both the arterial and myocardial portions of the tissue in the same manner as was done previously to evaluate DCELLed scaffolds.

Histological sections will be stained via H&E and Masson’s trichrome methods to visualize cellular dimensions, number, and distribution. Tissue morphology and maturation will be assessed with H&E, Movat’s pentachrome, Gomori’s trichrome, and Voerhoff van Geisen (VVG) stains (to facilitate semiquantitative measurement of cell types, elastin, and collagen content and structure). IHC and IF will be used to stain for cell type-specific markers (CM: cardiac MHC, actin, α-sarcomeric actinin, troponin, and desmin; CF: vimentin and discoidin domain receptor 1 (DDR-1); EC: CD31, Von Willebrand factor, VE-cadherin). Image analysis software (NIH) will be used to count nuclei in visual fields (taking into account that some CMs might be multinucleate), and the
principle of Delesse (area density=volume density) will be applied as described by Boyle\textsuperscript{[14]} and Levkau\textsuperscript{[15]} in order to determine cell density and proportions of specific cell types. In addition, small samples (1 mm\textsuperscript{3}) (n=6) of the constructs will be harvested, their masses will be recorded, and they will be subjected to a PicoGreen assay and gel electrophoresis to quantify DNA content as a measure of cell number. Cell viability will be assessed by a colorimetric assay for cellular respiration (MTT/MTS) on small samples (n=6) of the constructs and by performing Live/Dead staining on small samples (n=6). The induction of electrical integration will be evaluated via IF staining for connexin43 (characteristic marker of gap junctions), and contractile force generation will be measured via a force transducer connected to a data acquisition module. Additionally, the development of intercalated discs will be assessed semiquantitatively (using ImageJ) following staining with antibodies against N-cadherin.\textsuperscript{[16]}

\section*{7.3.2.1.6 Outcome Success Measures:}

Wherever possible, all measurements of construct characteristics will be compared with those of normal human myocardium, our control for these experiments. We have an IRB protocol in place and collaborate with local cardiac surgeons who would provide samples for us. We also have a contract with NDRI for obtaining human tissues. Cell density of at least 75\% of reported physiological cell density, or $> 1.5 \times 10^8$ cells/cm\textsuperscript{3}; percentages of specific cell types which fall within 15\% of those values reported in the literature.\textsuperscript{[11]} Cell viability $> 90\%$ and tissue morphology reasonably similar to that of normal human myocardium. Mechanical properties of constructs deviate from normal
myocardium by < 15%; generation of force > 0.5 N; calculated ratio of > 0.8 for number of intercalated discs: number of CMs; morphology that approximates that of native myocardium; positive staining for connexin43, actin, cardiac MHC, α-sarcomeric actinin, troponin T, and desmin; evidence of cells producing endogenous ECM, including collagen and basement membrane proteins.

7.3.2.1.7 Challenges and Alternative Strategies:

In the case of autologous stem cell therapies, hADSC are currently the population most readily translatable to clinical use. Human IPSC may be a viable option in the future, as many groups have recently demonstrated these cells’ ability to differentiate into a CM phenotype.[17, 18] Concerns remain, however, regarding the safety of using viral vectors and even small-molecule mediated methods to induce pluripotency, as well as the epigenetic stability of these cells following reprogramming.[19, 20] The aversion of regulatory agencies towards these practices is a barrier that must be overcome before IPSC can be pursued fully as a stem cell source for translational regenerative medicine and tissue engineering approaches. Should we encounter adverse outcomes with our planned studies involving low cell proliferation or differentiation, we will first attempt to alter our cell culture methods. We will use 3D cell culture in the form of the hanging drop method to form spheroids of hADSC for subsequent seeding onto scaffolds. Recent approaches for differentiating IPSC into CM have employed spheroid cultures as opposed to 2D monolayers, with the proposed benefit being that cells secrete endogenous ECM components and paracrine factors that contribute to more successful targeted
differentiation. With this approach, hADSC may be nearly differentiated or more primed for differentiation just prior to being seeded on the scaffolds.

We will also address another frequent concern of regulatory agencies—the use of animal sera—in alternative strategies for cell culture. We will explore the use of serum free media in culturing cells for seeding, which may not be as challenging as it sounds given that they proliferate very well in media formulations with low serum concentrations. Leakage and lack of cell engraftment or retention could be another challenge with seeding. In the event this becomes an issue, we will use fibrin glue to seal microvascular leaks and, as an alternative approach, lyophilize (freeze-dry) our scaffolds to encourage better infiltration of cells. If oxygen diffusion is limited within the scaffolds, we will improve this by adding oxygen carriers (perfluorocarbons) to the media. Access to sufficient human tissue to assemble data for control groups might be limited, although we do receive donated tissue periodically. This deficiency will be remedied by using figures and data published in the literature. In the event that constructs do not synchronously contract with a detectable force, we will analyze contractile force in individual CMs using a method described by Tasche et al.

7.2.4 Aim 4:

Animal studies should proceed as described in Chapter Six and as outlined in Figure 6.2.
7.4 References:


APPENDICES

Appendix A

Supplementary Figures
Figure 3.9: Continuous-flow, perfusion-immersion system for decellularization: A) (1) Inflow reservoir (2) Scaffold immersed in decellularization solution on multi-stirrer plate (3) Overflow and collection reservoir (4) Peristaltic pump. B) Expanded view of myocardial flap scaffold inside decellularization container.
Figure 3.10: **Continuous-flow, perfusion-immersion system for whole heart decellularization:** A) (1) Inflow reservoir (2) Scaffold immersed in decellularization solution on multi-stirrer plate (3) Overflow and collection reservoir (4) Peristaltic pump. B) Expanded view of a whole porcine heart being decellularized within a specially designed container. C) Whole porcine heart shown securely attached to an aortic cannula with zip-ties. All reagents flowed retrogradely across the aortic valve, into the left ventricle, and upward through the mitral valve into the left atrium. Pressure was held in these chambers by suturing or plugging the pulmonary veins. Reagents also flowed anteriogradely through the coronary circulation. D) CAD rendering of the specially designed decellularization vessel shown in A and B. The vessel and other parts in the assembly were designed using Solidworks software.