THE USE OF BIOMATERIALS FOR STEM CELL THERAPIES TO PREVENT MYOCARDIAL DAMAGE POST-IN FarCT

BY

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DISSERTATION

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Abstract

This thesis employed a stem cell encapsulating hydrogel patch to increase the amount of beneficial soluble factors that are delivered to the surface of damaged heart tissue following a myocardial infarction. While current medical practices to address the immediate aftermath of a myocardial infarction (MI) have evolved tremendously, there are few, if any, techniques currently administered to slow, cease, or reverse the negative side effects of an occluded artery, such as the replacement of functional myocardium with non-contractile scar tissue. Because of this scar formation, survival of the initial heart attack is commonly accompanied by a decrease in left ventricular functioning due to wall thinning and ventricular enlargement. As a result of the slow, or absent, ability of cardiomyocytes to divide and repopulate the infarcted area, the burden of heart function lies on the surrounding tissue; a load that exhausts the healthy tissue and decreases the quality of life of heart attack survivors.

Mesenchymal stem cells (MSCs) have emerged as a promising therapeutic avenue for post-MI treatment, in part due to the “survival signals” that they secrete. Previous work has shown that by increasing the amount of “survival signals” that are introduced to the damaged myocardium, the extent of cardiomyocyte cell death, and subsequent scar formation, can be decreased. While the therapeutic effects of these factors have been documented, one difficulty lies in the ability to maintain a constant flux of secreted factors to the damaged site. This project hypothesized that through the encapsulation of stem cells within an engineered hydrogel construct, the hurdle of soluble factor delivery at the site of injury could be overcome. A constant flux of paracrine factors to the heart surface would allow for cell recruitment to the site(s) of
damage, prevention of tissue degradation due to inhospitable environments, and promotion of neovascularization for sustainable tissue regeneration.

Using both a chick chorioallantoic membrane assay and a mouse model of MI, the following aims determined: 1) the vascularization potential of an MSC encapsulated patch, 2) the ability to deliver hydrogels containing pro-survival signals to the heart post-MI, and 3) the ability of these factors to decrease scar formation and improve cardiac function following a heart attack. Knowledge gained from this project will provide the basis for designing materials and strategies for similar studies in larger animal models and eventually for human clinical trials. Successful delivery of the MSC encapsulating patch, and subsequent decrease in myocardial degradation, will greatly improve the quality of life scores of individuals who have suffered heart failure. This increase in quality of life will aid in post-MI mobility and decrease the need for more intensive health care following the initial heart attack; overall decreasing the burden a strained heart has on both patients and the healthcare system.
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1. INTRODUCTION

1.1 Motivation and hypothesis

The objective of this thesis was to introduce a stem cell encapsulating hydrogel patch onto the surface of a damaged heart to prevent the degradation of tissue following a myocardial infarction (MI). An MI is defined as the occlusion of blood flow to a portion of the heart due to the rupturing of an atherosclerotic plaque. The lack of oxygen and nutrients delivered to the tissue presents an environment that is insufficient for proper tissue viability and remodeling. Within as little as 48h following occlusion, the extracellular matrix of the tissue begins remodeling and non-contractile scar tissue replaces functioning myocardial tissue, interfering with the ability of the heart to function properly. Mesenchymal stem cells (MSCs) have emerged as an encouraging therapeutic avenue shown to decrease tissue damage, increase cardiac output and promote vasculogenesis following acute myocardial infarction. Although such results are promising, current clinical delivery methods do not ensure MSC retention within the injured tissue. This research aims to overcome this critical barrier through the encapsulation of MSCs within a hydrogel patch using a specially modified Stereolithographic Apparatus (SLA). Previous work has shown that when the SLA is used to introduce microchannels into a fibroblast encapsulating hydrogel patch, neovessels were observed to correlate with microchannels, demonstrating controlled and directed neovascularization. This study was based on these promising results, with the motivation to keep the ischemic tissue alive long enough for angiogenesis to occur. We hypothesized that through the formation of a cell encapsulating hydrogel patch, cell to tissue interactions can be maintained.
over a physiologically relevant time course, decreasing the damage caused by a myocardial infarction and increasing angiogenesis in a directed manner.

1.2 Overview and specific aims

This approach was centered on three major aims:

Aim 1: Optimize MSC suspension in SLA patterned hydrogels to ensure long-term survival of encapsulated cells. As stated, preliminary data demonstrate that fibroblasts remain viable for up to 7d within a poly (ethylene glycol) dimethacrylate (PEGDMA) hydrogel patch, secreting beneficial soluble factors to the surrounding media or tissue. This same method of encapsulation will be applied to MSCs with the objective of providing an optimal environment within the hydrogel patch for long-term MSCs viability.

Aim 2: Deliver encapsulated MSCs and supporting cells to the surface of infarcted tissue to ensure cell to tissue contact over extended time course. Adhesion of a patch to the heart, with minimal residual damage, requires the formulation of a glue able to maintain patch-to-tissue fusion, and malleable enough not to interfere with heart function. The glue must be proven to be biocompatible with both the myocardium and the encapsulated cells. The use of this developed glue will permit maintenance of the hydrogel construct at the site of injury, ensuring its healing potential at the ischemic area.

Aim 3: Validate the beneficial properties of cell encapsulation in vivo through the establishment of a murine myocardial infarction model. To provide a test model, we will compile and modify murine myocardial infarction techniques from a number of established protocols and implement a procedural model. In order to test the efficiency of the model and ensure proper infarction damage, the
degradation of the myocardium will be assessed and the differences in treatment methods will be determined.

By accomplishing these aims of providing a means of encapsulating cells in a patch and placing that patch directly on the heart surface, a constant influx of paracrine factors to the ischemic tissue can be maintained over a suitable time course. The delivery of encapsulated cells to the surface of infarcted tissue for an extended period of time has proven to decrease or slow the degradation process while at the same time increasing angiogenesis, possibly in a directed manner. If demonstrated as clinically translatable, a new therapeutic avenue can be established that revolves around a non-toxic, immune friendly patch that delivers soluble factors to the site of injury.
2. BACKGROUND AND LITERATURE REVIEW

2.1 The Heart

The entire physiological balance of any living animal centers on the proper functioning of a single organ, the heart. The regular beating of the heart provides the body with a steady supply of nutrients and oxygen, without which would cause a total shutdown of all vital organs. The heart’s pumping action is accomplished by the collective beating of the organ-specific muscle cells, known as the cardiomyocytes, which, when taken together, form the myocardium, or cardiac muscle. Contraction of the cardiomyocytes occurs during the phase of the cardiac cycle known as systole, while relaxation, and subsequent re-filling of the heart chambers is a phase termed diastole. It is this constant circulation of systole and diastole that results in movement of the blood throughout the entire cardiovascular system\(^1\).

The chamber of the heart that is responsible for the force required to pump blood out of the heart and into the peripheral system is the left ventricle (LV), which, consequently, is the chamber that is most prone to cardiac dysfunction. The myocardium itself requires blood flow to maintain proper functioning, which is obtained through its own vascular system, the coronary vessels. It is an obstruction of these vessels that leads to one of the greatest causes of mortality worldwide, myocardial infarctions (MIs)\(^2\). The interruption of blood flow results in cell death at the site of ischemia, which is later replaced by non-functional scar tissue\(^3\). An interference with the functioning of the heart ultimately has systemic physiological consequences for the rest of the body, one of the major reasons
why cardiovascular disease is such a major hindrance on the overall health and well being of the world population⁴.

### 2.2 The Effect of CVD on the Body

Cardiovascular disease (CVD) is defined as pathological conditions that affect any aspect of the vascular system, including the heart, pericardium, and blood vessels. CVD is a far-reaching term, encompassing a wide range of etiologies including cardiac dysrhythmia, myocarditis, peripheral arterial disease, and coronary artery disease. Although it a broad-encompassing disease, there are very similar sources associated with it, with the most common being atherosclerosis and high blood pressure⁵. In fact, hypertension was determined to be the biggest contributor to the global burden of disease and to global mortality, in part due to its close ties with it accompanying risk of CVD and its debilitating side effects⁶. A large-scale analysis on over 83,000 presenting cases of CVD encompassing 12 distinct presentations showed a distinct increase in the lifetime risk of each type of CVD with an increase in patient hypertension⁷.

As with most major diseases, CVD has systemic implications. For example, the narrowing of the peripheral vessels, also known as peripheral artery disease (PAD), may cause immense pain that can limit walking, decreasing the patient’s ability to exercise, usually resulting in an increase in fat deposition and obesity and an accompanying decrease in possible interventions that might help with the patient’s overall health and well being. In some instances, the decrease in blood flow to an extremity may result in ischemia so severe it may require intervention in the form of amputation⁸. Additionally, patients presenting with PAD have a 4 to 5 times higher risk of cerebrovascular events such as a stroke⁹. The systemic implications, and secondary complications, make CVD a long-term disease that
is difficult to manage from a clinical perspective, and difficult to live with from a patient perspective.

One of the major players in CVD is coronary heart disease and, more specifically, MIs. Coronary heart disease is one of the leading causes of mortality in the United States, accounting for approximately one in every six deaths. It has been calculated that an American has a coronary event every 25s, with a death occurring approximately every minute of the day. Progressive buildup of plaque within the walls of a coronary artery eventually leads to total occlusion of the blood flow through the affected artery. This cessation of blood flow is what is defined as an MI one of the major contributors to CVD. Physiological changes begin to occur almost immediately following the vessel blockage, due almost entirely to the inability of the affected heart muscle to properly beat without the necessary nutrient and oxygen supply. From a symptomatic standpoint, a patient enduring an acute MI will have a sudden onset of chest pain, usually accompanied by a tingling or painful sensation running down their left arm. Patients sometimes feel fatigued and short of breath preceding the event. Within the heart itself, the myocardial tissue begins to remodel, replacing viable cells with a fibrous scar tissue. The changes in the heart tissue begin almost immediately following occlusion, and remodeling itself lasts for months after the initial assault. There are many factors that play a part in the response mechanism of an MI, some of which will be highlighted in the following section.

2.3 Timeline of Irreversible Myocyte Necrosis Following an MI Injury

2.3.1 Cellular view of cardiomyocyte death

A cellular view of myocardial infarction begins with fast accumulation of potassium in the extracellular fluid within 20s post-infarction and plateaus after 3-
10min. This rapid increase in potassium is due to a disturbance in the sodium/potassium pump that is involved in actively keeping intracellular potassium levels high and extracellular potassium levels low. After 20-30min of continuous ischemia, a second, larger increase in potassium is seen in the extracellular space, a phase that correlates to the beginning of irreversible myocyte death\textsuperscript{13}. At around the same time as the secondary potassium spike, enzymes associated with cardiac function, including glutamic oxaloacetic transaminase and succinic dehydrogenase, can be detected extracellularly, another determinate of myocyte degradation\textsuperscript{14}. Homogenized myocardial tissue taken 30m post-infarction showed a decrease in its oxidative metabolism ability, reinforcing that the death of myocytes begins as early 30m post-infarction\textsuperscript{15}.

From a pathophysiological perspective, the remodeling of the heart after an MI begins within min, with irreversible cell death occurring within 6h, and total remodeling occurring over the course of months. In a canine study aimed to determine the degree and timing of irreversible cell injury, the coronary arteries of animals were blocked for a varying amount of time, at which point they would be reopened and reperfusion was allowed to occur. Irreversible cell death was denoted as cells that exhibited swelling, mitochondrial calcification, prominent contraction bands, and membrane disruption upon the re-introduction of blood flow, a term denoted as contraction band necrosis (CBN)\textsuperscript{16}. It was observed that cells were able to fully recover to their pre-ischemic state if the occlusion of the artery lasted for 15min or less. After 15min, cell death began, with an increase in death as time progresses. Most CBN occurs over a 3h period, with total CBN development at 6h\textsuperscript{17}. Interestingly, necrosis begins as a wave beginning with the inner layer of the heart and gradually proceeding to the outer layers, owing to the fact that the outer layers still receive some blood flow from neighboring vessels,
whereas layers closer to the chamber are left without nutrients and oxygen supply. This gradient of necrosis is attributed to arterial collateral flow that is higher in the outer layers and decreases as you move closer to the ventricular chambers\textsuperscript{18}. If animals are left to live, areas that display CBN eventually remodel into scar tissue despite the re-introduction of blood flow to the area\textsuperscript{19}. Thus, there is a timeframe of 0-6h in which interventional therapy will have an effect on slowing the degradation of functional cardiac muscle into scar tissue.

2.3.2 Morphological changes following myocardial infarctions

On a gross morphological evaluation, all myocardial infarctions show the same characteristic progression of coagulative necrosis, followed by acute, then chronic inflammation, and finalized by fibrosis at the site of injury. At 12h post-infarction, there is usually no gross morphology that can positively identify a myocardial infarction, however, by 3h an infarct can be seen with Tetrazolium chloride, a stain that identifies areas of decreased enzyme activity, implicating the depletion of enzymes at the site of damage. Gross identification does not occur until 12-24h post-infarction, when a red-blue discoloration associated with coagulated blood due to a decrease of blood flow in the area\textsuperscript{1-3}.

The coagulative necrosis that is displayed in MIs is a type of necrosis in which the underlying tissue architecture is preserved, but cells begin their deterioration. While the cell structure and morphology remains in tact, the organelles, specifically the cell nuclei, begin to disappear. Enzyme denaturation accompanies structural protein breakdown, and inflammatory cells are recruited to the site to aid in the digestion of dead cells. This entire process usually begins at 4h post-infarction and lasts for approximately 3d. During this time, cardiomyocytes begin to lose their striations and begin to take on a wavy
appearance with edema present between necrotic cells. After approximately 14d, cardiomyocytes begin to be replaced with a loose connective tissue that provides the scaffold for eventual collagen deposition. The scar tissue begins to form at 2wk, with a dense collagenous scar and final fibrosis seen at 8wk post-infarction (Table 2.1). Histologically, scar tissue can be identified by Masson’s Trichrome staining as a light blue or deep purple color that denotes collagen and fibrin at the site of injury. Fibrous scarring begins on the border zone, or edge, of the infarct region, and progresses inwards until the entire ischemic area is remodeled into non-functional scar tissue. The heart was always thought of as a terminal organ, with a fixed number of cardiomyocytes from birth and an inability to regenerate lost muscle tissue, however, recent evidence suggests repopulation can occur under the proper circumstance, opening up a field of tissue regeneration and stem cell therapy that was once thought impossible.

2.4 Stem cell therapy to treat CVD

2.4.1 Clinical Trials

It is clear that CVD is a major overall public health issue. Early interventions must be implemented to help curb its onset and the debilitating side effects that CVD causes. Along with preventative measures, a number of interventional, stem cell based, therapeutics might aid in preventing the decrease of function that accompanies CVD events. Initial promise in laboratory studies resulted in a rapid onset of clinical trails to test the efficacy of stem cell treatments.

One of the major cornerstones in stem cell therapy for the treatment of MIs was the Reinfusion of Enriched Progenitor Cells And Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) clinical trial, a study that ran from 2004 to 2010. The REPAIR-AMI trial focused on the intracoronary delivery of bone-
marrow derived progenitor cells (BMCs) in 204 randomized patients with acute MI. Delivery of stem cells was done 3-7d post-reperfusion and patients were followed for up to 2yr. After 4min an enhanced contractive recovery of left ventricular function was observed, as well as a significantly greater improvement in regional contractility in the treated groups versus those of the placebo. When examined 2yr after the treatment, there was a significant change in recurring MIs, with an observation of 0 in the BMC treated group and a total of 12 in the placebo group, albeit some of those were multiple recurrent MIs. There was also a significant reduction in infarct size and regional contractility. Interestingly, at 2yr, there was a slight improvement in LVEF, but not as marked as at 4mo.

A similar trial, termed the BOOne marrOw transfer to enhance ST-elevation infarct regeneration (BOOST), was conducted in 2002. This randomized-control trial delivered intracoronary injection of autologous BMCs 3-6d post percutaneous coronary intervention in 60 patients. In contrast to the REPAIR-AMI trial, the BOOST trial showed no significant improvement in LVEF, but it did report an initial improvement in diastolic function at 6mo and 18mo post-treatment, a parameter not reported on in the REPAIR-AMI trial. The diastolic improvement diminished at 60mo post treatment, concluding that there was no sustained effect on the long-term follow-up of patients.

A more recent clinical trial involving autologous cardiac stem cells began in 2009 and is still ongoing. Stem Cell Infusion in Patients with Ischemic cardiomyopathy (SCIPIO) expanded autologous cardiac stem cells in culture and delivered them via intracoronary infusion 113d after coronary bypass surgery. The aim of this trial was to test the viability and safety of autologous cardiac stem cells, as well as determine any beneficial effects they might have. Aside from a proof of
concept regarding the safety of delivering autologous cardiac stem cells, the
treatment showed a marked improvement in LVED and a decrease in infarct size
in the time between 4mo and 12mo post-treatment within the treatment group.
While results appear promising, the trial did not report comparisons with the
untreated group, so direct comparison between efficacies of treatment versus
that of a placebo cannot be concluded

Finally, the CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar
dysfunction (CAUDECEUS) trial observed the effect of intracoronary injection of
autologous cardiosphere derived cells (CDC) 1.5-3mo post-infarction. The
conclusions from this proof-of-concept trial did not show any significant
improvement in heart function, but did show a smaller scar size and increased
myocardial thickness at 6mo and 12mo that was not seen in the control
patients. It is suggested that the effect of increased viable myocardial tissue in
this instance is both through direct physical contact as well as paracrine
stimulation to promote the regrowth of myocardium, although differentiation into
cardiomyocytes is not a suggested mode of regeneration.

All these studies taken together paint an interesting picture. Stem cell therapy
does show a marked effect in vivo across all trials, however the effects and the
parameters are different in each case. It is more intriguing that the type of stem
cell does not alter the overall outcome of myocardial protection or regeneration,
which brings up the question of what, exactly, mechanism of action is occurring
to promote cardiac repair or survival, and if there is a means to harness and
improve on that capability.
2.4.2 Mechanism of MSC therapy in cardiac repair: laboratory findings

The field of MSC therapy for ischemic heart disease suggests three modes of cardiac repair: 1) differentiation of MSCs into endothelial cells and vascular smooth muscle cells, 2) differentiation of MSCs into cardiomyocyte cells (CMC), and 3) cytoprotective and regeneration effects mediated through paracrine release. Initial studies utilizing stem cells to treat cardiac failure were focused on the hypothesis that injected stem cells would differentiate into a desired cell type, whether that type be cardiomyocytes, vascular smooth muscle cells, or endothelial cells. The differentiation of MSCs into cardiomyocytes was one of the earliest and most promising findings in the field that catapulted MSC therapy for heart disease from a far-reaching idea to a possibly attainable goal. In this breakthrough study, it was observed that after 1wk under supplemented culture conditions \textit{in vitro}, the morphology of approximately 30% of bone marrow stromal cells began to simulate the rod-like form of cardiomyocytes, including sarcomeres and centrally positioned nuclei. After 2wk in culture, the cells began to spontaneously beat, with synchronous beating occurring after 3wk in culture. In a separate study, when implanted into fetal sheep during early gestation, hMSCs underwent site-specific differentiation into cardiomyocytes in addition to their differentiation into a variety of other cell types, proving the multipotency of MSCs \textit{in vivo}. The finding of cellular caridomyoplasty, or cell based cardiac repair, was further shown in an adult murine heart, where human MSCs were labeled with lacZ and injected into the left ventricle of healthy mice. Over time, these labeled cells began to morphologically resemble cardiomyocytes, and expressed cardiac specific proteins. To test the ability of MSCs to differentiate at the site of injury post-MI, as opposed to in healthy myocardium, GFP labeled MSCs were infused into the bone marrow of irradiated mice. These mice were then given an MI and subsequently treated with granulocyte colony stimulating
factor (G-CSF) to promote the mobilization of injected MSCs into the bloodstream. Upon sacrifice at the 8wk time-point of the study, the ischemic myocardium of the mice showed evidence of GFP positive cells expressing the cardiac specific actinin marker, suggesting that the bone marrow MSCs not only homed to the site of injury, but further differentiated into CMCs in an attempt to aid in cardiac regeneration. The homing of MSCs to ischemic myocardium was further demonstrated via intravenous systemic injection of bone marrow MSCs 3h after coronary ligation. Transplanted MSCs differentiated into CMCs, as expected, but a separate population of cells expressed von Willebrand factor and formed vascular structures, suggesting a second form of differentiation with vessel-like characteristics, a finding that would help to further improve cardiac function following treatment after an MI. The conclusion of these, and a number of other studies, is that MSCs have the ability and differentiated into CMCs and vascular cells both in vivo and in vitro.

Despite these results, a growing body of evidence suggests that although MSCs have been shown to differentiate into functional cells that aid in repair, the percent of fused or differentiated cells is not sufficient to account for the amount of ventricular remodeling and vasculogenesis observed. Rather, paracrine mechanisms, driven by the secretion of soluble factors from MScs, are thought to be the driving factor in the repair process. The strongest indications of this are in vitro results that indicate that the cell culture medium of MSCs alone was sufficient to reduce apoptosis of cells in hypoxic conditions. Likewise, in vivo studies have shown a reduction in scar formation and extracellular matrix remodeling through the release of paracrine factors from MSCs. Finally, and most interestingly, recent studies suggest that the injection of MSCs promote the activation of resident cardiac stem cells, stimulating cardiomyocyte replication.
within the cardiac tissue itself, a finding that might re-open the long disputed concept of self-regeneration potential of the heart\textsuperscript{40-42}.

Although the study of the paracrine effects of MSCs on the surrounding myocardium is still a premature field, early experiments are beginning to lay the mechanistic foundation. A number of other key angiogenic, pro-survival factors have been attributed to MSC secretion in both normoxic and hypoxic conditions, including vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and stromal cell-derived factor-1 (SDF-1)\textsuperscript{37,43-45}. These cytokines play a variety of roles in tissue remodeling, but are in general responsible for increase in survival and contractility of CMCs, neovascularization, and differentiation of stem cells (Table 2.2). Additionally, BM-MSCs under oxidative stress release chemoattractants including macrophage inflammatory proteins (MIP-1\textsubscript{α}, MIP-1\textsubscript{β}) that are associated with wound repair and inflammatory responses\textsuperscript{46}. The expression of these proteins might help explain \textit{in vitro} studies that showed migration of CD14\textsuperscript{+} monocytes and endothelial cells, with additional mitogenic effect on endothelial cells, in response to BM-MSC cultured medium. Furthermore, when applied to a cutaneous wound model, groups treated with cultured medium showed an increase in CD4/80\textsuperscript{+} macrophages and an increase in endothelial cell and endothelial progenitor cell recruitment, further solidifying the role of BM-MSC secreted paracrine factors in cell recruitment and repair\textsuperscript{43}.

The body of knowledge surrounding the beneficial properties of MSCs is quite large, and growing. As stated, the results were deemed promising enough to quickly implement clinical trials, which have fallen short in terms of their long-term outcomes. The objective of future studies is to figure out a way to harness
the potential of MSCs to maximize their intended benefit. It is from this desire that the field of cardiac tissue engineering has emerged.

2.5 Cardiac tissue engineering

Simple injection of cells into the myocardium or coronary arteries have proven beneficial in decreasing myocardial remodeling, but their effects are diminished in longer-term studies, in part due to their limited cell survival at the ischemic area as well as their low cell retention within the myocardium\textsuperscript{47-49}. The field of cardiac tissue engineering has developed to address this concern. There are currently three general categories involved in cardiac tissue engineering; 1) The creation of functional tissue to replace \textit{ex vivo} to replace damaged myocardium, 2) improvement on the retention of injected cells, either by encompassing them in an injectable hydrogel or altering the properties of the cells themselves, or 3) the bioengineering of a three-dimensional epicardial patch that can either act as a scaffold for the regenerating heart, or a vehicle to deliver therapeutics to the site of injury\textsuperscript{49-52}.

Engineered heart tissue (EHT), or the creation of an \textit{ex vivo} functioning myocardial tissue, is arguably one of the more difficult subfields of cardiac tissue engineering. Strides have been made to grow engineered human myocardium on a 3D structure, biomimicking the human myocardium\textsuperscript{53}. Groups have also been able to incorporate an endothelial cell vascular network within the engineered tissue that pairs with host myocardium when placed on healthy hearts\textsuperscript{54}. The most promising result was an engineered heart tissue that, when placed on injured myocardium, showed electrical coupling to the native cardiomyocytes, helping to improve heart function \textsuperscript{55}. Despite this, a fully functional replacement
tissue has yet to be established in the clinical setting, part due to the complex nature of the myocardium and its supporting cell populations.

A parallel approach within the cardiac tissue engineering field is the exploitation of the body’s natural mechanisms to aid in either ceasing or reversing the damage following an MI. One tactic is the harnessing of the paracrine effects of MSCs through the encapsulation of the cells within varying constructs. Of these constructs, two major categories of scaffolding material, synthetic and naturally based, have emerged.

Synthetic scaffolds are appealing, in part due to the inherent control possibility they provide. For instance, hydrogels that are used in a variety of tissue engineering disciplines are chosen because their crosslinking nature provides the ability to utilize directed light or ionizing radiation to selectively crosslink the construct into a desired shape, surface morphology, and porosity. Additionally, the characteristic of hydrogels to swell while maintaining their 3D structure provides a framework for cell survival, while its inherent porosity permits adequate transport of nutrients and oxygen to allow the cells to proliferate.

There are a variety of complex and simple structures that have been developed to help aid in cell delivery post-MI (Table 2.3). A group out of Emory recently “double encapsulated” MSCs, first within alginate capsules, then transplanted those capsules into a poly-(ethylene glycol) (PEG) patch. Bioluminescence studies showed that the patch retained the cells for up to 5d at the site of implantation, peaking significantly at 3d with no trace of the cells by 7d. This is compared to the retention of simply injected cells, which showed a constant low level of cells, with total irradiation by 5d. When assessing cardiac function, an increase in both EF and SV was noted in the treatment group when compared to
the control group, as well as a decrease in scar size of the infarcted region and in increase in microvascular density in the peri-infarct region. In this study, PEG, a non-biodegradable polymer, was used as the delivery mechanism for MSCs, most-likely due to its cell retention capability after implantation. A different approach would be the use of a construct that is expected to degrade upon placement on the heart, thus releasing the cells within the construct for possible differentiation into cardiomyocytes and other advantageous cell types. This approach, when utilized on a poly(lactide-co-e-caprolactone) construct placed on a 10d post-MI rat model, was shown to enhance the effects of MSC therapy, suggesting that slow release of therapeutic cells might aid in decreasing cardiac remodeling, possibly due to differentiation into cardiac lineages\textsuperscript{60}.

The second classification of scaffolding material is one based on natural extracellular matrix proteins. Interest in this category of scaffolds has always been high due to the inherent biocompatibility of such devices. Fibrin and collagen have emerged as the most used foundation for regeneration, in part because of their biological association with wound healing. Fibrin glue has proven to aid in cell attachment, growth, and differentiation of cells in co-culture and encapsulated conditions, as well as protection of encapsulated cells from hypoxic environments\textsuperscript{61-63}. As an added benefit, the precursors for the fibrin-based glue, fibrinogen and thrombin, can be taken directly from the patient, providing a fully autologous scaffold\textsuperscript{64}. Simple entrapment of bFGF into a fibrin glue, followed by injection into an ischemic site, improved the EF, angiogenic density, and regional myocardial perfusion of the injured myocardium\textsuperscript{65}. Similarly, encapsulation of BMSCs within a fibrin glue and injection 1wk post-MI showed a marked improvement in cardiac function and angiogenesis\textsuperscript{61}. The results of a simple naturally occurring biomaterial to act as a scaffold has further been shown
in an acellular scaffold made of Type I collagen that has been manipulated to mimic the physiomechanical properties of the myocardium. The simple presence of the scaffold was enough to preserve some of the function of the heart post-MI\textsuperscript{66}. The mechanisms of this effect are attributed to the patch’s support of the injured myocardium, as well as its ability to facilitate cell migration and angiogenesis within the patch itself. The implications of this study are two-fold; 1) acellular cardiac protection is a viable means of cardiac tissue engineering, and 2) the vehicle of cellular delivery can itself help aid in cardiac regeneration if it properly primed to promote cell migration and angiogenesis.

These are just a few of the many ongoing laboratory studies involving the delivery of exogenous materials to help regulate of cardiac remodeling. Of the studies, some provide complex multilayered structures, while others are simple homogenous platforms. Many incorporate therapeutic cells, but a few rely on the scaffold itself to provide structure for remodeling. The framework of patches varies from proteins naturally found in the body, to engineered polymers. All have shown promise in their ability to diminish detrimental effects of MIs, however, the difficulty relies on the ability of such therapeutics to be translated into the clinical setting. The administration of the patch must be able to be done using minimally invasive techniques, preferably via laparoscopic surgery or coronary delivery, to ensure that collateral damage to the patient does not occur as a result of treatment. If this can be established, cardiac tissue engineering might prove either a therapeutic addition, or even an alternative, to conventional cardiac treatment.
2.6 Animal MI Models

Animal models play an imperative role in understanding the progression and treatment of CVD. There are currently a number of models for a wide range of CVDs, covering a variety of animals, from mice, to rabbits, to dogs, to pigs\textsuperscript{67}. Each model presents with its own unique characteristics as well as downfalls. The largest (porcine) and smallest (murine) models are discussed here.

Pigs are considered as an excellent animal model to explore human diseases and recapitulate clinical phenotypes due to their relative size, similar physiological characteristics, and comparable morphological anatomy to that of humans\textsuperscript{68}. The size of the pig allows occlusion of the LAD to be performed via cardiac catheterization, where the coronary arteries are approached via the femoral artery and obstructed using an angioplasty technique\textsuperscript{69,70}. This obliterates the need to perform open-heart surgery and decreases the possibility of additional ventricular remodeling that may be associated with such an invasive technique. Additionally, the coronary anatomy and organ structures are analogous to those of humans, decreasing the inevitable variably in action that is so often seen in translational medicine\textsuperscript{71}. Despite the advantages of a porcine model, the procedure is difficult to master, requiring full 24-hour veterinary staff supervision for both the surgical method as well as follow up monitoring. Due to this, and the overall cost of the porcine model, the use of pigs is best saved for later studies after therapeutics have proven themselves in smaller animal models.

One of the most relied upon small animal models for almost every diseased state is the mouse, thanks in part to the ease of genetic manipulation of the murine species. Recently, the mouse has emerged as a tool for studying cardiac
remodeling post-MI and provides a platform to test possible intervention strategies for CVD\textsuperscript{32,67,72}. The mice are small and easy to maneuver, with a full MI procedure lasting under 45m, allowing for a relatively large sample size without the restraints associated with larger animal models. However, just as with any other model, there are potential downfalls for the use of a smaller animal models. Specifically, the cardiovascular structure and coronary artery distribution is vastly different in the mouse compared to the human. The left coronary artery in the mouse does not divide into a left circumflex and left anterior descending artery, as in humans. Instead, the left coronary artery courses along the left ventricular free wall, and has a variable branching pattern as it descends along the free wall\textsuperscript{73}. As such, to emulate the occlusion of the LAD that is characteristic of an MI in humans, one must instead occlude the entire left coronary artery in mice, beginning immediately after it emerges past the tip of the left atrium. With this approach, a positive infarction can be identified as being accompanied by left ventricular dilation, a decrease in systolic function, and an increase in filling pressure\textsuperscript{74-76}. These can be qualitatively measured on their own, and can also be used in overall calculations of heart function, including a decrease in ejection fraction and stroke volume, an increase in the ventricular diameter, and a decrease in ventricular heart wall thickness, as determined by echocardiography and histological staining\textsuperscript{77-80}.

### 2.7 Conclusion and project overview

Current medical intervention post-myocardial infarction focuses on maintaining the remaining healthy heart tissue rather than preventing or reversing damage to the infarcted tissue. The overall goal of this project is to harness the therapeutic properties of stem cells through encapsulation in an engineered hydrogel in order to prevent tissue damage following a heart attack, and to test the efficacy of the
treatment using a murine MI model. This preventative measure will save the heart tissue from being replaced by non-contractile scar tissue, preserve heart function at or near pre-infarction levels, and increase the quality of life of heart attack survivors.

2.8 References


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### 2.9. Tables

<table>
<thead>
<tr>
<th>Time</th>
<th>Morphological Changes</th>
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<tbody>
<tr>
<td>0-1.5h</td>
<td>Reversible injury</td>
</tr>
<tr>
<td>3h</td>
<td>MI can be identified via enzymatic staining</td>
</tr>
<tr>
<td>4-12h</td>
<td>Begin coagulation necrosis</td>
</tr>
<tr>
<td>12-24h</td>
<td>Gross features of MI begin to become apparent. Dark mottling in ischemic region.</td>
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<tr>
<td>1-3d</td>
<td>Acute inflammation most apparent.</td>
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<tr>
<td>3-10d</td>
<td>Wave of macrophages responsible for removal of dead myocytes</td>
</tr>
<tr>
<td>10-14d</td>
<td>Early granular tissue formation</td>
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<tr>
<td>2w-8w</td>
<td>Collagen deposition</td>
</tr>
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<td>8w - ∞</td>
<td>Dense collagenous Scar</td>
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**Table 2.1: Overview of morphological changes characteristic of myocardial infarctions**
<table>
<thead>
<tr>
<th>Stem-Cell Secreted Paracrine Factors</th>
<th>Mechanism of Action</th>
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<tbody>
<tr>
<td>VEGF, HGF, IGF-1, SDF-1, bFGF, TGF-β, PTX-3, TSP-1</td>
<td>Survival</td>
</tr>
<tr>
<td>VEGF, bFGF, FGF2, HGF</td>
<td>Contractility</td>
</tr>
<tr>
<td>VEGF, IGF-1, SDF-1, PDGF-AA, TGF-β, TNF-α, IL-1, IL-8, Ang-1, Ang-2, MCP-1</td>
<td>Neovascularization</td>
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<tr>
<td>VEGF, IGF-1, HGF, TNF-α</td>
<td>Differentiation</td>
</tr>
<tr>
<td>IL-10, MMP-2, MMP-9, MCP-1, TGF-β, TIMP-1, TIMP-2, TIMP-9, HGF, IL-1, Activin A</td>
<td>Remodeling</td>
</tr>
<tr>
<td>MIP-1, MIP-2, MMP-9</td>
<td>Chemotaxis</td>
</tr>
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Table 2.2: Secretion factors and their role in remodeling and chemotaxis. Adapted from Mirotsou et al.
<table>
<thead>
<tr>
<th>Stem Cell Type</th>
<th>Biomaterial/Mode of delivery</th>
<th>MI Animal Model</th>
<th>Enhanced Angiogenesis</th>
<th>LV function improvement</th>
<th>LV remodeling attenuation</th>
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<td>Bone marrow cells</td>
<td>Muscle Patch</td>
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<td>+</td>
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<td>Polyglycolic acid cloth patch</td>
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<td>+</td>
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<td>Bone marrow mononuclear cells</td>
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<td>/</td>
<td>/</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>84</td>
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<td>Type I collagen-(GAG) patch</td>
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<td>+</td>
<td>/</td>
<td>/</td>
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<td>+</td>
<td>+</td>
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<td>Sliced acellular pericardia path and cell sheet</td>
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<td>/</td>
<td>/</td>
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<td>Sliced acellular pericardia patch and folded cell sheet</td>
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<td>-</td>
<td>+</td>
<td>88</td>
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<tr>
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<td>Intramyocardial injection of cell sheet fragments</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>Collagen injection</td>
<td>Acute in rats</td>
<td>/</td>
<td>-</td>
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<td>Porcine SIS patch</td>
<td>Chronic in rabbits</td>
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<td>+</td>
<td>+</td>
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<td>Acute in rats</td>
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<td>+</td>
<td>+</td>
<td>60</td>
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<td>Alginate capsules within Poly-(ethylene glycol) gel</td>
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<td>+</td>
<td>+</td>
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<td>Fibrin glue</td>
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<td>+</td>
<td>+</td>
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<td>Intrevenous injection</td>
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<td>66</td>
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<tr>
<td></td>
<td>bFGF in fibrin glue</td>
<td>Acute in canine</td>
<td>+</td>
<td>+</td>
<td>*</td>
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Table 2.3: Overview of cardiac patch in vivo experiments and results. (+) designates a positive result, (-) designates a negative result, (*) designates that the parameter was not tested, and (/) designates a parameter that is not associated with the study. Adapted from Martinez et al.
3. A HYDROGEL CONSTRUCT AND FIBRIN-BASED GLUE APPROACH TO DELIVER THERAPEUTICS IN A MURINE MYOCARDIAL INFARCTION MODEL.

3.1 Abstract

The murine MI model is widely recognized in the field of cardiovascular disease, and has consistently been used as a first step to test the efficacy of treatments in vivo\(^1\). The traditional, established protocol has been further fine-tuned to minimize the damage to the animal. Notably, the pectoral muscle layers are teased away rather than simply cut, and the thoracotomy is approached intercostally as opposed to breaking the ribs, preserving the integrity of the ribcage. With these changes, the overall survival rate for mice receiving an MI is near 70%.

Stem cell therapies aimed to alleviate the damage caused by MIs have shown promise over the years for their pro-angiogenic and anti-apoptotic benefits. Current approaches of delivering cells to the heart surface typically involve the injection of the cells either near the damaged site, within a coronary artery, or into the peripheral blood stream\(^2\)\(^-\)\(^4\). While the cells have proven to home to the damaged myocardium, functionality is limited by their poor engraftment at the site of injury, resulting in diffusion into the blood stream\(^5\). This obstacle can be overcome with the use of a cell-encapsulated hydrogel patch. The patch is fabricated prior to the surgical procedure and is placed on the injured myocardium immediately following the occlusion of the left coronary artery. To adhere the patch in place, a biocompatible external fibrin glue is placed directly on top of the patch, allowing for it to dry to both the patch and the heart surface.
This approach provides a novel adhesion method for the application of a delicate cell-encapsulating therapeutic construct.

### 3.2 Introduction

A myocardial infarction (MI) is defined as the interruption of blood to a region of the heart caused by the occlusion of a major coronary artery. The damage resulting from an MI is due to the remodeling of the viable heart tissue into non-functional scar tissue, which decreases the ability of the heart or, more specifically, the left ventricle, to beat properly. This results in a decrease in the volume of blood that can be delivered to the body with every heartbeat, known as the stroke volume, and the percentage of blood that is pumped out of the heart with each heartbeat, known as the ejection fraction. These, along with other diminished functions, increases the strain on the rest of the heart to maintain adequate function. Often, this increased strain can become so severe that it causes a second heart attack, a phenomenon seen in approximately 10% of individuals.

While medical practices have evolved to treat the immediate aftermath of an MI, no technique has been developed to halt, slow, or reverse the negative side effects of tissue remodeling. Stem cell therapies have emerged as a possible avenue for such a treatment, however, despite their promising potential, stem cells have not proven successful in the clinical setting. One theory for their shortcomings is the inability to ensure the beneficial cells remain at the site of infarction long enough to generate favorable results. It has been shown that no more than 24% of cells that are simply injected into the site of infarction survived and remained at the damaged site 1d post-delivery. A possible prospect to addressing this issue of cell retention is to develop biocompatible hydrogel
systems that encapsulates cells and can be delivered to the damaged site. This approach would ensure cell-to-tissue contact over an extended period of time, increasing the length of time the cells can provide beneficial factors to the underlying myocardium.

A bottleneck to the patch approach is the difficulty of adhering the patch to the heart surface. Many groups have overcome this through a variety of techniques, the most prevalent being a simple suture to tie the construct to the heart surface\textsuperscript{8,9}. This has proven successful in a number of cases in which the construct is made of a stiffer material, but fails when attempted on a hydrogel system, due to the high water concentration and delicate nature of the patch construct. To overcome this we have developed a fibrin glue external adhesive system that mimics the chemistry of clot formation. Fibrin glue has been used in numerous medical surgeries, including dura tears, bronchial fistulas, and corneal transplantation, highlighting the biocompatibility of the product as a wound sealant\textsuperscript{10-12}. Additionally, fibrin has been used in a variety of cardiac purposes, including surgical treatment of left ventricular ruptures and coronary artery bypass surgeries, however, its use as an adhesion glue for a cardiac patch is not commonly used \textsuperscript{13-16}. A simple formulation of thrombin and fibrinogen results in a biocompatible glue that can be placed directly on the outside of an external cardiac patch, providing a viable adhesion system to ensure patch to heart interaction.
3.3 Materials and Methods

3.3.1 Instrument Preparation
All non-disposable instruments used are autoclaved prior to the surgical procedure to ensure sterility. Instruments used multiple times in a session are sterilized with a glass bead sterilizer between uses.

3.3.2 Cell Preparation
Stem cells are thawed at least 24h prior to creation of the hydrogel construct. Approximately 5x10^6 cells are resuspended in 30mL of TheraPeak MSCGM-CD (Lonza) supplemented with 5% FBS and split into two T-75cm² flasks. The cells were incubated at 37℃ and 5 %CO₂ until ready for use.

3.3.3 Hydrogel Preparation
Patches are prepared 1d prior to the surgical procedure using a stereolithography apparatus (explained in detail in chapter4). In short, cells are resuspended in a pre-polymer solution of 20% Poly(ethylene-glycol)1000 dimethacrylate (Polysciences Inc). After creation, hydrogels are incubated overnight in cell culture medium to allow for cells to adjust to the environment within the patch. Patches are used within 24h of fabrication and transferred to the heart surface within 10m of removal from the incubator.

3.3.4 Fibrin-based glue
Fibrinogen (Sigma-Aldrich) and Thrombin (Sigma-Aldrich) were reconstituted into a 100mg/mL and 40mg/mL stock solution, respectively. Both solutions were kept on ice until mixing, at which time a 4:1 fibrinogen to thrombin solution was made. The formulation was mixed via gentle pipetting until desired viscosity was
reached, at which time it was transferred to the surface of the heart using a 10 µl pipette.

3.3.5 Echocardiography

Echocardiography is described in detail in Chapter 4. In brief, animals are placed under anesthetics by isoflurane inhalation and 2D M-mode echocardiographic traces are collected by the Ultrasound-Visual Sonics Vevo 2100.

3.3.6 Histological staining

Histological staining techniques are described in detail in Chapter 4. In brief, At the time of euthanasia, the hearts are collected from the animals and placed in a 10% formalin solution for 24h, after which time the hearts are transferred to 70% ethanol and kept at 4°C until tissue processing. Hearts are processed, embedded, sectioned, and stained with both a Hematoxylin and eosin (H&E) and Masson’s Trichrome stain.

3.4 Procedure

3.4.1 Surgical preparation

The final outcome of the surgical technique described below is the full occlusion of the left coronary artery of the mouse, the transfer of a hydrogel patch to the site of infarction, and the administration of a fibrin based glue to ensure patch adhesion at the desired site. Procedure is in full compatibility with IACUC protocol number 13302 and has been approved by the division of animal resources.

Each mouse is evaluated prior to the procedure to assure that the animal is in good health. Animals are placed into an anesthetic chamber and exposed to 5%
isoflurane with a 1 L/min O₂ support. The level of anesthesia is monitored by toe pinch reflex. Once the animal is properly anesthetized, they are weighed and placed on the intubation stand. A light source is directed towards the animal’s chest cavity and forceps are used to retract the laryngeal surface and expose the vocal cords. A 20G angiocath is carefully guided between the vocal cords, and smoothly inserted into the trachea. Proper insertion is detected by a mechanical movement of the chest cavity once the catheter is connected to the small animal ventilator. Ventilation settings are adjusted to the weight of the animal based on the manufacturers guidelines. Once intubated, the animal is placed in the supine position on a heating pad to prevent hypothermia. Hair is removed from the surgical site through the use of a depilatory cream and the site is sterilized by 3 alternating scrubs of betadine and ethanol. A drape is placed to expose just the surgical site, and vet ointment is placed on the eyes to prevent dryness during the procedure.

3.4.2 Thoracotomy and MI induction

The thoracotomy is performed on the left side of the animal, therefore, a skin incision is made approximately 1 cm to the left of the sternum, and runs the length of the sternum. Forceps are used to separate the muscle layer from the underlying ribcage in two steps. A delineating line is observed that represents the pectoralis major muscles of the animal. This muscle is slightly lifted and separated from the underlying external oblique muscle and retracted medially. The external oblique muscle is then freed from the underlying ribcage in the same manner, and retracted laterally, providing an unobscured view of the second, third, and fourth ribs (Figure 3.1a). The thoracotomy is performed between the third and fourth ribs. The fourth rib is gently lifted, and a cauterizer is used to open the chest cavity. Retractors are placed to further expose the cavity
and access the heart (Figure 3.1b). Forceps are used to rupture the thin pericardium of the heart. The left coronary artery is ligated with an 8-0 monofilament nylon suture. The suture is placed approximately 4mm from the apex of the heart, directly below the bottom tip of the left atrium. Proper suture placement can be determined by a blanching of the ventricular myocardium and an increase in the size of the left atrium after the suture has been tied.

3.4.3 Patch placement

Patches are kept at 37 °C and 5% CO₂ conditions until use. The patch is gently lifted using a flat-ended spatula and gently placed on the surface of the heart. To prevent slipping off the left ventricle, the patch can be held in place by lightly maintaining contact using either the spatula or the tip of a pair of forceps. The glue is prepared and mixed by repeated pipetting until it begins to thicken, usually within 1m after the beginning of the preparation. Once the solution viscosity reaches the desired level, approximately 10µL is quickly transferred to the patch surface. The clotting time of the fibrin glue is rapid, providing a small window of opportunity for efficient transfer of the glue. The chest cavity is left open for approximately 1-2m following the addition of the glue to allow for full crosslinking and minimize movement of the patch from the heart surface.

3.4.4 Closure

All closing sutures are done using a 6-0 monofilament nylon suture. The rib layer is closed with three to four individual interrupted sutures (Figure 3.1c). Before full closure of the intercostal layer, a PE-10 cannula is inserted into the incision to evacuate the chest cavity after closure is complete and reestablish proper intrapleural pressure (Figure 3.1d). The pectoral muscles are closed with three to four individual interrupted sutures and the skin layer is sealed with a continuous
suture (Figure 3.1 e,f). Following full closure, a 1.0mL syringe is attached to the end of the cannula and used to evacuate the chest cavity. A tissue adhesive is applied to the incision site to reinforce the suture site.

3.4.5 Post-surgical Treatment
Mice are given an injection of buprenorphine (0.05-1.0 mg/kg) and carprofen (2.2 mg/kg) subcutaneously immediately following the coronary ligation procedure and wound closure, but at least 20m prior to revival. Both drugs are administered 6-8h after surgery, and then given twice daily for up to 3d to control pain and distress. The animals are monitored continuously until conscious and every hour for the first 4h following surgery. A second checkup the night of the surgery occurs 2-4h later to administer a second dose of analgesic. Animals are monitored daily after the second day following the procedure, until animals appear stable.

3.4.6 Analysis of heart function and histology
Echocardiographs are performed on the mice 4wk following the procedure to determine the extent of damage following the MI. Mice are anesthetized with 4-5% isoflurane within an anesthesia chamber for induction of inhalation anesthesia, and then 2% isoflurane via face mask for maintenance anesthesia. Echocardiographic traces are collected and later analyzed for cardiac function assessment. Upon the termination of the experiment, animals are sacrificed and their hearts are collected for histological analysis. H&E stains are performed to assess overall heart morphology and cell population, while the Masson’s Trichrome stain can determine any fibrosis and collagen deposition that might have occurred due to ventricular remodeling.


3.5 Results

During the surgical procedure, ligation of the left coronary artery can be identified by a marked blanching downstream of the occluded artery. As a test before tying the knot, the suture can be tightened briefly to check if it is in the appropriate place. Additionally, since occlusion of the artery results in almost instantaneous decrease in the ability of the left atrium to properly beat, the left atrium will enlarge in response to a backflow of blood in the system.

M-mode echocardiography measurements taken as early as 2d post-infarction show a cessation of left wall movement, indicative of the muscle reconstruction (Figure 3.2 a,b). Qualitative calculations made from the data show a decrease in ejection fraction and stroke volume in the infarcted hearts. At the termination of the experiment, when the hearts are collected for histological purposes, a clear dilation of the left ventricle can be seen, along with a thinning of the left ventricular wall, and the deposition of collagen that denotes scar tissue deposition in place of functioning cardiomyocytes (Figure 3.2 c,d).

Care is taken in the transfer of the patch so as to ensure its location on the left ventricle (Figure 3.3 a). Administration of the glue aids in maintaining the patch location after chest closure. For our purposes, the adhesive system must be viscous enough to allow precise placement control and minimize subsequent runoff into surrounding organs, but malleable enough not to interfere with heart function. Preliminary tests were performed to calculate the viscosity, gelation time, and stiffness of various fibrinogen/thrombin ratios, in order to determine the proper combination that suits our needs. In vivo analysis was performed to test the ability of the fibrin glue to maintain patch-to-tissue adhesion while still allowing for full heart function (data not shown). Upon histological analysis, the
presence of the hydrogel construct can be seen thanks to the administration of the fibrin gel (Figure 3.3 b). It should be noted that the fibrin glue did not harm the myocardium, as evidenced by the lack of tissue remodeling or ventricular thinning at the site. The cardiomyocytes remain in tact despite the addition of both a cardiac patch and its accompanying glue. Additionally, viability testing confirmed that that the administration of fibrin glue to the external surface of a cell-encapsulating hydrogel patch did not affect cell survival within the patch (Figure 3.4).

3.6 Discussion

With this approach to the murine MI model, we have developed a system that minimizes the damage to non-myocardium areas that are associated with other murine MI techniques. These areas include damage caused by a tracheostomy, the cutting of the muscular layer, and the breakage of ribs to expose the chest cavity. These changes have resulted in a 70% survival rate. We believe this improvement is due to the care taken to keep as much of the major structures, including the ribs and muscle layers, in tact during the surgical procedure.

Fibrin glue is known in both the laboratory and clinical settings thanks to its ability to quickly aid in clot formation, as well as its potential to deliver and host MSCs\textsuperscript{17}. For our purposes, fibrin glue served as a biocompatible approach to adhering a water-based hydrogel construct to the surface of the heart. The use of a fibrin-based glue has proven successful in keeping the hydrogel construct at the damaged site for up to 8wk, with the possibility for tissue contact of a longer period of time. The use of this adhesion system allowed control for placement of the delicate construct without the need for additional sutures that harm the myocardium as well as the construct itself. We have found that the glue itself is
non-toxic to the encapsulated cells and does not negatively affect the underlying tissue.

3.7 References


3.8 Figures

Figure 3.1: Murine thoracotomy and subsequent chest closure. After the initial skin incision, two muscle layers are teased back to expose the 2nd, 3rd, and 4th ribs (a). The thoracotomy is performed between the 3rd and 4th ribs using a cauterizer, and the ribs are retracted to gain better access the heart (b). After the MI procedure and patch placement, the rib layers are sutured closed with 2-3 interrupted sutures (c). Prior to the final suture placement, a PE-10 cannula is inserted between the 2nd and 3rd ribs (d). The muscle layers are then sutured (e) followed by the suturing of the skin (f).
Figure 3.2: Left coronary artery ligation results in a decrease in cardiac function as measured by echocardiography. M-mode echocardiography of healthy (a) and infarcted (b) hearts. Infarcted hearts show a clear lack of wall movement where viable tissue has been replaced with scar tissue. This correlates with a measureable decrease in left ventricular ejection fraction and overall cardiac stroke volume. Histological analysis of healthy (c) and infarcted (d) hearts shows dilation of the left ventricle and thinning of the left ventricular wall of the infarcted myocardium, both signs of tissue remodeling and scar tissue deposition.
Figure 3.3: Application of fibrin glue ensures patch adhesion to the heart. The patch is placed on the surface of the heart while the chest cavity is exposed during the procedure (a). Once placed, a fibrin-based glue is added on top of the heart and allowed to dry. The patch can clearly be seen on histological sections of heart tissue that were collected at 1wk post-procedure (b). Results are consistent for up to 8wk of adhesion.
Figure 3.4: Fibrin-based glue does not harm encapsulated tissues. Cell viability was measured via MTT analysis 1wk after the administration of a fibrin-based glue. The viability of cells within the patch was not affected by the addition of the glue to the surface of the construct.
4. Incorporation of microchannel structures in mesenchymal stem cell encapsulated hydrogels improves their therapeutic capacity in the treatment of myocardial infarctions

4.1 Abstract
The major injury attributed to myocardial infarctions is due to the vast remodeling that occurs in the hypoxic environment post-occlusion of the coronary vessel. Over the past several years there has been an increasing interest in the use of mesenchymal stem cells (MSCs) to help cease or reverse the myocardial fate of collagen deposition and scar tissue formation, and help aid in the recovery of patients that suffer from debilitating heart attacks. In this study, we employ a poly(ethylene-glycol) dimethacrylate (PEGDMA) hydrogel construct to encapsulate MSCs and deliver them to the epicardial surface with the aim of concentrating their therapeutic benefits at the site of injury. A stereolithographic apparatus (SLA) was used to provide user control of the hydrogel fabrication and design parameters resulting in the incorporation of microchannels within the patch, creating multiple reservoirs in which soluble factors secreted from the MSC’s can concentrate and maximize their efficacy. Incorporation of the 500µm channels both increase vascular density and direct vascularization. Two cell concentrations were used to test the effect of cell number on angiogenesis in ovo and cardiac function in vivo. Placement of a cell encapsulated patch directly following the occlusion of the left coronary artery in a murine model showed significant improvement in the ejection fraction, fractional shortening, and stroke volume. Most importantly, the overall number of therapeutic cells required to display such a result were drastically lower than previous studies would suggest,
with significant improvement seen with as little as 1,400 MSCs/construct. This study demonstrates the ability to control the hydrogel construct to maximize its potential benefits in vivo in addition to suggesting that therapeutic effects of MSCs post-MI can still be obtained with a drastic decrease in the overall number of cells delivered to the myocardium.

4.2 Introduction
Despite medical progress in treatment and intervention, cardiovascular disease is still a leading cause of morbidity and mortality for both men and women worldwide. The immediate goal of current post-myocardial infarction (MI) treatments is to open the blocked coronary arteries and return blood flow to the injured cardiac muscle, with the hope that early recovery of blood flow will decrease permanent damage caused by the hypoxic insult\(^1\)-\(^3\). This approach is centered on supporting the remaining tissue, however, it does not actively prevent or reverse the degradation of the myocardium at the ischemic site\(^4\). In fact, an expected outcome of reperfusion therapy is collateral damage of injured tissue from reactive oxygen species that arise with a sudden influx of oxygenated blood to a previously hypoxic region\(^5\)-\(^7\). Recently efforts are increasingly made to harness the regenerative capabilities of mesenchymal stem cells (MSC) and using them to treat congestive heart failure through a number of mechanisms, including their secretion of pro-angiogenic and anti-apoptotic cytokines\(^8\)-\(^11\). Additionally, MSCs are a particularly promising cell type due to their relative ease of isolation and expansion from either bone or adipose tissue, as well as their immunoprivileged profile that allows them to be delivered to a host animal with little to no inflammatory response\(^12\).
There are three means currently being employed to deliver MSCs to infarcted
tissue: 1) direct intramyocardial injection to the injured site; 2) systemic
intravenous infusion, in which a fraction of transplanted cells have been shown to
home to the injured tissue; and 3) local intracoronary delivery via a catheter
placed within the coronary vessels. All three of these means have demonstrated
a decrease in infarct size, less severe ventricular remodeling, and improved
vascularization\textsuperscript{11, 13-15}. While these reports are encouraging, it has been reported
that 7d after transplantation, stem cell concentration at the site of injection
decreases by 10-fold, with no trace of stem cells after 3wks, suggesting massive
cell loss away from the injured tissue\textsuperscript{14, 16}. This restricts the efficacy of stem cell
therapy for ischemic heart disease and limits the full potential of MSCs, and
might help explain the limited efficacy of such therapies when applied to the
clinical setting\textsuperscript{17-21}.

One proposed solution to this challenge is the use of a cell-loading patch as a
vehicle to deliver the cells directly to the heart surface, ensuring that the MSCs
remain in close proximity to the injured tissue. There are a variety of approaches
to a cell-loaded patch for the purposes of tissue engineering and myocardial
regeneration\textsuperscript{22-26}. Many of these show increased retention of the cells at the site
of injury, as well as neovascularization and differentiation of implanted cells into
cardiomyocytes. What is less characterized in these studies is the role, if any,
secreted cytokines play in the regeneration capability of implanted cell-loaded
patches. Implanted patches should be rigid enough to maintain its structural
integrity against repeated cardiac contraction; however, a rigid matrix typically
presents limited permeability, resulting in the loaded cells to lose their viability
and, consequently, their secretion activities. We have focused not only on the
ability of maintaining cell viability within the patch, but the constitutive secretion of
cytokines from the patch into the surrounding environment. With this approach, the MSCs loaded in the patch should be able to sustainably secret cytoprotective factors, such as thrombospondin-1 (TSP-1), and angiogenic factors such as vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8)\textsuperscript{25,27-34}. The resulting angiogenesis within the ischemic tissue would keep the tissue viable long enough for the vasculature to regenerate and sustain myocardial function.

To resolve these challenges, in this study, we hypothesized that loading stem cells into a hydrogel implant we previously designed to be stiff and permeable would support cellular viability and secretion activities. Therefore, the resulting stem cell-loading hydrogel patch would be useful to promote vascularization and subsequently retain cardiac function of heart damaged by tissue ischemia. To examine this hypothesis, we loaded bone marrow derived mesenchymal stem cells (BMSCs) into a hydrogel formed from cross-linking of poly(ethylene glycol) dimethacrylate (PEGDMA). The cell-loading hydrogel was fabricated using a stereolithographic assembly (SLA) unit\textsuperscript{35}. Additionally, microchannels that can serve as a drainage of cell-secreting soluble factors to tissue, and in turn increase the angiogenic potential of the gel patch, were added to the construct structure\textsuperscript{36}. The cytokine secretion profile of the cells within the patch was explored and the angiogenic capability was determined. Altogether, we believe that this hydrogel patch would greatly serve to improve quality of cell therapies of various cardiovascular diseases due to its constitutive secretion of paracrine factors to heart wall, as well as its controlled angiogenic potential.
4.3 Materials and Methods

4.3.1 Cell preparation
VEGF secreting BM-MSCs (Lonza, Basel, Switzerland) were cultured in TheraPEAK MSCGM-CD mesenchymal stem cell medium (Lonza) supplemented with 5% fetal bovine serum (FBS), 100 U mL\(^{-1}\) penicillin, and 100 mg mL\(^{-1}\) streptomycin (Gibco, Carlsbad, CA, USA). Cells were incubated at 37°C and 5% \(O_2\) in 175 cm\(^2\) flasks to 80% confluence. When preparing for fabrication, cells were lifted with 0.25% trypsin and 0.04% EDTA in HBSS (Gibco, Carlsbad, CA, USA) and gently added to the pre-polymer solution.

4.3.2 Hydrogel fabrication
The hydrogel fabrication method was described in detail in a previous publication\(^1\). In brief, Poly(ethylene glycol) dimethacrylate (PEGDMA) of 1000 mol wt. (Sigma Aldrich, St. Louis, MO, USA) was dissolved in sterile 1x PBS to obtain a 20% w/v solution pre-polymer solution. The photoinitiator, 1-[4-(2-hydroxy-ethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (Irgacure 2959, Ciba, Tarrytown, NY, USA), was dissolved in DMSO and added to the pre-polymer solution for a final w/v of 0.5%. Depending on the desired condition, medium either with or without cells was mixed with the pre-polymer solution immediately before fabrication, resulting in a 20% concentration of cells/medium. The final pre-polymer solution consisted of 20% PEGDMA, 0.5% photoinitiator, and 20% cells/medium. A stereolithography apparatus (SLA, Model 250/50, 3D Systems, Rock Hill, SC, USA) was used to fabricate the hydrogel constructs. Computer aided design models were generated using AutoCAD 2009 (Autodesk, San Rafael, CA, USA) and exported to stereolithography (STL) format. The SLA software, 3D Lightyear v1.4 (3D Systems, Rock Hill, SC, USA), was used to slice the 3D models into a series of 2D layers from a user-specified thickness. Once
the fabrication procedure was finished, the hydrogels were rinsed multiple times with 2mL of culture media at each rinse to remove uncrosslinked polymer.

4.3.3 Viability tests

Each prepared hydrogel patch was transferred to a well of a 96-well plate and incubated in 200µL of media until viability testing was performed. Two forms of viability testing were done; a live/dead viability/cytotoxicity test (Life Technologies, Carlsbad, CA, USA) and an MTT assay. For the live/dead assay, a working solution consisting of 1µL of calcein AM and 2µL of Ethidium homodimer-1 added to 1mL of sterile 1x PBS was prepared immediately prior to performing the assay. The media was removed from each construct-containing well and 150µL of the working solution was added. After 20min, the patches were visualized under fluorescent microscopy. For quantitative analysis, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the media of the hydrogels at a ratio of 1:5 volume of MTT to volume of media and the patches were incubated for 4h. After the incubation period, absorbance was measured at 490 nm using a plate reader.

4.3.4 Secretion factor assessment

The supernatant was collected from cell cultures and measured for production of a variety of cytokines using a human angiogenesis antibody array kit (R&D systems, Minneapolis, MN, USA). The assay was run following the manufactures protocols. VEGF secretion was quantified using a VEGF human ELISA kit (Life Technologies, Carlsbad, CA, USA). Media was collected from cell encapsulating hydrogel constructs at days 1, 3, 5, and 7, and stored in -20°C prior to running the assay.
4.3.5 Chicken chorioallantoic membrane (CAM) assay

The ability of the patch to induce neovascularization was examined by implanting the cell-encapsulated patch onto a chicken chorioallantoic membrane (CAM) of a 1wk old chick embryo. Fertilized chicken eggs (Hy-Line W-36) were obtained from the University of Illinois Poultry Farm (Urbana, IL). The CAM was exposed by perforating a region of the egg shell and the construct was placed directly on the uncovered CAM. The perforated region of the shell was then sealed and the embryos were reincubated at 37°C and 5% O₂. The CAM was observed at varying time-points throughout the incubation period to ensure survival of the embryo. At the termination of the experiment, the embryos were exposed to 4% formalin solution to fix the structures, and the constructs and were located and excised from the remainder of the membrane. These constructs and their accompanying CAM were imaged and incubated for 24h in a 4% formalin solution, after which time they were processed, embedded, and sliced for histological analysis. Sections were stained with α-smooth muscle actin to highlight vessel structures. Digital images of the stained sections were collected with a NanoZoomer Slider Scanner/Digital Pathology System (Hamamatsu, Hamamatsu, SZK).

4.3.6 Myocardial Infarction (MI) model

Prior to surgery, mice were anesthetized in an anesthetic chamber with 5% isoflurane accompanied by and 1 L/min supportive O₂ flow. Their weight was recorded and mice were intubated with a 20G angiocath tube and connected to a small animal ventilator (160bpm), where 1.5% isoflurane and 1 L/min O₂ flow was administered. The mice were placed on a heating pad in the supine position. Hair was removed from the chest surgical site and an inch long vertical incision was made slightly to the left of center. Two muscle layers were teased back and
sutured in the retracted position. An intercostal incision was made between the third and fourth ribs and the heart was exposed. A single 8-0 monofilament suture (Johnson & Johnson, NJ) was placed to ligate the left coronary artery and occlude blood flow to the apex of the heart. Using a 6-0 monofilament suture (Johnson & Johnson, NJ), the ribs and muscle layers were closed by interrupted suture and the skin layer incision was closed by a continuous suture. The isoflurane administration was ceased before the removal of the tracheal tube from the mouse, and animals were monitored until full recovery. Four conditions of MI’s were tested: no patch placement (n=3), patch without cells (n=3), patch with \(0.4 \times 10^6\) cells/mL cell concentration (n=3), patch with \(2.0 \times 10^6\) cells/mL cell concentration (n=4). Additionally, control animals in which no surgical procedure was performed were used as negative controls (n=27).

4.3.7 Echocardiography

A VisualSonics Vevo 2100 (VisualSonics, Toronto, Canada) small animal ultrasound was used to perform echocardiography on the mice at 4wks post-operation. Animals were placed in an anesthetic chamber and 5% isoflurane was administered with 2 L/min of supportive O\(_2\). Once properly anesthetized, the animals were moved to a nose cone and the percent of isoflurane was decreased to 1.5-2%. The imaging area was cleared using a depilatory cream and 2D M-mode echocardiography was performed on the animals. All heart function calculations were made using the Vevo 2100 software.

4.3.8 Histological analysis of heart sections

Animals were sacrificed at 4 and 8 wk post-surgery and their hearts were collected, fixed in a 10% formalin solution, and embedded in paraffin. Tissue was sliced and cross-sections were stained for Hematoxylin and Eosin and Masson
Thrichrome Blue. Digital images of the stained sections were collected with a NanoZoomer Slider Scanner/Digital Pathology System (Hamamatsu, Hamamatsu, SZK).

4.4 Results

4.4.1 Cell encapsulated hydrogel

The hydrogel was constructed from a poly(ethylene glycol) dimethacrylate (PEGDMA) polymer using a stereolithography apparatus (SLA). The SLA approach focuses a UV light directly on the polymer solution, allowing for controlled crosslinking at the site of light focus (Figure 4.1). This technology provides a platform for layer-by-layer fabrication of a cell-encapsulating hydrogel via in situ polymerization. The precision of the photocrosslinking allows for detailed control of the three-dimensional features of the construct, providing a mechanism to incorporate patterns within the patch.

With this control, we incorporated 9 separate microchannels that span the thickness of the patch, with two separate conditions of diameters 500µm and 1000µm alongside a patch with no additional features (Figure 4.2). By incorporating BM-MSCs within the pre-polymer solution, we were able to entrap the cells in a uniform distribution throughout the construct (Figure 4.2). The concentration of cell incorporation varied between no cells (herein referred to as blank), \( 0.4 \times 10^6 \) cells/mL (low concentration), and \( 2.0 \times 10^6 \) cells/mL (high concentration). Overall, through the variation of microchannel diameter and cell number, there were 9 conditions tested throughout the experimental procedures, each with differing surface to volume ratios and total cell numbers (Table 4.1).
4.4.2 Cell Survival and Cytokine Secretion

Long-term cell viability within the constructs was analyzed by two methods. An MTT assay was used for quantitative analysis of cell survival, and a live/dead assay was used to visualize and relate the ratio of live cells to apoptotic cells. Constructs of 500µm diameter and high cell concentration were cultured for up to 7d and cell viability was tested at days 1, 3, 5, and 7 of culture using the MTT assay. There was no decrease in cell viability within the patch for up to 3d post-encapsulation, at which time the viability within the patch began to decrease, with an overall survival of 60% at day 7 post-fabrication (Figure 4.3). To obtain a broader picture of the cell population and its changes over time, a qualitative live/dead cell viability stain was performed at days 1, 7, and 14. There appears to be a relative abundance of dead cells at day 1, with a relatively even ratio of dead cells to live cells, however, the number of dead cells does not seem to increase with time, as the MTT analysis would suggest (Figure 4.4). Instead, we see leakage of cells out of the patch, which can be identified in the live cell images by their morphological change that is characteristic of plastic adherence of MSCs. Thus, the decrease in cell numbers can be attributed to a gradual cell escape from the construct instead of merely cell death within the patch.

Next, the secretion factor profile of the encapsulated cells were examined to determine their role in cardiac remodeling and angiogenesis. The permeability of the PEGDMA hydrogel allows for the release of cytokine secretion from the construct into the culture media. Testing the culture media on a cytokine array, we found that BM-MSCs encapsulated within the construct secreted a variety of pro-angiogenic factors, including interleukin-8 (IL-8), Pentraxin-3, vascular endothelial growth factor (VEGF), and Activin A (Figure 4.5). Additionally two proteins, TIMP metallopeptidase inhibitor -1 (TIMP-1) and plasminogen activator
inhibitor (PAI-1), which have been associated with a decrease in left ventricular remodeling and cardiac fibrosis post-MI, and thrombospondin-1 (TSP-1), which is a known anti-apoptotic cytokine (Table 4.2). These results suggest that several pathways might be collaborating to the potential cardiotherapeutic properties of the encapsulated cells.

We focused on one protein, VEGF, to test the difference in secretion levels of constructs with differing diameter and cell density. As expected, there is an increase in secretion in relation to number of cells encapsulated for all microchannel diameter conditions (Figure 4.6). When comparing microchannel diameters to one another, we found that the secretion level was statistically indistinguishable between the patches that contained no microchannels and those with 500µm diameter microchannels, however, there was a distinct drop in secretion from the patches containing 1000µm diameter microchannels. This presumably is due to the relative number of cells within the patches, and the decrease in cell number that accompanies the incorporation of a channel that cuts through the patch itself. To test this hypothesis, we normalized the secretion levels to the calculated cell number within each patch. This normalization brought all secretions levels to a more comparable level. Again, no significant difference was seen between the zero and 500µm microchannel diameters, however, the 1000µm still shows a lower, and statistically significant, secretion rate than the 500µm condition, suggesting a diminishing return of cytokine secretion in regards to the surface to volume ratio of the constructs.
4.4.3 Controlled vascularization via microchannel incorporation

To assess their vasculogenic capabilities, the constructs were placed on a chick chorioallantoic membrane (CAM). At 10d post-transplantation, a clear, directed neovascularization was seen at the location of the microchannels in the patches containing 500µm diameter microchannels, a phenomenon that diminishes when the microchannel diameter is increased to 1000µm (Figure 4.7). While blood vessels were indeed seen on the CAM of samples containing 1000µm microchannels, the vessels were much smaller and more immature than those seen in constructs with either no microchannels or 500µm microchannels.

After imaging, the construct-containing region of the membrane was dissected and fixed for further histological analysis. The sections were stained with α-smooth muscle actin to identify vessels within the membrane. The area directly below the construct was isolated and vessels with an area of 50µm² or greater were counted. A clear increase in overall vessel area density was seen with increasing cell numbers (Figure 4.8). Observing the vessel density difference between microchannel diameter, holding the cell number constant, the highest density is seen in constructs of 500µm diameter, with a density decrease when the microchannel diameter was increased to 1000µm. These results indicate, as suggested by the secretion profile, show diminishing returns of microchannel incorporation when the diameter is increased to 1000µm, independent of the cell density that is contained within the hydrogel patch. Interestingly, while VEGF secretion between no microchannels and 500µm microchannels is not significantly different, there does seem to be a trend in increasing vessel density with the incorporation of the channels, suggesting that that concentration of the soluble factors might play a role in the vasculogenic capabilities of the construct.
4.4.4 Mouse MI model
A mouse myocardial infarction model was used to test the efficacy of the microchannel containing hydrogel patch in helping to cease the remodeling of the heart following ischemic injury. The left coronary artery was occluded to mimic the blockage of blood flow to the left ventricle that is characteristic of the majority of myocardial infarctions. A blanching of the myocardium downstream of the occluded vessel can be seen almost instantaneously, aiding in the identification of the effected tissue. Immediately following the occlusion, the patch was placed directly on the ischemic tissue, and a fibrin-based glue was applied to the exterior of the patch to ensure adherence to the myocardium (Figure 4.9). Echocardiogram measurements were then performed to analyze the heart function with and without treatment.

4.4.5 Induction of MI
The initial LAD occlusion resulted in a significant drop in both the ejection fraction (from 60.7%±9.2% to 15.9%±11.0%), the fractional shorting (from 31.2%±6.2% to 7.2%±5.0%), and the stroke volume (from 27.7%±6.2% to 13.7%±6.6%) (Figure 4c, d, e). Histological analysis of the excised hearts showed a marked dilation of the left ventricular diameter with increase in luminal volume and diameter, and ventricular aneurysms with a decrease in left ventricular wall thickness along with collagen deposition at the site of ischemia, confirming positive infarction induction and scar tissue deposition.

4.4.6 Treatment of MI
Following initial in vitro experiments, it was concluded that the efficacy of the 500µm microchannel diameter constructs would be tested in both lower and higher cell concentrations. The lack of angiogenic potential of the 1000µm
microchannel diameter constructs negated any further in vivo testing. To maintain the focus of the efficacy of the patch on secreted cytokines, rather than on transplanted and integrated cells, the no diameter patch was also kept out of in vivo testing.

Patches were placed on the heart directly following LAD ligation. Echocardiograph measurements taken 4-5wks post-operatively showed a significant increase in the ejection fraction and stroke volume of hearts treated with a cell-encapsulated patch. Although not statistically significant, a step-wise improvement was noticed with an increase in cell density. Patches containing no cells displayed cell function similar to the MI only group, confirming that the cells within the construct are responsible for cardiac protection, and not the presence of the construct itself (Figure 4.10).

Animals were sacrificed at 8wk to visualize the cellular and morphological changes attributed to the treatment. Histological analysis showed that the patches containing a low density of cells caused a slight decrease in cardiac fibrosis with a decrease in left ventricular dilation, while animals treated with the high density cell construct showed very little necrosis at the site of injury with little to no ventricular dilation and wall thinning, as well as an increase in neovascularization. This was in clear contrast with the untreated animals where left ventricular dilation, wall thinning, and clear collagen deposition and fibrosis are seen (Figure 4.11). Together, all these results are characteristic of cardiomyocytes necrosis and scar tissue formation. Animals exposed to a patch with no encapsulated cells showed similar results as untreated animals. Quantitatively, these results can be summarized by the left ventricular and septal
thicknesses, which, when normalized to the ventricular diameter, both showed an increase compared to the negative controls (Figure 4.11).

4.5 Discussion

We have shown that MSCs can be maintained within a PEGDMA hydrogel construct without losing their ability to secrete beneficial cytokines to its surroundings. The growing body of knowledge suggests that stem cell treatment for cardiovascular disease is dependent more on the secretion of soluble factors from the cells rather than direct differentiation of the cells. By controlling the environment and delivery of these cells via user-generated 3D manipulation of the vehicular constructs, we can further increase their therapeutic potential. In previous studies, we have established that the introduction of microchannels produces "wells" of concentrated secreted cytokines. This concentration in turn allows for greater, and directed, angiogenic potential at the site of the microchannels\(^{36}\). Although an MSC containing hydrogel construct has been shown to improve heart function by a number of other groups, we hypothesize that the added benefit of controlled vascularization restores the heart to within reach of its pre-MI state.

Upon analysis of the histological sections obtained at \(8\)wk post-implantation, the animals that either received no patch or a blank patch without cells showed no inflammatory response at the site of injury. When cells were added as a treatment, florid inflammatory reactions were still present \(8\)wk post-operatively. This finding suggests that the prolongation of the inflammatory process in the treated animals, as well as the increase in neogenesis, are two contributing mechanisms to the protection of the myocardial muscle from the effect of post-MI fibrosis. Whereas terminal scar formation was seen in animals that received no
treatment, a robust immune response was still present with the addition of stem cell therapy, prolonging the window of opportunity to cease or reverse the negative effects of the hypoxic environment on the cardiomyocytes.

With this design, we have also shown the importance of cell density when conducting these studies. While most groups use a cell density of $1 \times 10^6$ cells/condition or more, we cut this amount drastically, with our most beneficial patches containing only 7,100 cells. This drastic decrease in cell number might help alleviate the translational burden of collecting and culturing MSCs for clinical use.

Utilizing an established stereolithography apparatus, we have developed a platform in which complex, three-dimensional, cell encapsulated structures can be fabricated. We have previously shown that fibroblasts can both remain viable and secrete factors for up to 7d post encapsulation\textsuperscript{35}. Additionally, the user-controlled nature of the platform was utilized to incorporate microchannels into the cardiac patch. These channels serve as a well for soluble factors to congregate within, increasing the surface area available for release of these beneficial factors, and in turn, increasing the angiogenic potential of the patch and allowing for directed neovascularization at the site of the microchannels\textsuperscript{36}. This hydrogel system provides a means to deliver the therapeutics to the site of injury, introducing the prospect of using the material for treatment of ischemic heart failure. Through the replacement of fibroblasts with MSCs, we can deliver cells that have been proven successful in post-MI therapy directly to the site of infarction, while ensuring maximum soluble factor secretion at the site of injury via microchannel incorporation. This would thus provide a cytokine driven
therapeutic approach to aid in the prevention of detrimental heart remodeling following a myocardial infarction.

4.6 References


4.6. Figures and Tables

Figure 4.1: Schematic representation of the stereolithography apparatus (SLA) procedure. A 3D computer aided design (CAD) is developed and converted into the stereolithography format. The SLA then focuses a UV light source on a cell-containing pre-polymer solution. The focusing of the light results in cross-linking and subsequent encapsulation of the cells in the pre-defined design.
Figure 4.2: Construct designs and microchannel diameters. Three designs were chosen, one lacking microchannels, and two with 9 evenly spaced channels, one of 500\(\mu\)m in diameter, and another of 1000\(\mu\)m diameter. Each construct, aside from the blank controls, contained encapsulated cells that were “trapped” within the crosslinking PEGDMA.
Table 4.1: Conditions used throughout the experiment. A total of 9 conditions were tested. Each construct was delineated by three different microchannel diameters (0\(\mu\)m, 500\(\mu\)m, and 1000\(\mu\)m) as well as three different cell concentrations (no cells, 0.4\(\times\)10\(^6\) cells/mL, and 2.0\(\times\)10\(^6\) cells/mL).
Figure 4.3: Cell viability in the constructs post-encapsulation. MTT data taken at days 1, 3, 5, and 7 from constructs with 500 µm diameter microchannels and high cell density show sustained viability for up to 3d post-encapsulation, at which point relative cell viability decreases within the patch. By day 7, the population drops to 60% when compared to day 1 post-encapsulation.
Figure 4.4: Live and dead cell imaging of encapsulated cells. Live/dead analysis of constructs containing high density of cells show a relative high percentage of dead cells within the patch at day 1. Instead of an increase in dead cells, as would be expected, there seems to be a leakage of cells from within the patch to the surrounding area by days 7 and 14, evident by the change of some live cells into fibroblast-like morphology that is typical of plastic adherence of MSCs.
Figure 4.5: Paracrine factors secreted by encapsulated cells. An angiogenesis antibody array was used to detect the relative levels of cytokines secreted out of a $500\mu$m microchanneled construct containing a high cell density. Of the 55 angiogenesis-related proteins present on the membrane, 12 could be detected. The action of these cytokines range from potent promoters of angiogenesis to inhibitors of cardiac remodeling.
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Function</th>
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<tbody>
<tr>
<td>Activin A</td>
<td>Increases expression level of type-1 collagen.</td>
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<tr>
<td>IGFBP-1, 3</td>
<td>Lengthens half-life of circulating IGF in tissues.</td>
</tr>
<tr>
<td>IL-8</td>
<td>Potent promoter of angiogenesis.</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Promotes cell metastasis from the bone marrow. ECM protease.</td>
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<tr>
<td>PTX-3</td>
<td>Anti-apoptotic</td>
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<tr>
<td>PAI-1</td>
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<tr>
<td>PEDF</td>
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<td>Promotes plasminogen to plasmin conversion for ECM breakdown</td>
</tr>
<tr>
<td>VEGF</td>
<td>Promoter of angiogenesis</td>
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**Table 4.2: Brief description of proteins secreted from MSC encapsulated patch**
Figure 4.6: Cells within the patch show sustained secretion of paracrine factors for up to 7d post-encapsulation. Focusing on a single secreted cytokine (VEGF), a distinct increase in secretion was seen with an increase in encapsulated cell number for all three microchannel diameter conditions (a). The disparity seen between the 1000µm microchanneled patches and those of the 500µm and no microchannel patches are quite large, however, the differences diminish when the secretion levels are normalized to number of cells encapsulated within the patch (b).
Figure 4.7: Directed vascularization of cell encapsulated constructs with 500µm diameter microchannels. Brightfield images of construct containing CAM 10d post-implantation show distinct neovascular structures at the site of 500µm microchannels. This phenomenon is diminished when the microchannel diameter is increased to 1000µm.
Figure 4.8: Vessel density differences between hydrogen construct conditions and cell densities. Construct containing portions of the CAM were excised, sliced, and stained to quantitatively determine vessel densities. Vessels of 50µm² were counted, and an increase in vessel density was seen with increasing cell numbers within the constructs. When comparing microchannel diameters in constructs containing high cell density, we again saw the greatest number of vessel density in constructs with 500µm microchannels, with lesser numbers in the no-channel containing and 1000µm channel constructs. Constructs made of 500µm microchannel diameter and high cell concentration showed the greatest vessel density. Representative images of stained CAM are shown below their respective quantitative data bars. Asterisks (*) represent statistical significance of p = 0.5.
Figure 4.9: Patch placement on the murine heart post-MI induction. A live-image of the final placement of the construct on the epicardial surface of the heart clearly shows the suture placement directly below the left atrium and the microchannel incorporation within the patch.
**Figure 4.10: Cell encapsulated patches improve cardiac function post-infarction.** Echocardiogram measurements show a comparative measurement of cardiac function between animals receiving no treatment (MI only) and those with constructs containing no cells (Blank). The addition of a small amount of cells (low) showed significant improvement over controls, but the greatest difference was seen when a higher concentration of cells was added to the construct (high). Results from the higher cell density constructs are nearing those of healthy controls (No MI). Asterisks (*) represent statistical significance of $p = 0.5$. 
Figure 4.11: Cell encapsulated patches show a decrease in left ventricular remodeling 8wks post-infarction. Representative 8wk histological images of each condition are shown along with their respective functional measurements (a). The constructs can clearly be seen, and a decrease in fibrosis and collagen deposition (blue staining) can be appreciated with increasing cell densities. Quantitative analysis of left ventricular free wall thickness (b) and septal thickness (c) normalized to the overall heart diameter show an increase in each with the addition of cells to the constructs, indicating a decrease in myocardial remodeling.
5. **SUMMARY, ONGOING WORK, AND FUTURE DIRECTIONS**

5.1 **Summary**

As highlighted throughout this dissertation, we have developed a stem cell centered cardiac patch that, when delivered to the site of injury, helps to decrease cardiac fibrosis and improves cardiac function post myocardial infarction assault. Cardiac tissue engineering is an emerging field, with a number of current and ongoing studies being done to determine the best scaffold, cell type, and delivery method to treat alleviate cardiac remodeling and degeneration. While this project is just a piece of the larger picture, I believe it provides a number of benefits over similar studies.

One such advantage is the use the non-immunocompetent mice in our studies. Many groups that have shown a decrease in cardiac fibrosis in fact ran their *in vivo* tests in severe combined immunodeficiency mice or nude rats\(^1\)-\(^3\). While these studies provided a necessary baseline and framework for future studies, they also must be approached with some level of caution since the introduction of lymphocytes and the complete complement system might alter the animal's response to therapy. By using C57BL/6J mice, we have removed the question of immune response from any final conclusions we can make about the effectiveness of our constructs.

Another benefit of our model is the overall number of cells that were used and delivered to the heart wall. We chose to encapsulate a much smaller number of cells, between 1,400 to 7,000, within each construct, as opposed to almost every other study that encapsulated or delivered 1x10\(^6\) cells (see Chapter 1 for related
citations). This drastic decrease in the number of cells might possibly alleviate the burden or necessity of isolating and expanding stem cells if/when this technique arrives in the clinical setting.

Finally, we utilized the control that the SLA provides to incorporate microchannels within our constructs. These channels increased the surface to volume ratio of the hydrogels, allowing for greater secretion of paracrine factors, and subsequently increased the vascularization potential of the encapsulated cells.

5.2 Ongoing Work and Future Directions

5.2.1 Unveiling the underlying mechanisms of BM-MSC protection

Overview: One major question that still needs to be addressed is the exact mechanism that accounts for the protection attributed to the BM-MSC therapy. There has been plenty of work in the field to attempt to answer this question, with a number of possible theories emerging. The pathway most attributed to cardioprotection is that of the phosphoinositide 3-kinase (PI3K)/Protein kinase B (AKT) pathway\(^4\)\(^-\)\(^7\). The activation of the pathway may be from a variety of mechanisms. Some suggest integrin-linked kinase (ILK) might play a role in AKT activation, while others point to a protein complex that consists of growth factor receptor-bound protein 2 (Grb2), focal adhesion kinase (Fak), the proto-oncogene tyrosine-protein kinase Src (cSrc), and a specific subunit of PI3K (PI3K-p85)\(^5\)\(^,\)\(^7\)\(^,\)\(^8\). No matter the mechanism, activated AKT is thought to activate the protein complex known as nuclear factor kappa-light-chain enhancer of activated B cells (NF\(\kappa\)B) which has a direct result of regulating genes involved in cell survival\(^5\). AKT can also concurrently block the phosphorylation of Bcl-2-
associated death promoter (BAD), caspase 9, and forkhead transcription factors, slowing down their associated apoptotic pathway\(^7\).

Determining the mechanistic properties of our cardiac patch: Seeing as there are a variety of mechanisms that might play a role in MSC protection, it would be beneficial to add our own piece of information into the growing body of knowledge. Future work can be done to strengthen the model highlighted in this dissertation by providing a more detailed view of the pathways associated with the pro-angiogenic and survival results that we highlighted with the patch therapy.

5.2.2 Stromal derived factor-1 (SDF-1) incorporation within the construct

Overview: The body naturally tries to prevent the degradation of heart tissue following an MI event via the recruitment of BM-MSCs or similar stem cells through cytokine secretion. A number of cytokines and enzymes have been suggested to play a role in the recruitment of stem cells to the damaged tissue site including, interleukin’s -1, -6, and -8 (IL-1,-6,-8), granulocyte colony-stimulating factor (G-CSF), transforming growth factor-β (TGF-β) matrix metalloproteinase’s (MMP’s), and elastases\(^9\)-\(^{12}\). A growing body of evidence suggests that SDF-1 plays the most prominent role in the homing of stem cells that express the CXCR4 ligand\(^13\)-\(^{17}\). We have hypothesized that a patch doped with stem cell homing factors, specifically SDF-1, may provide a means to upregulate the naturally occurring homing mechanisms of stem cells. If proven successful, this doped patch can be placed similarly to our cell encapsulated patch, but instead of therapy by an external cell population, we would be able to utilize the body’s own resident stem cells for protection and regeneration post-MI.
Fabrication of an RGD-alginate gel: Alginates are polysaccharides that are naturally found in algae, and are widely used to encapsulate a number of different cells types in the field of bioengineering (refer to Chapter 1 for more details)\textsuperscript{18,19}. Cells do not attach to alginate itself, thus, a bioactive peptide must be added to promote attachment of cells to the construct\textsuperscript{20,21}. For these purposes, we have added an RGD peptide to the alginate backbone to promote cell retention after mobilization to the patch.

Creation of horizontal microchanneled, cell capturing gels: Mimicking similar work done in our lab, horizontal microchannels were incorporated into a hydrogel patch to increase the surface area available for cytokine secretion and direction vascularization in a parallel fashion\textsuperscript{22}. Using a copper plate cooled by liquid nitrogen, the patches were placed on their edge, such that directional freezing was achieved within the patch (Figure 5.1). This directional freezing created ice channels of, on average, 100µm in diameter. The patches were then lyophilized, allowing for the ice columns to dissipate and leave behind horizontal microchannels of approximately the same diameter.

Incorporation of SDF-1 PLGA microparticles: Previous work in our lab has focused the incorporation of drug-releasing poly(lactic-co-glycolic acid) (PLGA) microspheres within cell-encapsulating gels\textsuperscript{23}. These microparticles are small enough to not interfere with the integrity of the gel, but allow the inclusion of desired materials via a permeable vehicle. Microparticles of 1-30µm in diameter containing SDF-1 were prepared using a double emulsion/evaporation process and incorporated into hydrogel patches. The goal was to utilize these microparticles as a module to systematically deliver SDF-1 to the surrounding tissue, creating a gradient that would encourage stem cells to home to the patch.
SDF-1 was encapsulated within the PLGA microparticles and secretion from the particles was tested and proven positive (data not shown). These particles were then added to a pre-polymer solution and incorporated into our hydrogel constructs.

As stated, the constructs were either directionally freeze dried to introduce horizontal microchannels, or simply placed in liquid nitrogen to produce a microporous structure. The patches were then dehydrated via lyophilization, and imaged under a scanning electron microscope (SEM) to determine the construct’s topology and composition. The introduction of horizontal microchannels can be appreciated as a smooth groove within the construct, while the PLGA microparticles can clearly be recognized as beaded structures throughout the construct (Figure 5.2 a,b). In contrast to the microchanneled topology, the microporous structures showed a randomized linking within and around the PLGA microparticles (Figure 5.2 c).

SDF-1 secretion from the constructs: While we were able to identify PLGA microsphere’s within the construct there was no trace of SDF-1 secretion upon re-hydration of the patch, despite the detection of SDF-1 secretion out of the non-encapsulated PLGA microparticles (Figure 5.3). In an attempt to address this issue, the lyophilized patches were re-hydrated with a 100µg/mL solution of SDF-1 for 10min, followed by emersion in media that was changed and collected at varying time points. With this method, SDF-1 secretion was observed and maintained for up to 7d, providing a possible avenue for SDF-1 secretion in future studies (Figure 5.3).
Homing of autologous stem cells to the construct: By providing a means of releasing SDF-1 from our constructs into the surrounding tissue, we aim to enhance the natural SDF-1 gradient that is responsible for stem cell homing to the site of injury, and naturally observed in the myocardium post-MI. To test the ability of our patches to attract stem cells, the constructs that either contained SDF-1/PLGA microspheres or were rehydrated with SDF-1 cytokine were incubated atop an MSC-containing collagen gel. After 10d of incubation, we excised the patch and imaged the cells with confocal microscopy. The patches that showed constitutive secretion of SDF-1 from the patch (those that were rehydrated with SDF-1) interestingly showed less migration to, and adhesion onto, our constructs than those that did not appear to secrete any detectible amount of cytokine into the surrounding medium (Figure 5.4). Although it requires further work to test the hypothesis, one possible explanation relates to the concentration gradient created by the constructs: If SDF-1 is released very actively into the surrounding environment, cells will not be stimulated to home to the material due to a lack of concentration gradient. In contrast, with localized secretion from the PLGA particles into just the construct of the environment, the cells can be signaled to home to within the construct. In other words, an increase in secretion of SDF-1 into the surrounding tissue obliterates the gradient that is needed for chemotaxis, whereas a localized release confined to the construct alone will allow for stem cell homing and mobilization to the construct itself.

With this preliminary data, we envision the incorporation of SDF-1 into a bioengineered construct to promote the enhancement of stem cell recruitment to the site of injury, embellishing the body’s natural mechanism of cardiac protection via stem cell mobilization.
5.2.3 Greater control through emerging micro-SLA technology

Overview: Work is actively being done to scale-down the resolution capabilities of the SLA to a nanometer scale. This capability will open the door for even greater control of the construct, including the ability to incorporate more accurate designs within the patch.

Creating vascular gradients: Greater control of the patch morphology and structure allows for a wide range of future possibilities. One vision would be the introduction of a gradient of nano- and micro-channels. As such, we can promote vascularization at the border zone first, with smaller and more densely populated nanochannels, and gradually increase the diameter and decrease the density of the channels, promoting the merging of smaller vessels into larger ones that can help sustain the myocardium. In such a way, we can address the gradient of necrosis that is observed in post-MI hearts.

Altering the 3D microenvironment: We can further use the micro-SLA to alter the environment within the hydrogel system, possibly by incorporating support cells, cytokine containing microparticles, or different scaffolding materials within the patch. As it stands, our constructs are made of a single material, PEGDMA, however, it has been documented that the 3D environment in which cells are grown alters their proliferation and secretion properties\(^{24,25}\). The micro-SLA platform will afford greater capability to alter and regulate the cell binding properties and characteristics that accompany different scaffold properties. Additionally, through the incorporation of support cells and/or cytokine containing microparticles, we can further control the secretion and growth properties of the encapsulated cells.
5.2.4 Less-invasive patch delivery via a self-unrolling patch

Overview: Work has emerged from our group demonstrating the ability to manipulate hydrogel gels to produce constructs that self-fold\(^2\). By pairing layers of poly(ethylene glycol) diacyrlates of different expansion ratios and elastic moduli, self-folding can be accomplished thanks to the different absorption properties of the two layers. Moreover, constructs were altered as such that one layer secreted encapsulated cytokines, while the other did not, resulting in directional delivery of incorporated cytokines.

Laparoscopic patch delivery with directed cytokine delivery: Utilizing the same bilayer approach, it is hypothesized that we can create a patch that unfurls upon contact with the heart surface. With this technology, a patch can be rolled into a thin cylinder, and placed within a needle or other delivery device to be delivered via conventional laparoscopic surgery. This approach would provide a non-invasive delivery method, addressing concerns of clinical translation of our hydrogel construct. In addition, the directed delivery that was detailed in the preliminary study could translate into a sustained release out of one surface of our patch, that which is closest to the heart. This would result in a sustained release towards the heart only, instead of our current construct, which doesn’t delineate the direction in which soluble factors diffuse out.

5.2.5 Large animal stem cell mobilization

Overview: The pig is an excellent pre-clinical model for a variety of procedures, diseases sates, and damage models (see Chapter 1). The biological similarities between pigs and humans are numerous and include similar organ to body weight ratio, heart physiology, and reproductive organs. Porcine models have been developed to test tissue engineering for skeletal defects, myocardial
infarction treatment, and skin defects. Additionally, with the sequencing of the entire porcine genome, we are able to integrate comparative genomics into clinical outcomes and physiological responses to a variety of different treatments in the pig. Overall, there is a great therapeutic potential in the use of the pig as a pre-clinical animal model. Despite this promise the identification and characterization of porcine stem cells in hampered by a lack of commercial reagents and antibodies.

Hematopoietic stem cells are chosen as a therapeutic cell type to treat a number of human diseases. They are capable of repopulating the blood and immune system, making them a likely candidate for cell transplantation, and they are known to produce beneficial growth factors and cytokines, a trait of critical importance for clinical therapeutics. Release of hematopoietic stem cells into the periphery, termed mobilization, is a naturally occurring phenomenon that can be reproduced clinically by a number of cytokines, chemokines, and chemotherapeutics (Table 5.1). While early bone marrow transplants required invasive procedures to collect the stem cell containing bone marrow of donors, new technologies often rely on this simple mobilization of stem cells from the bone marrow niches into the blood stream. Once present in the peripheral blood, simple collection and isolation techniques allow for relatively pain free isolation of the cells that can then be injected into the recipient patient. In the clinic, one of the most widely used mobilization techniques is the chemical induction by granulocyte colony stimulating factor (G-CSF). G-CSF act to cleave the stem cells from their niche within the bone marrow and migrate into the surrounding vasculature. While the use of G-CSF is the standard method of collection of human hematopoietic stem cells, a pig specific form of the growth factor has yet to be available commercially.
Mobilization and collection of pig HSCs: Our group has developed a pig specific G-CSF protein that, when injected into the bloodstream, shows mobilization of cells almost immediately, with sustained mobilization for up to 4d post-injection (Figure 5.5). Moreover, we have shown an increase in both white blood cell counts and CD34+ (hematopoietic stem/progenitor) cells in response to Plerixafor (AMD3100), the CXCR4 blocking drug, mobilization that has not previously been performed in pigs before (Figure 5.6). Equipped with ability to mobilize stem cells in the pig, we have the ability to collect and encapsulate autologous stem cells for a large animal model.

5.3 Cardiac Patch Outlook
Taken together, the suggestions above paint a rather exciting future for the cardiac hydrogel construct:

1) The microSLA technology will allow us to better control the designs being incorporated into the patch. With this, we can test better techniques to promote vascularization in a small animal model. Furthermore, by better understanding the mechanisms of stem cell protection, we can enhance the construct, either by the addition of supporting cells or by the encapsulation of specific cytokines, to upregulate the desired survival and angiogenic pathways that are already being tapped into.

2) The incorporation of SDF-1 into a patch construct will allow the natural recruitment of therapeutic stem cells from the body into the patch. With this, we envision a construct that contains both therapeutic stem cells as well as SDF-1 secreting microparticles. This would allow the encapsulated
cells to immediately perform their angiogenic and survival capabilities, while the addition of SDF-1 would enhance stem cell recruitment and homing to the site of injury, providing a second manner of therapeutics that relies on the body’s natural ability to fight the negative effects of hypoxic injury.

3) Large animal stem cell mobilization, paired with the SDF-1 doped patch, allows for a full model for autologous stem cell therapy without ever having to extract, isolate, expand, and re-introduce the cells into the body. The self-unrolling technology developed in our lab recently will further decrease the invasiveness of the procedure, allowing for laparoscopic delivery of our patch to the animal. If proven successful, such an approach would be the least-invasive and quickest model for stem cell driven cardiac treatment.

5.4 Materials and Methods

5.4.1 PLGA microparticles

SDF-1α encapsulating PLGA microparticles were prepared by a water-in-water double emulsification method. A 100µg/mL stock solution of SDF-1α (R&D systems) was further diluted in 50µL PBS to create a final concentration of 0.1µg/mL. This solution is referred to as the initial water phase (W1). Concurrently, 0.1g of PLGA (Sigma-Aldrich) was dissolved in 1mL of dichloromethane, a phase termed the oil phase (O). The primary emulsion was obtained by mixing W1 with O, followed by a 20s vortex. The resulting emulsion was poured into a 0.1 (w/v) aqueous poly(vinyl alcohol) (Sigma-Aldrich) solution (W2 phase) and stirred vigorously for 4h under a hood to allow the
dichloromethane to evaporate. The resulting solution should now consist of the SDF-1$\alpha$ containing microparticles in an aqueous solution. The solution is placed in a lyophilizer overnight to dry out the microparticles. For microparticle encapsulation into a hydrogel construct, the PLGA microparticles were dispersed in the pre-gel solution at a concentration of 1% (w/v) prior to the hydrogel-forming crosslinking reaction.

5.4.2 Microchanneled and microporous alginate hydrogels
Alginate gel manufacturing and microchannel/micorporous structure integration were performed following previously published protocols$^{22}$. In brief, Microparticle encapsulating RGD-Alginate gels were produced by mixing PLGA microparticles sulfonated N-hydroxysuccimide (Sulfo-NHS; Thermo Scientific), adipic acid dihydrazide (AAD; Sigma-Aldrich), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Thermo Scientific). The pre-gelled mixture was spread between two glass plates with a 1mm spacer in between. Once gelled, disks of 5mm in diameter were punched out and incubated in DI water at room temperature overnight. To create the horizontal microchannels, the constructs were precariously placed on their edge atop a copper plate that whose temperature was decreased to -196°C by liquid nitrogen to induce column growth throughout the patch. Constructs with micropores, as opposed to microchannels, were not directionally frozen, but rather exposed to liquid nitrogen by direct submerging. All frozen gels were then lyophilized overnight to evaporate any aqueous solution within the gel and create the final microporous or microchanneled structures. When needed, the dehydrated gels were re-hydrated by the addition of aqueous solutions.
5.4.3 SDF-1α secretion studies
Dehydrated gels were rehydrated with 10µl of either a 100µg/mL solution of SDF-1α (R&D systems), or sterile 1x PBS. Gels were left to rehydrate for 10m, after which time aqueous media was added to the constructs. Media was collected and changed at the 30m time point, as well as every consecutive day for a total of 7d. An SDF-1α ELISA (R&D Systems) was then run on the samples following the manufacturer’s protocol.

5.4.4 Cell migration studies
Constructs were embedded into a cell containing collagenous gel to test the ability of SDF-1 doped constructs to promote cell migration into the patch. D1 mouse MSC’s were re-suspended in culture media at a concentration of 5x10⁴ cells/mL. The cell containing media was then added to the collagen (Pure Col; Advanced BioMatrix) in a 1:1 ratio. Patches were immediately placed on the solution following mixing and incubated for 2h at 37°C 5% O₂. Following the 2h incubation, warm media was added to the now-gelled cell containing collagen solution and incubated. Cells adhered to the construct were stained by fluorescent phallodiin and DAPI to image their actin filaments and nuclei, respectively. Constructs were then imaged with a laser scanning confocal microscope (Leica, LSM700).

5.5 References
2 Otto Beitnes, J. et al. Intramyocardial injections of human mesenchymal stem cells following acute myocardial infarction modulate scar formation


Anderson, T. S., Berit L.; Formo, Kjetil; Alsberg, Eben; Christensen, Bjorn E. *Carbohydrate Chemistry: Chemical and Biological Approaches*. (Royal Society of Chemistry, 2011).


5.6 Figures and Tables

Figure 5.1: Creation of micro-grooves within hydrogels. Horizontal microchannels can be incorporated within hydrogel constructs by directing the freezing of ice crystals. By laying the constructs on their edge atop a copper plate cooled with liquid nitrogen, the crystals begin to form at the contact point and freeze upwards. Here you can see the frontline of the directed freezing.
Figure 5.2: Incorporation of PLGA microparticles. Cytokine containing microparticles are encapsulated within the hydrogel structure in much the same way cells were encapsulated in earlier experiments (Chapter 4). SEM images of a micorchanneled gel with no microparticles (a) show distinct grooves without any interfering structures, in contrast to the a microchanneled gel with PLGA microparticles incorporated within it (b). A PLGA encapsulating gel that lacks the distinct horizontal microchannels is porous in nature, with indiscriminate crosslinking throughout (c).
Figure 5.3: SDF-1 secretion from re-hydrated and PLGA containing constructs with and without horizontal microchannels. The secretion of SDF-1 from constructs with either bulk SDF-1 (SDF) or SDF-1 contained in PLGA microparticles (PLGA) were tested for their secretion profiles. Rehydration with bulk SDF-1 showed a constitutive secretion into the surrounding media, in stark contrast to the constructs encompassing SDF-1 containing PLGA microparticles, which showed no detectible secretion into the surrounding media. Further delineation was made between microchannel incorporation (MC) or microporous structures (MP), showing no difference in secretion between the two construct conditions.
Figure 5.4: SDF-1 upregulates stem cell mobilization *in vitro*. Microporous and microchanneled constructs with either bulk SDF-1 or SDF-1 containing microparticles were placed on an MSC-seeded collagen gel to test their ability to mobilize stem cells to within the patch. Confocal images taken 10d after implantation show directed mobilization in all microchanneled constructs, with the greatest concentration of cells seen in constructs containing the SDF/PLGA microparticles (a). Quantitative analysis further verifies that the presence of SDF1/PLGA microparticles increases cell mobilization into the constructs (b).
<table>
<thead>
<tr>
<th>Designation</th>
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<td>Cytokines</td>
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<tr>
<td>Chemokines</td>
<td>SDF-1, IL-8, MIP-1α, GROb</td>
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<tr>
<td>Chemotherapeutics</td>
<td>Cyclophosphamide, Paclitaxel, Plerixafor</td>
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Table 5.1: Molecules attributed to stem cell mobilization
Figure 5.5: G-CSF mediated cell mobilization in a porcine model: Porcine specific G-CSF was administered as a bolus injection to assess the functionality of the species-specific cytokine. Two separate concentrations were administered, alongside a negative control of 1xPBS injection. Subsequent leukopheresis was performed over a 4d timecourse and a white blood cell count was obtained. Results show a clear increase in mobilization of cells from the bone marrow into the surrounding circulation with each dose administered, with the greatest mobilization seen at 10 µg/kg concentration 24h after initial injection.
Figure 5.6: Dose response of AMD3100 in cell mobilization. AMD3100 was administered in three different doses to three different pigs. A leukapheresis machine (COBE Spectra) was used to collect large numbers of white blood cells (WBCs) after mobilization. A marked increase in WBCs was seen in a dose dependent manner (a). The serum was then tested for the presence of the stem cell marker CD34, the same dose dependent response was seen (b), suggesting that along with the overall WBC response, mobilization with AMD3100 triggered a subpopulation of stem cells into the peripheral blood.