INVESTIGATING SCAFFOLD DESIGNS FOR PROGENITOR CELLS-BASED CELL THERAPY FOR CARDIAC REPAIR

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INVESTIGATING SCAFFOLD DESIGNS FOR PROGENITOR CELLS-BASED CELL THERAPY FOR CARDIAC REPAIR

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To my family who have
given me love and support beyond measure and
are the source of my strength and inspiration

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LIST OF SYMBOLS AND ABBREVIATIONS

ADSC	Adipose-derived stem cells
ANOVA	Analysis of variance
BM-MNC	Bone marrow derived mononuclear cells
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CDC	Cardiosphere derived cell
CPC	Cardiac progenitor cell
CSC	Cardiac stem cell
CVD	Cardiovascular disease
EB	Embryoid body
ECM	Extracellular matrix
EF	Ejection fraction
EGFP	Enhanced green fluorescent protein
ESC	Embryonic stem cell
EthD	Ethidium homodimer
FGF	Fibroblast growth factor
FS	Fractional shortening
GFP	Green fluorescent protein
HA	Hyaluronic acid
hCPC	Human cardiac progenitor cells
HGF	Hepatocyte growth factor
HNA	Human nuclear antigen
iPSC	Induced pluripotent stem cell
LV	Left ventricle/left ventricular
LVEF	Left ventricular ejection fraction
MI	Myocardial infarction
MSC	Mesenchymal stem cell
PCI	Percutaneous coronary intervention
PCL	Polycaprolactone
PDGF	Platelet-derived growth factor
PEG-MAL	Maleimide-crosslinked poly(ethylene glycol)
PEG-4MAL	4-arm PEG-MAL
PLGA	Poly(lactic-co-glycolic acid)
rCPC	Rat cardiac progenitor cells
SA	Sarcomeric actin
SCF	Stem cell factor
SEM	Standard error of mean
TCPS	Tissue culture grade polystyrene
VEGF	Vascular endothelial growth factor

SUMMARY

Cell therapy for congestive heart failure has shown promising results in preclinical studies, but results of clinical trials suggest the need for this modality to be optimized. C-kit+ cardiac progenitor cells (CPCs) are adult stem cells that have been shown to differentiate toward lineages of the heart and exert beneficial paracrine effects. Their injection in humans resulted in moderate but insufficient improvements in cardiac function after myocardial infarction. In this project, we studied two strategies for enhancing CPC based-cell therapy using tunable maleimide-crosslinked poly(ethylene glycol) (PEG-MAL) hydrogels.

In the first strategy, CPCs were encapsulated in PEG-MAL hydrogels presenting vascular endothelial growth factor (VEGF). Although activation of ERK signaling was observed in CPCs encapsulated in VEGF presenting PEG gels, this strategy failed to induce endothelial differentiation or modulate paracrine effects of CPCs *in vitro*. Different growth factor doses, cell populations and biomaterial density and degradation rates were tested. In the second strategy, CPCs were encapsulated in integrin-specific hydrogels. α2β1-specific hydrogels induced cardiomyocyte differentiation of CPCs accompanied by a reduction in expression of secreted factors *in vitro*. Interestingly, following injection in rats undergoing ischemia-reperfusion, treatment with CPCs encapsulated in non-adhesive hydrogels resulted in the greatest preservation of cardiac contractility and attenuation of post-infarct remodeling.

Overall, this work adds to our knowledge of CPC behavior in presence of stimuli relevant to pragmatic design of regenerative therapies, as well as broadens our understanding of design principles that may be used to augment effects of cell therapy for myocardial repair.

CHAPTER 1. LITERATURE REVIEW

1.1 Myocardial infarction

Myocardial infarction (MI) is defined as myocardial cell death due to prolonged ischemia according to the Third Global MI Task Force. It also involves elevation of blood levels of biomarkers including cardiac troponin [1].

1.1.1 Prevalence and impact

Cardiovascular disease is the leading cause of death globally. Cardiovascular diseases are responsible for over 30 percent of all global deaths, with 80 percent of those deaths taking place in low- and middle-income countries [2]. About 610,000 Americans die from heart disease each year accounting for 1 in every 4 deaths. 35% of deaths because of cardiovascular diseases occur before the age of 75 years, which is younger than the current average life expectancy of 78.8 years. The economic impact of heart disease in the US is about $207 billion every year in cost of health care services, treatments, and lost productivity. Every year, an estimated approximately 660,000 Americans have a new coronary attack, approximately 305, 000 have a recurrent attack and an additional 160,000 silent myocardial infarctions are estimated to take place. Someone has a heart attack every 42 seconds in the US. Coronary heart disease is the most common type of heart disease and was responsible for killing about 365,000 people in 2014. While the rates of death attributable to cardiovascular diseases have decreased in the United States in the past decade, the burden remains significant [3]. This warrants the need for better prevention, early detection, and management and treatment strategies.

1.1.2 Pathophysiology

Etiology: More than 75% of acute myocardial infarcts are the result of thrombotic occlusion of a coronary vessel caused by rupture of a vulnerable plaque [4]. MI is the first diagnostic presentation of coronary artery disease (CAD) in about 50% patients suffering from CAD [5]. Coronary artery disease involves the gradual development of an

atherosclerotic plaque in the coronary artery and depends on various risk factors including genetics, cigarette smoking, hypertension, obesity etc.

Aftermath: Myocardial ischemia results in large scale cardiomyocyte death by necrosis and apoptosis, which commences a cascade of compensatory remodeling mechanisms. The pathological remodeling following MI is a common cause for heart failure. After acute MI, severe loss of functioning myocytes occurs. The death of myocytes takes place through necrosis, apoptosis and autophagy mechanisms in the hypoxic environment. The loss of myocytes starts within minutes of the occlusion at the subendocardium and extends through the ventricular wall under an hour in small animals like rats and within a few hours in larger animals like humans. Gross morphological changes do not become apparent until a few hours but cellular biochemical and structural changes are noted in less than an hour. Signals released by dying myocytes, danger-associated molecular patterns (DAMPs), and their intracellular contents as well as signals from the damaging extracellular matrix incite an immunological response. Ischemia-mediated generation of reactive oxygen species (ROS) is also an important contributor to the activation of inflammatory signals at the infarct. Leukocytes (neutrophils and mononuclear cells) infiltrate the infarct site within few hours of the infarction. Pro-inflammatory subpopulations of macrophages are also present during the inflammatory phase. The acute inflammatory phase involves clearing up of the wound and lasts for hours to few days. Matrix metalloproteases (MMPs) released by the neutrophils and other proteases degrade intermyocyte fibrillar collagens resulting in wall thinning and loss of myocyte alignment and slippage, contributing to impaired heart function. Infarct expansion takes place within hours of myocyte injury. Within a certain window of time of about 12 hours, damage to myocytes at the infarct border can be reversed by reperfusion; reperfusion limits infarct expansion initiated by the MI but causes additional injury, known as ischemia-reperfusion injury. Simultaneously with myocyte death and infarct expansion, the left ventricular pump function deteriorates and there is reduced stroke volume and increased end diastolic pressure. Adaptive mechanisms are activated to preserve the stroke volume. Contractility in the remote non

infarcted myocardium increases triggered by neurohormonal signaling in response to altered Frank/Starling relations. Wall thinning increases stress which activates mechanoreceptors that together with the neurohormonal signaling and Renin-Angiotensin system activation set off a signaling cascade leading to hypertrophy of surviving cardiomyocytes, showing an increase in cross-sectional area by up to 70%. However, unlike exercise or pregnancy induced physiological hypertrophy, pathological hypertrophy is associated with depressed cardiac function over time, termed as hypertrophy decompensation. Macrophage subpopulations secrete factors that recruit myofibroblasts and vascular cells to the infarct. Fibroblasts differentiate into myofibroblasts, migrate to the infarct, proliferate and secrete a matrix prominently composed on collagen. This fibrotic tissue is soft, vascularized and prone to rupture in the first week after MI. Over the next few weeks, the microvasculature recedes, majority of myofibroblasts undergo apoptosis and the scar crosslinks and matures. The collagen scar increases the tensile strength of the myocardium and diminishes wall stress along with hypertrophied myocytes initially. However, these compensatory mechanisms are maladaptive and are associated with heart failure. The scar is noncontractile and contributes to ventricular dysfunction and arrhythmia. The extent of fibrosis is a strong prognosis marker and has been found to correlate with morbidity and mortality due to arrhythmias and sudden cardiac death [6–8].

1.1.3 Current treatments

With existing treatments, patients are treated with the goal of preventing progression of CAD, left ventricular (LV) remodeling, sudden death, and reoccurrence of infarction. The management strategies include lifestyle management, pharmacological treatment (ACE inhibitors, Angiotensin receptor blockers, β-adrenergic blockers, calcium channel blockers etc.), electrophysiological devices (implantable cardioverter-defibrillators, cardiac resynchronization therapy), and revascularization strategies (coronary artery bypass graft, percutaneous coronary intervention) [9]. These treatment strategies aim at reducing preload, afterload, neurohumoral activation, and mineralocorticoid

dysregulation. They slow the progression of the syndrome but the underlying loss of cardiomyocytes and the fibrosis that follows MI are not reversed [10]. Endogenous regeneration of cardiomyocytes takes place at the rate of <1% annually in adults according to several studies although higher estimates have been reported as well [11]. These endogenous mechanisms are not sufficient to regenerate the injured heart and CAD progresses to heart failure. The only cure for heart failure is heart transplantation which is an unrealistic solution given the large discrepancy between the need and availability of organ donors.

1.1.4 Preclinical models

Animal models serve as important tools to study the disease etiology, test diagnostic and treatment strategies and understand their mechanisms of action. Small and large animals in which heart damage is induced by different modes such as chemical (isoproterenol administration), physical (overlapping burns, cryo-injuries), surgical (transient or permanent coronary artery ligation, aortic banding, balloon occlusion) are frequently used prior to advancing to clinical trials [12,13].

1.2 Cardiac cell therapy

Motivation: Cells as therapeutic agents are complex and are being studied for their potential to change the paradigm from chronic heart failure management to healing by regeneration. Stem cells can secrete a multitude of factors including cytokines, exosomes etc. and target different cells and receptors simultaneously; can transform themselves and dynamically react to their environment. Therefore, cells are considered to be suitable for developing a complex regenerative therapy approach. Supplying cells exogenously as building blocks for reconstruction of the damaged heart is a motivation as the intrinsic regeneration capabilities of the heart by cardiomyocyte proliferation and differentiation of endogenous stem cells are insufficient [14].

1.2.1 Mechanism of action

Differentiated cardiomyocytes as well as stem and progenitor cells of various types have been used in preclinical and clinical studies. Cells are believed to elicit beneficial effects by (1) secreting paracrine factors and (2) integrating with the host tissue as functional cardiomyocytes.

Paracrine effects: Cells secrete paracrine factors of various forms including chemokines, cytokines and microRNAs, which drive many processes affecting remodeling such as activation of host progenitor cells (e.g. HGF, IGF-1), cardiac differentiation (e.g. FGFb, VEGF, IGF1, HGF), angiogenesis/arteriogenesis (e.g. PlGF, VEGF, bFGF, PDGF, Ang-1, Ang-2, HGF, IGF-1, IL-1β), cardioprotection by attenuation of apoptosis and necrosis (e.g. SFRP2, IGF, HGF, VEGF, TNFα), proliferation (VEGF, bFGF) stem cell homing (VEGF, SDF1α, HGF, G-CSF), immunomodulation (IL4, IL6, IL8, CXCL6, MCP1, TNFα) and further promotion of growth factor secretion by host and transplanted cells [15,16]. For example, cardiac resident stem cells and early committed cells expressing c-kit, Sca-1 and MDR1 secrete HGF and IGF-1 that promote stem cell migration, survival, cardiomyocyte differentiation and further stimulate growth factor expression[17,18]. Bone marrow derived mononuclear cells (BM-MNCs) express bFGF, VEGF, Ang-1, Ang-2, PDGF, IL-1β and TNF-α that are implicated in inducing angiogenesis and cytoprotection of various cell types. Injection of BM-MNCs also further stimulates production of bFGF and Angiogenin by host cardiac cells, and leads to an increase in physiological blood flow, infarct size reduction and cardiac function improvement [19,20]. Endothelial progenitor cells secrete VEGF, FGF-2, Ang-1, Ang-2, PlGF, HGF, IGF-1, PDGF, SDF-1 and their implantation in ischemic myocardium leads to higher capillary density, higher proliferation of myocardial cells, reduction in cardiomyocyte apoptosis, reduced infarct size and improved function [21,22]. Interestingly, following injection of EPCs in animal hearts, the paracrine factor expression by transplanted cells peaked at d1 after which it faded. However, a prolonged increase in expression of the paracrine factors sustained for up to 2 weeks was seen in the host cells [22]. These observations demonstrate that injected cells act

directly through their secreted paracrine factors, and also indirectly by stimulating sustained generation of paracrine factors by the host cells. The effectiveness of cell therapy in spite of very low retention in the heart is believed to be through their powerful paracrine effects. MSCs injected in hearts after MI do not differentiate into contracting cardiomyocytes but reduce the stiffness of the subsequent scar: $E_{infarcted\ tissue}$= 55 ± 15 kPa, $E_{MSC\text{-}hearts}$= 40 ± 10 kPa, E_{Sham}= 18 ± 2 kPa. Attenuation of post-MI cardiac remodeling and improvement in function without direct differentiation of the injected cells could be an outcome of the paracrine factors secreted by the cell as well [23].

Direct integration/differentiation: Although seen infrequently, transplanted cells also integrate in the host myocardium. Injected fetal cardiomyocytes juxtapose with host cardiomyocytes and connect with existing myocardium through Connexin43 without showing any arrhythmias [24,25]. However, the supply of a patient's cardiomyocytes is low since they have very limited proliferation potential. Skeletal muscle satellite cells can be expanded *in vitro* to form skeletal myoblasts that possess force generating capabilities. However, skeletal myoblasts unfortunately had rhythm disparity with host myocardium and failed to improve echocardiographic function in MAGIC phase I clinical trials possibly due to lack of formation of gap junctions [26]. Approaches involving stem/progenitor cells that differentiate *in situ* after transplantation because of environmental cues as well as those that are differentiated prior to transplantation using various regimens are ongoing in preclinical research and clinical trials. Exogenously delivered cells do not differentiate into fully mature cardiomyocytes even if they engraft. For example, an average of 14% of bone marrow derived mesenchymal stem cells that engrafted in the heart exhibited evidence of cardiomyocyte commitment by expression of early or late stage differentiation markers. Interestingly, they also showed coupling to host myocardium via connexin43 and were of similar size as other myocytes [27] In another study, GFP+ CPCs were injected in infarcted hearts and later found to express cardiomyocyte marker α-sarcomeric actin (SA). However, these cells appeared immature and were smaller than other myocytes [28]. In a study comparing the engraftment and myocyte differentiation of different human cell types injected

intramyocardially in mice hearts, about 0.5-3% of HNA+ cells expressed α-SA, with the highest engraftment and differentiation seen in the cardiosphere derived cells group [29]. Implanting predifferentiated cardiomyocyte-like cells presents challenges of arrhythmia and questionable contribution by their force generation due to lack of maturity. A cardiac patch was made with iPSC derived cardiomyocytes and pericytes, and transplanted in rats after MI. Most transplanted and engrafted cardiomyocytes showed elongation and circumferential alignment but were very small in size, indicating their immaturity. They formed gap junctions among themselves but not much with the host cardiomyocytes [30]. Cell fusion of delivered stem cells with host cardiomyocytes has also been proposed as a potential mechanism by which transplanted cells integrate with the host. However, this theory is controversial as in Cre-recombinase based models, progenitor cells can cross the membrane of the recipient cell, mimicking cell fusion [31]. Along with cardiomyocyte differentiation, transplanted cells have also been reported to differentiate into vascular cells. Direct differentiation into endothelial or/and vascular smooth muscle cells in the in vivo environment has been reported following injection of MSCs [32], ADSCs [33], CD34+ cells [34] and cardiac stem cells (CSCs) [35,36].

Paracrine effects vs. direct integration: Increasing evidence is suggesting the paracrine mechanism to be the primary driver of effects of stem cell therapy and differentiation of the transplanted cells a minor contributor. The retention of injected stem cells is very low, yet they are able to induce improvement in host hearts. For example, only 12.7% of the injected CPCs were still present in the heart at 24 h, and only about 1000 cells remained after 35 days. Significant numbers of injected CPCs were found in the lungs and kidneys, but only in the first 24 h, indicating recirculation of CSCs initially retained in other organs. However, in spite of the low retention and rapid disappearance of CPCs, LV function was significantly improved at 35 days [37]. In a study measuring the outcome of CPC injection after 1 year, a significant improvement in function was seen but only some of the transplanted cells or their progeny persisted (4-8% of all nuclei); most were still proliferative and few appeared to show a mature cardiomyocyte phenotype. CPC transplantation, however, triggered endogenous cells perhaps by

paracrine factors resulting in increased formation of endothelial cells, proliferation of endogenous CPCs and small cells that expressed cardiomyocyte proteins (α-sarcomeric actin) but not a mature cardiomyocyte phenotype [38]. Multipotent cells have been shown to integrate with the host vasculature as well. In studies comparing the effect of direct differentiation versus paracrine effects on injection of cells based on contribution of implanted cells in new vasculature or cardiomyocytes, 20-25% of observed effects were accounted for by direct differentiation in case of human CDC transplantation [39], 9% following ADSC injection [40] and about 30% following CDC transplantation with a platelet gel biomaterial [41]. While it is largely believed that paracrine factors are the primary mechanism by which exogenous stem/progenitor cells act, there is no consensus on whether the conditioned media of cells would suffice as effective treatment. For example, some studies showed that MSC conditioned media alone is not sufficient to reduce infarct size and improve cardiac function comparable to stem cell treatments [40,42–44]. However, another study reported that a single injection of neonatal CPC-derived total conditioned medium is more effective than transplanted neonatal CPCs or their exosomes [45].

1.2.2 Enhancing cell therapy effects

Motivation: Clinical trials to test cell therapy for cardiac repair have shown modest benefits at best and the results have been mixed. The ischemic heart after IR is not a suitable environment for cell survival because of high inflammation, oxidative stress and damaged ECM. The barriers to successful clinical translation of cell therapy include (1) limited knowledge of mechanism of action and therefore decisions of optimal dose, cell fate and pharmacokinetics; (2) poor engraftment and survival of cells in the ischemic heart; and (3) risk of tumorigenicity, immunogenicity and arrythmogenicity [46]. Several preclinical and clinical studies are underway to refine the method in attempts to achieve better functional outcomes. These involve selection of the optimal cell type(s); dosage; timing, frequency and mode of administration; as well as various pretreatment and co-treatment strategies with exosomes, growth factors, biomaterials etc. to enhance the

therapeutic effects of delivered cells. The Cardiovascular Cell Therapy Research Network (CCTRN) and other groups also provide recommendations on designing and conducting clinical trials in this burgeoning field to aid clinical translation of stem cell therapies [47,48].

1.2.2.1 Cell type

Different types of stem cells including totipotent cells, ESCs and iPSCs, which can differentiate into cells of all three- ectoderm, endoderm and mesoderm cell lineages, and multipoint/unipotent cells, such as CSC/CPCs, BM-MNCs, MSCs etc., which differentiate only into a few closely related cell types, are being studied.

c-kit+ cardiac progenitor cells: Discovery of stem cells in the heart over a decade ago was very exciting as it refuted the long held notion that the heart is a terminally differentiated organ without self-renewal potential [35]. Cardiac ckit(+) cells represent the most primitive population in the rodent heart [49]. These cells, expressing c-kit and lacking hematopoetic markers, are self-renewing, clonogenic and multipotent with the ability to differentiate into cardiomyocyte, endothelial and smooth muscle lineages *in vitro* and *in vivo* [35,50]. For these reasons, these cells were chosen as candidates for cardiac cell therapy. Preclinical studies showed improvement in cardiac function following delivery of these cells [51,52], even in hearts with mature scars treated 30-days after MI [51]. Like other stem cells, the benefits of CPC therapy appear to be driven by paracrine effects primarily. Following EGFP+ CPC injections in rat hearts, only an average of 2.6% EGFP+ cells were found engrafted in the host myocardium after 5 weeks, which is not sufficient to explain the benefits in cardiac function improvement. While the presence of injected cells was low, implantation of CPCs led to activation of endogenous CPCs, perhaps via paracrine factors [51].

Following the encouraging results in preclinical studies, these cells were tested in phase 1 clinical trials. The phase 1 trial, SCIPIO, a randomized open label trial, involved isolation of c-kit$^+$ cells from the right atrial appendage[53] of patients undergoing open heart surgery for coronary artery bypass grafting (CABG). The harvested CPCs were

expanded *in vitro* and then infused back into the patient heart via the coronary arterial circulation 3-5 months after CABG. The autologous treatment was found to be safe and feasible. While efficacy needs to be investigated by later clinical trial phases, preliminary results from the SCIPIO trial showed improvement in left ventricular function, reduction in infarct size and increase in viable tissue, consistent with preclinical studies [54]. While these cells were originally proposed to be used autologously, they are currently under investigation for potential use as allogeneic therapy. Delivery of allogeneic cells has been found to be safe in porcine models [55] and is being investigated in phase 1 clinical trials, CAREMI. Tolerogenic immune behavior is regulated by PD-L1-dependent allogeneic-driven immunomodulation and it may be possible to use PD-L1 expression as a marker to identify and select low-risk high-benefit allogeneic cardiac repair cells [56]. Allogeneic cell therapy would be more cost-effective, provide faster access and better efficacy if a patient's own cells have limited regenerative potential. These cells are also being studied as part of a stem cell combination therapy. The CONCERT-HF trial is an ongoing phase 2 trial to test the effects of CSCs, MSCs both alone and in combination delivered via transendocardial injection in patients with ischemic cardiomyopathy. This is based on promising results from preclinical studies in which it was found that MI size reduction was 2-fold greater in combination versus either cell therapy alone, along with significantly higher improvement in left ventricular ejection fraction (LVEF) and 7-fold greater engraftment of stem cells. MSC-enhanced proliferation and differentiation of CSCs could explain the synergistic effects of the MSC-CSC combination [57,58].

Human CPCs are mostly isolated from right atrial appendage. The right atrial appendage is a rich source of these cells [53,59] and making small approved incisions is sufficient to isolate and expand cells to get enough yield for injection. Note that rat CPCs used in literature have been isolated from different parts of the heart or whole hearts. The number and functionality of these cells depends on donor characteristics. Higher numbers have been found to be present in women, and neonates have the highest number of CPCs among children. However, age was found to not be an influencing factor on number of CPCs in adulthood [53,59]. About 5.2% of cells isolated from right

atrial appendage of neonatal donors [59] and about 3.6% from those of adult donors [60] have been reported to be c-kit+. Their number increases during certain conditions such as pregnancy, following exercise, ischemia reperfusion and pediatric end-stage heart failure [61–63].

Effectiveness of CPCs depends on donor age and health state. For example, CPCs obtained from neonatal donors less than a week old were found to induce greater function improvement than those from older child donors in an animal model of pediatric right ventricular failure [64]. Neonatal CPCs show stronger *in vitro* proliferative capacity and are more effective than adult CPCs at recovering cardiac function post-MI. Neonatal CPCs showed greater levels of several cytokines and growth factors, and Heat Shock Factor-1 was found to be an important regulator of differences in secretome [45]. CPCs from diabetic donors are lower in number and demonstrate inferior proliferative capacity [65]. However, it may be possible to ameliorate these issues. For example, it was found that genetic modification of CPCs to overexpress Pim-1 kinase mitigates senescent characteristics and enhances youthful properties like proliferation in CPCs [66]. While reduced numbers and pluripotency of CPCs is observed in rats with chronic heart failure; their differentiation potential could be augmented by inhibiting TGF-β signaling [67].

Cardiosphere derived cells: Cardiosphere derived cells are a natural mixture of stromal, mesenchymal, and progenitor cells and are obtained by culturing percutaneous endomyocardial biopsies, which yield spherical multicellular clusters called ‘cardiospheres’. They are multipotent, clonogenic and immunopriveleged making them attractive candidates for clinical translation. Rats and pigs treated with allogeneic CDCs after MI showed restoration of heart function, scar reduction and increase of viable tissue [68,69]. CDCs were established to be safe in CADUCEUS and ALLSTAR phase 1 clinical trials [70] and phase 2 ALLSTAR trial for testing allogeneic CDCs is underway [71]. In a direct comparison between CDCs and other stem cell types- BM-MSCs, BM-MNCs, ADSCs and CPCs, CDCs showed the greatest potential for myogenic and angiogenic differentiation, resistance to oxidative stress and relatively high production of various angiogenic and antiapoptotic-secreted factors. Injection of CDCs into the

infarcted mouse hearts resulted in the greatest improvement in cardiac function, the highest cell engraftment, apoptosis reduction, myogenic differentiation rates, and LV wall thickness after 3 weeks [29]. Note that CPCs were obtained by selecting c-kit+ cells from CDCs, which is different from how CPCs are obtained in our lab and other primary CPC literature cited wherein c-kit+ cells are selected from digested atrial tissue. In addition to CPCs and CDCs, other populations of cardiac stem cells characterized by Sca-1, Isl-1 expression, side population cells are also being investigated for cardiac cell therapy in preclinical studies [72].

Other cell types: Bone marrow mononuclear cells, a mixed population of single nucleus cells including monocytes, lymphocytes, hematopoetic stem cells, endothelial progenitor cells, mesenchymal stem cells, are the most studied cell source for cardiac cell therapy. Several preclinical and clinical studies are investigating the role of specific cell populations constituting BM-MNCs, such as CD34+ cells, endothelial progenitor cells, in order to determine the most efficacious treatment [31]. In spite of exciting results of preclinical and early clinical studies, BM-MNC administration failed to show significant improvement in cardiac function after MI irrespective of timing of administration (TIME, LateTIME and SWISS-AMI clinical trials. The results of BAMI phase 3 clinical trials to test the effect of intracoronary reinfusion of BM-MNC on all cause mortality in MI patients are awaited [73]. Mesenchymal stem cells from various tissue sources, including bone marrow and non-bone marrow tissues are being tested for cardiac cell therapy since they are multipotent, easy to obtain from patients and expand *in vitro* as well as immunopriveleged. After exciting results in animal models, the therapeutic effects of MSCs and ADSCs are now being tested in clinical trials (ATHENA). Skeletal myoblasts were thought to have the ability to act as surrogates for cardiomyocytes because of their force generating capabilities but they unfortunately caused arrhythmias and did not improve cardiac function in MAGIC phase I clinical trials. Embryonic stem cells and pluripotent stem cells have been used to obtain mature cardiomyocytes by differentiation and can also form blood vessels because of their multilineage potential. While injection of ESC derived cardiomyocytes improved cardiac function in non human primates,

arrhythmias were observed [74]. A single patient trial with ESC derived Isl-1+ SSEA+ cardiovascular progenitors, however, showed functional improvement and no arrhythmia complications in the 3 month follow-up [75]. The efficacy and safety of this treatment will need to be investigated in controlled randomized trials. Induced pluripotent stem cell-based treatments need to eliminate risk of arrhythmias and tumorigenicity before clinical translation [76] and attempts toward that are underway [77].

1.2.2.2 Dosage, administration route, frequency and timing

Dosage: It is not known whether a low or high cell dosage yields the greatest benefit as conflicting results have been observed in both preclinical and clinical studies [78]. In clinical trials administering CD34+ bone marrow cells, the high doses of ≥10 million cells showed the greatest benefit, and not the low dose of 5 million cells [79]. Whereas in another human trial with hMSCs, the lower dose of 20 million cells showed significantly greater cardiac function and infarct reduction than the 200 million cell group [80]. There is not a direct relation between cell dosage and clinical effect and therefore, the dosage needs to be optimized for the specific cell type and route of administration. In a dose-response study for CSCs delivered intracoronarily in an ischemia-reperfusion rat model, the lowest dose of 0.3 million cells had no effect, medium doses of 0.75 million, 1.5 million and 3 million improved LV function to a similar extent, and the high dose of 6 million cells was harmful as increased mortality was seen [81].

Route of delivery: Intracoronary and intramyocardial routes of cell delivery have been employed in cell therapy clinical trials. The difficulty of homing cells to the damaged heart when delivered through the intravenous route makes it unattractive in spite of the ease and low cost. Following systemic intravenous delivery of BM-MSCs to rats after MI, the majority of cells are trapped in the lungs [82]. Intracoronary delivery method has been widely used in clinical trials and has been established to be safe. In this approach, cells are injected into a coronary artery through a balloon catheter placed in the coronary artery [83]. So it can be coupled with percutaneous coronary intervention (PCI) in which a catheter is placed in the coronary vasculature, and can also be tailored

to target a specific coronary region. The injected cells are required to transmigrate through the capillaries to reach the myocardium in the presence of strong coronary flow. Intramyocardial delivery can be done via minimally invasive thoracoscopic procedure or catheter-based needle injections. This route is the most direct and reliable for directly delivering cells to the myocardium. However, it requires more expensive equipment and specialized expertise; cell spillage can take place at the injection site and the delivery to only targeted areas could limit global function improvement [14]. A study comparing distribution of radiolabeled MSCs following intramyocardial, intracoronary, and interstitial retrograde coronary venous delivery in an ischemic swine model found significantly greater retention in the myocardium following intramyocardial injection (11+/-3%) versus intracoronary delivery route (2.6+/-0.3%) [84]. However, in another study performing direct comparison of intramyocardial and intracoronary approaches for CSC delivery showed similar improvement in global and regional LV echocardiographic parameters after MI. The intracoronary delivery led to greater uniformity of cell distribution, myocyte regeneration, and amount of viable tissue in the risk region [28].

Frequency: Few studies have measured the effect of multiple cell injections in comparison to a single injection, which has been the regimen in most clinical trials. Repeated injections were found to exert greater benefits than a single injection in case of CSCs injected in rats [85], BM-MNCs in humans [86,87] and skeletal myoblasts in pigs [88]. Another study delivering bone marrow cells to mice hearts after MI noted that the timing of cell delivery is more important and that repeat injections did improve ejection fraction but not infarct size reduction [89].

Timing: The timing of cell delivery after MI has been found to be a critical determinant of treatment efficacy, which is not surprising knowing that a sequence of distinct events follow infarction. The intracoronary injection of CPCs in pigs alleviated myocardial dysfunction whether they were treated the same day of MI or 7 days later. However, greater reduction of LV remodeling was seen in the day 7 treatment group [55]. Bone marrow cell treatment at 3 days after MI showed greater LVEF improvement than day 7 or day 14 injections [89]. TIME and Late-TIME trials compared the effect of

cell delivery timing as 3-7 days versus 2-3 weeks after AMI. Neither trial found the cell therapy treatment to elicit any benefit unfortunately [90]. Ascertaining the optimal timing of delivery is an aim of various phase 3 clinical trials [83]. In a rat model study, MSCs were injected at 1h, 1 week or 2 weeks after MI. The greatest function improvement and engraftment were observed in the 1 week group. This suggests that injection too early may lead to poor cell engraftment due to strong inflammatory response, and cells injected too late may suffer from lack of homing signals in the injured myocardium.[91]

1.2.2.3 Treatments to enhance cell therapy

Approaches utilizing biological, pharmacological agents and biomaterials, and their combinations have been investigated to enhance the effects of cell therapy. The mechanisms by which they act include improving the survival, engraftment of cells; pharmacokinetics of cell delivery, modulating cell differentiation state and/or paracrine factors.

1.2.2.3.1 Biomaterials

Biomaterials can improve retention of cells, provide a protective environment to enhance cell survival, provide biophysical and biochemical cues that modulate the cell behavior (paracrine factors [92], differentiation) and modify wall stresses in an infarcted heart. Different types of biomaterials have been studied including natural derived and synthetic biomaterials and can be delivered as injectable materials or patches. Many variants of biomaterials in terms of size, mechanical properties, topography, porosity, degradation rate and chemistry have been investigated. Biochemical and biophysical properties of biomaterials greatly influence their mode of action.

1.2.2.3.1.1 Types of biomaterials

Examples of natural biomaterials include collagen, fibrin, Matrigel, hyaluronic acid, gelatin, tissue derived extracellular matrix etc. Natural-derived biomaterials have inherent bioactivity and integrin-binding proteins that promote cell attachment and signaling, can be degraded by endogenous proteins and release peptides or other

molecules. Their biochemical activity can be enhanced by coupling with additional signaling molecules [93]. Natural biomaterials generally are softer than native myocardium. Their mechanical properties can be adjusted within a range by certain methods such as increasing concentration, chemical crosslinking [94] and combining with a stiffer synthetic or natural material such as mixing softer collagen with stiffer gelatin [95]. While they are rich in biologic information, they have very limited tunability to achieve tight control of cellular behavior and tissue maturation. The very limited understanding at the molecular level of the *in vivo* response to materials incorporating native molecules is another limitation [96]. Synthetic biomaterial examples are poly(ethylene glycol), polylactide-caprolactone, peptide nanofiber scaffolds etc. Synthetic biomaterials can be modified to deliver bioactive agents via physical encapsulation or chemical immobilization. Synthetic biomaterials also provide greater possibilities of tuning mechanical and biochemical properties which in turn regulate cell morphology and behavior [97]. Along with acting as therapeutic agents and drug/cell delivery vehicles, synthetic material and their hybrids with natural molecules are important tools for understanding the mechanisms that affect cell and tissue behavior.

Biomaterials can be delivered to the heart via injection or as a patch. In the injectable approach, biomaterials are delivered in the liquid state as a direct injection in the ventricular wall (epicardial injection) or through a percutaneous catheter (transendocardial injection). Cell suspension can be mixed with the liquid form of the biomaterial before injection. The biomaterial immediately gels after transplantation to prevent cells from getting washed out. Design of such injectable biomaterials is often based on their sensitivity to gel at physiological conditions such as temperature and/or pH. Another injectable biomaterial strategy involves microspheres that can encapsulate cells or proteins. While the injectable biomaterials strategy circumvents the need for open-chest surgery and supports integration of the delivered materials with the myocardium, not all materials can be made injectable while maintaining their beneficial physical and biochemical properties necessary for repair and shear stress may be exerted on the cells. In the patch-based approach, a tissue-like structure is created *in*

vitro and then transplanted *in vivo*. The advantage of this approach is that the cells can be cultivated under precise culture conditions before in vivo delivery and then positioned precisely on the infarcted region. However, apart from the need for invasive surgical application, nutrient diffusion can limit the thickness of the constructs and the patch-based approach supports limited integration of the graft with the host myocardium [14].

PEG hydrogels: Poly(ethylene glycol) is a hydrophilic polymer synthesized by the ring-opening polymerization of ethylene oxide. It is biocompatible and several PEG based formulations have been approved by the FDA. The biocompatibility, high water content, solute permeability, tissue like elasticity of PEG hydrogels make them an attractive candidate for delivery of cells and biomolecules [98]. PEG hydrogel can be made via different crosslinking mechanisms including photopolymerization, click chemistry, enzymatic crosslinking and supramolecular assembly [99].

PEG-4MAL hydrogels are based on 4-arm PEG macromers utilizing maleimide crosslinking click chemistry via Michael-type addition reaction. They maintain high viability of encapsulated cells, possess the ability to immobilize macromolecules/ligands and can be functionalized with cell-adhesive sites such as RGD and GFOGER, and crosslinked with protease sensitive peptides [100]. Therefore, PEG-4MAL hydrogels also have the capability to present the stem cells with a niche-like environment in terms of mechanical and biochemical cues. Mechanical properties of these gels can be tuned within a range by adjusting the PEG macromer size and density. In addition, these hydrogels are injectable, and possess low thrombogenicity and immunogenicity [101]. Therefore, they are synthetic hydrogels that have the advantage of greater control and consistency and can be functionalized with natural materials inspired bioactive molecules.

1.2.2.3.1.2 Mechanism of action

Codelivery of cells with biomaterials has shown improvements in their effects. Biomaterials impart the improvement through one or more of the following mechanisms:

improvement in retention; survival and engraftment; differentiation; proliferation; modulation of paracrine factors; and mechanical effects.

Retention: The retention of cells following injection to the LV is very low. Less than 10% cells are retained in 24 hours irrespective of injection method and cell type and more than 90% of the retained cells die within a week. Even though there is accruing evidence supporting the paracrine mechanism of action of stem cells, a correlation has been seen between stem cell engraftment, even though low, and functional effects [102]. Improvement in retention and engraftment of exogenous cells has been seen when delivered with many biomaterials. In a study comparing retention of MSCs delivered with saline injection (current clinical standard), 2 injectable hydrogels (alginate, chitosan/β-glycerophosphate) and 2 epicardial patches (alginate, collagen), all four biomaterials retained 50-60% of cells after 24 hours compared to 10% for the saline control [103]. Cardiac stem cells encapsulated within low melt agarose supplemented with fibronectin and fibrinogen exhibited higher cell retention and engraftment in the infarcted myocardium as compared to cells only group (10 ± 1% vs. 4 ± 1% CSCs retained after 3 weeks, respectively; $p \leq 0.03$) and greater improvement in cardiac function as well [104]. Retention of transplanted stem cells is closely related to the remaining biomaterial and depends on degradation rate of degradable materials, which can be modulated by several methods such as chemical crosslinking [105]. Longer retention of exogenous cells may enable more sustained release of their paracrine factors as well.

Cell survival and engraftment: The post-infarct microenvironment is hostile and limits cell survival and engraftment. Presence of significant oxidative stress and damaged myocardium lead to anoikis and death of transplanted cells. Biomaterials can provide a custom microenvironment for transplanted cells. For example, a previous study used nanofiber scaffolds to create an injectable pro-survival microenvironment for transplanted endothelial cells or cardiomyocytes in an infarcted myocardium. The greatest differentiation of implanted MSCs into TnI+ cells was seen in the RGDSP + nanofiber scaffold. The improved survival and differentiation with the RGD stimulation

was attributed to promotion of cell adhesion and prevention of anoikis, and stimulation of integrins relevant to early cardiac development α5β1 and αVβ3 [106]. In another study, culturing CDCs on platelet gels derived from venous blood led to higher viability of cells *in vitro* (CDCs on platelet gel: 1.8% EthD+ dead cells, CDCs on TCPS: 5.2% EthD+ cells) and promoted recruitment of endogenous stem cells *in vivo*. Hearts treated with CDC-seeded platelet gel showed the greatest improvement in LVEF, reduction of adverse left ventricular remodeling and the highest number of new vessels and cardiomyocytes in the infarct region as compared to hearts transplanted with platelet gel only or vehicle controls [41]. Physical properties such as Matrix architecture, topography, shear stress and stretch [107] also influence cell morphology and infiltration. For example, by introducing porosity between PCL electrospun fibers, cell infiltration and proliferation were improved. This enabled successful infiltration without compromising the mechanical stability by using materials such as PLGA that undergo faster hydrolytic degradation [108]. Biomaterial characteristics affect not just individual cells but higher level constructs as well. For example, degradable scaffolds also allow vascular invasion and integration of the graft with the host [109].

Cell differentiation: Biomaterials can induce differentiation of delivered cells through biochemical or physical cues initiated-signaling. For example, MSCs encapsulated in hydrogels made of N-isopropylacrylamide, N-acryloxysuccinimide, acrylic acid and poly(trimethylene carbonate)-hydroxyethyl methacrylate with 6% type 1 collagen added to improve biocompatibility showed cardiomyocyte differentiation *in vitro*. Scaffolds with varying moduli (16, 45, 65 kPa) were prepared and differentiation extent was found to depend on mechanical properties. MSCs in the hydrogel with the 65 kPa modulus had the highest cardiomyocyte differentiation efficiency (76% after 14 days) and they even expressed Connexin43 [110]. Myoblasts fuse into myotubes independently of substrate stiffness but further maturation involving myosin/actin striations emerge later only on gels with stiffness typical of normal muscle (passive Young's modulus, $E \sim 12$ kPa). [111]. Biomaterial degradation also influences cell differentiation via affecting cell spreading and traction. hMSCs encapsulated within HA

hydrogels that permit cell-mediated degradation exhibited high degrees of cell spreading and high tractions, and favored osteogenesis. Adipogenesis was favored in MSCs cultured in HA matrices of equivalent mechanics but restrictive of cell mediated degradation and therefore limiting cell spreading and traction. Interestingly, switching the permissive hydrogel to a restrictive state through delayed secondary crosslinking led to reduction of further hydrogel degradation, suppressed traction, and caused a switch from osteogenesis to adipogenesis. Pharmacological modulation of tension induced signaling had the same effect on cell fate determination [112]. Numerous studies have found that several biochemical and biophysical stimuli act synergistically to drive maturation of stem cells. Using a PEG-based hydrogel allowing control of biochemical and biophysical cues presented to encapsulated pluripotent P19 EC cells, the authors found that initial cardiac muscle commitment was enhanced by the matrix elasticity, but integrin stimulation and degradability was necessary along with matrix elasticity for further maturation. Soft matrices (E~322 Pa) mimicking the elasticity of embryonic cardiac tissue increased the fraction of cells expressing the early cardiac transcription factor Nkx2.5 compared to embryoid bodies (EB) in suspension. In contrast, stiffer matrices (E~4036 Pa) decreased the number of Nkx2.5-positive cells significantly. Further indicators of cardiac maturation were achieved by stimulation of integrins relevant in early cardiac development (α5β1, αvβ3) using RGDSP ligand in combination with the MMP-sensitivity of the soft matrix compared to EB in suspension. Stiffer matrices promoted skeletal muscle differentiation of the EBs [113].

Paracrine effects modulation: Biomaterials can also modulate the paracrine effects of cells through biochemical or physical cues they provide to cells. For example, in comparison with ADSCs cultured in monolayers, cells cultivated in alginate show higher mRNA expression of HGF and bFGF mRNA expression, which could enhance local angiogenesis and cell survival. Conditioned media from ADSC-alginate system also inhibit lymphocyte proliferation more than monolayer ADSCs [114]. In another study, conditioned media from MSCs adherent to polyacrylamide hydrogel functionalized with fibronectin, collagen I, or laminin was applied to 3D matrigel cultures containing human

microvascular endothelial cells. It was found that the extent of tubulogenesis, which depended on paracrine factors, was a function of the material composition and stiffness and gene expression of several cytokines depended on material stiffness. Through an experiment wherein the cell spreading was controlled by the area of fibronectin matrix layer underneath the cells, the authors found the secretion of cytokines to be a function of cell spreading on stiff substrates but not on soft substrates [115].

Mechanical effects: Biomaterials can provide a mechanical environment that supports maturation and functioning of the delivered cells in a beneficial manner and also provide mechanical support to the weakened LV wall post-MI.

Following myocardial infarction, beating cardiomyocytes are replaced by a fibrotic scar which is much stiffer (E~35-70 kPa vs E~10 kPa in healthy tissue) [23]. Cellular differentiation is dependent on the microenvironment mechanical properties [97]. In response to the post-infarct mechanical cues from the scar, MSCs transplanted in the post infarction environment start differentiating as demonstrated by troponin T expression but stop short of full differentiation into beating cardiomyocytes [23] and even run the risk of calcification [116]. Matrices that mimic the elasticity of the developing myocardial microenvironment are optimal for stem cell differentiation and maturation [111,117]. Chicken embryonic pre-cardiac cells were cultured on hydrogels stiffening over time to mimic developmental changes in the ECM. The dynamic collagen-coated HA hydrogels showed a 3-fold increase in mature cardiac specific markers and formed up to 60% more maturing muscle fibers than they do when grown on compliant but static polyacrylamide hydrogels over 2 weeks [118].

After myocardial infarction, the border zone expands chronically, causing ventricular dilatation. In an ovine model, following an MI, the LV wall decreased by 11% and endocardial curvature decreased by 55%. By Laplace's law, wall stress is inversely proportional to the product *K.h* (curvature.radius) so the geometric changes cause increased dynamic wall stress, which likely contributes to border zone expansion and remodeling [119]. It is believed that biomaterials also provide mechanical support and increase wall thickness, thereby reducing the wall stresses. Simulations support this

theory [120]. In a previous study, injectable HA hydrogels formulations possessing similar degradation and tissue distribution upon injection but differential moduli (G'=~8 versus ~43 kPa) were injected into the infarcted heart of an ovine model. The modulus of the tissue/hydrogel composite was greater than explanted cardiac tissue alone for the MeHA High group but not for the MeHA Low group. Treatment with both hydrogels significantly increased the wall thickness compared with the control infarct. However, only the higher-modulus (MeHA High) treatment group had statistically smaller systolic and diastolic end volumes, and infarct area compared with the control infarct group. The high modulus group also tended to show the greatest function preservation [121]. This suggests the role of mechanical properties of biomaterial in mitigating LV remodeling. In another study, by measuring the effects of an inert PEG hydrogel (G'=500 Pa) injected in the LV, the authors decoupled the effects of mechanical support and bioactivity of biomaterials. The PEG hydrogel injection increased wall thickness but the passive structural intramyocardial support by itself does not prevent negative LV remodeling or maintain cardiac function. No cell infiltration was seen in the PEG hydrogel. This and another study with similar results suggests the importance of other mechanisms such as bioactivity and/or cell infiltration in the inhibition of LV remodeling and cardiac function improvement [122,123].

1.2.2.3.2 Physical/Pharmacological/biological factor pretreatment or co-stimulation

Methods to activate important survival or functional activity using physical, biological or pharmacological agents have been used as well, sometimes even in combination with each other or biomaterials. For example, exposing cells to hypoxic environment activates HIF-1α signaling which turns on survival pathways including increase in Akt phosphorylation and p38MAPK activity [124]. Consequently, improved survival in the infarcted microenvironment *in vivo* is seen after hypoxic preconditioning of MSCs [125]. Heat shock treatment at 42^{o}C before implantation improves survival in cardiac stem cells [126] and skeletal muscle cells [127] *in vivo*. Small molecules such as Necrostatin-1 reduce necrosis in cardiac stem cells [128]. Pretreatment with vasodilator prostacyclin

increases retention of ADSCs, perhaps by better extravasation of stem cells from vasculature into myocardium [129]. Codelivery or priming cells with biological factors such as VEGF, IGF-1 initiate pro-survival signaling pathways [130,131]. ADSCs pretreated with Exendin-4 demonstrate better survival and adhesion under oxidative stress *in vitro* and greater cardiac function preservation along with transplanted cell survival, angiogenesis and matrix remodeling after MI [132].

1.2.2.3.3 Genetic manipulation

Many studies have performed genetic manipulation of cells to enhance their functional properties or survivability in the hostile myocardium after MI. For example, in one study, rat MSCs transfected with hVEGF165 using adenovirus mediated gene transfer were delivered to rat hearts after MI. Reduction in infarct size, and improvement in left ventricular dimensions, ejection fraction and capillary density of the infarcted region was more enhanced than non-transfected MSCs or vehicle control [133]. The overexpression of VEGF in CPCs increases telomerase expression of VEGF, which suggests the importance of regulated expression of the factor [134]. The overexpression of Pim-1 in CPCs increases telomerase expression and activity, which enables telomere elongation in the cells. This in turn leads to enhanced regenerative potential of the CPCs via enhanced proliferation, metabolic activity and differentiation in vitro [135] and infarct reduction *in vivo* [66]. Treatment of MSCs with miR-210 improves their survival under anoxic and hypoxic conditions *in vitro*, and cardiac function preservation and infarct size control after MI *in vivo* [136,137]. Overexpressing integrin lined kinase (ILK) in MSCs [138,139] or Sca1+ [140] cardiac progenitor cells before transplantation enables them to better preserve cardiac function, reduce fibrosis and increase angiogenesis in the host after MI [140].

1.2.2.3.4 3D aggregates

Three dimensional aggregates of stem cells enable greater cell-cell contact which can promote their functional effects. 3D cell aggregates are commonly generated by

culturing cells on non-adhesive surfaces or on highly porous biodegradable polymers among other methods. 3D aggregation of CPCs shows enhanced cardiac differentiation and resistance to oxidative stress [141]. Delivery of 3D aggregates of CPCs showed superior survival of implanted cells *in vivo* in comparison to cell suspension in an ischemia-reperfusion model [142]. MSCs in spheroids show enhanced expression of immunomodulatory factors and growth factors including TNFα and HGF [92].

1.2.3 Other regenerative therapies

Other regenerative therapies are being studied as well including acellular biomaterial based-, protein based- and gene based-therapies, or their combinations.

Biomaterials are being tested for their ability to improve cardiac function after MI not just as a cell carrier but acellular biomaterials, sometimes carrying a drug, as well. They offer the advantage of being simpler to translate than cell therapies. Both natural and synthetic materials have been tested including alginates, fibrin, hyaluronic acid, decellularized tissues, PEG hydrogels, nanofiber scaffolds etc. Their physical and chemical properties enable them to have beneficial effects and they have been able to reduce infarct expansion, leading to reduction in ventricular wall stress and attenuated ventricular remodeling. For example, acellular porcine decellularized matrix-based patch induces significant cardiac function improvement following MI. The graft recruited host progenitor, myocyte cells and vasculature. The recruited progenitor cells expressed both early and late cardiomyocyte differentiation markers and recruited cardiomyocyte-like cells showed a partially striated and immature muscle fiber arrangement and expressed connexin43. Presence of the patch also induced an increased M2/M1 macrophage subtype ratio in comparison with animals that did not receive any treatment [143]. Notch activating Jagged-1 functionalized nanofiber scaffolds improved cardiac function, decreased fibrosis, showed greater angiogenesis and cell proliferation [144]. In a study done with a relatively inert material PEG of rheological properties commonly used in animal studies for cardiac repair, to ascertain if the mechanical properties of biomaterials would be sufficient, cardiac function of infarcted rats did not improve. This

suggests that bioactive properties of biomaterials may be important for their ability to enhance cardiac repair [122].

Protein and gene therapy, by themselves or in combination with biomaterials are also being explored in the search for simple but potent regenerative strategies. Protein therapies are also expected to be less challenging to translate to the clinic given established expertise of the industry and regulatory bodies with recombinant proteins; and simpler storage, transport, maintenance requirements and greater ease of standardization than cells; and get an off the shelf therapy not possessing the risk of immunorejection. However, protein therapies may not be as dynamic and responsive to the microenvironment, and as rich as cells. A conclusion on whether cell therapies or protein-based therapies show superior results has not been made. Biomaterials are often used as delivery platforms to achieve controlled, sustained delivery of proteins possessing short half lives and/or deleterious effects in high doses, such as VEGF [145]. Delivery of VEGF and HGF via protease degradable PEG hydrogels improved chronic cardiac function in rats and limited remodeling [146]. With the knowledge that different proteins may serve different functions and act synergistically, and that cells exert their benefits primarily through a multitude of paracrine factors in cell therapy, cocktails of growth factors have also been tested. A direct comparison study reported that a single injection of neonatal CPC-derived total conditioned medium is more effective than transplanted neonatal CPCs or their exosomes [45]. Exosomes that carry microRNAs and proteins have been showing exciting results as well. Exosomes derived from CPCs improved cardiac function in infarcted rats and attenuated post-MI remodeling [147,148].

CHAPTER 2. INTRODUCTION

2.1 Motivation

Cardiovascular disease is the leading global cause of death. In the United States cardiovascular diseases are responsible for 1 in every 4 deaths and pose an economic burden of over $200 billion every year. The significant morbidity, mortality and economic burden associated with coronary artery disease and heart failure motivate development of better prevention and therapeutic strategies. Coronary artery disease (CAD) involves the gradual development of an atherosclerotic plaque in the coronary artery and depends on various risk factors including genetics, cigarette smoking, hypertension, obesity etc. CAD often manifests as myocardial infarction. More than 75% of acute myocardial infarcts are the result of thrombotic occlusion of a coronary vessel caused by rupture of a vulnerable plaque [4]. Myocardial ischemia results in large scale cardiomyocyte death by necrosis and apoptosis, which commences a cascade of compensatory remodeling mechanisms. The pathological remodeling following MI is a common cause for heart failure. Briefly, after acute MI, loss of functioning myocytes occurs, followed by myocardial fibrosis and ventricular dilatation. Neurohormonal signaling and LV remodeling lead to progressive deterioration of the remaining viable myocardium, eventually leading to development of heart failure [6,149]. The existing treatment strategies aim at reducing preload, afterload, neurohumoral activation, and mineralocorticoid dysregulation. However, the underlying loss of cardiomyocytes and the remodeling that follows are not reversed [10]. The extent of fibrosis is a strong prognostic marker and correlates with morbidity and mortality due to arrhythmias and sudden cardiac death [150].

Cell therapy for congestive heart failure has shown promising results in preclinical studies but results in clinical trials have been mixed and generally below expectations. This suggests the need for the modality of cell therapy to be optimized in order to enhance the regenerative potential of stem cells. C-kit+ cardiac progenitor cells

(CPCs) are adult stem cells that have been shown to differentiate into cardiomyocytes, endothelial cells and vascular smooth muscle cells [35,151], and exert beneficial paracrine effects[45]. They are safe for delivery as determined in phase I clinical trials, do not pose ethical issues, are easily isolated and expanded, and can be delivered autologously or allogeneically to patients [54,55]. These attributes make CPCs an attractive choice for cell therapy for heart failure and other cardiovascular diseases. Despite the positive outlook, injection of CPCs in humans in phase I trials resulted in moderate but insufficient improvements in cardiac function after myocardial infarction.

The poor outcome of cell therapy is attributed to the rapid wash out of injected cells as well as the hostility of the environment in the infarcted myocardium. Extensive damage in the myocardial extracellular matrix architecture and biochemical changes in addition to high oxidative stressors induce death of transplanted cells and provide insufficient regenerative cues to them. Delivering cells with well-designed biomaterials is a promising approach with the potential to overcome the hurdles listed above. Biomaterials can improve the retention of CPCs allowing controlled and sustained release of cells. In addition, various molecules of interest can be physically or chemically linked to biomaterials. Delivering cells embedded in biomaterials creates an opportunity to provide them with a custom microenvironment that protects them from the hostile chemical, mechanical and topographical signals in the infracted myocardium, and provide beneficial cues that enhance the regenerative potential of the transplanted cells[152].

Maleimide-crosslinked poly(ethylene glycol) (PEG-MAL) hydrogels are synthetic hydrogels that maintain high cell viability, are injectable and highly tunable in terms of their mechanical properties, degradation rate and ability to present sites for linkage of bioactive ligands for cell adhesion and stimulation [100]. Synthetic biomaterials-based strategies provide increased control over biochemical and physical properties as compared to natural ones; therefore, synthetic biomaterials can complement studies using natural biomaterials with cells and act as useful research tools to learn about the complex mode of cell therapy that has produced mixed results in clinical trials. In

addition, synthetic biomaterials can be used to develop therapeutic strategies with greater quality control and localized specific effects.

2.2 Specific aims

The *central hypothesis* of this dissertation is that *customized bioactive synthetic scaffolds can be used to present microenvironmental cues to progenitor cells that modulate their behavior to enhance their therapeutic effects*. The *goal* of this dissertation is *to design and test PEG-MAL hydrogels of specific densities and functionalized with specific biochemical ligands that were hypothesized to promote the beneficial effects of CPC-based cell therapy*. 4-arm PEG-MAL (PEG-4MAL) hydrogels are customizable allowing some orthogonal control of parameters like viscoelasticity and ligand density. CPCs are interesting candidates for cell therapy as they have shown encouraging, albeit insufficient, improvement in phase 1 trials. Therefore, developing PEG-4MAL scaffold designs to enhance the effects of CPCs has the potential to develop important therapies for humans.

Aim 1: VEGF functionalized scaffolds for enhancing CPC angiogenic behavior.

The hypothesis of this study is that degradable hydrogels functionalized with VEGF enhances angiogenic behavior of encapsulating CPCs. Endothelial differentiation of CPCs, tube formation characteristics in hydrogel and modulation of paracrine factors in the presence of VEGF were measured. Different growth factor doses, cell populations and biomaterial density and degradation rates were tested.

Aim 2: Integrin specific hydrogels for activation of regenerative signaling in CPCs.

The hypothesis of this study is that encapsulating CPCs in integrin-specific hydrogels improves adhesion and activates signaling involved in reparative processes. Basal integrin expression on CPCs and rheological properties of hydrogels were characterized. Expression of lineage markers to assess differentiation of CPCs and secreted factors *in vitro* following encapsulation in integrin-specific or non-adhesive control gels were measured. *In vivo* measurement of effects of these constructs on cardiac function in an

ischemia-reperfusion model and *ex vivo* analyses of cardiac fibrosis, angiogenesis, hypertrophy and engraftment of exogenous cells were performed.

CHAPTER 3. VEGF FUNCTIONALIZED SCAFFOLDS FOR ENHANCING ANGIOGENIC BEHAVIOR OF CARDIAC PROGENITOR CELLS

The objective of this study is to determine how VEGF presenting hydrogels modulate behavior of encapsulated CPCs *in vitro* in terms of endothelial differentiation, tube formation tendency, and secretion of paracrine factors. Different growth factor doses, cell populations, and biomaterial density and degradation rates were tested.

3.1 Motivation

Therapeutic angiogenesis following MI: Myocardial infarction (MI) is characterized by myocyte death, scar formation, ventricular remodeling, and potential progression to heart failure. Various treatment options currently in use aim to re-establish blood flow to the ischemic tissue. Early stage disease is managed by lifestyle changes and pharmacological agents that reduce the heart rate to decrease the amount of oxygen it requires and/or increase blood flow by vascular smooth muscle dilation. As the disease progresses, patients require coronary revascularization by percutaneous coronary intervention (angioplasty) or bypass surgery. A significant number of patients are ineligible for these surgical procedures as they continue to suffer from associated comorbidities and symptoms of MI after treatment because of widespread blockages as in diffuse coronary artery disease [153]. Stimulating angiogenesis at the ischemic site using proangiogenic molecules and/or cells has the potential to aid such patients who do not benefit from existing therapies [154]. An estimated 30-60% of patients suffer from microvascular dysfunction or “no reflow”, preventing proper perfusion of the entire myocardium. Angiogenesis can restore the dysfunctional microcirculation and prevent the progression to heart failure [155]. Additionally, tissue engineered vascular grafts are expected to be superior to autografts and synthetic grafts because they have the potential to satisfy the need for non-morbid, easily available

and non-thrombogenic grafts. Therefore, inducing therapeutic angiogenesis after an MI is believed to greatly improve patient outcomes. A large body of preclinical evidence has shown improvement in perfusion and myocardial function after inducing angiogenesis in animal models of myocardial infarction and hind limb ischemia. Neoangiogenesis in the infracted heart results in beneficial hypertrophy of myocytes [156], reduced apoptosis of hypertrophied myocytes, improved survival of viable myocytes and reduction in collagen deposition [157], thereby contributing to improved myocardial function.

Cell therapy for therapeutic angiogenesis: Different populations of resident stem cells have been found in the heart but they are either insufficient in numbers, have inadequate differentiation potential or receive improper cues to drive sufficient regeneration after MI. Cells that secrete angiogenic factors or are precursors to vasculogenesis have been studied for implantation for therapeutic angiogenesis. Cell therapy has been shown to result in altered LVEF ranging from -7% to +25%, depending on cell type, number of cells, site of infarct etc., in large animals [158]. Insufficient cardiac improvement after cell therapies in clinical trials is attributed primarily to low retention and survival of cells and insufficient pro-regenerative cues in the ischemic region [159]. In the event of an MI, the architecture of extracellular matrix is also damaged along with cells, which further limits engraftment of transplanted cells [160]. Biomaterials provide the opportunity to present cells with a regenerative microenvironment in terms of physical and biochemical signals. Delivering cells to an infarcted heart via biomaterials has previously been shown to result in retention and survival of 50-60% cells after 24 h compared to 10% with saline [103]. Some recent studies have used a combination of growth factors, cells and scaffold. Cord blood derived vasculogenic progenitor therapy was found to result in greater vessel density when codelivered with VEGF, HGF and Ang-1 [161]. Similar improvement was seen on delivering VEGF with EPCs via PCL scaffold

[162]. Co-delivery of FGF-10 with iPSCs via a nanofiber scaffold resulted in greater number of differentiated cardiomyocytes suggesting the role of codelivered growth factor in differentiating the encapsulated cells [163].

CPCs for therapeutic angiogenesis: CPCs are adult stem cells isolated from the heart, first discovered in 2003 [35]. These cells have been shown to have the potential to differentiate into vascular lineages (endothelial, vascular smooth muscle cells) as well as into myocardial cells [36,151]. Therefore, they are an attractive population to study for regeneration of vasculature as well as vascularized myocardium. Flk1+ CPCs have a greater tendency to form endothelial and vascular smooth muscle cells and Flk1- CPCs to form cardiomyocytes [36]. Injection of CPCs in the SCIPIO trial resulted in unprecedented LV improvement and decreased fibrosis [164]. CPCs are isolated from the heart by selecting c-kit+ cells from the atrial appendage, which is removed as part of the routine procedure for coronary artery bypass graft and can be cloned and expanded in culture [54] and the autologous cells delivered to the patients in about six weeks. Clinically relevant number of functional CPCs can be isolated from patients with advanced heart failure as well [165]. These cells have been found to be necessary and sufficient for cardiac regeneration [166]. Injection of CPCs in clinical trials has established their safety [164]. Genetic mapping studies suggest that the majority of these cells may be vascular progenitors and differentiate into endothelial cells in vivo [167]. For these reasons, CPCs are an exciting alternative population that is worth exploring for vascular tissue engineering.

PEG-MAL hydrogels for stimulating CPCs and supporting angiogenesis: PEG-4MAL hydrogels are synthetic biomaterials that maintain high viability of encapsulated cells, are able to immobilize macromolecules possessing free thiols, can be functionalized with cell-adhesive RGD sites and crosslinked with protease cleavable peptides [100]. Because of these properties, PEG-4MAL hydrogels have

the capability to present stem cells with a niche-like environment in terms of mechanical and biochemical cues. Mechanical properties of these gels can be tuned by adjusting the PEG macromer size and density. In addition, these hydrogels are injectable and show low thrombogenicity and immunogenicity [101]. Therefore, PEG-4MAL hydrogels are synthetic hydrogels that have the advantage of greater control and consistency, together with the ability to be functionalized with natural material-inspired bioactive molecules such as RGD and VEGF. RGD supports angiogenesis because it is a ligand for αVβ3 integrins that are highly expressed in endothelial cells undergoing angiogenesis [168]. Because of the protease cleavable sites, these hydrogels allow for sprouting and branching of blood vessels. These hydrogels have been shown to release immobilized VEGF in a cell-demanded manner [146].

VEGF: VEGF is one of the most studied proangiogenic growth factors, perhaps due to its role in vascular development and tumor vascularization. VEGF-A promotes endothelial cell survival, proliferation and migration and drives vasculogenesis and angiogenesis primarily through VEGFR2/Flk-1 [169]. Administration of VEGF leads to neoangiogenesis and improved blood flow in a rodent model [170] as well as improved cardiac function in a porcine model of MI [171]. VEGF induces endothelial differentiation of numerous stem cells including ESCs [172], PSCs [173], MSCs [174] and amniotic fluid-derived stem cells [175]. Several isoforms of hVEGF exist including soluble VEGF-121, that diffuses freely and heparin binding VEGF-165, which is mostly present as bound to the extracellular matrix and cell surface. The mitogenic activity of VEGF-121 is lower than that of VEGF-165 [169].

Effects of VEGF on CPCs: VEGF promotes migration of CPCs [146,176,177] via activation of PI3K/Akt. It also improves adhesion of CPCs to extracellular matrix and endothelial cell-mediated adhesion systems in vitro and engraftment *in vivo* via VEGFR-PKCα-VCAM-1 pathway stimulation. Inhibitors of both VEGFR1 and VEGFR2 attenuate CSC migration. Interestingly, additional mechanisms are involved as inhibitors for both

VEGFR1 and VEGFR2 did not completely block the VEGF induced CSC migration. Both VEGFR1 and VEGFR2 inhibitors blocked the VEGF induced CSC adhesion as well. CSC adhesion to endothelial cells was increased in a dose dependent manner with VEGF121, VEGF165, or VEGF189 treatment. Further, delivery of VEGF treated CPCs to infarcted hearts led to improved cardiac function, perhaps because of enhanced engraftment and homing [178].

3.2 Rationale and hypothesis

CPCs have been shown to differentiate into endothelial cells *in vitro* and *in vivo* [36,151]. Co-delivering CPCs with VEGF will provide them a proangiogenic environment. It has been seen with other cell types (EPCs [162], cord blood derived vasculogenic progenitor cells [161]) that a combination of VEGF with these progenitor cells was superior to cells only in neovasculature density. VEGF induces endothelial differentiation of many stem cells and is expected to drive VEGFR2 expressing CPCs down the endothelial lineage as well. VEGF is also expected to improve survival of these cells in the hypoxic environment post MI [179]. Like endothelial cells, CPCs are known to migrate in response to a VEGF concentration gradient [177], which is essential to angiogenesis.

3.3 Approach

CPCs were encapsulated in PEG-MAL hydrogels with or without VEGF. A schematic of the synthesis process is shown in Figure 1. Cell viability of CPCs in PEG hydrogels was ascertained. mRNA and protein expression of endothelial differentiation markers, tube formation characteristics in hydrogel and modulation of secreted paracrine factors in the presence of VEGF were measured. Effect of VEGF supplementation with different growth factor doses (100, 250, 5000 ng/mL), cell populations (rCPCs- rat CPCs, nhCPCs- neonatal human CPCs, chCPCs- child human CPCs), biomaterial density (4%, 5% w/v) and degradation rates (fast degrading ‘VPM’, slow degrading ‘GPQ’) were tested. For testing the effect of VEGF supplementation in these various combinations,

mRNA expression of endothelial differentiation markers was used as the main surrogate; paracrine factors also were assayed for gels of different degradation rates. The constructs were made with 5% w/v PEG, 2 mM RGD, 10 million CPCs/mL, 0 or 5 ug/mL VEGF unless otherwise specified.

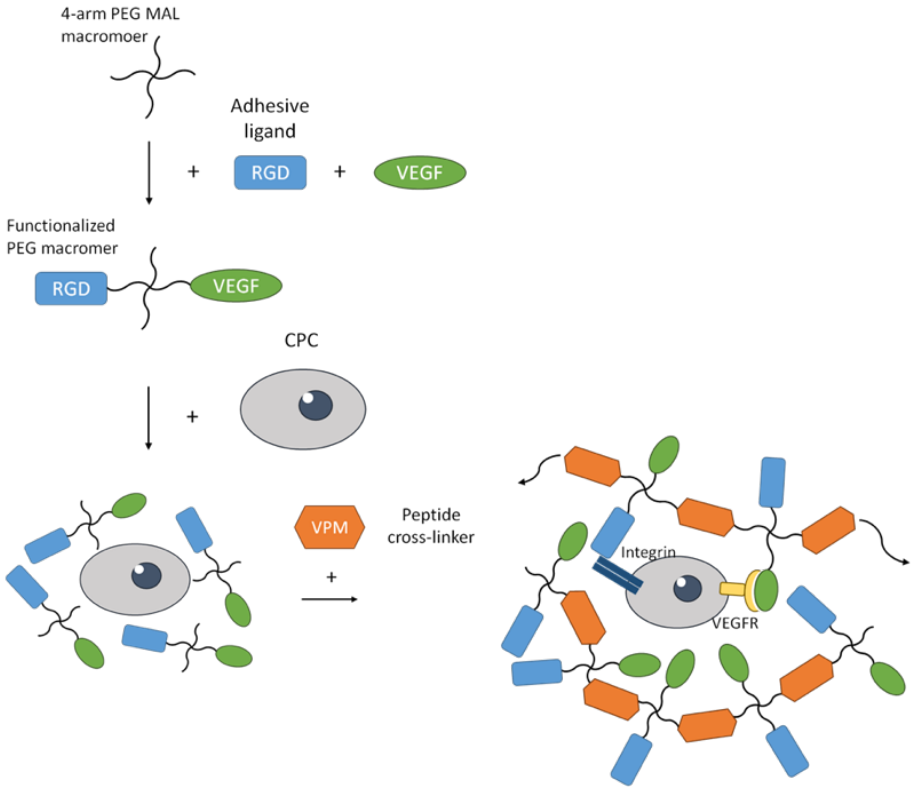


Figure 1. Schematic detailing synthesis of VEGF functionalized PEG hydrogels. 4- arm PEG macromers were incubated with RGD and VEGF (or no VEGF in control samples), then mixed with cell suspension and finally crosslinked using VPM. Substitutions of RGD or VPM were made in some experiments and are described where that was the case.

3.4 Results

3.4.1 Viability of encapsulated rCPCs

PEG-4MAL hydrogels have been found to support high viability of other cell types, but we tested viability of encapsulated CPCs to confirm its fit as a cell carrier for CPCs. We used Calcein AM/EthD-1 based live/dead assay on hydrogel constructs 3 days after encapsulation. Live cells uptake hydrophilic, cell permeating, fluorescent Calcein which

is generated following hydrolysis of non-fluorescent Calcein AM by intracellular esterases. EthD-1 cannot pass through the intact membrane of live cells but passes through the compromised membranes of dead cells and its fluorescence amplifies upon binding to nucleic acids. We tested hydrogels of different densities (4%, 5%, 6% w/v) and found approximately 80% viability in all hydrogel densities. The cells that were stained by Calcein AM only were counted. Specifically, 84.07±2.11% cells were Calcein AM+EthD- in 4% hydrogels, 83.99±6.86% in 5% hydrogels and 85.13±2.92% in 6% hydrogels (n=3).

3.4.2 Activation of signaling in rCPCs in VEGF-immobilized hydrogels

We measured ERK phosphorylation as a surrogate for determining successful activation of VEGF-stimulated signaling because VEGF binding to VEGFR2 activates the ERK/MAPK pathway. Measurements were made using cell lysates obtained 1 h post-encapsulation (10 min gelation + 10 min + 40 min Collagenase I treatment to release cells) as ERK phosphorylation is seen within minutes of cell stimulation in 2D. 18 h cell lysates were also tested to assess if ERK activation is sustained longer in immobilized VEGF-stimulated cells. ERK phosphorylation was determined by immunoblotting for phospho-ERK and total ERK and densitometry of obtained bands. Ratio of pERK/ERK signals was normalized by dividing by time-matched control (0 ng/mL VEGF) pERK/ERK signals ratio. As shown in Figure 2, ERK phosphorylation was 2.59±0.77 times higher than the control ($p<0.05$) in 250 ng/mL VEGF carrying hydrogels 1 hour after encapsulation but not 18 hours later. A lower dose of 100 ng/mL VEGF not lead to increased ERK phosphorylation at either time point.

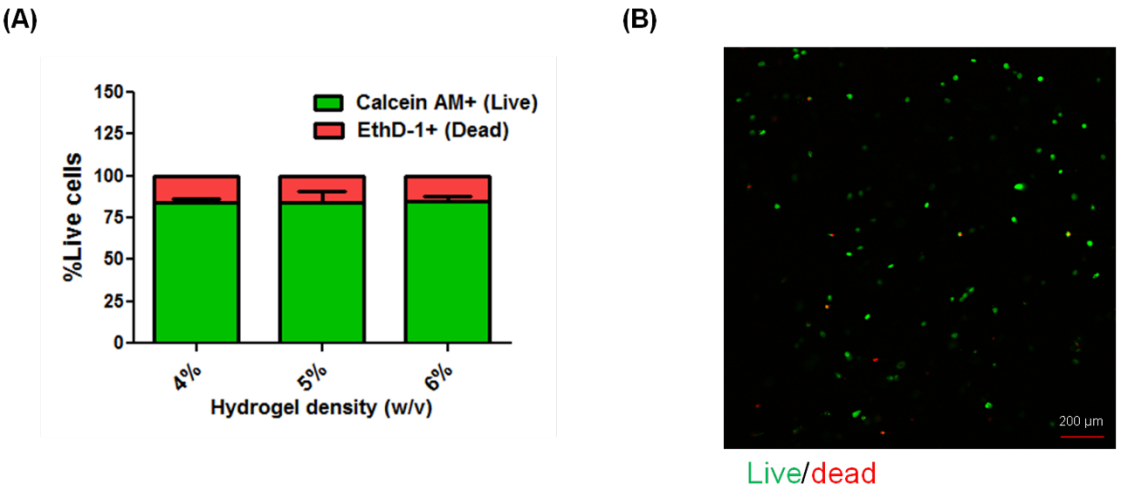


Figure 2. Cell viability in hydrogels. Three days after encapsulating cells in hydrogels of different densities, they were incubated with Calcein AM and EthD-1 dyes which stain live and dead cells respectively. (A) Number of Calcein AM (green) and EthD-1 (red) stained cells were counted and percentage of cells stained by Calcein AM only were computed. Values are mean ± SEM; n=3. (B) Representative micrograph of cells in PEG-MAL hydrogel stained with Calcein-AM and EthD-1 show green, red and colocalized red and green colors; scale bar equals 200 µm.

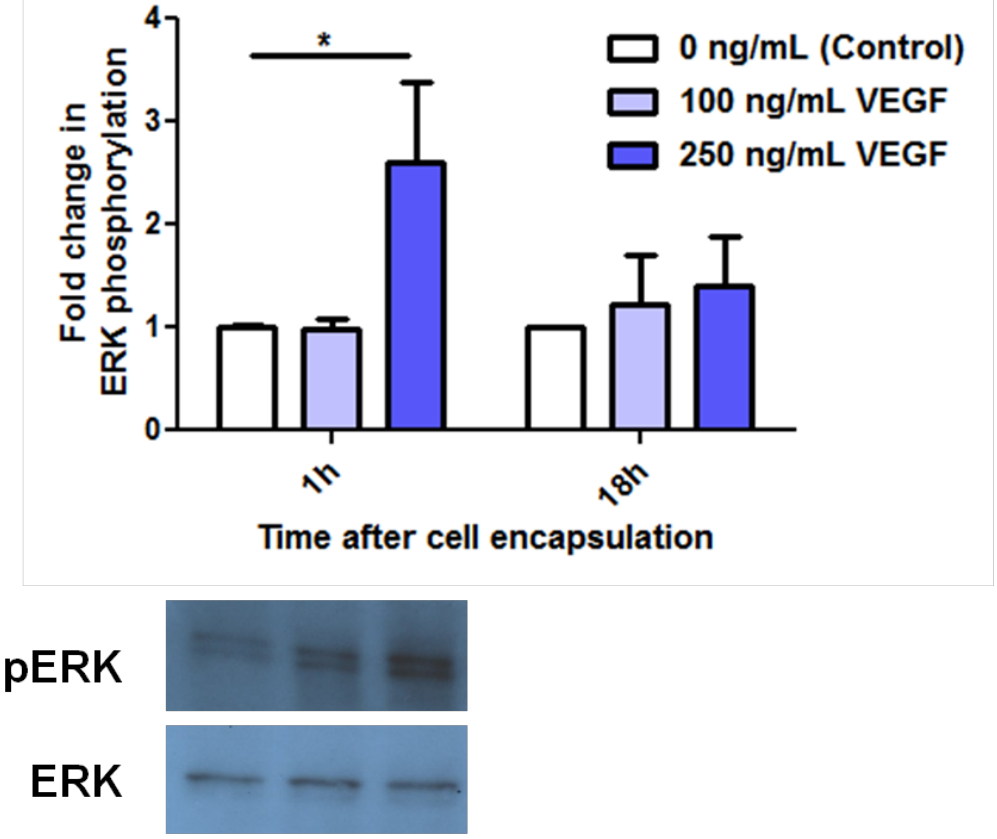


Figure 3: ERK phosphorylation. rCPCs were encapsulated in hydrogels immobilized with 0 (control), 100 and 250 ng/mL VEGF-165. The hydrogels were degraded using Collagenase treatment and cells lysed 1 h or 18 h after encapsulation. ERK phosphorylation was determined by immunoblotting for phospho-ERK and total ERK and densitometry of obtained bands. Ratio of pERK/ERK signals was normalized by dividing by time-matched control (0 ng/mL VEGF) pERK/ERK signals ratio. Values are mean ± SEM; statistical significance of difference between means was measured using ANOVA and Dunnett's post-hoc test comparison to control, *:$p<0.05$; n=4-10. Representative blot at 1 h is also shown.

3.4.3 Expression of lineage markers in rCPCs

Differentiation of VEGF-PEG gel encapsulated CPCs into endothelial cells was assessed by measuring mRNA and protein expression of endothelial markers (flk1, tie2, cdh5 and vwf) 7 days after encapsulation, using real-time PCR and flow cytometry, respectively. Various doses were tested, 100 ng/mL, 250 ng/mL, 5 ug VEGF/mL hydrogel, for real-time PCR and for flow cytometry, all doses except the highest dose of 5 ug/mL were tested. We had noted heterogeneity in Flk-1 expression in CPC populations among different clones ranging from approximately 3-80% (data not shown). Only the clones showing Flk expression by 70% or more cells were used following preliminary studies appearing to suggest a correlation of Flk expression in cell populations and increase mRNA expression of endothelial markers response to VEGF (data not shown), as well a report from literature showing Flk1+c-kit+ cells to be vascular progenitors and Flk1-ckit+cells to be myogenic progenitors[36]. As shown in Figure 4(A), neither dose of VEGF led to statistically significant increase in mRNA expression of either endothelial marker (n=2-10). As shown in Figure 5, protein expression levels of endothelial markers Flk1, Pecam1, VE-cadherin and vWF were also not different in VEGF gel treated rCPCs than those from control gels (n=3). Flk-1 was expressed in 23.69±3.79% cells obtained from control gels and in 24.29±3.81% and 45.47±10.40% cells harvested from 100 ng/ mL and 250 ng/mL VEGF functionalized hydrogels respectively. No differences in means were seen between control group and either

treatment dose in percentage of cells expressing endothelial markers VE-cadherin, Pecam-1 and vWF. About 98% cells expressed VE-cadherin and vWF and about 75% cells expressed Pecam-1. Statistical significance of difference between means was evaluated by one-way ANOVA followed by Tukey's post test with significance level set at $\alpha=0.05$.

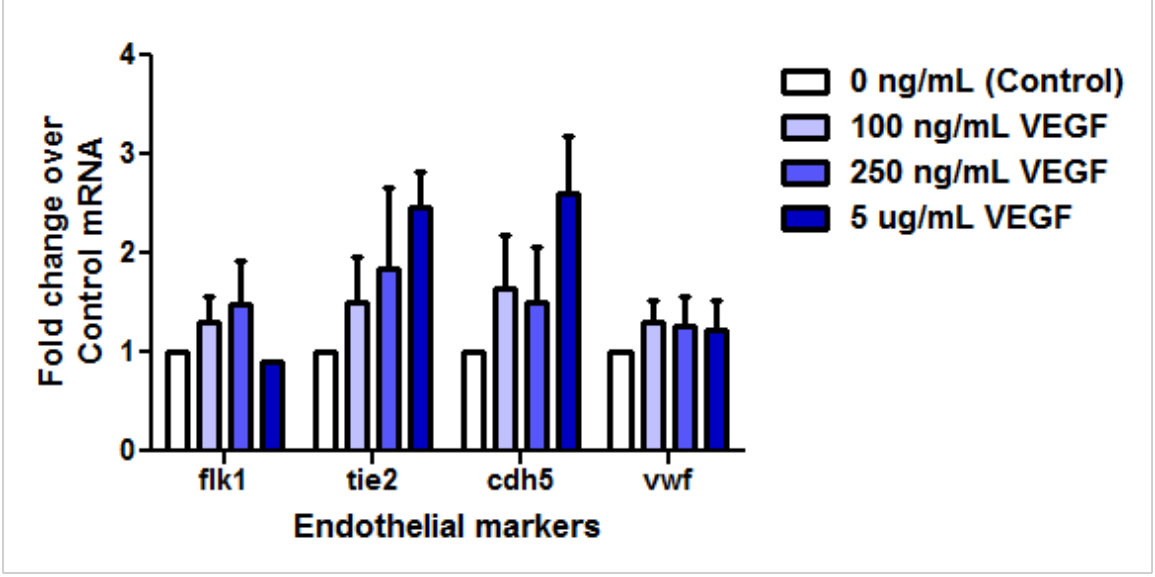


Figure 4: mRNA expression of endothelial markers in rCPCs. mRNA expression of endothelial lineage markers (encoding protein) *flk1* (VEGF Receptor 2), *tie2* (TEK Receptor Tyrosine Kinase), *cdh5* (VE-Cadherin), *vwf* (von Willebrand Factor) was measured 7 days after encapsulating hCPCs in PEG-MAL gels immobilizing 2 mM RGD and 0, 100, 250 or 5000 ng/mL VEGF using real-time PCR. Data are expressed as fold change over 0 ng/mL VEGF control. Values are mean ± SEM; n≥8 for 100 and 250 ng/mL groups and n=2 for 5 ug/mL group.

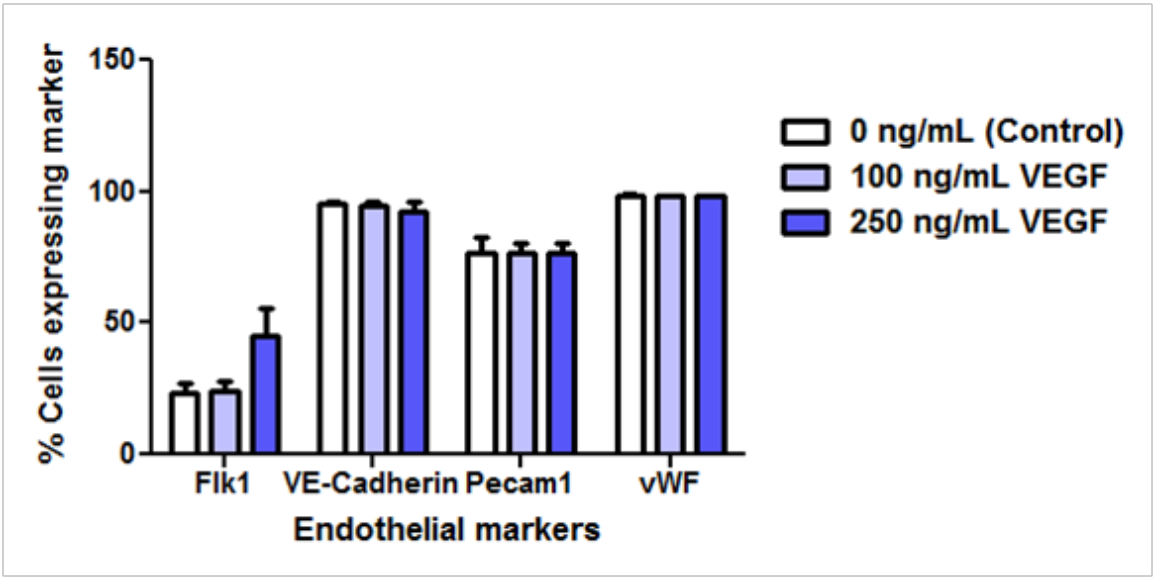


Figure 5: Protein expression of endothelial markers. (A) Protein expression of endothelial lineage markers Flk1 (VEGF Receptor 2), VE-Cadherin, Pecam-1 and vWF (von Willebrand Factor) was measured 7 days after encapsulating hCPCs in PEG-MAL gels immobilizing 2 mM RGD and 0, 100 or 250 ng/mL VEGF using flow cytometry. Values are mean percentage of cells expressing the specific protein ± SEM; n=6.

3.4.4 Tube formation characteristics in rCPCs

Hydrogels of different densities (4, 5, 6% w/v) were tested for their suitability for formation of vascular structures with and without VEGF conjunction. The Young's modulus of each of these hydrogels lies in the range of 450-800 Pa, that has been reported to enable tube formation by endothelial cells[180]. Since the most suitable hydrogel stiffness for stem cell differentiation and tube formation is dependent on the specific cell type and adhesivity of the gel[181],[182], we tested gels of different stiffness for our application. Only a limited range of biomechanical environments are suitable for tube formation. Less stiffer gels are likely to support greater branching and sprouting [181][176] but too compliant matrices result in formation of unstable lumens[184]. Number of branching points presented by CPCs was measured in hydrogels with and without 100 ng/mL VEGF 7 days after encapsulation. Figure 6(A) demonstrates that lower density gels show a trend of greater branch points per frame. The effect of hydrogel density was statistically significant (n=2-5, p=0.0012) by two-way

ANOVA analysis. Number of branch points per frame was not influenced by VEGF supplementation in hydrogels of either density. Figure 6(B) shows examples of branch points counted by pointing with white arrows.

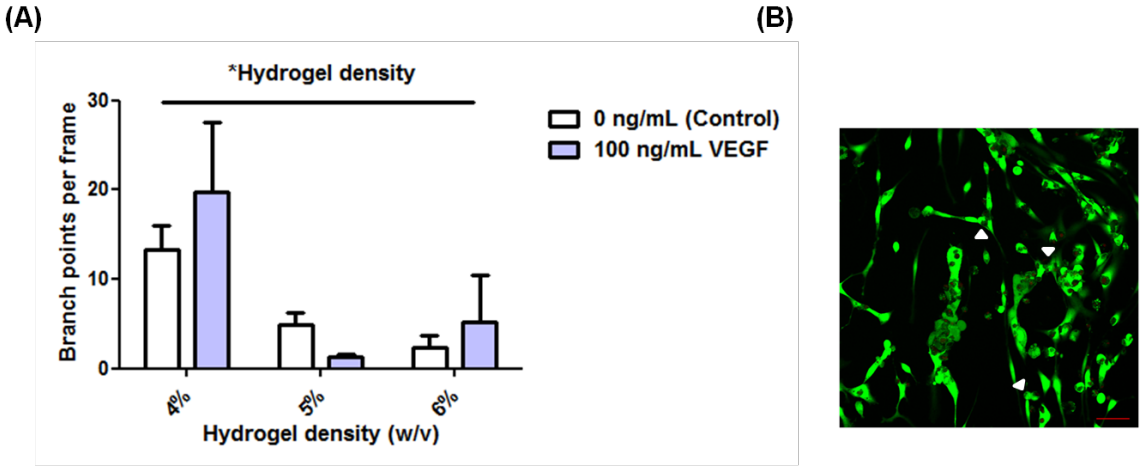


Figure 6: Tube formation characteristics by CPCs in hydrogel. (A) Number of branching points presented by CPCs was measured in hydrogels with or without 100 ng/mL VEGF 7 days after encapsulation. Values are mean ± SEM; statistical significance of differences was assessed using ANOVA; *:$p<0.05$. (B) White arrows point to examples of branch points in this image.

3.4.5 Expression of lineage markers in hCPCs

We had been using different clones of rat CPCs in the experiments so far. The results from rat cells showed a lot of heterogeneity making it difficult to make conclusions reliably. We switched to more relevant human CPCs and used a pool of cells obtained from 3 different patients to address these issues. These were obtained from patients of age 1 week or less and are will be referred to as neonatal hCPCs (nhCPCs). Following observation of endothelial cells by majority of rCPCs obtained from control gels lacking VEGF (Figure 5) and cells not receiving any kind of treatment (data not shown), basal expression of endothelial markers in untreated nhCPCs was tested as well. Figure 7 shows representative histograms of populations stained with antibodies for endothelial markers Flk1, Pecam1, VE-cadherin and vWF. More than 95% of cells express mature endothelial markers Pecam-1, VE-cadherin and vWF. Based on these observations, we

measured mRNA expression in subsequent experiments to determine increase in endothelial lineage commitment.

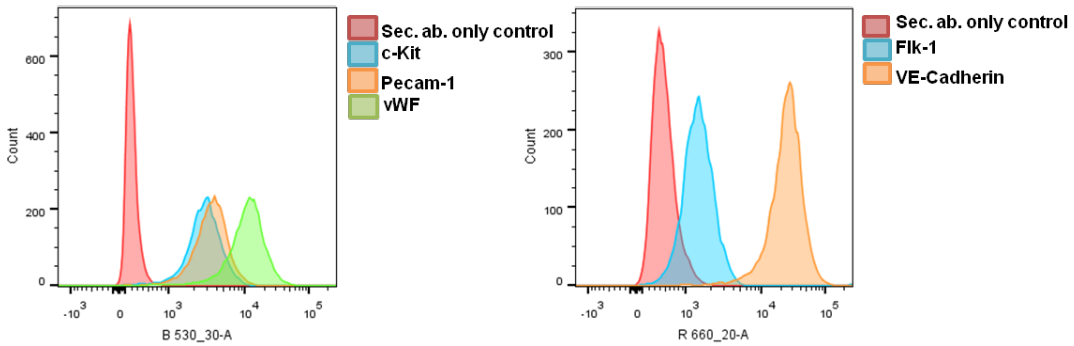
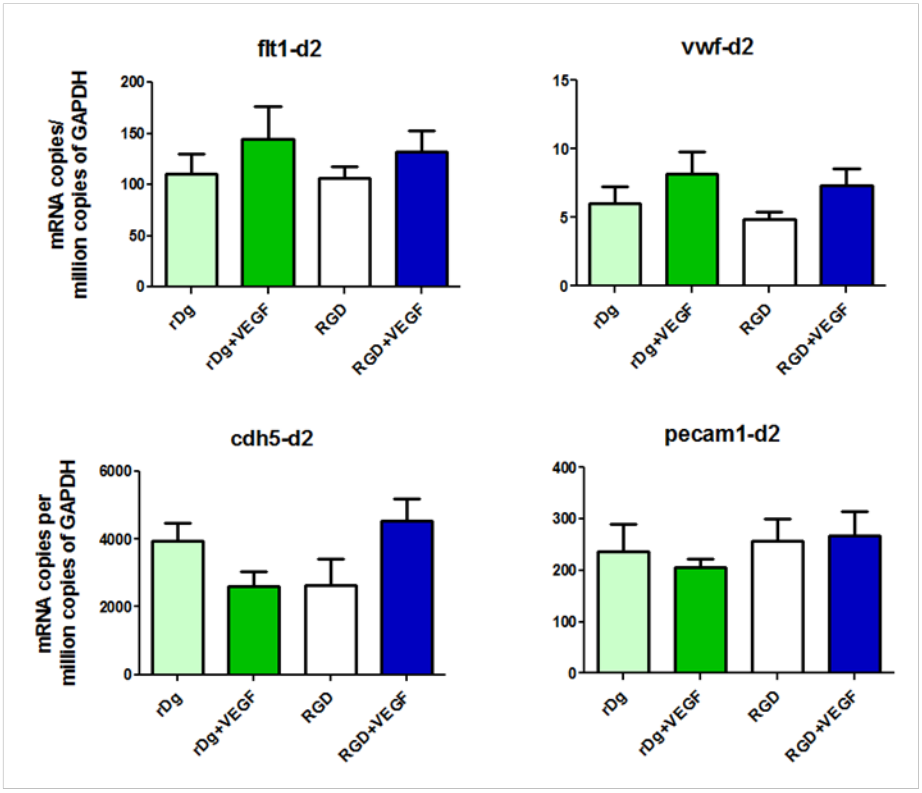


Figure 7: Basal expression of endothelial markers on nhCPCs. Basal expression of stem marker c-Kit and endothelial markers Pecam-1, vWF, Flk-1 and VE-Cadherin in unstimulated nhCPCs was measured using flow cytometry. Histograms showing count of cells on Y-axis and fluorescence intensity on X-axis are shown for the analytes and secondary antibody only negative control; n=1.

RGD has been shown to support angiogenesis therefore we included scrambled rDg with and without VEGF groups in order to determine the role of RGD in inducing endothelial differentiation of CPCs. mRNA expression of endothelial markers at d2 and d5 were measured. Figure 8 panels (A) and (B) show mRNA expression of endothelial lineage markers *flt1, vwf, cdh5 and pecam1* at d2 and d5 respectively. There was no statistical difference between means of any group suggesting that presence of neither RGD (compared to rDg) nor VEGF (compared to respective control groups without VEGF) affects mRNA expression of endothelial markers.

(A)


flt1-d2
vwf-d2
cdh5-d2
pecam1-d2
mRNA copies/ million copies of GAPDH
mRNA copies per million copies of GAPDH
rDg
rDg+VEGF
RGD
RGD+VEGF

(B)

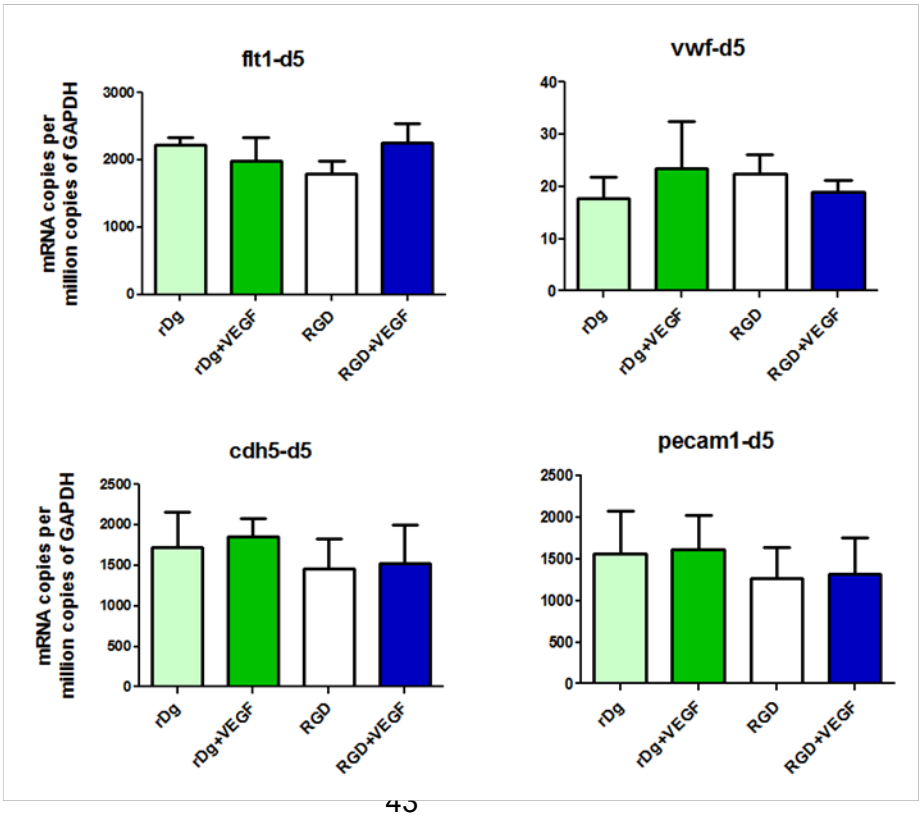

flt1-d5
vwf-d5
cdh5-d5
pecam1-d5
mRNA copies per million copies of GAPDH
rDg
rDg+VEGF
RGD
RGD+VEGF

Figure 8: mRNA expression of endothelial markers in neonatal hCPCs (nhCPCs). mRNA expression of endothelial lineage markers (encoding protein) *flt1* (VEGF Receptor 1)*, vwf* (von Willebrand Factor), *cdh5* (VE-Cadherin) and *pecam1* (Pecam-1) was measured (A) 2 days and (B) 5 days after encapsulating neonatal hCPCs in 5% w/v PEG-MAL gels immobilizing 2 mM rDg, 2 mM rDg + 5 μg/mL VEGF, 2 mM RGD or 2 mM rDg + 5 μg/mL VEGF using real-time PCR. A standard curve was prepared using human LV atrial tissue total mRNA to measure mRNA copy numbers. Values are mean mRNA copies per million copies of GAPDH ± SEM; n=4.

Lower density hydrogels are more likely to be conducive for tube formation because of lower stiffness which supports endothelial differentiation and higher porosity that allows cells to migrate and form tube networks. Our results from studies with rat cells (Figure 6) and preliminary studies with human cells (Figure 9(A)) showed greater elongation of CPCs, sprouting and formation of tip-like cells in lower density gels. Therefore, we tested mRNA expression of endothelial markers following VEGF supplementation in lower density 4% w/v gels. As shown in Figure 9(B), there was no significant difference between means of mRNA expression of endothelial markers between rDg, rDg+VEGF, RGD and RGD+VEGF groups in 4% w/v gels in any marker of endothelial or vascular smooth muscle lineage tested- *flt1, cdh5, pecam1, vwf, tagln, acta2*.

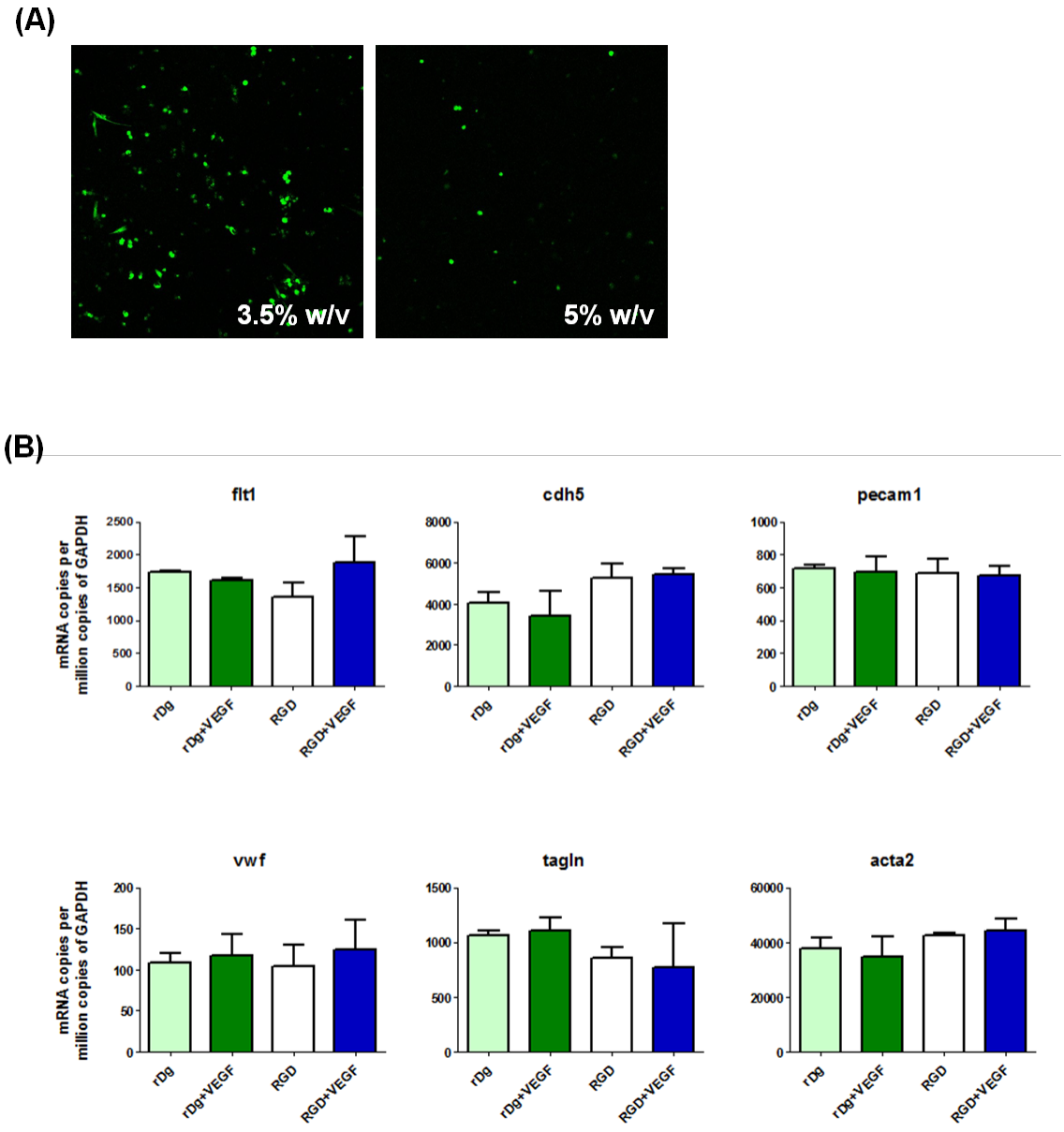


Figure 9: mRNA expression in neonatal hCPCs in low density gels. (A) Micrographs showing morphology of neonatal hCPCs encapsulated in low density 3.5% or high density 5% w/v gels. (B) mRNA expression of endothelial lineage markers (encoding protein) *flt1* (VEGF Receptor 1), *cdh5* (VE-Cadherin), *pecam1* (Pecam-1), *vwf* (von Willebrand Factor), *tagln* (Transgelin), *acta2* (Aortic smooth muscle actin) was measured 2 days after encapsulating neonatal hCPCs in 4% w/v PEG-MAL gels immobilizing 2 mM rDg, 2 mM rDg + 5 μg/mL VEGF, 2 mM RGD or 2 mM RGD + 5 μg/mL VEGF using real-time PCR. Values are mean mRNA copies per million copies of GAPDH ± SEM; n=2.

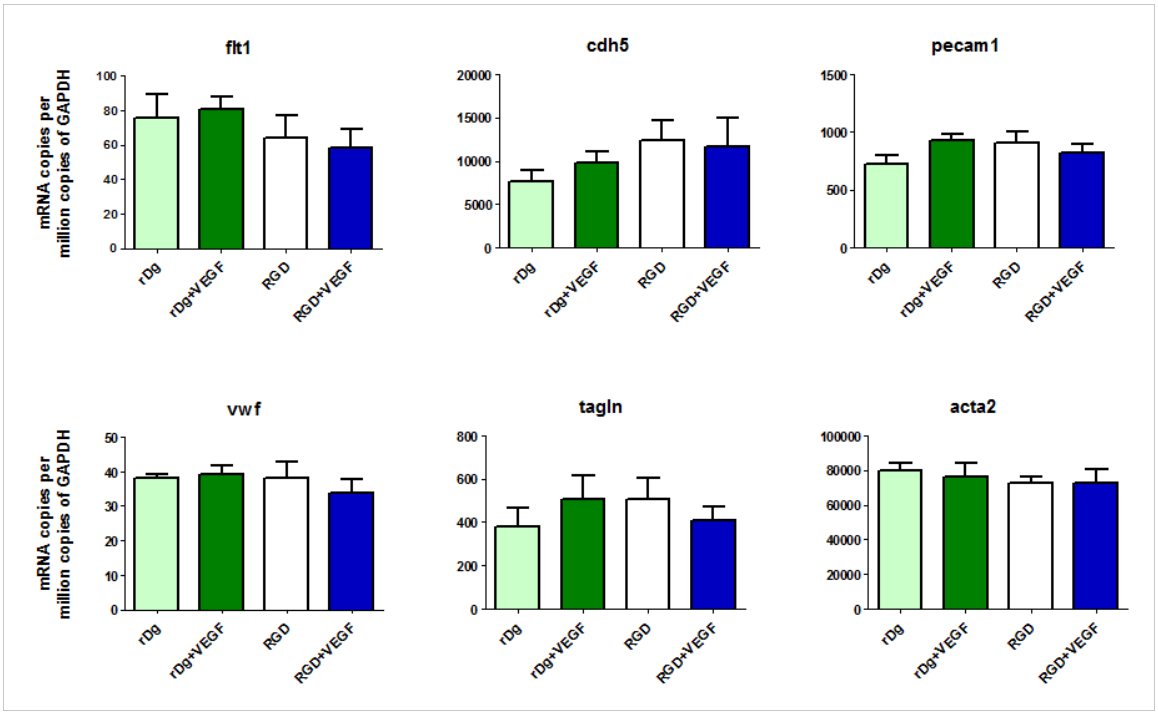


Figure 10: mRNA expression in hCPCs from older child patients. mRNA expression of endothelial lineage markers (encoding protein) *flt1* (VEGF Receptor 1), *cdh5* (VE-Cadherin), *pecam1* (Pecam-1), *vwf* (von Willebrand Factor), *tagln* (Transgelin), *acta2* (Aortic smooth muscle actin) was measured 2 days after encapsulating neonatal hCPCs in 4% w/v PEG-MAL gels immobilizing 2 mM rDg, 2 mM rDg + 5 µg/mL VEGF, 2 mM RGD or 2 mM RGD + 5 µg/mL VEGF using real-time PCR. Values are mean mRNA copies per million copies of GAPDH ± SEM; n=6.

Through a previous study from our lab and others in literature, CPCs from neonatal patients (nhCPCs) have been found to show possess greater regenerative potential than cells from older infant/child patients. For this reason, we tested the effect of VEGF supplementation on CPCs from older children patients 1 year or older in age (chCPCs), to determine if VEGF stimulation would impart more benefit to the less regenerative population. As shown in Figure 10, no significant differences were found between means of mRNA expression of endothelial markers between the treatment groups in 5% w/v gels using child CPCs in any marker of endothelial or vascular smooth muscle lineage tested.

We hypothesized that the hydrogels may be degrading too fast to allow immobilized VEGF to interact with CPCs and stimulate them for enough duration to induce endothelial differentiation or modulate their paracrine factors. So we tested effect of VEGF supplementation in slow degrading 'GPQ' gels. GPQ is a protease sensitive crosslinker with a lower rate constant of the protease mediated cleavage reaction [185]. However, as shown in Figure 11, mRNA expression of the cardiac lineage commitment (*nkx2.5, gata4*), endothelial (*flt1, cdh5, pecam1, vwf*) and vascular smooth muscle (*tagln, acta2*) markers was similar between VEGF supplemented samples and their respective controls with matched crosslinkers but no immobilized VEGF. Therefore, VEGF supplementation did not affect mRNA expression of these genes in slow degrading GPQ gels as well. There were differences in *cdh5* (encoding VE-cadherin) gene expression between VPM and GPQ gels in the absence or presence of VEGF.

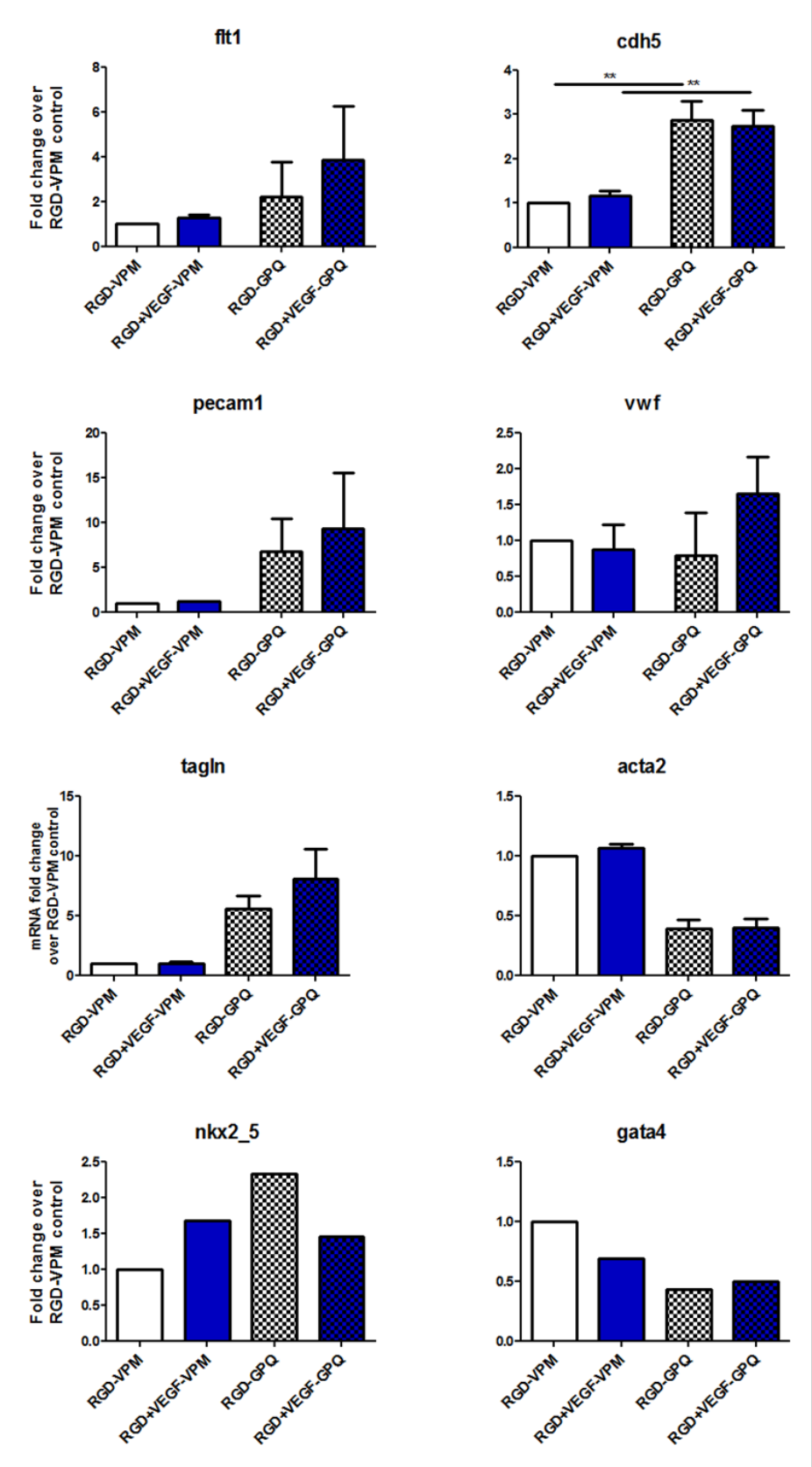
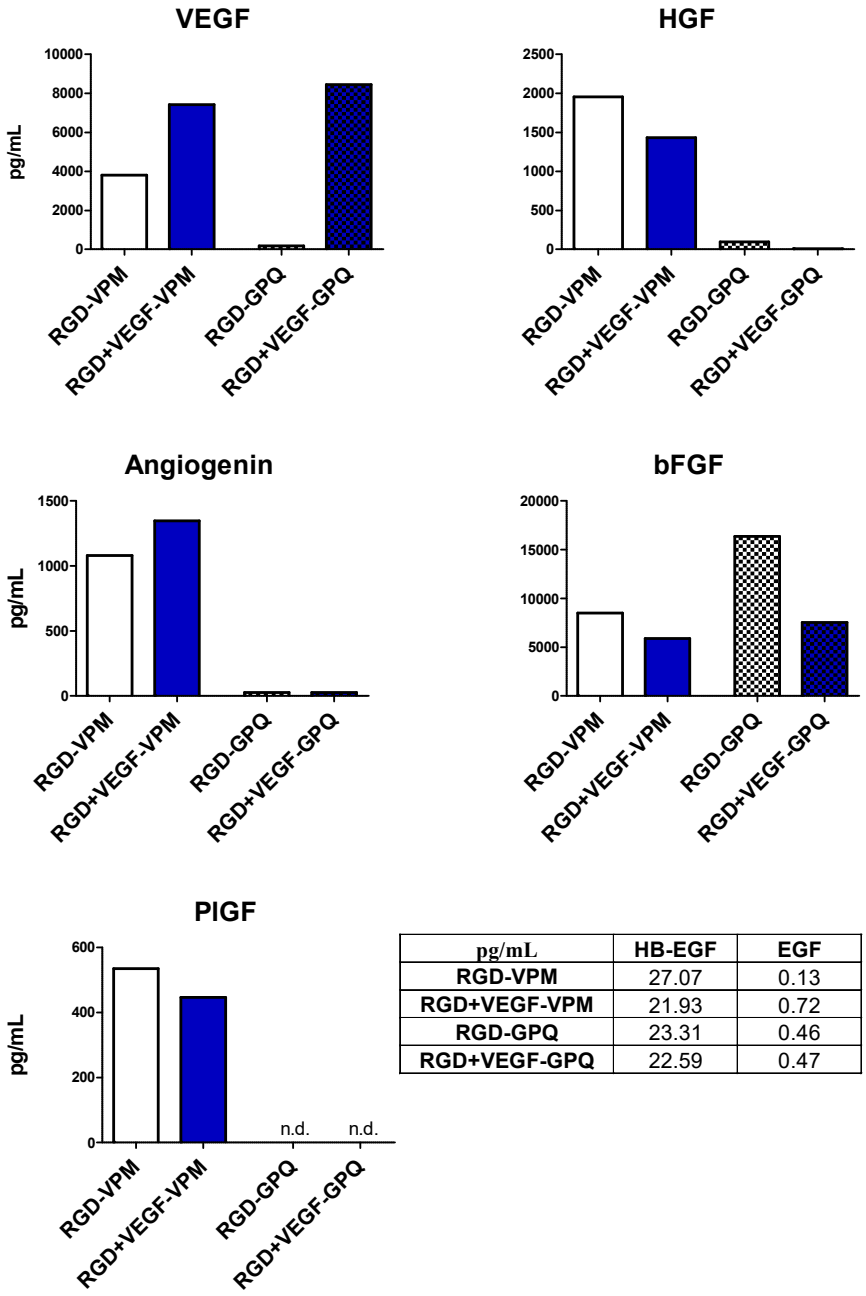

flt1
Fold change over RGD-VPM control
RGD-VPM
RGD+VEGF-VPM
RGD-GPQ
RGD+VEGF-GPQ
cdh5
**
**
pecam1
vwf
tagln
mRNA fold change over RGD-VPM control
acta2
nkx2_5
gata4

Figure 11: mRNA expression in in slow degrading PEG gels. mRNA expression of lineage markers (encoding protein) *flt1* (VEGF Receptor 1)*, cdh5* (VE-Cadherin), *pecam1* (Pecam-1), *vwf* (von Willebrand Factor), *tagln* (Transgelin), *acta2* (Aortic smooth muscle actin), *nkx2-5* (NK2 Homeobox 5), *gata4* (GATA Binding Protein 4) was measured 2 days after encapsulating neonatal hCPCs in 5% w/v PEG gels crosslinked with fast degrading crosslinker VPM or slow degrading GPQ. Data are reported as fold change over VPM-RGD control; statistical significance of difference between means was measured using ANOVA and Tukey's post-hoc test, *: $p<0.05$; n=5.

3.4.6 Paracrine factors

VEGF can also modulate the secretome of stimulated cells. We measured the effect of VEGF supplementation on paracrine factors in both fast degrading VPM and slow degrading GPQ gels using ELISA. Concentration of Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF), Angiogenin, Basic Fibroblast Growth Factor (FGFb), Placental Growth Factor (PlGF), Heparin Binding-EGF like Growth Factor (HB-EGF) and Epidermal Growth Factor (EGF) in d3 conditioned media obtained from neonatal hCPC encapsulating 5% PEG gels crosslinked with fast degrading VPM/slow degrading GPQ and with/without immobilized 5 ug/mL VEGF were measured using an ELISA array. As shown in Figure 12, VEGF supplementation did not have an effect on levels of secreted paracrine factors Angiogenin, HGF, bFGF, PlGF, EGF and HB-EGF. VEGF concentration in d3 conditioned media obtained from VEGF-PEG gels appears to be higher than gels without VEGF in case of both GPQ and VPM gels; the elevation in conditioned media VEGF on immobilization of VEGF to PEG gels appears to be higher in GPQ gels than VPM gels (RGD-VPM: 3806±430.5, RGD+VEGF-VPM: 7413±619.2, RGD-GPQ: 175±8.112, RGD+VEGF-GPQ: 8448±543.6 pg/mL). However, this experiment only consisted of 2 data points so statistical significance cannot be established.



pg/mL	HB-EGF	EGF
RGD-VPM	27.07	0.13
RGD+VEGF-VPM	21.93	0.72
RGD-GPQ	23.31	0.46
RGD+VEGF-GPQ	22.59	0.47

Figure 12: Secreted factors in conditioned media. Concentration of Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF), Angiogenin, Basic Fibroblast Growth Factor (FGFb), Placental Growth Factor (PlGF), Heparin Binding-EGF like Growth Factor (HB-EGF) and Epidermal Growth Factor (EGF) in conditioned media of neonatal hCPC encapsulating 5% PEG gels crosslinked with fast degrading VPM/slow degrading GPQ and with/without immobilized 5 ug/mL VEGF at d3 were measured using an ELISA array. Values are mean; n=2.

3.5 Discussion

The angiogenic, mitogenic and survival-enhancing effects of VEGF are well known. We therefore proposed and tested VEGF conjugated PEG hydrogels (made with 5% w/v PEG, 2 mM RGD, 10 million CPCs/mL, 0 or 5 ug/mL VEGF unless otherwise specified) for delivery of CPCs as a strategy to enhance CPC-based cell therapy. It has previously been shown that VEGF-165 conjugated to PEG-MAL gels retains its biological activity, and VEGF conjugated PEG gels enhance network formation in HUVECs [186]. We used ERK phosphorylation as a surrogate for assessing activation of VEGF-induced signaling because of its known participation in growth factor stimulation signaling pathways [187] and found that CPCs encapsulated in VEGF conjugated gels indeed show increased ERK phosphorylation.

Despite increased ERK activity, expected downstream outcomes (RNA and protein expression) as well as functional outcomes (tube formation) were not significantly altered. We tested commonly seen VEGF-stimulation effects including mRNA and protein expression of endothelial markers, and tube formation characteristics in the hydrogel. The PEG hydrogel was degradable and functionalized with RGD, which made it a suitable platform to test tube formation characteristics of CPCs in presence or absence of VEGF. We tested these features separately in rat (rCPCs) and human CPCs (hCPCs). Human CPCs comprised of a pool of cells obtained from 3 different neonatal (nhCPCs) or child (chCPCs) patients. Neonatal and child hCPCs were tested because of known dependence of age in regenerative potential of CPCs. We hypothesized that less regenerative child CPCs will benefit more from VEGF supplementation, as opposed to neonatal CPCs which are naturally more regenerative as has been seen in various injury models[64,188]. Despite the increased ERK phosphorylation (tested in rCPCs only), VEGF conjugated PEG gels did not show increased mRNA or protein expression of endothelial markers in either cell population. Additionally, VEGF conjugated PEG gels failed to show higher tube formation metrics than those without VEGF in rat or human neonatal CPCs. It is worth noting that we tested both low (100, 250 ng/mL) and high (5

μg/mL) doses of VEGF, and both doses had similar outcomes ruling out the possibility that the lack of response is due to low VEGF dose.

Even though neonatal and older child CPCs are known to exhibit different regenerative potentials, we did not observe any difference in their expression of endothelial mRNA and protein expression levels under VEGF stimulation. Computational analyses have previously shown the activity of angiogenic pathways in both neonate and children cells are similar[64]. It could be that the regenerative potentials of neonatal and child CPCs are differentiated by other mechanisms while their angiogenic capacities are similar. This could why we did not see any differences between the two cell populations in this context.

The gels we used in our study were functionalized with RGD, which has been shown to support angiogenesis. To isolate the effects of RGD and VEGF in inducing endothelial differentiation of CPCs, we tested RGD and scrambled rDg with and without VEGF in a 2x2 design. Neither VEGF nor RGD in isolation or in combination had an effect on mRNA expression of endothelial markers.

We observed that hydrogel density did have an effect on tube formation with lower density gels showing greater tube length when tested with 4, 5, 6% w/v gels. Moreover, both rat cells (Figure 6) as well as human cells (Figure 8(A)) showed greater elongation of CPCs, sprouting and formation of tip-like cells in lower density gels. The low density gels are more likely to be conducive for tube formation because of lower stiffness which supports endothelial differentiation [97] and higher porosity that allows cells to migrate and form tube networks[189]. Based on the observed favorability of low density gels for tube formation, we tested mRNA expression of endothelial markers following VEGF supplementation in lower density (4% w/v) gels. However, we failed to see an increase in mRNA expression of endothelial markers on VEGF supplementation in nhCPCs in the low density gels as well.

Concerned that the hydrogels may be degrading too fast to allow immobilized VEGF to interact with CPCs and stimulate them for enough duration to induce endothelial differentiation or modulate their paracrine factors, we tested effect of VEGF

supplementation in slow degrading ‘GPQ’ gels. However, VEGF supplementation did not have an effect on mRNA expression of endothelial markers, nor secreted paracrine factors including angiogenin, HGF, bFGF, PlGF, EGF and HB-EGF.

We saw that GPQ gels showed a trend towards higher mRNA expression than VPM gels with or without VEGF at day 2. GPQ gels also showed a trend for lower expression of growth factors VEGF, angiogenin, HGF, bFGF and PlGF. These observations could be due to the different mechanical properties of the GPQ and VPM gels and differing degradation rates. Matrix mechanics are known to affect the fate and modulate secretory profile of cells [115].

Collectively these experiments show that the presence of VEGF increased ERK activation in CPCs and the biomaterial construct may also have increased VEGF availability post-implantation, but despite these changes the CPCs failed to exhibit a categorical transition toward the endothelial lineage. To further explore this seemingly paradoxical behavior, we assessed the baseline expression of endothelial markers in our rat and human CPC populations by flow cytometry. The results showed that a large fraction (~90%) of the CPCs were basally positive for endothelial markers; however, this experiment is qualitative in nature and does not clearly establish whether the CPCs, although expressing endothelial markers in varying strengths, were sufficiently committed to the endothelial lineage. At the same time, this result does raise the possibility that our CPCs may not be amenable to further differentiation by VEGF treatment. High expression of endothelial markers by CPC populations has been reported by other groups as well [190–192]. Some recent genetic mapping studies have suggested that majority or all of the CPCs are cardiac vascular progenitors, and one study even suggests that these are mature endothelial cells [167,193]. A previous study reported an increase in endothelial gene expression in culturing CPCs in comparison with directly isolated cells [190], raising the possibility that our cultured cells may have differentiated to endothelial cells. It is also possible that VEGF secretion by CPCs in PEG gels (with rDg or RGD) is high enough to saturate signaling such that recombinant VEGF supplementation does not induce any additional effects.

Although ERK phosphorylation was significantly higher in VEGF-functionalized gels, it is a nonspecific downstream kinase whose activity is influenced by many stimuli and could have been activated by alternative pathways, such as a possible change in mechanical properties of PEG gels on VEGF immobilization [194]. It is also possible that the little or no expression of αvβ3 integrin on CPCs may be acting as a weak link in the VEGF signaling pathway. The cross-activation of VEGFR2 and β3 are important for mediating the mitogenic effects of VEGF [195]. Delivery of other growth factors like HGF and IGF with CPCs has shown to enhance their regenerative behavior *in vitro* and *in vivo* but we did not see an effect with VEGF. VEGF conjugated PEG-MAL gels have also shown regenerative effects in the context of MSCs and endothelial cells for bone repair [186] and cell free gels for cardiac repair [146]. In addition to some of the possible reasons inherently related to CPCs mentioned above, there may have been some study design flaws because of which we did not see interesting behaviors in our constructs. For example, potentially insufficient VEGF stimulation duration by an appropriate dose of VEGF, non-optimal cell density for VEGFR stimulation, neglecting to measure other possible outcomes such as proliferation etc. Further discussion of potential limitations and future directions can be found in Section 5.1.2.

There is great interest in cardiac stem cells for cardiac repair given their encouraging results in phase 1 clinical trials and ongoing phase 2 studies. However, our understanding of their mechanism of action and ways in which the benefits of CPCs can be enhanced is limited. In this project, behavior of CPCs encapsulated in VEGF functionalized hydrogels was studied with several combinations of cell populations, scaffold properties and growth factor dosage. Findings from this project add to our knowledge of behavior of CPCs in response to stimuli relevant to practical design of regenerative therapies and emphatically highlight both the pitfalls and potential avenues of further exploration for technologies and methods reliant on growth factor encapsulated CPCs for cardiac regeneration.

CHAPTER 4. INTEGRIN SPECIFIC HYDROGELS FOR ACTIVATION OF REGENERATIVE SIGNALING IN CARDIAC PROGENITOR CELLS

The objective of this study is to determine modulatory effects of integrin-specific hydrogels on encapsulated hCPCs *in vitro*, to study the effects of injecting these constructs on cardiac function *in vivo,* and to investigate the mechanism driving the observed effects.

4.1 Motivation

Integrins: Integrins are an important family of cell-surface-adhesion receptors that are involved in mediating cell adhesion with extracellular matrix proteins and other cells. They are heterodimers of non-covalently associated α and β subunits, each of which is a transmembrane glycoprotein and has a short cytoplasmic tail linked to intracellular cytoskeleton. 18 α subunits and 8 β subunits have been discovered and they can form 24 different heterodimeric structures. One ligand may interact with different integrin receptors and one integrin may recognize multiple types of ligands. The ligand-binding site forms in a region at the intersection of the integrin α-chain β-propeller and the βI domain, with the α chain being central in determining ligand specificity. Integrins with an αI domain bind ligands via the αI domain; however, since this ligand-binding leads to conformational changes in the I domain, it affects the conformation of the β subunit in turn as well [196]. Along with their critical role in adhesion, integrins also act as transducers of cellular environmental conditions as integrin engagement triggers intracellular signaling in cells, known as outside-in signaling. Ligation of integrins by ECM results in integrin clustering, followed by focal adhesion protein complex formation, actin polymerization and then actin-myosin stress fiber formation, ultimately providing rigidity to the cell and a mechanosensitive link between the extra- and intra-cellular environments. Adhesion strength, focal adhesion characteristics and type of integrins engaged are determinants of cell signaling which enable integrins to inform cells about

both the chemical and mechanical properties of the environment and modulate cell responses. Integrin adhesion triggered cellular responses are self-renewal, migration, survival, differentiation, and response to other inputs such as through growth-factor [186] or G-protein-coupled receptors. Integrins also participate in inside-out signaling in which conformation of their extracellular domains changes in response to intracellular signaling to regulate their affinity for extracellular ligands. Because of these regulatory abilities, integrins play a critical role in development, tissue maintenance and repair [197].

Integrin ligation mediated differentiation induction: 2D or 3D interaction with extracellular matrix can lead to induction of differentiation of stem or progenitor cells via integrin-mediated signaling in a substrate chemical composition, mechanical properties and topography dependent manner. Integrin expression also changes during cell differentiation and maturation. Embryonic stem cells cultured on combinatorial matrix composed of fibronectin and laminin differentiated into cardiomyocytes, with the greatest differentiation efficiency of ~75% achieved at 70:30 composition. The gene expression of β4 and β5 integrin subunits increased in cells differentiated by fibronectin + laminin or gelatin treatment and differentiation was inhibited by blocking either or both β4 and β5 integrin subunits [198]. Encapsulating ESC embryoid bodies in decellularized porcine ECM and collagen I combination hydrogels in hypoxic conditions promoted differentiation into contracting cardiomyocytes with striations. Extent of differentiation achieved using 75:25 ECM and Col I gels was comparable to that achieved with Col I supplemented with VEGF and DKK-1 (Dickopf related protein 1) [199]. Cardiomyoblast cells differentiated into cardiomyocytes on fibroblast-derived matrix (FDM) consisting of fibronectin, collagen and laminin. Differentiation efficiency was increased on stiffer crosslinked form of the matrix (Y= 8 kPa, 10X higher than uncrosslinked matrix) and was associated with a decrease in vinculin area and α5 mRNA expression [200]. Collagen-β1 interaction is necessary for cardiac differentiation of miPSC-derived embryoid bodies [201]. Fibronectin has been shown to promote mesodermal differentiation via β1 integrin by activating the Wnt/β-catenin pathway [202]. Differentiation of myoblasts on fibronectin was inhibited when α5-RGD interaction was blocked. Percentage of differentiated cells

and proliferation were a function of substrate conformation as well; fibronectin adsorbed on bacterial polystyrene, tissue culture polystyrene, and collagen demonstrated differences in fibronectin conformation [203].

Integrins in cell therapy: Integrin mediated signaling induced cell responses such as differentiation, growth, proliferation and survival have been implicated in cell therapy. Circulating angiogenic cells co-delivered with collagen matrix improved engraftment of delivered cells and led to enhanced myocardial function, perfusion and infarct reduction as compared to collagen or cells alone groups. These *in vivo* effects were blocked when cells with blocked α2 integrins were used in animal studies and cell adhesion, proliferation, and paracrine factors were found to decrease in *in vitro* studies. α5 was found to be necessary for the angiogenic potential of circulating angiogenic cells on collagen [204]. Sca-1^{+}/Lin^{-} hematopoietic progenitor cells isolated from β2-integrin–deficient mice were found to have lowered homing ability to sites of ischemia and of improving neovascularization when injected in mice with hind limb ischemia. Pre-activating β2-integrins expressed on endothelial progenitor cells by activating antibodies prior to transplantation in animals led to enhanced homing and neovascularization *in vivo* [205]. Blockade of β2-integrins in exogenously delivered EPCs significantly reduced their engraftment, homing to ischemic myocardium and ability to preserve cardiac function following myocardial infarction [206]. High-mobility group box 1 (HMGB1) released extracellularly on cell necrosis and tissue damage stimulates migration of EPCs on fibronectin and fibrinogen. Blocking β1 and β2 integrins on EPCs reduces this migration and pre-stimulating EPCs with HMGB1 improves their homing and adhesion by increasing integrin affinity and polarization [137]. Overexpressing integrin lined kinase (ILK) in MSCs [138,139] or Sca1+ [140] cardiac progenitor cells before transplantation enables them to better preserve cardiac function, reduce fibrosis and increase angiogenesis in the host after MI. ILK overexpressing cells exhibited better viability, migration, proliferation, survival, adhesion to myocardium *in vitro* and greater retention in the peri-infarct area 3 days later, but not 4 weeks after MI *in vivo*.

RGD: The tripeptide Arg-Gly-Asp (RGD) was first identified as a cell adhesion motif within fibronectin. Since then the RGD motif has been found in many other proteins including fibrinogen, vitronectin, osteopontin etc. and supports cell adhesion in many, but not all proteins such as collagen and laminin because of inaccessibility or unsuitable conformation. A subset of the integrins recognize the RGD motif including all five αV integrins, three β1 integrins (α5, α8, α3), and αIIbβ3 with varying affinities [96,207]. RGD binds at an interface between the α and β subunits- the R residue fits into a cleft in a β-propeller module in the α subunit, and the D coordinates a cation bound in a von Willebrand factor A-domain in the β subunit [208]. RGD has been found to be effective at promoting the attachment of numerous cell types with many diverse materials *in vitro.* *S*ynthetic RGD peptides have been used to support cell adhesion on natural-derived [93,209] and synthetic biomaterials including PEG-MAL hydrogels [100,210,211]. RGD concentrations ranging from 25 μM to 3.5 mM have been used with PEG hydrogels [100]. Ligation of RGD to β1 integrin on pancreatic islet cells enhanced cell survival via Akt phosphorylation although this effect was not RGD specific and was induced with anti-β1 antibodies as well [212,213]. Spacing of RGD ligands influences cell spreading, migration, efficiency of integrin activation, proliferation and stem cell differentiation [214,215]. RGD-integrin ligation triggered signaling acts synergistically with other signaling processes such as stem cell differentiation in the presence of differentiation cues [216,217]. RGD conjugated PEG-MAL hydrogels have been used to deliver HGF and VEGF to infarcted hearts [146]. Their simplicity, low cost, ability to maintain functionality after manufacturing and be coupled to biomaterials in a controlled manner make RGD peptides attractive for clinical translation [96]. *In vivo*, RGD conjugated biomaterials have produced mixed results with some resulting in lending improvement [218–220] and some showing no additive benefit [146,194,221–224] in various tissue engineering applications including cardiac tissue engineering.

GFOGER: GFOGER is a triple helical collagen-mimetic peptide containing the hexa-peptide sequence GFOGER and the full sequence of synthetic peptide used is $GGYGGGPC(GPP)_5GFOGER(GPP)_5GPC$ [225]. GFOGER sequence is present in

fibrillar collagens and is recognized by α1β1, α2β1 and α10β1 integrins [226]. Three loops on the upper surface of the I domain that coordinate a metal ion also engage the collagen, with a collagen glutamate completing the coordination sphere of the metal. The change in metal coordination leads to a reorganization of the upper surface and together they create a complementary surface for binding collagen. Conformational changes affect the opposite pole of the domain as well [227]. Recognition of this sequence by integrins found to be dependent on the triple helical structure by amino acid substitution experiments [228] and the synthetic peptide has been shown to maintain that structure at physiological temperatures by circular dichroism studies [229]. It also remains active following reaction with PEG-MAL [186,230]. GFOGER has shown notable effects in orthopedic tissue engineering applications. GFOGER stimulation induces osteoblastic differentiation of immature osteoblast-like MC3T3-E1 cells like collagen I *in vitro* [231] and accelerates bone formation in non-healing critical size bone defects [186,225,230]. GFOGER presenting degradable PEG hydrogels exhibited greater proliferation, and chondrogenic differentiation of MSCs in presence of chondrogenic media than RGD presenting unmodified PEG gels [232].

4.2 Hypothesis

Implanting numerous cell types with collagen [199,204,233,234] or collagen alone [235,236] results in improved cardiac function after MI. Similarly, cell delivery with fibrin enhances their reparative effects. The improvement in cardiac function noted on delivering collagen-chondroitin sulfate matrix plus circulating angiogenic cells (CACs) to infarcted mice hearts was negated (LVEF: ~61% vs ~45%) when α2β1 integrin was blocked on the cells. Reduction in cell proliferation and paracrine factors secretion was found *in vitro* following the integrin blocking. These observations point toward the role of α2β1 integrin in matrix-CAC interaction and synergistic effects. Blocking α5 integrin reduced the angiogenic potential, proliferation and angiogenic potential of CACs [204]. ESCs differentiate into cardiomyocytes on ascorbic acid treatment through a required step of collagen synthesis [237]. CPCs cultured on collagen show increased expression

of connexin43 suggesting they may be driven to cardiomyocyte lineage [238]. Culturing CPCs on fibronectin induces their proliferation and protection via β_1-integrin-focal adhesion kinase-signal transducer [238,239]. GFOGER-α2β1 and RGD-α5β1 ligation are prominent cell interaction mechanisms with collagen and fibronectin, fibrin etc. We hypothesized that α2β1 integrin stimulation of CPCs by encapsulating CPCs in GFOGER presenting PEG gels will drive their differentiation and enhance their cardiac repair potential; α5β1 integrin stimulation of CPCs by encapsulating CPCs in RGD presenting PEG gels will enhance their proliferation and angiogenic potential, and thereby enhance their cardiac repair potential. In addition to testing the proposed therapeutic strategy, this study would provide a controlled experimental setup to investigate the effects of specific integrin stimulation on CPCs *in vitro* and integrin specific hydrogels as delivery vehicles *in vivo*.

4.3 Approach

PEG hydrogels functionalized with α2β1-specific GFOGER, α5β1- and αvβ3-specific RGD or non-adhesive rDg ligands, and encapsulating hCPCs were synthesized. Figure 13 shows a schematic detailing the synthesis procedure. Basal integrin mRNA and surface protein expression on CPCs and rheological properties of hydrogels were characterized. mRNA and protein expression of lineage markers to assess differentiation of CPCs and concentration of secreted factors *in vitro* following encapsulation in integrin-specific or non-adhesive control gels were measured. *In vivo* measurement of effects of these constructs on cardiac function in an ischemia-reperfusion model and *ex vivo* analyses cardiac fibrosis, angiogenesis and hypertrophy were performed. Neonatal human CPCs were used, ‘rDg/RGD/GFOGER gels’ stand for 5% PEG gels presenting the specified ligand and 10 m CPCs/mL hydrogel unless otherwise specified.

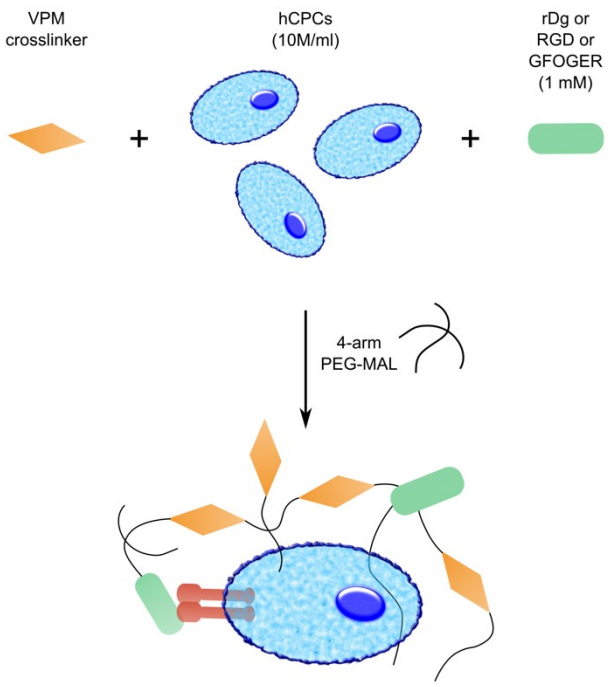


Figure 13: Synthesis of integrin-specific or non-adhesive PEG hydrogels. A solution of VPM crosslinker, cell suspension and integrin specific (RGD, GFOGER) or non-adhesive (rDg) ligands was prepared. The gels were crosslinked by mixing with 4-arm PEG-MAL macromer solution.

4.4 Results

4.4.1 Integrins expressed by human CPCs

In order to assess the availability of integrin receptors as first point of contact with integrin specific hydrogels, mRNA expression of relevant integrins and protein expression on surface of hCPCs were measured. GFOGER is known to interact with α1β1, α2β1, α10β1 (found primarily in cartilage) and α11β1. RGD is a ligand for αvβ1, αvβ3, αvβ6, αvβ5, α5β1, α8β1, α11β3, α3β1 integrins. α2β1 and αvβ3/α5β1 are the most relevant integrins for GFOGER and RGD stimulated signaling, respectively. A pool of hCPCs from three neonatal patients at passages ranging from P3-10 was tested. mRNA expression of α1, α2, α3, α5, α8, α10, α11, αV, β1, β3 and β5 subunits was measured using real-time PCR. A standard curve was prepared using human LV atrial tissue total mRNA to measure mRNA copy numbers. Values shown in Figure 14(A) are mean mRNA copies per million copies of GAPDH ± SEM;n=4-7. Protein expression of α1, α2, α3, α4, α5, αV, β1, β2, β3, β4, β6, αVβ5 and α5β1 subunits/integrins was measured using an ELISA array based on colorimetric detection and the results are shown in Figure 14(B). Values are mean OD_{550nm} ± SEM after subtracting negative control (non-antibody lined plate wells) values; n=4. Absolute values of integrins expressed on surface could not be measured because of lack of a standard curve. CPCs show prominent expression of β1 at both mRNA (2.9±1.3 $X10^5$ copies /million copies of GAPDH) and surface protein level. They also express α2, αV and α5, integrins relevant to RGD and GFOGER ligands. The expression level of β3, an important ligand for RGD binding was below detection level in assay of both mRNA and surface protein.

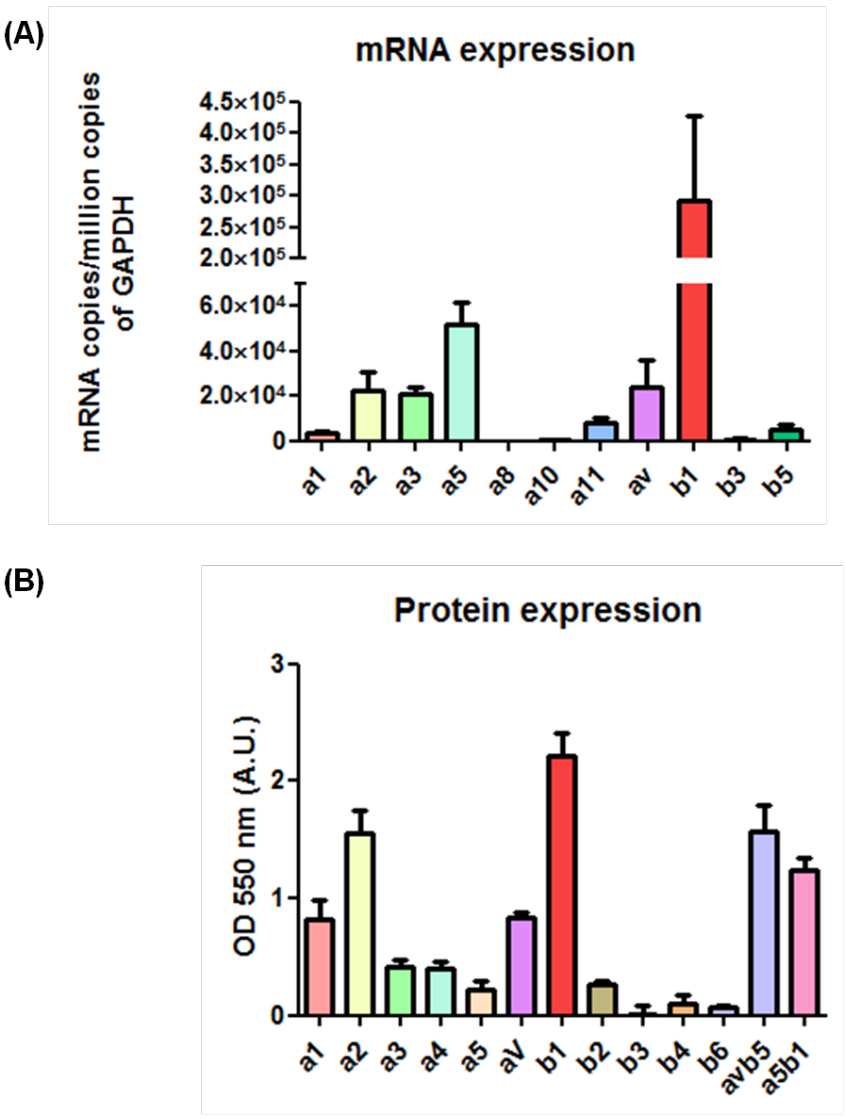


Figure 14. mRNA and protein expression of integrins in unstimulated hCPCs. (A) mRNA and (B) protein expression in hCPCs of integrin subunits involved in adhesion to RGD and GFOGER molecules were measured. A pool of hCPCs from three neonatal patients at passages ranging from P3-10 was tested. (A) mRNA expression of α1, α2, α3, α5, α8, α10, α11, αV, β1, β3 and β5 subunits was measured using real-time PCR. A standard curve was prepared using human LV atrial tissue total mRNA to measure mRNA copy numbers. Values are mean mRNA copies per million copies of GAPDH ± SEM; n=4-7. (B) Protein expression of α1, α2, α3, α4, α5, αV, β1, β2, β3, β4, β6, αVβ5 and α5β1 subunits/integrins was measured using an ELISA array based on colorimetric detection. Values are mean OD_{550nm} ± SEM after subtracting negative control (non-antibody lined plate wells) values; n=4.

4.4.2 Mechanical characterization of PEG-MAL hydrogels

Mechanical properties of biomaterials are known to influence stem cell differentiation and therefore the mechanical properties of these hydrogels would impact the fate of transplanted cells as well as host progenitor cells that migrate to these hydrogels *in vivo*. In addition, biomaterials are thought to dissipate wall stresses in infarcted hearts for which their mechanical properties are relevant as well. Rheological characterization of hydrogels was performed to measure their mechanical properties because of their viscoelastic nature. Storage modulus (G′) measures the deformation energy stored during imparted stress on the hydrogel, representative of the stiffness of the material and loss modulus (G″) is representative of the energy dissipated during shear, i.e. the flow or liquid-like response of the material [240]. PEG-MAL hydrogels of 4% or 5% w/v densities, and immobilizing 1 mM rDg, RGD or GFOGER ligands were prepared and allowed to swell overnight in PBS. The linear viscoelastic region was determined by performing amplitude sweeps. Oscillatory frequency sweeps were performed in the linear viscoelastic region and obtained storage (G') (Figure 15(A)) and loss moduli (G'') (Figure 15(B)) values were averaged. Comparing density matched groups with different ligands, the storage moduli of rDg and RGD gels were similar (4%rDg: 48±8.72, 4%RGD: 55.7±7.54, 5%rDg:86.34±7.7, 5%RGD: 83.73±5.27 Pa; differences non-significant between density matched rDg and RGD gels) but these were significantly different ($p<0.05$, n=6-8) than GFOGER gels (4%GFOGER: 87.81±7.6, 5%GFOGER: 119.7±3.352 Pa). Except for RGD, differences between means of 4% and 5% w/v gels for matched ligands were also significant ($p<0.05$). G'' was found to be comparable between all groups with the differences between means to be non-statistically significant. G'' values were approximately 4 Pa.

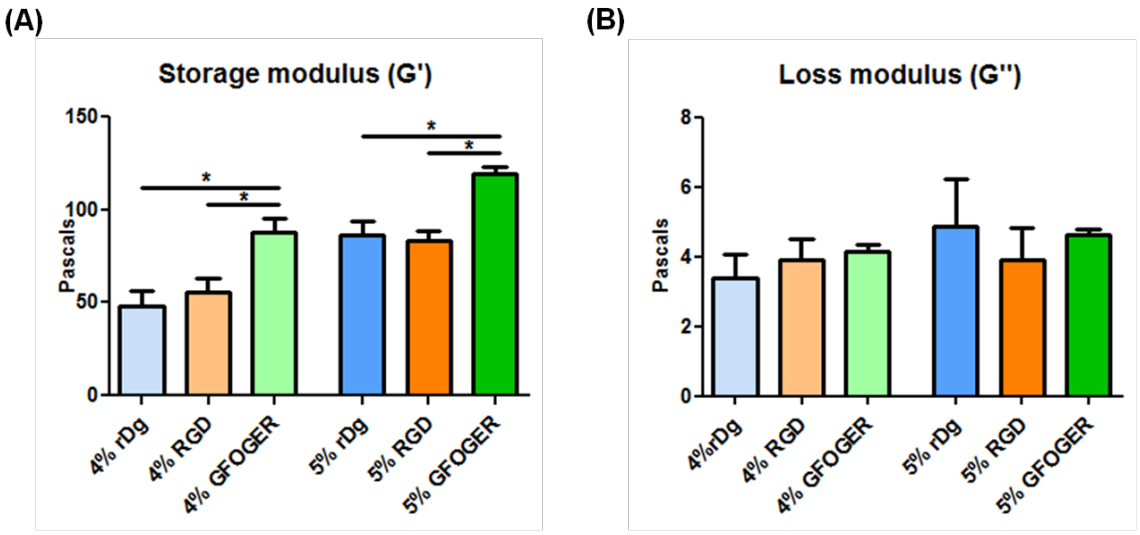


Figure 15: Rheological characterization. (A) Storage moduli (G') and (B) loss moduli (G'') of PEG-MAL hydrogels of 4% or 5% w/v densities, and immobilizing 1 mM rDg, RGD or GFOGER ligands were measured using rheology. Hydrogels were prepared and allowed to swell overnight before performing rheology. The linear viscoelastic region was determined by performing amplitude sweeps and values obtained in the linear viscoelastic region during frequency sweep were averaged. Values are mean ± SEM; statistical significance of difference between means was measured using ANOVA and Tukey's post-hoc test, *: $p<0.05$; n=6-8.

4.4.3 Expression of lineage markers in encapsulated CPCs

In order to assess the integrin-specific gels to induce differentiation of CPCs via integrin signaling activation, a screen of mRNA expression of several lineage markers for cardiomyocyte, endothelial and vascular smooth muscle was performed (Figure 16). Protein expression of cardiomyocyte markers and cardiac transcription factor were also measured following findings of cardiomyocyte differentiation at mRNA level (Figure 17).

Cellular hydrogels were homogenized in Trizol to isolate RNA. mRNA expression of genes of various lineages 2 days post-encapsulation were measured using real-time PCR using primers for *flt1* (VEGF Receptor 1)*, cdh5* (VE-Cadherin), *pecam1* (Pecam-1), *vwf* (von Willebrand Factor), *tagln* (Transgelin), *acta2* (Aortic smooth muscle actin), gata4 (GATA binding protein 4), *nkx2-5* (NK2 Homeobox 5), *mef2c* (Myocyte enhancer factor 2C), *myl2 (Myosin light chain 2)*, *tnni3* (Cardiac type Troponin I3), *myh6* (Myosin heavy chain 6), *tnnt2* (Cardiac type Troponin 2), *myh7* (Myosin heavy chain 7) were measured. mRNA expression of cardiac transcription factors *nkx2_5* and *mef2c*, cardiomyocyte specific structural proteins *myh6, myh7, myl2, tnnt2, ctnni3* and vascular smooth muscle and cardiomyocyte marker suggested to be involved in calcium interactions and contractile properties of the cell [241], *tagln* was upregulated in GFOGER gels 2 days after encapsulation. No significant differences between groups were found in other markers tested (data not shown), suggesting induction toward cardiomyocyte lineage in GFOGER gels.

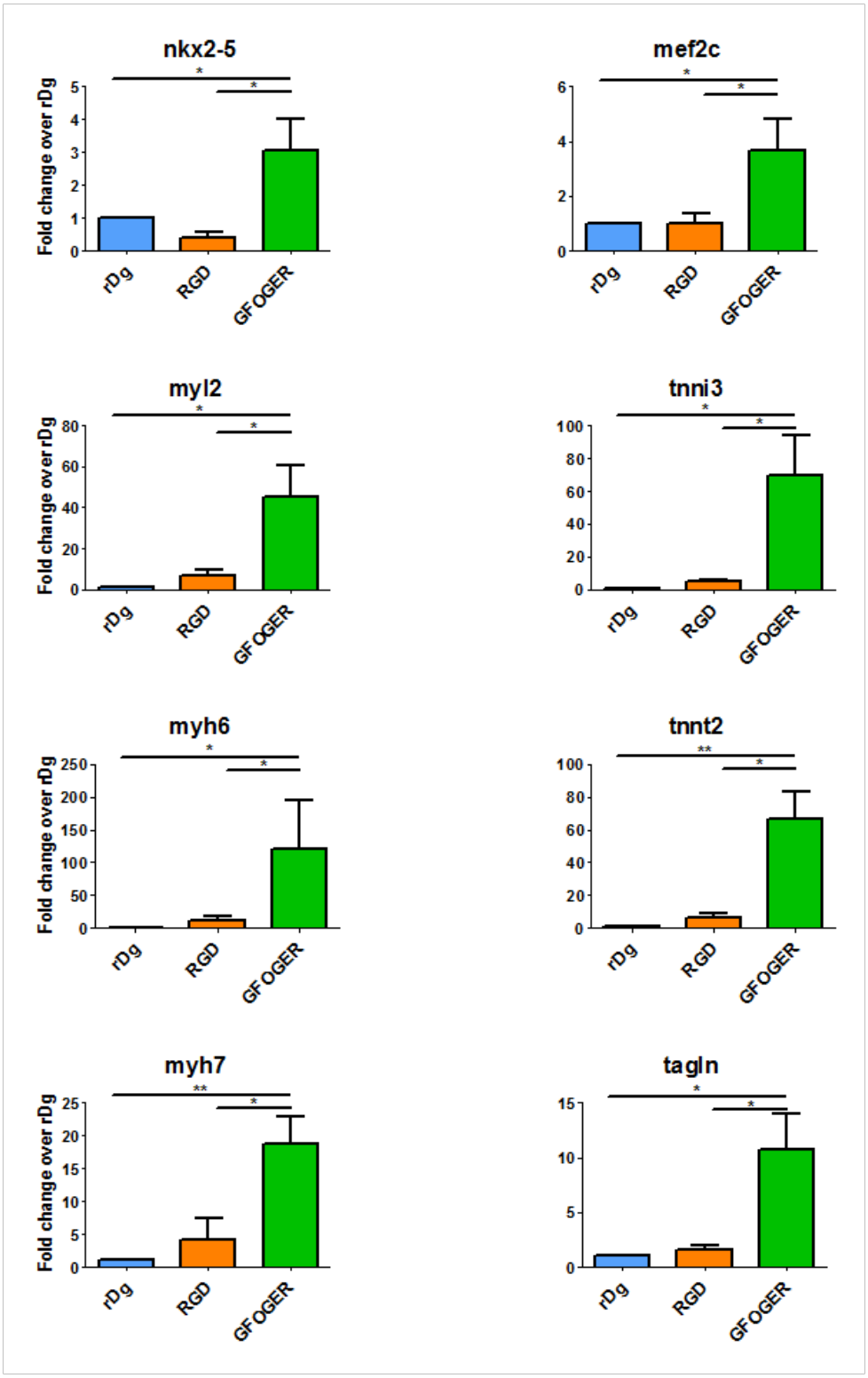


Figure 16: mRNA expression in encapsulated hCPCs. mRNA expression of cardiomyocyte lineage marker genes (encoding protein) *nkx2-5* (NK2 Homeobox 5), *mef2c* (Myocyte enhancer factor 2C), *myl2 (Myosin light chain 2)*, *tnni3* (Cardiac

type Troponin I3), *myh6* (Myosin heavy chain 6), *tnnt2* (Cardiac type Troponin 2), *myh7* (Myosin heavy chain 7), *tagln* (Transgelin/Smooth muscle protein 22-alpha) was measured 2 days after encapsulating hCPCs in PEG-MAL gels immobilizing 1mM rDg, RGD or GFOGER ligands using real-time PCR. Data are expressed as fold change over rDg control. Values are mean ± SEM; statistical significance of difference between means was measured using ANOVA and Tukey's post-hoc test, *:$p<0.05$, **:$p<0.001$; n=3-4.

For protein expression, hydrogels were degraded by incubation with Collagenase I to release the cells which were then lysed using NP-40. The solublized membrane and cytoplasmic fractions of cell lysate were run separately on SDS-PAGE gels. Protein expression of cardiac type troponin I and myosin heavy chain (MHC) was assessed 5 days after encapsulating hCPCs in PEG-MAL gels immobilizing 1mM rDg, RGD or GFOGER ligands by Western blotting. Proliferating cell nuclear antigen (PCNA) and Beta-actin (β-Actin) were used as loading controls for membrane fraction and cytoplasmic fraction respectively. n=4. Western blotting results show CPCs obtained from GFOGER gels 5 days after encapsulation expressed cardiomyocyte specific Troponin I and Myosin Heavy Chain, whereas those from rDg and RGD gels did not. Early cardiac transcription factor Nkx2.5 expression levels were similar between different groups (data not shown).

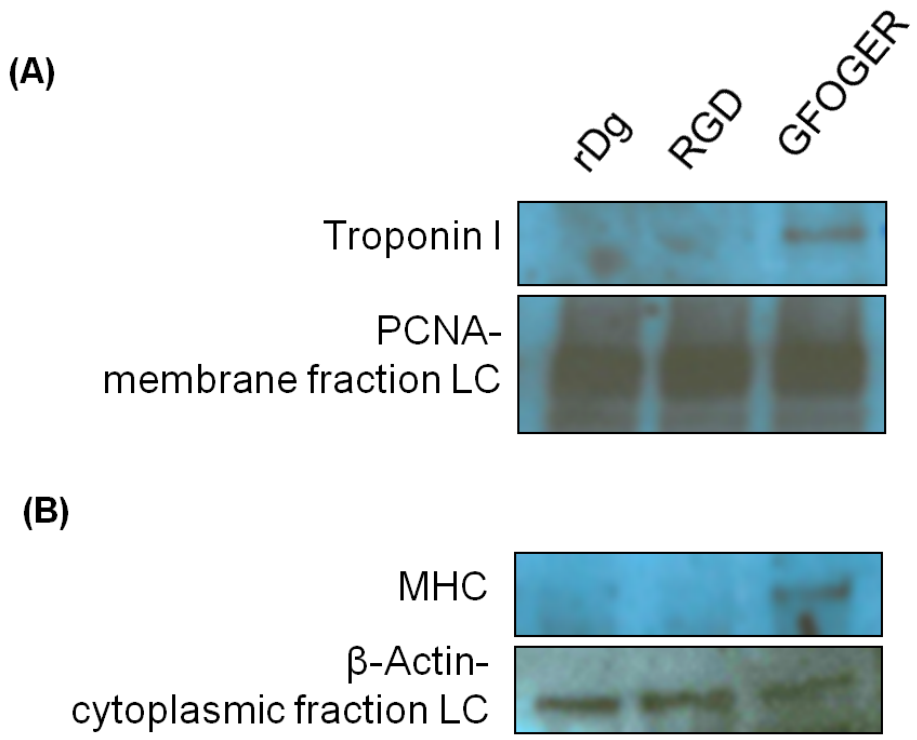


Figure 17: Protein expression in encapsulated hCPCs. Protein expression of (A) Cardiac type Troponin I and (B) Myosin heavy chain (MHC) was assessed 5 days after encapsulating hCPCs in PEG-MAL gels immobilizing 1mM rDg, RGD or GFOGER ligands by Western blotting. The solublized membrane and cytoplasmic fractions of cell lysate were run separately on SDS-PAGE gels. Proliferating cell nuclear antigen (PCNA) and Beta-actin (β-Actin) were used as loading controls for membrane fraction and cytoplasmic fraction respectively. n=4.

4.4.4 Paracrine factors

Paracrine factors have been shown repeatedly to be a very important mode by which stem cells including CPCs exert their effects [45]. The modulation of paracrine factors by integrin-specific specific hydrogels was tested by measuring concentrations of several analytes in conditioned media obtained at d2 and d5. Concentration of Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF), Basic Fibroblast Growth Factor (FGFb), Matrix Metalloproteinase 9 (MMP9), Matrix metalloproteinase 2 (MMP2), Interleukin 10 (IL10), platelet derived growth factor-BB (PDGF-BB), Stromal cell-derived factor 1 (SDF1) in conditioned media at d2 and d5 were measured using a Luminex bead-based multiplex assay. Obtained individual protein concentrations were normalized for sample loading by dividing by total protein in conditioned media. Normalization by total protein concentration of cells secreting these molecules was also computed (not shown) and similar trends were observed. As shown in Figure 18, the levels of paracrine factors VEGF, HGF and FGFb were significantly lower in cellular GFOGER gels' conditioned media as compared to that from rDg and RGD at both d2 and d5. rDg and RGD had similar levels of these factors. IGF-1, MMP2, MMP9, IL10, PDGF-BB and SDF1 concentrations were outside the range of detection. Mean±SEM values are as follows: d2-rDg-VEGF:1325±98, d2-RGD-VEGF: 1562±109, d2-GFOGER-VEGF: 27±2, d5-rDg-VEGF:2381±168, d5-RGD-VEGF: 2692±205, d5-GFOGER-VEGF: 75±22 pg/mg; d2-rDg-HGF:405±42, d2-RGD-HGF: 337±50, d2-GFOGER-HGF: n.d., d5-rDg-HGF:792±68, d5-RGD-HGF: 628±106 pg/mg, d5-GFOGER-HGF: n.d.; d2-rDg-FGFb:1439±287, d2-RGD-FGFb: 751±286, d2-GFOGER- FGFb:834±82, d5-rDg-FGFb: 1979±250, d5-RGD-FGFb: 2238±412, d5-GFOGER- FGFb: 569±132 pg/mg.

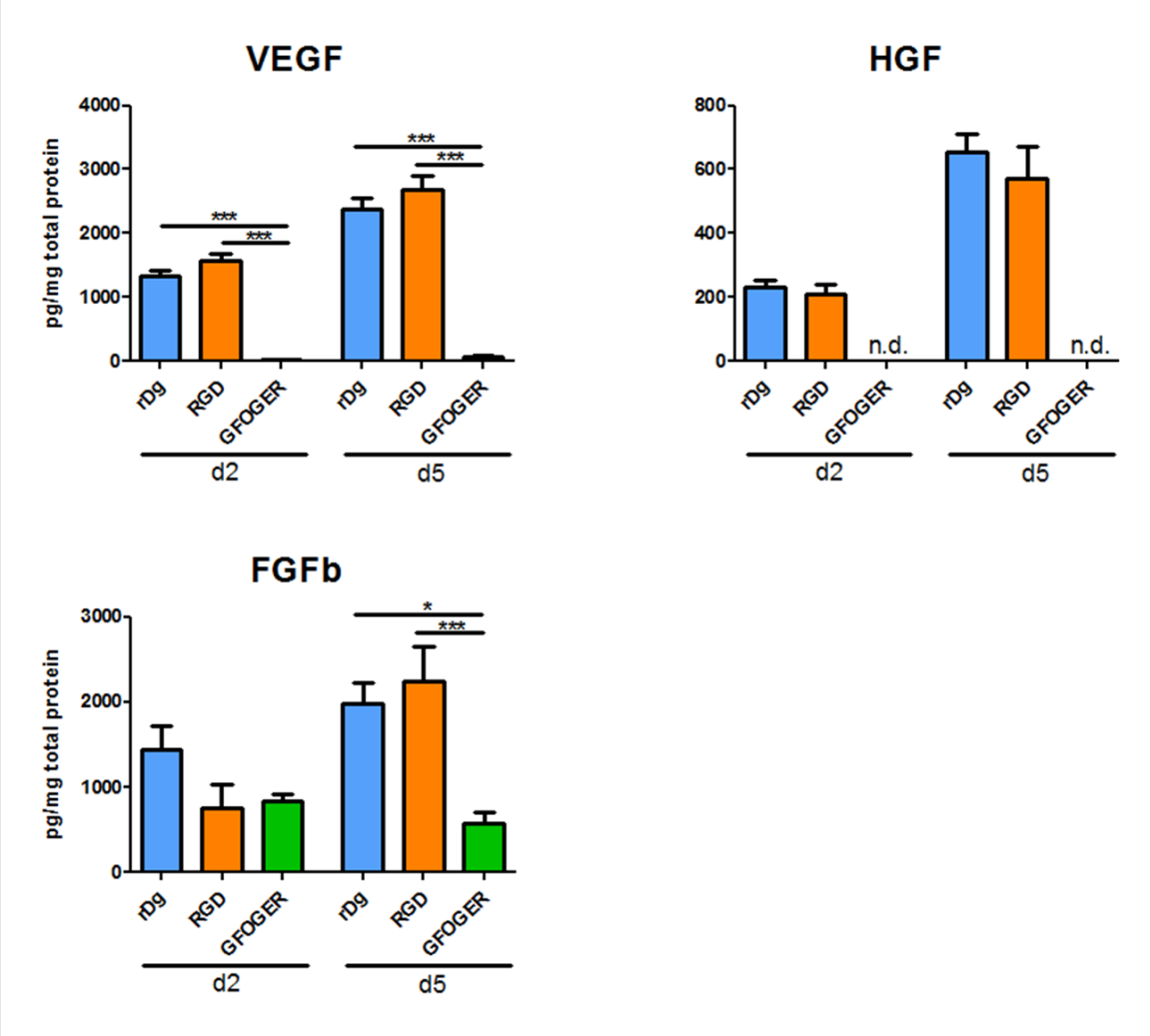
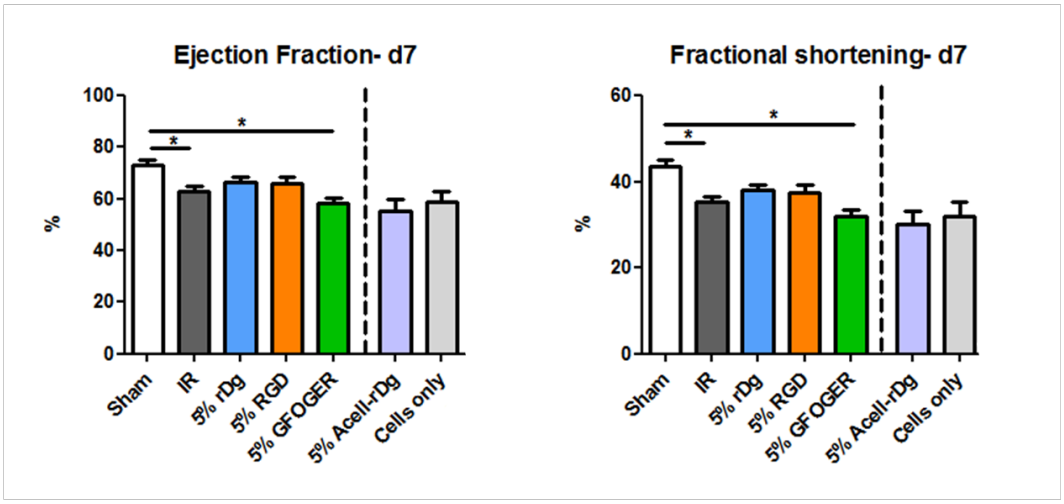


Figure 18: Secreted factors in conditioned media. Concentration of Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF) and Basic Fibroblast Growth Factor (FGFb) in conditioned media at d2 and d5 were measured using a Luminex bead-based multiplex assay. Obtained individual protein concentrations were normalized for sample loading by dividing by total protein in conditioned media. Values are mean ± SEM; statistical significance of difference between means was measured using ANOVA and Tukey's post-hoc test, *: $p<0.05$, **: $p<0.001$; n=4-8.

4.4.5 Cardiac function

Studies in *in vivo* models are critical expected performance of a cell therapy strategy in patients. We used nude rats in order to perform studies with human cells. 6-8 week old athymic rats (Crl:NIH-*Foxn1rnu*) underwent ligation of left anterior descending coronary artery for 30 minutes followed by reperfusion. Treatments were injected immediately after reperfusion. Echocardiography was performed at d7 and d28 after surgery and the results are presented in Figure 19. 7 days after surgeries, animals treated with cellular GFOGER gels showed a significant reduction in EF (58.15±2.39%) and FS (31.81±1.64) in comparison to sham animals (EF: 73.12±2.06, FS: 43.49±1.75%) ($p<0.05$). Cellular rDg (EF: 66.67±1.66, FS: 37.96±1.36%) and RGD (EF: 66.02±2.30, FS: 37.57±1.84%) groups show a trend of slightly better function than IR (ischemia-reperfusion) only group (EF: 62.88±1.88, FS: 35.25±1.27%) that did not receive any treatment, although the difference between means are not statistically significant. But EF and FS values in rDg and RGD groups at d7 are not significantly inferior to the sham group either. At d28, cellular rDg group EF (72.21±2.26%) and FS (43.03±2.10%) values were significantly higher than IR group (EF: 63.30±2.84, FS: 34.38±2.40%) ($p<0.05$). RGD (EF: 50.65±7.76, FS: 27.10±4.85%) and GFOGER (EF: 49.91±4.82, FS: 27.91±3.88%) groups showed inferior function and their EF and FS values were significantly lower than those of sham animals (EF: 77.92±1.9, FS: 48.19±1.86%). Controls acellular rDg (d7-EF: 55.40±4.53, d7-FS: 30.14±3.05%, d28-EF: 46.72±6.83, d28-FS: 24.98±4.55%), cells only groups (d7-EF: 59.06±3.61, d7-FS: 31.88±3.50%, d28-EF: 61.02±4.12, d28-FS: 35.39±3.29%) and 4% GFOGER (d28-EF: 61.02±4.12, d28-FS: 35.39±3.29%) showed lower means than sham group at both time points.

(A)



(B)

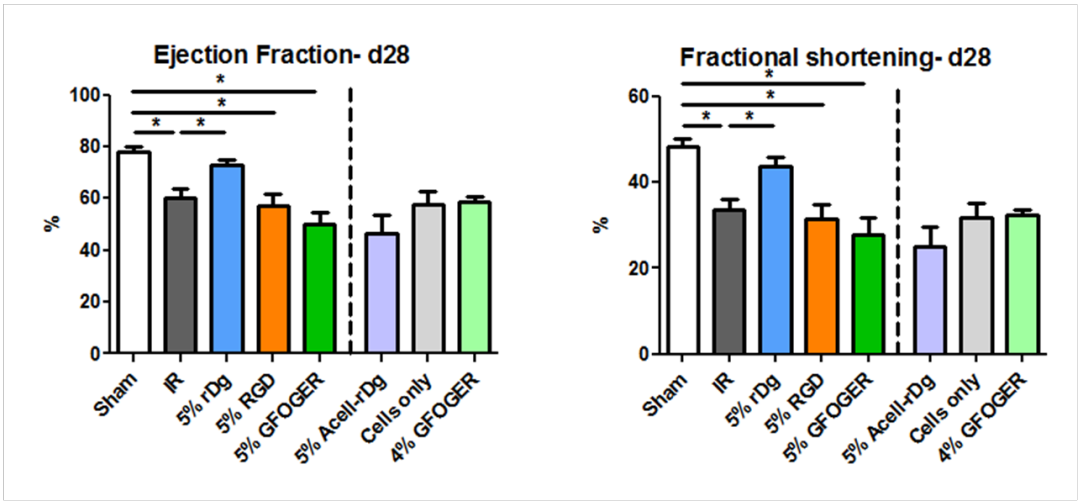


Figure 19: Cardiac function following treatment with integrin-specific hydrogels. Ejection fraction and fractional shortening were obtained using M-mode echocardiograms of rat hearts (A) 7 days and (B) 28 days following treatment. Values are mean ± SEM; statistical significance of difference between means was measured using ANOVA and Dunnet's post-hoc test comparisons with Sham and IR groups, *: $p<0.05$; $n\geq3$ for 4% GFOGER, Acell-rDg, IR (d7) and $n\geq5$ for other groups.

4.4.6 Histological evaluation

To understand the mechanism behind observed cardiac performance following various treatments, histological evaluation of fibrosis, angiogenesis, hypertrophy and engraftment of transplanted cells were done. Formalin fixed paraffin embedded hearts from rats sacrificed 28 days following treatment were stained with different stains- picosiruius red for fibrotic area measurement; fluorescence labeled-isolectin for angiogenesis; fluorescence labeled-wheat germ agglutinin for cross-sectional area to determine hypertrophy and human-specific anti-mitochondria (MTCO2) antibody for measuring engraftment of transplanted human cells.

For measurement of fibrotic area, picosiruius red staining which stains collagen red was performed. Percentage of scar tissue stained dark red was measured. As shown in Figure 20, the percentage of scar tissue in cellular rDg gel group (9.45±0.92%) was significantly lower than IR only group (28.14±2.32%) ($p<0.05$). The means of fibrotic area in other treatments did not were not significantly different from IR only group. %Scar area in other groups: RGD: 32.51±6.65%, GFOGER: 34.96±2.02%, Sham: 4.48±0.88%. Representative picosiruius red stained heart sections for all groups are shown in Figure 20(B).

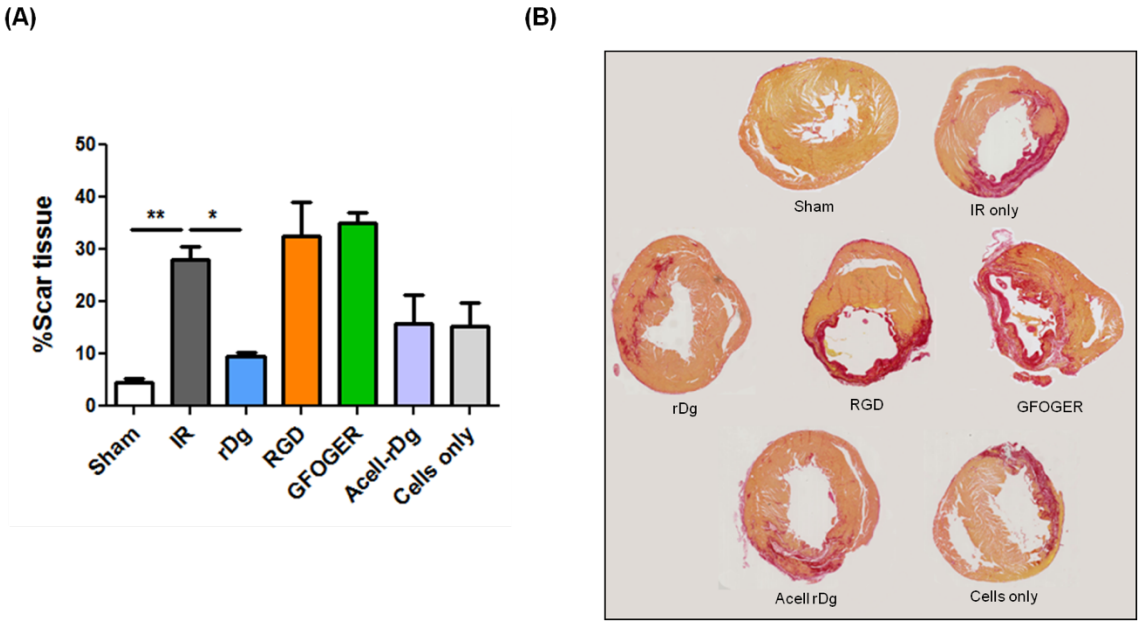
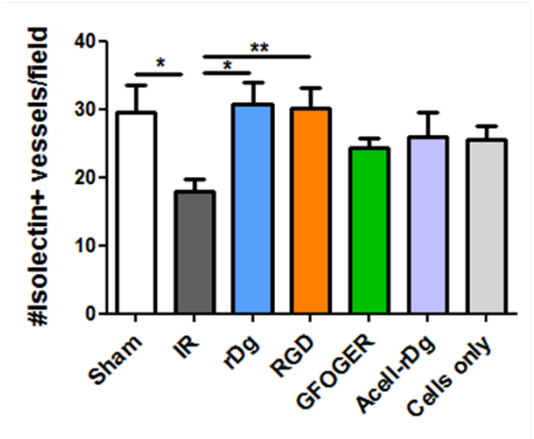


Figure 20: Fibrosis in rat hearts following treatment with integrin-specific hydrogels. Formalin fixed paraffin embedded hearts from rats sacrificed 28 days following treatment were stained with picosiruius red. (A) Percentage of scar tissue stained dark red was measured using Aperio ImageScope software. Values are mean ± SEM; statistical significance of difference between means was measured using ANOVA and Tukey's post-hoc test, *: $p<0.05$; $n \geq 3$ for GFOGER, Acell-rDg, Cells only and $n \geq 5$ for other groups. (B) Representative picosiruius red stained heart sections for all groups.

Angiogenesis was measured by staining sections with isolectin that stains rodent endothelial cells and other cells expressing terminal α-galactosyl residues. Three images per section in the infarct border region were captured and number of isolectin-positive vessels per field was counted. Both rDg (30.75±3.09/field) and RGD (30.21±2.834/field) groups showed higher number of isolectin-positive vessels than IR only group (17.94±1.89/field) at d28 ($p<0.05$), but mean of GFOGER group (30.21±2.83/field) was not significantly different from IR group. Average number of isolectin+ve vessels/field ±SEM for other groups were Sham: 29.50±4.0, acell-rDg: 25.86±3.74, cells only: 25.49±2.016 vessels/field. A representative isolectin-IB4 stained infarct border region micrograph highlighting some examples of isolectin-positive vessels marked by arrows is presented in Figure 21(B).

(A) (B)



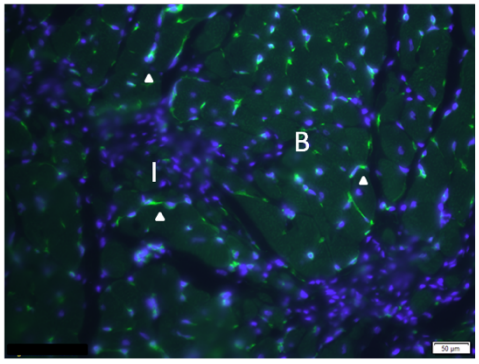


Figure 21: Angiogenesis following treatment with integrin-specific hydrogels. Formalin fixed paraffin embedded hearts from rats sacrificed 28 days following treatment were stained with fluorescent molecules tagged isolectin-IB4. (A) Three images per section in the infarct border region were captured and number of isolectin-positive vessels per field was counted. Values are mean ± SEM; statistical significance of difference between means was measured using ANOVA and Tukey's post-hoc test, *:$p<0.05$, **:$p<0.001$; $n\geq3$ for GFOGER, Acell-rDg, Cells only and $n\geq5$ for other groups. (B) Representative isolectin-IB4 stained infarct border region micrograph shows some isolectin-positive vessels, I: Infarct, B: Border; scale bar: 50 μm.

To determine hypertrophy of cardiomyocytes, cross-sectional areas were measured by tracing cell membranes stained by fluorescently-tagged wheat germ agglutinin. One image per section in the infarct border region was captured and the cross-sectional area of 7-10 randomly selected myocytes in the infarct border zone were recorded as shown in Figure 22(B). Figure 22(A) demonstrates that the cross-sectional area of myocytes in rDg treated animals (557±27.55 sq. micron) were significantly lower than those of animals that did not receive any treatment (IR) (759.5±38.11 sq. micron). Mean of RGD treated animals (637.9±26.30 sq. micron) group did not differ significantly from IR only animals. Cross-sectional areas were significantly GFOGER (907.3±53.85 sq. micron) treated animals were significantly higher than the IR only group too. Average area ±SEM in sham group was 378.90±25.20 sq. micron.

(A) (B)

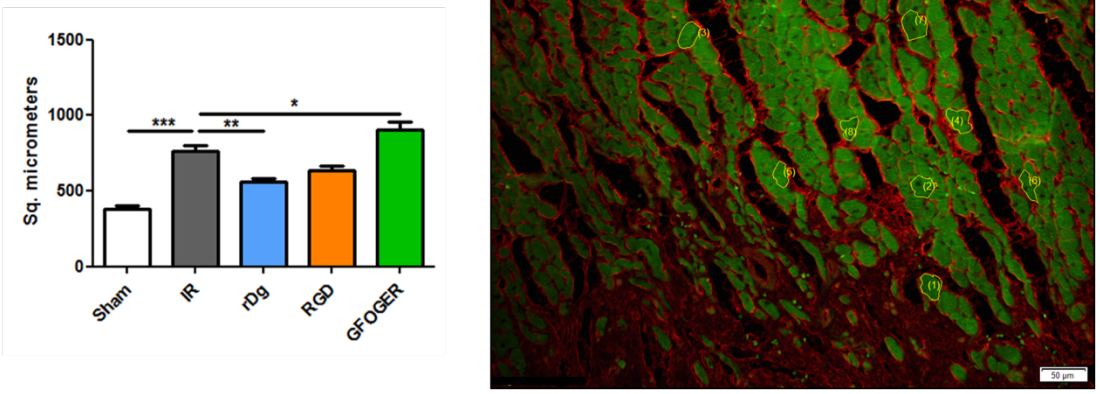
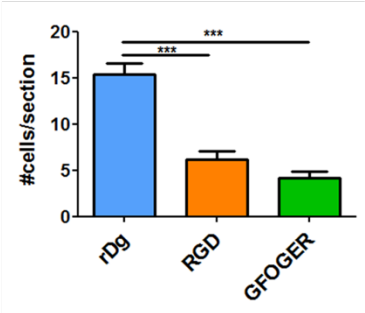


Figure 22: Hypertrophy following treatment with integrin-specific hydrogels. Formalin fixed paraffin embedded hearts from rats sacrificed 28 days following treatment were stained with fluorescent molecules tagged wheat-germ agglutinin (WGA). (A) One image per section in the infarct border region was captured and the cross-sectional area of 7-10 randomly selected myocytes in the infarct border zone as determined by marking of cell membranes by WGA staining. Values are mean ± SEM; statistical significance of difference between means was measured using ANOVA and Tukey's post-hoc test, *:$p<0.05$, **:$p<0.001$; n=4, 7-10 cells per animal. (B) Representative WGA stained infarct border zone showing measurement method of cell cross-section outlines; scale bar: 50 µm.

Engraftment of transplanted cells at d28 was measured by counting cells stained with human specific anti-mitochondria antibody. As shown in Figure 23(B), Whole slide scans at 40X magnification were taken and manually inspected for hMTCO2+ cells. Only the cells in left ventricular wall were included in the analysis. 1-2 sections were used per animal. Figure 23(A) shows that significantly higher number of human CPCs was found in sections from rDg treated animal hearts (15.25±1.031) as compared to RDG (5.62±0.82) and GFOGER (4.17±0.73) gel treated hearts($p<0.0001$). Biological and experimental negative controls did not show any positive staining.

(A)



(B)

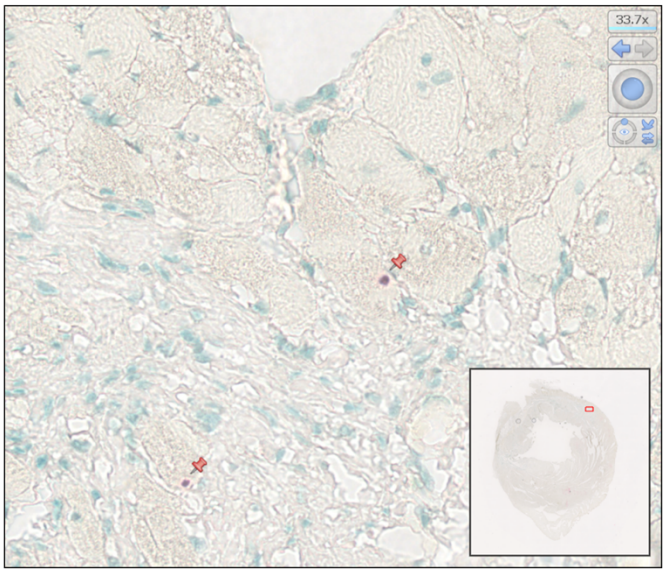


Figure 23: Engraftment of hCPCs in rat hearts following treatment. Formalin fixed paraffin embedded hearts from rats sacrificed 28 days following treatment were stained with human specific anti-mitochondria antibody (pink) and methyl green nuclear counterstain. (A) Whole slide scans at 40X magnification were taken and manually inspected for hMTCO2+ cells. Only the cells in left ventricular wall were included in the analysis. 1-2 sections were used per animal. Biological and experimental negative controls did not show any positive staining. Values are mean ± SEM; statistical significance of difference between means was measured using ANOVA and Tukey's post-hoc test, *:$p<0.05$, **:$p<0.0001$; n=4. (B) Representative figure showing a part of a whole slide scan in the LV at ~33X magnification. Pins are placed at cells included in the analysis.

4.5 Discussion

Cell therapy is undergoing clinical trials and numerous preclinical and clinical studies are being pursued to understand the mechanisms of action and test strategies to enhance benefits of cell therapy for treating heart failure. Biomaterials-based strategies have shown potential to improve outcomes of cell therapy because of the ability of biomaterials to enhance retention of cells, act as reserves of growth factors that can be presented to the cells and create a custom microenvironment that promotes regeneration instead of the hostile environment in the heart post-MI. Natural biomaterials have been demonstrated to enhance cell therapy effects; however, their mechanism of action are unclear. Synthetic biomaterials-based strategies provide much greater control of biochemical and mechanical properties. Therefore, they can be instrumental in testing hypotheses and answering specific questions to help learn about the complex mode of cell therapy that has produced mixed results in clinical trials. In addition, synthetic biomaterials modified with biomimetic peptides can be used to develop therapeutic strategies with greater quality control and localized specific effects.

In this work, we studied encapsulated human neonate derived c-kit+ cardiac progenitor cells in integrin specific PEG hydrogels presenting fibronectin mimetic α5β1 and αvβ3 ligating RGD, collagen mimetic α2β1 ligating GFOGER or scrambled rDg peptide. We characterized the integrins expressed by human cardiac progenitor cells and performed rheology of the hydrogels. The effects of integrin specific hydrogels on modulation of encapsulated hCPCs were determined by measuring mRNA and protein expression of lineage markers and secreted factors. These hydrogel-cell constructs were transplanted in an animal model of ischemia-reperfusion and consequent cardiac function indicative measurements were recorded. To understand underlying mechanisms, *ex vivo* histological analyses were performed on rat hearts to measure fibrosis, angiogenesis, hypertrophy and engraftment of transplanted cells.

We found that the CPCs express α5β1 and α2β1 but show little expression of αvβ3. GFOGER gels were found to have greater storage modulus than rDg and RGD hydrogels of the same PEG backbone density. GFOGER gels induced cardiomyocyte differentiation of CPCs as seen by mRNA and protein expression of cardiomyocyte structural markers. Cell differentiation in GFOGER gels correlated with decrease in secreted paracrine factors by those cells in comparison with rDg and RGD gels. RGD gels maintained comparable lineage marker gene and protein expression and paracrine factor levels in conditioned media as rDg gels. Following injection in rat hearts that underwent ischemia-reperfusion, CPC-carrying rDg and RGD gels showed a trend of improving cardiac function at day 7. At day 28, cardiac contractility preservation was maintained by rDg gels only. *Ex vivo* histological evaluation of rat hearts sacrificed at day 28 showed significant reduction in fibrosis only in cellular rDg gels group, increase in isolectin-positive vessels in both rDg and RGD groups, decrease in hypertrophy only in cellular rDg group in comparison with animals receiving ischemia-reperfusion only. The number of delivered CPCs engrafted at d28 was significantly higher in rDG group than GFOGER and RGD groups.

Expression of integrins relevant to RGD and GFOGER ligands adhesion were measured at the mRNA level by real-time PCR and at protein level using ELISA. RGD is known to bind to all five αv integrins, three β1 integrins (α3, α5, α8), and αIIbβ3. GFOGER, on the other hand, binds to α1β1, α2β1 and α10β1 integrins. α5β1 and α2β1, which are important participants of cell adhesion with RGD and GFOGER, respectively, were present on the CPCs. β1 was found to be the most abundant subunit through both real-time PCR and ELISA measurements, although the exact concentration of protein could not be measured in the absence of a standard curve. β1 integrins are important participants in reparative mechanisms associated with cell therapy. For instance, the β1 subunit has been shown to be responsible for mediating fibronectin-induced proliferation and protection of CPCs *in vivo* and *in vitro*, which happens via β1 integrin-FAK-Stat3-Pim1 pathway independent of Akt [239]. β1 integrins have been found to be necessary for differentiation, adhesion and migration of other stem cells. For example, in ESCs,

lack of β1 was compensated for at early stages but not later stages of cardiac differentiation and led to impaired ventricular cardiomyocytes [242,243]. Blocking β1 inhibits embryoid body formation and cardiomyogenic iPSC differentiation [201], extracellular matrix-mediated maturation in PSC monolayers [244] and inhibits proliferation and adhesion on Matrigel [245]. Differentiation of ASCs in carbon nanotube-collagen substrates was reduced on blocking β1 integrins. β1 is also required for CDC-contact mediated cardiomyocyte proliferation [246]. β1 is also involved in vascular smooth muscle differentiation of Sca1+ progenitor cells [247]. MSCs use β1 integrins to adhere to HUVECs *in vitro* [248]. β1 is involved in the migration and adhesion of BM-MSCs in the infarcted myocardium [249,250] and of HSCs in an *in vitro* model of myocardium [251]. However, cardiomyogenic differentiation of EPCs stimulated by contact with cardiomyocytes was not inhibited by blocking β1[252], suggesting the involvement of β1 may be cell and stage specific. These observations from previous studies illustrate that the β1 subunit has an important role in multiple aspects of stem cell function; the presence of this subunit in our CPCs suggests that they are good candidates for cardiac repair cell therapy.

β3, an important integrin involved in RGD ligation was found not to be expressed on our cells. Another study in which c-kit+ cardiac stem cells were isolated by a slightly different explant method made similar observations about β3 expression. To isolate the cells used in that study, the authors minced atrial appendages of patients with undisclosed characteristics, cultured in serum and mercaptoethanol containing bFGF lacking media for 7 days, and then sorted the cells into c-kit+/CD90-, c-kit-/CD90+ and c-kit-/CD90- groups. The c-kit+/CD90- cells showed no expression of β3 subunit at mRNA or protein level [104]. Ligands of β3 include fibronectin, fibrinogen, von Willebrand factor, thrombospondin, vitronectin, osteopontin, collagens and tenascin. β3 integrins are important participants in the blood clotting mechanism and knockout mice show impaired platelet aggregation and clot formation [253]. αvβ3 is involved in collagen V induced cardiomyogenic differentiation of MSCs [254]. In the absence of αVβ3, α5β1 integrins may be upregulated as a compensatory mechanism for adhesion to fibronectin substrate

although they serve different roles in adhesion and downstream signaling [242,255]. It is possible that the abundant expression of α5β1 may be a part of a compensatory mechanism to make up for attenuated β3 expression in hCPCs.

To understand mechanical properties of the hydrogels constructs, rheological characterization of 4% and 5% w/v hydrogels conjugated to rDg, RGD or GFOGER was performed. The loss modulus was determined to be negligible and of much smaller magnitude than the storage modulus so these gels are elastic. The storage moduli of denser 5% gels were higher than 4% gels. GFOGER gels exhibited statistically significantly greater storage modulus than rDg and RGD gels of the same PEG density. These observations conflict with those reported by Garcia *et al.* wherein GFOGER gels were found to have comparable G' values as rDg and RGD gels [186]. Given the larger size of GFOGER peptide than RGD and rDg peptides that could interfere with hydrogel crosslinking, this is a surprising finding. Possibly under our reaction conditions, conformational changes occur and more than one cysteine per triple-helical GFOGER participates in crosslinking to some extent along with VPM. This can cause the storage modulus to increase beyond what would be expected with only one cysteine bound to PEG like a pendant, as in the case of rDg and RGD peptides. Another possibility is that batch-to-batch differences and insufficient precision of the peptide concentration measurements to capture these differences may have introduced additional variability.

Irrespective of the above differences, all our hydrogel constructs are softer than heart (G' ~ 0.1 kPa). Solvent swollen murine heart is reported to have a storage modulus of ~5 kPa and loss modulus of ~1 kPa [256]; the elastic modulus of neonatal rat heart is 4-11.4 kPa, that of adult rat heart is 11.9-70 kPa, and adult human's is 0.02-0.5 MPa [257]. Native tissue like stiffness of biomaterials is most conducive to differentiation [111,258]. However, softer hydrogels are commonly used in studies perhaps because they are more likely to permit diffusion of nutrients and viability of encapsulated cells [259]. Also, optimal mechanical properties vary with stage of differentiation [113] and do not influence stem cell fate independently, since biochemical and other physical properties such as topography properties play a significant role as well. We observed

induction of cardiomyocyte differentiation of CPCs and reduction in secreted paracrine factors in GFOGER gels. Along with biochemical factors, mechanical stimulation plays a significant role in inducing cardiomyocyte differentiation as well as modulation of paracrine factors. Dependence of lineage induction on scaffold mechanical properties has been reported for CPCs as well as other stem cell types. A previous study found that CPCs cultured in 1% w/v (E=500 Pa) self-assembling peptide hydrogels differentiated toward endothelial and smooth muscle lineages and those in 2% w/v (E=1800 Pa) scaffolds differentiated toward the cardiomyocyte lineage. These behaviors were reported to correlate with increased notch activation in the higher density stiffer gels and were attributed to mechanotransduction–based notch activation. Hydrogel mechanical properties also influenced CPC proliferation and released paracrine factors; greater PDGF release was seen in 1% jagged (ligand to notch) presenting gels and greater SCF secretion and CPC proliferation were seen in 2% jagged presenting hydrogels [144]. In a study performing a systematic evaluation of substrate stiffness on CPC differentiation, neonatal or adult CPCs encapsulated in fibrin and ECM-fibrin scaffolds with E= 2, 8, 14 and 32 kPa did not show an upregulation in troponin mRNA at 21 days. They did, however, find upregulation in expression of endothelial and vascular smooth muscle cell markers on 32 kPa gels in adult and neonatal CPCs respectively. Since the authors were unable to see cardiomyocyte differentiation of CPCs at either condition, they intend testing stiffer matrices in the future. [260]. Another study reporting substrate induced CPC differentiation did 2D culture of CPCs on an electrospun polymer mesh with a tensile modulus of ~9 MPa [261]. Note that E~3G for most hydrogels [262]. In contrast to these reports where much stiffer gels were required to elicit differentiation of CPCs, we found that soft GFOGER gels with G’ value of ~120 Pa were sufficient to induce mRNA and protein expression of troponins and other cardiomyocyte structural proteins. Since the stiffness of our gels is too low to solely drive this behavior, our observations suggest that the biological signal initiated by α2β1 integrin stimulation may be playing an important role. Extent of integrin-mediated signaling activation is a function of the substrate’s mechanical properties and is an important contributing factor to the influence

of matrix stiffness on stem cell behavior. Substrate rigidity affects cell behavior through regulation of integrin-mediated focal adhesion complexes and downstream intracellular signaling [263]. Our observation of *in vitro* cardiomyocyte differentiation induction in GFOGER gels and not rDg or RGD gels, and differences in paracrine factor levels is likely a function of both mechanical and biochemical properties of the $\alpha 2\beta 1$ specific GFOGER gels.

For the duration before degradation of the hydrogels, their mechanical properties affect behavior of not just the delivered cells but also host cells including infiltrating stem and progenitor cells. For example, it was reported that injection of alginate gels functionalized with adhesive peptides RGD, YIGSR or non-adhesive RGE surprisingly led to worse cardiac function and extent of scar than unmodified alginate gels [194]. There were no significant differences between blood vessel density, myofibroblast or macrophage infiltration or cell proliferation between the experimental groups. A suggested explanation for these observations was based on the different mechanical properties of modified and unmodified scaffolds. Layering this observation on top of our previous arguments, there is a distinct possibility that our *in vitro* and *in vivo* observations are a complex function of hydrogen stiffness, properties of active biological agents embedded in the gel, and the modulatory effect of gel stiffness on delivered cells as well as host cells.

CPCs encapsulated in GFOGER exhibited cardiomyocyte differentiation in the absence of any supplemental growth factors or differentiation media. mRNA expression of cardiac transcription factors *nkx2_5* and *mef2c*, cardiomyocyte specific structural proteins *myh6, myh7, myl2, tnnt2, ctnni3*, and vascular smooth muscle and cardiomyocyte marker suggested to be involved in calcium interactions and contractile properties of the cell [241], *tagln* was upregulated in GFOGER gels 2 days after encapsulation. CPCs obtained from GFOGER gels 5 days after encapsulation expressed cardiomyocyte specific troponin I and myosin heavy chain. It would be interesting to see if the GFOGER presenting gels can push the cells to express more

mature markers like connexin43 and calcium channel proteins at this or a later time point.

While cardiomyogenic differentiation of CPCs by other stimuli including substrates has been seen before, α2β1 stimulation as a factor driving cardiomyocyte differentiation of CPCs is a novel finding. Other biochemical and physical cues have been shown to induce cardiomyogenic differentiation of CPCs. For example, CPCs cultured on poly(ester-urethane urea) aligned electrospun scaffolds showed cardiomyocyte differentiation after 31 days [261], although earlier time point measurements were not shown and it is not clear what the relative contributions of the aligned topography and chemical composition of the scaffold were. miR-708 overexpression induced cardiomyocyte differentiation of CPCs showing 8-fold mRNA expression of early marker nkx2.5 and 4-fold expression of mature marker tnnt2 after one week of treatment [264]. Epigenetic modification of CPCs using class I histone deacetylase (HDAC1) inhibition interestingly showed significant cardiac differentiation with 75-fold higher mRNA expression of Troponin T and ~23% cells expressing Troponin T following 7 day treatment [67]. HDAC4 inhibition also led to cardiomyocyte differentiation of CPCs [265] and cardiac function improvement was seen following injection of modified CPCs in both cases. Treating with 5-Azacytidine, by itself or in combination with TGF-β leads to cardiomyocyte differentiation of CPCs [60,266,267]. However, the untargeted nature of 5-Aza, which is a general demethylating agent that leads to unmasking of genes that are not expressed due to promoter hypermethylation raise safety issues. Dexamethasone treatment induces cardiovascular lineage commitment [36] and its differentiation inducing effect (along with proliferation) is further enhanced in Pim-1 kinase overexpressing [268] and Pin-1 overexpressing CPCs [269]. HGF and IGF-1 and their combination have been shown to induce cardiomyocyte differentiation as well; ~26% cells express cardiac troponin I after 7 days. Coculture with cardiomyocytes drives CPCs to differentiate into beating cells [18]. Mechanical stretch is another stimulus that has been shown to influence cardiomyocyte differentiation of CPCs [238,270]. As these examples illustrate, while CPC differentiation induced by substrates

has been reported before, the nature of biochemical cues driving the differentiation is not absolutely clear. In that context, our finding that stimulation of α2β1 integrins may be involved in CPC differentiation is novel.

We found significantly lowered expression of the paracrine factors HGF, VEGF and FGFb by cells in GFOGER gels as early as day 2 and day 5. rDg and RGD had similar levels of these proteins. These factors play pivotal roles in cardiac regeneration. HGF, VEGF and FGFb show cardio-protective, mitogenic effects [271] and direct stem cell homing to ischemic myocardium [272–274]; VEGF and FGFb are angiogenic factors [275]. In our study, differentiation of CPCs correlated with an accompanying decline in release of beneficial paracrine factors. Such an observation has been made with c-kit+ BM-MSCs wherein 5-Aza treatment increased their cardiomyocyte differentiation as marked by increased expression of Nkx2.5, Gata4, cTnT and Cnx43 but led to reduction in VEGF (400 vs. 180 μg/pg) and bFGF (2100 vs. 600 μg/pg) expression by the cells [276]. Another study comparing levels of secreted factors from rat BM-MSCs and cardiomyocytes noted lower levels of VEGF, bFGF, SDF and IGF (VEGF: 8 vs 3, bFGF: 30 vs 5, SDF: 0.15 vs 0.02, IGF: 80 vs 10pg/μg protein) in conditioned media of cardiomyocytes [277]. On the other hand, differentiating ESCs in EBs showed coincident increased expression of the growth factors IGF, VEGF, BMP-4, FGF and PDGF, perhaps reflecting the secretome in that stage of embryogenesis [278]. These reports collectively suggest that the relation of paracrine factors and differentiation state may be cell or stage dependent. It is also possible that these processes may be independent and occurring simultaneously without one having any mechanistic bearing on the other.

On transplantation of integrin-specific hydrogel-cell constructs, we found rDg gel + CPCs combination to drive the greatest cardiac function improvement at 28 days after ischemia-reperfusion. Integrin specific RGD and GFOGER gels carrying CPCs failed to improve cardiac function which was unexpected as these ligands were expected to stimulate regenerative signaling in cardiac progenitor cells based on literature and our *in vitro* data. Acellular rDg gels did not increase cardiac function and neither did cells delivered without a hydrogel carrier (cells only group), suggesting the important role of

both the rDg hydrogel carrier and cells in driving the improvement in EF and FS. Delivering CPCs with the rDg hydrogel must have improved their retention, also allowing sustained delivery of their paracrine factors. 4% GFOGER gels were also tested but they failed to improve cardiac function at day 28 even though the 4% GFOGER hydrogels had comparable mechanical properties as 5% rDg gels. These data point to the role of CPCs, PEG hydrogels and the non-adhesive ligand rDg presented by the hydrogels in synergistically leading to cardiac function improvement of infarcted rat hearts.

These results were unexpected. Given the cardiomyogenic differentiation of CPCs in GFOGER gels *in vitro*, we were expecting CPC + GFOGER gels to improve cardiac function more than the other combinations. This assessment was based on some prior studies using CPCs that have shown differentiation inducing strategies to support greater cardiac function improvement. In a previous study from our lab, culturing rat CPCs in jagged-1 presenting hydrogels induced cardiomyocyte differentiation of the cells *in vitro* and *in vivo* implantation of this construct led to improvement in cardiac function post-MI. Interestingly, conditioned media of the differentiating cells showed higher SCF, CPC migration and proliferation and cardiomyocyte proliferation. So the same group had superior paracrine factor levels and more differentiated phenotype and led to improved cardiac function *in vivo* [144]. Inhibition of histone deacetylase 4 (HDAC4) induced cardiomyocyte differentiation of CPCs *in vitro* and injection of HDAC4 siRNA-treated CPCs showed greater improvement in cardiac function than control CPCs. Quantification of paracrine factor secretions *in vitro* were not done [265]. On the other hand, some other studies based on other cell types have shown the less differentiated cells to demonstrate greater improvement in cardiac function. 5-Aza treatment of BM-MSCs increased their cardiomyocyte differentiation *in vitro* but led to reduction in VEGF, bFGF expression by the cells. Following *in vivo* implantation of cells, it was observed that the less differentiated untreated c-kit+ BM-MSCs showed greater improvement in cardiac function after MI than 5-Aza differentiated cells. The undifferentiated group showed higher secretion of growth factors *in vitro* and greater angiogenesis, recruitment of endogenous cardiac stem cells and proliferation of

cardiomyocytes *in vivo*. The 5-Aza pretreated cells showed greater cardiomyocyte differentiation of transplanted cells *in vivo* as well, but did not improve function [276]. In another study, less lineage committed skeletal muscle-derived stem cells (MDSCs) showed greater and more persistent engraftment, induced more neoangiogenesis through graft expression of VEGF, showed greater resistance to oxidative stress, prevented cardiac remodeling, and elicited significant improvements in cardiac function in comparison with more differentiated myoblasts [279]. iPSCs differentiated to an optimal extent toward the cardiac phenotype showed the greatest engraftment in infarcted hearts in comparison with less and more differentiated cells [280]. We saw that GFOGER gels induced cardiomyogenic differentiation of CPCs and a reduction in paracrine factor levels *in vitro*. *In vivo*, GFOGER gels encapsulating CPCs could not preserve cardiac function and had low angiogenesis, and high fibrosis and hypertrophy 4 weeks after treatment. The inability of GFOGER + CPC group to rescue cardiac function despite showing the greatest differentiation potential of all combinations studied points to the role of lowered paracrine effects observed in this group as the potential cause. Our results and those discussed above reinforce the suggestion of paracrine factors to be the main mechanism of action of cell therapy induced benefits.

The *in vitro* paracrine factor profile and differentiation state of CPCs, and dynamic modulus measured by rheology were comparable between RGD and rDg PEG gel groups, so they cannot explain the differences between the two groups *in vivo*. At relatively early time point of day 7, both rDg and RGD gels codelivered with CPCs showed a trend of better cardiac function than rats that did not receive any treatment, although these differences were not statistically significant. However, distributions of EF and FS in cellular rDg and RGD gel receiving rats were not statistically significantly dissimilar than sham group either at d7. Adding biological replicates may help make a reliable conclusion. At day 28, only rDg gels encapsulating CPCs showed significant function improvement than untreated control group. At the 28 day time point, both rDg and RGD gels encapsulating CPCs showed significantly improved angiogenesis than ischemia-reperfusion only group, but significantly lower fibrosis was seen only in the

cellular rDg group and not the RGD group. Note that isolectin-positive cells were marked as surrogates for angiogenesis, which labels not just mature perfused large and microvessels but endothelial cells of immature vessels as well [281]. Lower hypertrophy in comparison with IR only group was also seen only in rDg treated rats. Hypertrophy was even higher than IR only group in GFOGER gel treated rats. We also measured engraftment of transplanted CPCs in rat hearts at day 28 and found rDg group to have higher retention of transplanted CPCs than RGD and GFOGER groups. While the number of human cells found in the rat heart at that time point were very low in all groups (~5 for RGD and GFOGER groups and ~15 for rDg groups), there were statistically significant differences between means of integrin-specific RGD, GFOGER functionalized gels and non-adhesive rDg gels groups.

One possible reason for these observations is that the ligands RGD and GFOGER may be blocking the integrins on hCPCs, even after cleavage of the VPM peptides, and preventing them from adhering to the infarcted myocardium. This in turn may be limiting the engraftment of exogenous cells and their benefits to treat infarcted hearts as has been observed before. For example, EPCs adhere to the infarcted myocardium via CD18-ICAM interaction. Blocking CD18 on EPCs using antibodies prior to injection in infarcted animal hearts significantly reduced their presence in the heart and abolished EPC induced cardiac wall thickening, prevention of remodeling and neovascularization 2 weeks after injection [206]. Similarly, blocking β1 integrin on BM-MSCs using anti-CD29 mAb reduced the accumulation of the injected cells in the infarcted heart by about 40% 72 hours later [249]. The resulting impact on function was not measured in this particular study. But these data suggest that blocking molecules that mediate adhesion between the implanted cells and myocardium negatively affect their retention in the infarcted heart and possibly, cell-mediated functional improvements. This hypothesis could be tested *in vitro* by measuring adhesion of cells released from rDg, RGD and GFOGER gels to a matrix such as reconstituted porcine ECM. Note that this model will not reflect the post-IR environment known to modify expression of adhesion molecules on transplanted cells and myocardium. For instance, in the post-IR

environment increased ROS levels downregulate integrin-related adhesion molecules and inhibit cellular adhesion of MSCs [282]. Another way of testing the hypothesis would be measuring engraftment *ex vivo* at an early time point such as 1 week after transplantation. A control group with anti-β1 antibody treated CPCs delivered through non-adhesive rDg gels could be used to help confirm this explanation. According to our observations, the number of retained transplanted cells at day 28 is very low which is in agreement with other studies done using CPCs and other cell types. Even though the number of retained transplanted cells was low, nonetheless, a correlation was found between the number of retained cells and improvement in cardiac function following cell delivery. Such a correlation between engraftment, even though low, and functional benefit has been observed with other cell types before [27,206,283,284]. Another possibility is that RGD and GFOGER gels may be anchoring the delivered cells and preventing them from migrating through the infarct. *In vitro* studies have shown that cells migrate along RGD gradient [285]; this haptotactic behavior is cell and ligand concentration specific [286]. This hypothesis can also be tested by tracking implanted cells about at an earlier time point such as d7 after transplantation. RGD and fibronectin have been reported to induce pathological hypertrophy of cardiomyocytes similar to pressure-overload leading to increased cross sectional area and ANP, NFAT expression by cardiomyocytes [287–289]. So we also examined that as a possible mechanism of dysfunction in RGD treated hearts. However, since the rat heart myocytes had maladaptive hypertrophy following MI, it was hard to determine if RGD stimulation caused additional hypertrophy. rDg PEG gels are likely to show sufficient adhesion of cells *in vivo* through proteins adsorbed on the gels. Acellular rDg-PEG gels are reported to show comparable protein adsorption as acellular RGD-PEG gels *in vitro* and *in vivo.* However, acellular RGD gels invite greater macrophage adhesion and are surrounded by a thinner fibrous capsule [290]. However, this difference is unlikely to explain the *in vivo* functional improvement by cellular rDG gels and not RGD gels.

Overall, non-adhesive rDg gels lead to the significant improvement in cardiac function of infarcted rats which is sustained for at least 4 weeks. rDg gels also show

significant attenuation of fibrosis and hypertrophy, and increased angiogenesis than animals receiving no treatment following IR. CPCs delivered with rDg gels show higher engraftment in host tissue at day 28 than those delivered with GFOGER and RGD gels. These findings suggest that too much interaction with the carrier biomaterial may interfere the cells' incorporation into the host tissue in addition to negatively affecting their paracrine effects.

Nude animals were used in our study in order to avoid immune rejection for study of hCPCs which leads to destruction of the xenograft. Immune cells are closely linked to the repair and regeneration processes in the heart. Different rates of repair and mechanisms due to lack of T cells are seen on implanting grafts in athymic animals in comparison to syngeneic or allogeneic animals. Some examples of these varied responses between immunodeficient and immunocompetent animals, as well as cardiac repair processes involving T cells are provided in the section 5.2.2. In order to understand the detailed mechanisms of cell therapy in cardiac regeneration, utilizing both immunodeficient animal models (with human cells) and immunocompetent animal models (with animal cells) to understand the effect of species difference as well as immunological response is justifiable.

Overall, this study reveals novel insights about the behavior of CPCs in integrin-specific or non-adhesive scaffolds and response to these constructs in an animal model of ischemia-reperfusion. These findings add to our understanding of factors that influence the outcome of cell therapy and will help in future design of strategies for enhancing cell therapy for cardiac repair.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

As novel therapeutic approaches based on cell therapy to prevent and treat heart failure are evolving, it is imperative that we advance our understanding of the processes underlying cell therapy to enable rational design of the most optimal therapeutic approaches. Such efforts should include characterizing specific designs of the cell therapeutic modality to distinguish what types of designs are more likely to be successful and what are the potential pitfalls. At a deeper level, it is also critical that we develop a mechanistic understanding of why certain approaches are more successful than others. This dissertation focuses on biomaterial-based strategies for enhancing the regenerative effects of CPCs, and on understanding how the complex interactions between the biomaterial, transplanted cells and the host system determine outcome.

Cardiovascular diseases are the leading global cause of death. They are responsible for 1 in every 4 deaths in the United States and an economic burden of over $200 billion every year. The significant morbidity, mortality and economic burden associated with heart failure motivate development of better prevention and therapeutic strategies. Cell therapy for congestive heart failure has shown promising results in preclinical studies but results in clinical trials have been mixed and lower than expectations. This suggests the need for this modality to be optimized in order to enhance the regenerative potential of stem cells. In this dissertation, biomaterials-based strategies for enhancing effects of CPCs were designed and tested.

5.1 Aim 1: VEGF functionalized scaffolds for enhancing CPC angiogenic behavior

5.1.1 Summary

The hypothesis of this study was that degradable hydrogels functionalized with VEGF will enhance angiogenic behavior of encapsulated CPCs. CPCs encapsulated in VEGF conjugated gels showed increased ERK phosphorylation. VEGF conjugated PEG gels did not show higher tube formation metrics than those without VEGF in experiments

using both rat and neonatal human cells. The hydrogel density did have an effect with lower density gels showing greater tube length among the 4, 5, 6% w/v gels. VEGF gels also failed to show any significant increase in mRNA or protein expression of endothelial markers in rat cells or human cells from neonate or older children donors. We tested both low (100, 250 ng/mL) and high (5 ug/mL) doses of VEGF and found neither to result in increased endothelial marker expression. Neither VEGF nor RGD had an effect on mRNA expression of endothelial markers as determined by using rDg conjugated gels with or without VEGF. Endothelial mRNA expression in CPCs with VEGF supplementation also did not increase in lower density (4% w/v) gels, which we saw to support cell elongation and sprouting, and tube formation. Even with slow degrading GPQ gels, VEGF supplementation did not have an effect on mRNA expression of endothelial markers, nor secreted paracrine factors angiogenin, HGF, FGFb, PlGF, EGF, HB-EGF. VEGF concentration was higher in conditioned media from VEGF conjugated gels using both VPM and GPQ crosslinkers, although it cannot be said whether this higher concentration is due to only the exogenously delivered 5ug/mL VEGF conjugated to the gel or increased secretion from the CPCs stimulated by autocrine signaling. The expression of endothelial markers was found to be quite high (>90%) in rat and human CPC populations as assessed by our flow cytometry experiments.

5.1.2 Limitations and future directions

Most experiments, including all experiments using human cells, were done using 5 ug VEGF/mL hydrogel however activation of VEGF signaling was not tested at that dose. ERK phosphorylation was used as the surrogate for measuring activation of VEGF induced signaling in CPCs. However, ERK is a nonspecific, downstream kinase whose activity is influenced by many stimuli and could have been activated by alternative pathways, such as a possible change in mechanical properties of PEG gels on VEGF immobilization [194]. Instead, VEGFR1 and VEGFR2 phosphorylation would have been better markers as they are more specific to activation by VEGF ligand. VEGFR2 phosphorylation occurs in the presence of stimuli other than VEGF also, including shear

stress [291], but that would not be a concern in our controlled experimental setup. Also, it would have feasible to measure in our setup as VEGFR2 phosphorylation may not have subsided one hour after encapsulation when we lysed the cells after digesting the hydrogels.

Due to release of VEGF in the media due to protease mediated hydrogel degradation and alternate day media changes, CPCs may not have received sufficient concentration of VEGF for the required duration for successful stimulation. We tested slower degrading GPQ gels with the rationale that they would enable slower crosslinker degradation-mediated release of VEGF and longer stimulation of encapsulated CPCs with immobilized VEGF. However, GPQ gels did not show enhanced endothelial gene expression or paracrine factor release in VEGF immobilized groups, except for a possible autocrine regulated increase in VEGF release. Whether this increase in VEGF release was truly due to paracrine effects can be clarified by measuring mRNA expression of VEGF by the cells. While VEGF immobilized on PEG-MAL gels has been shown to maintain activity when immobilized or released [146,186], we did not confirm that in our hands. VEGF-VEGFR2 mediated signaling is sensitive to cell density and it could be that the cell density and ligand concentration used in our experiments was not supportive for signaling activation [292].

We only measured some surrogates including endothelial differentiation, modulation of some paracrine factors and tube formation characteristics. However, we did not measure other possible outcomes of VEGF stimulation such as proliferation, survival under hypoxic stress etc. We did see increase in VEGF in conditioned media of VEGF conjugated PEG gels encapsulating CPCs. This higher concentration of VEGF released to the environment may result in enhanced regenerative behavior *in vivo* by exerting chemotactic and angiogenic effects on host progenitor cells and maybe enhance engraftment of exogenously delivered CPCs. However, we did not test the outcome of in vivo transplantation of CPC encapsulating VEGF-PEG gels.

The expression of endothelial markers was found to be quite high (>90% cells were positive at baseline) in rat and human CPC populations as assessed by our flow

cytometry experiments. High prevalence of endothelial marker positive cells in CPC populations has been reported by other groups as well. Sandstedt *et al.* found ~65% CPCs to express Pecam-1 (CD31) directly after isolation and mRNA expression of CD31 increased further after monolayer culture [191]. They also found ~81% cells to express mature endothelial marker vWF [190]. Matuszczak et al. found ~84% cells to express CD31 [192]; although they used different culture conditions of 20% FBS and culturing on fibronectin. Note that there are some variations in surface markers expression in heart derived c-kit+ cardiac progenitor/stem cells studied in different groups and could be because of differences in donor, anatomic source [293], isolation and culture conditions [60,151,190,192,260,268,294–298]. Some recent studies have performed genetic mapping to understand the origin and fate of endogenous CPCs. It has been suggested that the majority of c-kit+ cells in the heart are vasculogenic cardiac progenitor cells [167,299]. One study has also suggested that the majority of $cKit^+$ cells in the heart are mature endothelial cells [193], although observations of two other studies disagree with this; this discrepancy between observations could be due to differences in detection sensitivities of reporter alleles used in the studies [167,299,300]. While these studies looked at *in vivo* mapping of c-kit+ cells, one study has reported an increase in mRNA expression of *pecam-1* after *in vitro* culture of cells in comparison to directly isolated CPCs [191]. This could mean that our *in vitro* expanded CPCs may not be amenable to further differentiation by VEGF treatment. Our cell culture growth media includes FGFb, a factor that has been reported to induce endothelial differentiation of CPCs [297]; even after removal of FGFb in treatment media, there might be a memory effect causing cells to maintain their differentiated state like memory of mechanical stimuli has been shown [301]. Along with high expression of endothelial markers, our cells also readily form tubes on Matrigel (not shown), although that behavior has been seen with non-endothelial cells like fibroblasts as well [302].

It is also possible that VEGF secretion by CPCs in PEG gels (with rDg or RGD) may be high enough to saturate signaling such that recombinant VEGF supplementation does not induce an additional effect. Studies have shown VEGF treatment to change

CPC behavior such as induce migration or increase adhesivity [146,176,177]. However, these studies involved VEGF treatment of CPCs in 2D, and those findings may not apply to our 3D culture which is known to modify cells' secretion profile [92]. CPCs may perhaps benefit from supplementation with a factor they are deficient in, e.g., HGF and IGF have been shown to enhance their regenerative behavior [303]. Perhaps VEGF treatment would benefit from use with a system where VEGF is depleted, such as in GFOGER PEG gels encapsulating CPCs, as seen in Section 4.4.4 (Figure 18). We saw that α2β1 integrin specific GFOGER gels induced cardiomyocyte differentiation of CPCs but were accompanied by reduction in VEGF and other paracrine factors. We hypothesize that the inability of cellular GFOGER gels to rescue cardiac function after IR may be because of the lowered paracrine factor levels. PEG gels functionalized with VEGF and GFOGER may provide a treatment strategy that can overcome this limitation and deliver CPCs differentiating to the relevant cardiomyocyte cells because of GFOGER stimulation along with better cell survival, chemotaxis of endogenous progenitors and angiogenesis mediated by VEGF [177,178].

Another possible explanation is that the little or no expression of αvβ3 integrin on CPCs may be acting as a weak link in VEGF signaling pathway. Our results (Figure 14) and those by another group [104] have shown below detection levels expression of β3 subunit on CPCs. αvβ3 integrin plays a very important role in VEGF stimulation and downstream endothelial differentiation, proliferation and angiogenesis [304]. In endothelial cells, binding of VEGF-A to VEGFR2 triggers autophosphorylation, leading to complex formation by interaction between cytoplasmic tails of both receptors and leading to integrin αvβ3 phosphorylation at Y747 in the β3 cytoplasmic tail [305]. Introducing mutation of β3 Y747 to phenylalanine impaired VEGF-induced activation of αVβ3 and downstream endothelial responses including adhesion and migration. β3 integrin tyrosine phosphorylation is required for maximum tyrosine phosphorylation of VEGFR-2. Plating endothelial cells on the αvβ3 ligand vitronectin led to VEGFR2 phosphorylation and this was augmented on VEGF treatment. However, endothelial cells maintained in suspension or plated on collagen or laminin, matrices that primarily do not adhere

through integrin β3, showed insignificant VEGFR2 phosphorylation increase in the presence of VEGF stimulation, implicating VEGFR2 and β3 to participate in cross activation. The cross-activation of VEGFR2 and β3 receptors leads to the recruitment and activation of Src kinases [306] which mediate the mitogenic effects of VEGF [195]. On the other hand though, in β3 knockout mice, elevated levels of VEGFR2 and angiogenesis have been noted [306–308] suggesting dual roles of β3 or activation of alternative mechanisms in β3 knockout animals. In another study, blockade of αvβ3 integrins on CD34+ cells cultured on fibronectin did not inhibit their VEGF induced differentiation and migration [309], perhaps because of association of α5β1 with VEGFR2 in presence of fibronectin [310]. These interactions involve VEGF binding domains on fibronectin, not RGD so our RGD presenting PEG gels may not support an α5β1 dependent, αvβ3independent VEGF-induced differentiation mechanism.

Some studies in the literature have reported certain treatments to enhance endothelial differentiation of CPCs. Note that the phenotype of untreated CPCs in some of these studies was different from that of ours. Methods include treatment with bFGF [297] (ICC, untreated control not shown), H_2O_2 (mRNA) [311], jagged presenting nanofiber scaffolds (mRNA) [144], fibrin (mRNA) [260], macrophage migration inhibitory factor (Western, ICC) [298], dexamethasone (ICC) [36]. Perhaps endothelial differentiation in our cells could be induced using stimuli other than VEGF because of the impaired VEGFR2-β3 signaling in CPCs. VEGF induced- but not bFGF induced-angiogenesis is inhibited on introducing mutation in integrin β3 cytoplasmic tail [306]. Exogenous addition of agents that activate Wnt/β-catenin signaling has been found to augment endothelial differentiation in cardiovascular progenitor cells obtained from ESCs [312] and iPSCs [313], which can act as models of development. Supplementing VEGF treatment with lithium chloride (LiCl), a Wnt/β-catenin activator, enhanced the expression of endothelial markers in ESC-derived c-kit+ progenitor cells [312]. Wnt pathway modulation has been shown to induce endothelial differentiation of ESCs independent of VEGF as well, with VEGF not lending any significant additive effect [313] suggesting that they may be acting through a common pathway, as has been observed

in VEGF induced differentiation of MSCs and ESCs [314]. Other mechanisms that have been shown to enhance endothelial differentiation synergistically with VEGF or independently include combination of growth factors [315], shear stress [315–319], epigenetic modulation [320,321], and hypoxic [315] and oxidative stress [311], and could be tested with CPCs.

5.2 Aim 2: Integrin specific hydrogels for activation of regenerative signaling in CPCs

5.2.1 Summary

The hypothesis of this study was that encapsulating CPCs in integrin-specific hydrogels will improve adhesion and induce signaling involved in reparative processes. CPCs express GFOGER interacting α2β1 and RGD activating α5β1 but not αvβ3. GFOGER gels were found to have greater storage modulus than rDg and RGD gels of the same PEG backbone density hydrogels. GFOGER gels induced cardiomyocyte differentiation of CPCs as seen by mRNA and protein expression of cardiomyocyte structural markers. Cell differentiation in GFOGER gels was accompanied by a decrease in secreted paracrine factors by those cells in comparison with rDg and RGD gels. RGD gels maintained comparable lineage marker gene and protein expression and paracrine factor levels in conditioned media as rDg gels. Following injection in rat hearts that underwent ischemia-reperfusion, CPC carrying rDg and RGD gels showed a trend of improving cardiac function at day 7. At day 28, however, cardiac contractility preservation was maintained by rDg gels only. Ex vivo histological evaluation of rat hearts sacrificed at day 28 showed significant reduction in fibrosis only in cellular rDg gels group, increase in isolectin-positive vessels in both rDg and RGD groups, decrease in hypertrophy only in cellular rDg group in comparison with animals receiving ischemia-reperfusion only. The number of delivered CPCs engrafted at d28 was significantly higher in rDG group than GFOGER and RGD groups.

5.2.2 Limitations and future directions

The extent of differentiation of CPCs on GFOGER gels was not tested. Testing more mature markers including gap junction (e.g. Cnx43) and calcium handling proteins (e.g. SERCA, L-type-calcium channels) can help assess that. Culturing CPCs in α2β1-specific gels for longer duration, possibly on slower degrading or non-degradable gels and then assessing the extent to which α2β1-stimulation is able to induce cardiomyocyte differentiation will be informative as well. These hydrogels are much softer than native myocardium and also degrade quickly losing stiffness further. Yet they were able to induce cardiac differentiation of CPCs through $\alpha 2\beta 1$ signaling. Native tissue like stiffness of biomaterials are most conducive to differentiation and maturation [111,258]. It would be interesting to see the effect of α2β1-integrin activation on stiffer matrices as they may support greater extent of cardiac differentiation. Also, CPC behavior in healthy and infarcted hearts could be studied by using α2β1-specific hydrogels with a greater range of stiffness to mimic collagen in healthy myocardium and scar. Cells experience mechanical and biochemical signals from both neighboring cells and extracellular matrix [322]. Integrin-specific PEG gels culturing same type or different types of cells such as cardiomyocytes-CPCs in contact could be used as models to study how cells integrate signals originating from neighboring cells and matrix and respond, especially in different mechanical environments such as healthy myocardium and scar tissue.

Activation of downstream signaling due to RGD stimulation on CPCs was not examined and we cannot be sure if RGD acted at all on the CPCs under the conditions studied. RGD behaved similarly to rDg gels *in vitro* in terms of rheology, CPC differentiation and release of paracrine factors. This could be either because RGD does not stimulate these effects in CPCs or that RGD ligand spacing, concentration or adhesion force due to hydrogel stiffness were not conducive to CPC activation. This could be tested by measuring activation of early signaling molecules in integrin-activation pathway like FAK, ILK or proliferation, which is a known effect of fibronectin on CPCs mediated via $\alpha 5\beta 1$ integrins [239]. More potent cyclic RGD, cRGD could also be used. In comparison with linear RGD, cRGD has been found to better mimic the

conformation of RGD sequence in the cell-binding domain of fibronectin exposed at the tip of a loop with a spatial constraint that results in increased affinity for cell binding [323].

We used nude animals in our study in order to avoid immune rejection for study of hCPCs which leads to destruction of the xenograft. The nude mutation in the *Foxn1* gene in both mice and rats causes hairlessness and lack of T cells when homozygous. Athymic rats do have B cells and can even possess enhanced concentration and activity of natural killer (NK) cells [324] as well as enhanced macrophage function [325], although these depend on the strain and method of generation of immunodeficiency [326]. In a study analyzing the immune response against a complex rat cell-fibrin matrix graft in syngeneic, allogeneic, and immunodeficient rat hearts, it was seen that no systemic immune response was elicited in the immunodeficient rats and there was minimal infiltration of CD3+ lymphocytes and CD68+ macrophages, unlike in immunocompetent syngeneic (low infiltration) and allogeneic (high infiltration) rats [327]. However, comparative function and fibrosis assessment over time was not done in that study. Faster healing has been noted in immune-deficient animal models than immunocompetent animals because of lack of inflammatory cytokines and their effect on the survival of transplanted cells. In musculoskeletal tissue engineering applications, T cells were found to inhibit the ability of exogenous BM-MSCs to induce bone repair. This inhibition is mediated by IFN-γ-driven downregulation of the osteogenic Runx-2 pathway and upregulation of TNF-α signaling in the stem cells which triggers their apoptosis [328]. In a study directly comparing compact bone derived progenitor cells-driven bone formation in immunocompetent and immunodeficient mice, new bone area and the number of differentiated progenitor cells were greater at 4 weeks in nude animals than in immunocompetent mice, a response that subsided at 8 weeks. Greater inflammatory cell infiltration and expression of TNF-*α* and IL-4 were evident at 1 week after injections in the immunocompetent animals and could have inhibited the repair process [329]. hESC-derived pancreatic progenitor cells matured faster in nude rats compared with SCID-

beige mice [330]. These studies show that host response plays a very important role in cell fate post-transplantation. As immunological reactions depend on animal species and vary between strains, the findings from this study cannot simply be applied to studies using other animal models. In the heart, immune modulation is closely tied to stem cells and regeneration [331,332]. $CD4^+$ T-cell deficient mice showed increased cardiac dilation, impaired neovascularization, and collagen deposition after MI [333]. Anti-CD25 treatment and subsequent Treg deletion significantly enhanced postischemic neovascularization [334]. This suggests that the cardiac regeneration process must be different in athymic rats than immunocompetent animals. In order to understand the detailed mechanisms of cell therapy in cardiac regeneration, utilizing both immunodeficient animal models (with human cells) and immunocompetent animal models (with animal cells) to understand the effect of species difference as well, such as immunological response, is important.

Tracking of exogenously delivered cells was not performed at an earlier time point. A previous study of delivery of ILK overexpressing Sca1+ cells exhibited better cardiac function and exercise ability than control Sca1+ cells in animals getting MI at 4 weeks post injection. This correlated with greater retention of the ILK overexpressed group in peri-infarct area 3 days later, but not 4 weeks after MI *in vivo* when there were no differences in retention between control and ILK overexpressed groups [140]. This suggests that cell retention at early time points may be more important for function improvement and may therefore be worth measuring with our constructs as well.

Two of the hypotheses that we have proposed to explain the results of our *in vivo* study could be tested by measuring cell retention. The first of these hypotheses is that the ligands RGD and GFOGER may be blocking the integrins on hCPCs even after cleavage of the VPM peptides and preventing them from adhering to the infarcted myocardium. The second hypothesis is that RGD and GFOGER gels may be anchoring the delivered cells and preventing them from migrating through the infarct. Tracking delivered cells at an early time point and measuring their number and location could indicate if any of these explanations were true. To test the former hypothesis, a control

group with anti-β1 antibody treated CPCs to block the β1 integrins on them, delivered through rDg gels could be used. This hypothesis could be tested *in vitro* by measuring adhesion of cells released from rDg, RGD and GFOGER gels to a matrix such as reconstituted porcine ECM, although this will not truly reflect the post-IR environment, which is known to modify expression of adhesion molecules on transplanted cells and myocardium[282].

Bioluminescence imaging of luciferase+ CPCs would have been very useful to track retention of transplanted cells in rat hearts over several time points. We attempted to transduce CPCs with a lentivirus expressing firefly luciferase and blasticidin resistance under the control of the constitutively active cytomegalovirus and respiratory syncytial virus promoters, respectively. However, this severely affected the growth rate of cells negatively rendering them unfit for use. We tested three different concentrations of transducing lentivirus but additional treatment concentration or vectors to induce successful transduction while maintaining cell growth rate could have been tested. We also did preliminary studies with lipophilic near-IR dye DiR labeled cells (data not shown) but got erratic results, perhaps because of the leakiness of DiR causing host cells to be fluorescent as well and showing false positives [335].

We are proposing that the inability of cellular GFOGER gels to improve cardiac function may be due to reduction in released paracrine factors. A confirmatory study to test this hypothesis could be to deliver pre-differentiated cells with the non-adhesive rDg gel. However, the limitations of such an experiment would be that the extent of differentiation at time of injection and later, presence of the differentiation inducing-stimulus after injection and paracrine factors profile may be different when using an agent like dexamethasone or HGF+IGF or even GFOGER to pre-differentiate cells before delivering with rDg gels. A systematic study, as have been done using other cell types such as iPSCs [280], is desirable wherein CPCs are differentiated to different extents and transplanted to determine the optimal differentiation extent for *in vivo* function improvement.

Additional mechanisms that could have been important for cellular rDg gel induced cardiac function improvement were not tested. Some examples are cardiomyocyte proliferation, survival, macrophage infiltration, M1/M2 macrophage ratio, host progenitor cell infiltration. rDg and RGD gels secrete paracrine factors VEGF, HGF and FGFb that are known to influence these processes and could be contributing mechanisms. We stained day 28 heart sections with a macrophage marker CD11b (data not shown) and got comparable numbers between various groups including IR only and sham controls. Perhaps the macrophage response in the infarct was resolved by day 28 or was minimal any way in the athymic rats used [327]. An earlier time point would have been better for assessing role of macrophages in observed effects of various treatments.

Isolectin-positive cells were marked as surrogates for angiogenesis which label not just mature perfused large and microvessels but endothelial cells of immature vessels as well [281]. Perfusing hearts with isolectin just before sacrificing the rat hearts and then counting isolectin+ vessels, instead of using post-fixation stained heart sections, would have helped quantify functional vasculature. Also, there was variability in infarct size in the control IR only group suggesting there must have been variability in the other treatment groups as well. LAD ligation for creating an infarct is a technically challenging model and this variability has been reported by others as well [336]. Our and others' studies work around this by using sufficiently powered studies, but employment of *in vivo* infarct measurement tools such as contrast agents for MRI [337] to measure infarct size before assigning treatment could further help to limit experimental error.

Measurements of rheological parameters were done *in vitro* on acellular gels only a day after swelling the gels, which act as important surrogates to inform us of the behavior of these hydrogels at the onset of the study in comparison with each other and other studies in literature. But they do not reflect the mechanical properties of cellular hydrogels over the course of time in the *in vivo* environment; the degradation rate of different ligand presenting gels could be significantly different from each other because of the associated microenvironmental protease activity, affecting their mechanical properties. Degradation rate of materials affects functional outcomes by influencing

extent of angiogenesis [338] among other mechanisms. Slower and faster degrading rDg gels as well as gels of different densities could be tested to find the optimal physical properties for imparting greatest improvement by rDg gels.

The contribution of 3D encapsulation of CPCs on the observed effects was not determined. 'Cells only' group was only tested in *in vivo* studies for cardiac function determination. However, the cells only group was not included in other experiments such as differentiation and paracrine factors assessments *in vitro*. Encapsulation and 3D culture of cells has been shown to affect cell behavior [92,339]. For example, ADSCs encapsulated in PEG-HA gels experienced inhibited proliferation but enhanced growth factor secretion [339]. MSCs in spheroids show enhanced expression of immunomodulatory factors and growth factors including TNFα and HGF [92].

5.3 Conclusion

Delivery of c-Kit+ cardiac progenitor cells has shown encouraging but insufficient improvement in clinical endpoints for patients with ischemic cardiomyopathy. Motivated by the need to enhance the therapeutic benefits of cell therapy for cardiac repair, this dissertation focused on designing and testing of biomaterials-based strategies to enhance c-kit+ cardiac progenitor cell-based therapy. The findings add to our knowledge of CPC behavior in the presence of stimuli relevant to pragmatic design of regenerative therapies including VEGF functionalized and integrin specific hydrogels, as well as enhance our understanding of *in vivo* response to these constructs. Apart from testing clinically translatable technologies, this work also advances our mechanistic understanding of factors that influence outcomes of cell therapy. While CPCs and PEG-hydrogels were used in these experiments, lessons learned in this project could help generate hypotheses for cell therapy strategies based on other cell types and biomaterials as well. Overall, this work broadens our understanding of design principles that may be used to augment effects of cell therapy for myocardial repair.

APPENDIX

A.1. Cell isolation and culture

Donors: Neonatal human c-kit+ cardiac progenitor cells (nhCPCs) were isolated from human atrial tissue obtained from children aged 1 week or less undergoing corrective surgery at Children's Healthcare of Atlanta. Child CPCs (chCPCs) were obtained from children aged 1 year or older. For hCPCs (nhCPCs and chCPCS), cells from three donors were pooled at the first passage and used for our experiments. Rat CPCs were isolated from whole heart. Rat CPCs were clonally expanded after isolation.

Isolation of cells from tissue: The tissue was rinsed with cold Hank's Buffered Salt Solution (HBSS) and chopped into small pieces. The chopped tissue was further digested with sterile 1 mg/mL Collagenase II (Worthington) solution and kept on a rocker in the cell culture incubator maintained at $37^{O}C$, 5% CO_2 for 30 minutes for enzymatic degradation of extra cellular matrix. The solution was then passed through a 70 μm strainer (BD). The cells were pelleted by centrifugation at 1500 g for 5 minutes. Magnetic beads (Dynal) conjugated to anti-c-kit antibodies (Santa Cruz H-300) were prepared. The cells were mixed with beads conjugated to anti- c-kit antibodies and incubated on a rocker in a humidified cell culture incubator for 2 hours. Magnetic beads were incubated with digested tissue to bind c-kit+ cells and then separated using a magnet, interspersed with two washes. The separated c-kit+ cells were expanded and expression of c-kit in the collected population was measured by flow cytometry to ensure they were at least 90% positive.

Culture of CPCs: The cells were passaged up to passage 12 and they maintained c-kit expression at least up to that passage. Human CPC cell culture media included Ham's F-12 base media (Corning), Pennicillin-Streptomyocin cocktail (Cellgro), L-glutamine (Cellgro), 0.1 μg/mL basic fibroblast growth factor (Sigma) and 10% heat inactivated

fetal bovine serum (Hyclone). Rat culture media had the additional supplement of Leukemia Inhibitory Factor (10 ng/mL). For treatments, serum free media comprising Ham's F-12 base media, Pennicillin-Streptomyocin cocktail, L-glutamine and Insulin-Transferrin-Selenium (ITS) (Cellgro) was used. Media was refreshed every 2-3 days.

A.2. Hydrogel synthesis

VEGF functionalized hydrogels: Michael-type addition PEG-4MAL hydrogels were formed by reacting 20 kDa 4-arm functionalized PEG-macromer (Laysan Bio) with 2mM GRGDSPC (New England Peptide) or GRDGSPC (AAPPTec) and specified dosage of VEGF (Cell Signaling Technology) (or PBS in case of control gels). CPCs were then suspended in the hydrogel followed by cross-linking with the protease degradable peptide VPM (New England Peptide) at a molar concentration equal to the remaining reactive MAL groups. The reactions were carried out in 2 mM, pH 7.4 Triethanolamine (Sigma) in PBS.

Integrin specific hydrogels: Poly(ethylene glycol)-based hydrogels encapsulating cells and different bioadhesive/non-adhesive ligands were prepared. Components were resuspended in pH 6.0 10 mM HEPES in DPBS++. A premixed solution of the protease-sensitive crosslinker peptide, 'VPM' (GCRDVPMSMRGGDRCG) (New England Peptide) , cells (10 million/mL hydrogel) and non-adhesive ligand 'rDg' (GRDGSPC) (AAPPTec) or 'RGD' (GRGDSPC) (New England Peptide) or 'GFOGER' (GGYGGGP(GPP)5GFOGER(GPP)5GPC) (AAPPTec) was mixed with 4% or 5% w/v 20 kDa PEG-MAL (Laysan Bio). The final concentration of the rDg/RGD/GFOGER was 1 mM and the concentration of VPM (New England Peptide) was equal to the balance maleimide sites remaining after accounting for maleimides theoretically reacting with the 1 mM adhesive/scrambled ligands.

The solutions were mixed in a syringe as a mould and allowed to gel at 37^{o}C for 5-15 min in a 1 mL syringe barrel as mould, transferred to a 24 well plate containing 300-500

μL/well Ham's F-12 media with ITS, P/S and L-glutamine (CPC treatment media). Hydrogels made for microscopy as end-point studies are gelled directly on 1.5 mm glass bottom dishes (MatTek). Media was changed every 2-3 days.

A.3. Rheology

As described in a previous publication [186], cellular hydrogels were made, swollen in DPBS++ overnight and their storage and loss moduli measured using dynamic oscillatory strain and frequency sweeps performed on a MCR 302 stress-controlled rheometer (Anton Paar, Austria) with a 9 mm diameter, 28 cone, and plate geometry. The hydrogels were loaded between the cone and plate, and the measuring system was lowered to a 39 mm gap. Initial strain amplitude sweeps were performed at ω=10 rad/s to determine the linear viscoelastic range of the hydrogel. Oscillatory frequency sweeps (0.5–100 rad/s) were then used to examine the storage and loss moduli at a strain of 1%.

A.4. Real time PCR

Cells were harvested in Trizol (Life Technologies) and total RNA was isolated by following the manufacturer's protocol. For isolating RNA from cells encapsulated in hydrogels, the hydrogels were homogenized in Trizol using a homogenizer. RNA quantification and purity were determined by absorbance readings at 260 and 280 nm (BioTek Synergy2 Spectrophotometer). cDNA was prepared from the mRNA using a MuMLV reverse transcriptase-based reaction described as follows. RNA was mixed with 0.1 μg random hexamers (Thermo Scientific), 0.1 μg oligo dTs (Fermentas), 25 nmol dNTPs (Fermentas) and RNase free water (Hyclone) to a final solution volume of 12 μL. Controls were performed by using RNase free water instead of RNA. Samples were heated at 65°C for 5 minutes to denature the RNA, followed by 25°C for 10 minutes to allow hexamers and oligos to anneal. First strand buffer (Invitrogen), 0.2 μmol DTT (Invitrogen), 40 units RNaseOUT Inhibitor (Invitrogen) and 200 units M-MLV (Invitrogen) were then added to the samples making up the volume to 20μL. Samples were heated at

37°C for 60 minutes for reverse transcription, followed by 70°C for 15 minutes to deactivate the enzyme. Real-time polymerase chain reaction was performed and analyzed using Step One Software (Applied Biosystems). Each reaction mixture contained 7.5 μL Power SYBR Green (Invitrogen), 5.1 μL RNase free water (Hyclone), 1.4 μL of the forward and reverse primers at 1μM (IDT) and 1 μL 1:3 diluted cDNA. The polymerase chain reaction protocol was 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A melt-curve was calculated at 2°C intervals with the same cycling conditions. Each sample was run in triplicate. $\Delta\Delta C_t$ method was used to obtain fold change values over the specified control or standard curve method was used where mRNA copy numbers are provided. 18s was used as housekeeping gene in rCPC studies and GAPDH was used in hCPC experiments.

A.5. Western blotting

Cells were homogenized in NP-40 lysis buffer supplemented with protease inhibitor and phosphatase inhibitor cocktails. For analyzing cells encapsulated in hydrogels, the hydrogels were degraded by incubation with 1mg/mL collagenase I (Worthington) solution for 40 minutes and the released cells were pelleted by centrifugation. The lysate was centrifuged at 10k g for 10 minutes and the supernatant was stored for further analysis. Equal total protein amount samples, as measured by micro BCA, were loaded on SDS-PAGE gels and they were run at 120 V. The polyacrylamide gel was transferred on to nitrocellulose membranes, blocked using 5% BSA-TBST at RT for 1 h, incubated with 1:1000 v/v primary antibody at 4^{O}C overnight, washed with TBST three times for 10 minutes each, incubated with 1:5000 v/v horseradish peroxidase tagged secondary antibody (Bio-rad) at RT for 1 h, washed three times for 10 minutes each, incubated with the substrate ECL (Denville Scientific) or ECL Prime (GE) and then exposed to an X-ray film (Denville Scientific). The films were scanned at 300 dpi resolution and obtained bands were quantified by densitometry analysis in ImageJ (NIH).

A.6. Flow cytometry

For analyzing cells encapsulated in hydrogels, the hydrogels were degraded by incubation with 1mg/mL collagenase I (Worthington) solution for 40 minutes and the released cells were pelleted by centrifugation. Plated cells were dislodged using TryplE for analysis. Cells were fixed with 2% paraformaldehyde, permeabilized using methanol for intracellular antigens, blocked with goat serum and incubated with primary antibody (1:100 v/v) overnight at $4^{O}C$ followed by incubation with secondary antibody (1:300 v/v) (Alexa Fluor 488 conjugated antibodies, Life Technologies). Samples were analyzed using a flow cytometer.

A.7. Conditioned media immunoassay

Conditioned media from hydrogels were separated, centrifuged at 10k g for 10 minutes to remove particulate matter and supernatants stored for further analysis. 1:2 dilutions of the samples were processed using a Luminex kit (R&D Systems- LXSAH) following manufacturer's protocol. Briefly, the samples were added to a mixture of color-coded beads, pre-coated with analyte-specific capture antibodies. Biotinylated detection antibodies specific to the analytes of interest were added and they formed an antibody-antigen sandwich. Phycoerythrin (PE)-conjugated streptavidin was added and it bound to the biotinylated detection antibodies. The beads were analyzed on a Luminex 100 instrument. Concentrations were obtained from fluorescence readings by mapping on to a standard curve.

A.8. Animal studies

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Emory University. Athymic rats (Crl:NIH-*Foxn1rnu*) (~250gm, 6-8 weeks old) were obtained from Charles River Laboratory. Rats were anesthetized with 2% of isoflurane, orally intubated, and ventilated. The left anterior descending coronary artery was ligated for 30 mins followed by reperfusion. During reperfusion, hydrogels 60 µL hydrogels were injected into the myocardium at three border zones using 27G insulin syringe (BD). Cardiac function was evaluated over the course of time after

injection of various treatments by echocardiography (Acuson Sequoia 512 with a 14 MHz transducer) to assess cardiac function. All functional evaluations were conducted and analyzed by investigators blinded to the animal treatment group. Ejection fraction and fractional shortening were obtained by analyzing scans in M-mode (Vevo 2100). The rats were euthanized 4 weeks after surgery and their hearts were excised and processed for histological analyses.

A.9. Histology

The hearts were fixed in 4% paraformaldehyde at $4^{O}C$ overnight or RT for 4 hours, dehydrated in ethanol and embedded in paraffin using Leica TP1020 tissue processor. 5-7 µm thick sections were made. The paraffin-embedded heart tissue sections were dewaxed in Histoclear (National Diagnostics) followed by a series of washes in ethanol.

Picosirius red: The sections were stained with pico-sirius red solution for 1 hour (Sigma), washed in acidified water and ethanol and mounted with resinous medium (Cytoseal). Whole slide scans were taken (Hamamatsu) and the percentage of fibrosis was quantified from low resolution images using Aperio software as the ratio of fibrotic tissue (stained red) to total tissue.

Isolectin: The sections underwent antigen retrieval in pH 6 citrate buffer for 10 min and incubated with FITC-tagged isolectin (Vector) for 1 h at RT or $4^{O}C$ overnight. Three randomly chosen sections in the infarct border region were photographed and the number of fluorescent cells also positive for DAPI was manually counted.

Wheat germ agglutinin: The sections underwent antigen retrieval in pH 6 citrate buffer for 10 min and incubated with Rhodamine-tagged isolectin (Vector) for 30 min at RT. The cross-sectional area of 6-8 myocytes in the infarct border region were measured by manual tracing (CellSens software).

Human mitochondria: The sections underwent antigen retrieval in pH 6 citrate buffer for 20 minutes, permeabilization with 0.1% Triton X for 10 minutes and incubated with anti-human mitochondria antibody (1:50 v/v, Abcam) overnight at $4^{O}C$, followed by incubation with alkaline phosphatase tagged secondary antibody (1:50 v/v, Sigma). The sections were incubated with Vector Red (Vector) substrate for 10 minutes, counterstained with methyl green, dehydrated in ethanol and mounted with resinous medium (Cytoseal). Whole slide scans were taken (Hamamatsu) and cells positive for both pink (developed by alkaline phosphatase-Vector Red reaction) and green (stained nuclei by methyl green) were counted (NDP view software).

A.10. Statistics

All data are expressed as mean ± SEM (standard error of mean). To determine significance of difference between means, either a One-way or Two-way analysis of variance (ANOVA) followed by the appropriate post-hoc test, or Student's t-test was performed using GraphPad Prism5, as specified with each result. $p \leq 0.05$ was considered statistically significant.

A.11. List of primers for real-time PCR

Animal	Target gene	Forward primer (5'->3')	Reverse primer (5'->3')
Rat	flk1	GCCAATGAAGGGGAACTGAAGAC	TCTGACTGCTGGTGATGCTGTC
Rat	tie2	TGCCACCATCACTCAATACCA	AGGCTGGGTTGCTTGATCCT
Rat	cdh5	TCCTCTGCATCCTCACTATCACA	GTAAGTGACCAACTGCTCGTGAAT
Rat	vwf	CCCACCGGATGGCTAGGTATT	GAGGCGGATCTGTTTGAGGTT
Rat	18s	TTCCTTACCTGGTTGATCCTGCCA	AGCGAGCGACCAAAGGAACCATAA
Human	gapdh	GTGGACCTGACCTGCCGTCT	GGAGGAGTGGGTGTCGCTGT
Human	gata4	GGAGATGCGTCCCATCAAGAC	GGAGACGCATAGCCTTGTGG
Human	nkx2_5	ACCCTGAGTCCCCTGGATTT	TCACTCATTGCACGCTGCAT
Human	myh7	GGCAAGACAGTGACCGTGAAG	CGTAGCGATCCTTGAGGTTGTA
Human	tnnt2	GCGGGTCTTGGAGACTTTCT	TTCGACCTGCAGGAGAAGTT
Human	myh6	TCTCCGACAACGCCTATCAGTAC	GTCACCTATGGCTGCAATGCT
Human	tnni3	CCAACTACCGCGCTTATGC	CTCGCTCCAGCTCTTGCTTT
Human	mef2c	TAACTTCTTTTCACTGTTGTGCTCCTT	GCCGCTTTTGGCAAATGTT
Human	mlc2v	CCTTGGGCGAGTGAACGT	GGGTCCGCTCCCTTAAGTTT

Human	flt1	GACTAGATAGCGTCACCAGCAG	GAAACCGTCAGAATCCTCCTC
Human	cd31	GAGTCCTGCTGACCCTTCTG	ATTTTGCACCGTCCAGTCC
Human	vwf	TGTCTGGCTGAGGGAGGTAA	GTACATGGCTTTGCTGGCAC
Human	cdh5	TTTCCAGCAGCCTTTCTACCA	GGAAGAACTGGCCCTTGTCA
Human	tagln	AAATGCCCCGGATGACTTGG	GGGGAAAGCTCCTTGGAAGT
Human	acta	AATACTCTGTCTGGATCGGTGGCT	ACGAGTCAGAGCTTTGGCTAGGAA
Human	itga1	AGGATTTCTGGCTTGTGGG	ACTATGTCCAGTTGAGTGCTG
Human	itga2	GACCTATCCACTGCCACATG	TGTGAGAAAACCTCCAGTTCC
Human	itga3	GGAACAGCACCTTCATCGAG	AATGTCCACAGAGAACCACG
Human	itga5	GGAACCTCACTTACGGCTATG	ACCAGCAAGTCATCCAGC
Human	itga8	ACAGGCTCACATTCTGGTG	TCCTTCCCCTTCATTTCTTGC
Human	itga10	CTTCAGTTCTGGGATATGTGCC	CCAGTCTTCGTAGGAAGGTCT
Human	itga11	GTGCCTATGACTGGAATGGAG	CGACCGATGTGACTGTGTAC
Human	itgav	GCAGTGTGAGGAATTGATAGCG	AAGTAGAATGTGAGCCTGTCG
Human	itgb1	TGTAAGGAGAAGGATGTTGACG	CAACCACACCAGCTACAATTG
Human	itgb3	CCCTGCTCATCTGGAAACTC	CGGTACGTGATATTGGTGAAGG
Human	itgb5	GCTCGCAGGTCTCAACATATG	TCTCTATCTCACCTCCACAGC

A.12. List of antibodies

Animal	Target protein	Antibody/ stain	Application	Dilution (v/v)
Rat/human	c-kit	H-300	CPC isolation	10 uL antibody added to 50 uL beads Dynabeads M-280 Sheep anti-rabbit IgG
			FC	1:100
Rat	pErk	CST #9101	WB	1:1000
Rat	Erk	CST #9102	WB	1:1000
Rat/human	Nkx2.5	H-114	WB	1:1000
Rat/human	CD31	M-185	FC	1:100
Rat/human	Flk1	ab9530	FC	1:100
Rat/human	VE-cadherin	ab166715	FC	1:100
Rat/human	vWF	ab6994	FC	1:100
Human	MHC	ab50967	WB	1:1000
Human	Troponin I	H170	WB	1:1000
Rat	CD11b	CBL1512	IHC	1:50
Human	MTCO2	ab92824	IHC	1:50
Rat	Isolectin	FL-1201	IHC	1:50
Rat	Wheat germ agglutinin	RL-1022	IHC	1:250
WB: Western blotting, FC: Flow cytometry, IHC: Immunohistochemistry				

References

1. Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD. Third universal definition of myocardial infarction. Nat Rev Cardiol. Nature Publishing Group; 2012;9: 620–633. doi:10.1038/nrcardio.2012.122
2. World Health Organization. WHO | Global status report on noncommunicable diseases 2014. WHO. World Health Organization; 2015; 298. Available: http://who.int/nmh/publications/ncd-status-report-2014/en/
3. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. Heart Disease and Stroke Statistics—2016 Update. Circulation. 2015;
4. Falk E, Shah PK, Fuster V. Coronary Plaque Disruption. Circulation. 1995;92.
5. Manfroi WC, Peukert C, Berti CB, Noer C, Gutierres D de A, Silva FTBGC da. Acute myocardial infarction: the first manifestation of ischemic heart disease and relation to risk factors. Arq Bras Cardiol. 2002;78: 392–5. Available: http://www.ncbi.nlm.nih.gov/pubmed/12011955
6. Sutton MGSJ, Sharpe N. Left Ventricular Remodeling After Myocardial Infarction. Circulation. 2000;101.
7. Frangogiannis NG. Pathophysiology of Myocardial Infarction. Comprehensive Physiology. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2015. pp. 1841–1875. doi:10.1002/cphy.c150006
8. Frangogiannis NG. The inflammatory response in myocardial injury, repair, and remodelling. Nat Rev Cardiol. NIH Public Access; 2014;11: 255–65. doi:10.1038/nrcardio.2014.28
9. Gheorghiade M, Sopko G, De Luca L, Velazquez EJ, Parker JD, Binkley PF, et al. Navigating the Crossroads of Coronary Artery Disease and Heart Failure. Circulation. 2006;114.
10. Assmus B, Dimmeler S, Zeiher AM. Cardiac Cell Therapy. Circ Res. 2015;116.
11. Bergmann O, Jovinge S. Cardiac regeneration in vivo: Mending the heart from within? Stem Cell Res. 2014;13: 523–531. doi:10.1016/j.scr.2014.07.002
12. Zaragoza C, Gomez-Guerrero C, Martin-Ventura JL, Blanco-Colio L, Lavin BB, Mallavia BB, et al. Animal models of cardiovascular diseases. J Biomed Biotechnol. Hindawi Publishing Corporation; 2011;2011: 497841. doi:10.1155/2011/497841
13. Houser SR, Margulies KB, Murphy AM, Spinale FG, Francis GS, Prabhu SD, et al. Animal Models of Heart Failure. Circ Res. 2012;111.
14. Feyen DAM, Gaetani R, Doevendans PA, Sluijter JPG. Stem cell-based therapy: Improving myocardial cell delivery. Adv Drug Deliv Rev. 2016;106: 104–115. doi:10.1016/j.addr.2016.04.023
15. Baraniak PR, McDevitt TC. Stem cell paracrine actions and tissue regeneration. Regen Med. NIH Public Access; 2010;5: 121–43. doi:10.2217/rme.09.74
16. Ranganath SH, Levy O, Inamdar MS, Karp JM. Harnessing the Mesenchymal Stem Cell Secretome for the Treatment of Cardiovascular Disease. Cell Stem Cell. 2012;10: 244–258. doi:10.1016/j.stem.2012.02.005
17. Urbanek K, Rota M, Cascapera S, Bearzi C, Nascimbene A, De Angelis A, et al. Cardiac Stem Cells Possess Growth Factor-Receptor Systems That After Activation Regenerate the Infarcted Myocardium, Improving Ventricular Function and Long-Term Survival. Circ Res. 2005;97.
18. Ellison GM, Torella D, Dellegrottaglie S, Perez-Martinez C, Perez De Prado A, Vicinanza C, et al. Endogenous cardiac stem cell activation by insulin-like growth factor-1/hepatocyte growth factor intracoronary injection fosters survival and

regeneration of the infarcted pig heart. J Am Coll Cardiol. Elsevier Inc.; 2011;58: 977–986. doi:10.1016/j.jacc.2011.05.013
19. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, et al. Implantation of Bone Marrow Mononuclear Cells Into Ischemic Myocardium Enhances Collateral Perfusion and Regional Function via Side Supply of Angioblasts, Angiogenic Ligands, and Cytokines. Circulation. 2001;104.
20. Takahashi M. Cytokines produced by bone marrow cells can contribute to functional improvement of the infarcted heart by protecting cardiomyocytes from ischemic injury. AJP Hear Circ Physiol. 2006;291: H886–H893. doi:10.1152/ajpheart.00142.2006
21. Urbich C, Aicher A, Heeschen C, Dernbach E, Hofmann WK, Zeiher AM, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol. 2005;39: 733–742. doi:10.1016/j.yjmcc.2005.07.003
22. Cho H-J, Lee N, Lee JY, Choi YJ, Ii M, Wecker A, et al. Role of host tissues for sustained humoral effects after endothelial progenitor cell transplantation into the ischemic heart. J Exp Med. The Rockefeller University Press; 2007;204: 3257–69. doi:10.1084/jem.20070166
23. Berry MF, Engler AJ, Woo YJ, Pirolli TJ, Bish LT, Jayasankar V, et al. Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. Am J Physiol - Hear Circ Physiol. 2006;290.
24. Soonpaa MH, Koh GY, Klug MG, Field LJ. Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science. 1994;264: 98–101. Available: http://www.ncbi.nlm.nih.gov/pubmed/8140423
25. Koh GY, Soonpaa MH, Klug MG, Pride HP, Cooper BJ, Zipes DP, et al. Stable fetal cardiomyocyte grafts in the hearts of dystrophic mice and dogs. J Clin Invest. 1995;96: 2034–2042. doi:10.1172/JCI118251
26. Menasche P, Alfieri O, Janssens S, McKenna W, Reichenspurner H, Trinquart L, et al. The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) Trial: First Randomized Placebo-Controlled Study of Myoblast Transplantation. Circulation. 2008;117: 1189–1200. doi:10.1161/CIRCULATIONAHA.107.734103
27. Quevedo HC, Hatzistergos KE, Oskouei BN, Feigenbaum GS, Rodriguez JE, Valdes D, et al. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. Proc Natl Acad Sci U S A. National Academy of Sciences; 2009;106: 14022–7. doi:10.1073/pnas.0903201106
28. Li Q, Guo Y, Ou Q, Chen N, Wu W-J, Yuan F, et al. Intracoronary administration of cardiac stem cells in mice: a new, improved technique for cell therapy in murine models. Basic Res Cardiol. NIH Public Access; 2011;106: 849–64. doi:10.1007/s00395-011-0180-1
29. Li T-SS, Cheng K, Malliaras K, Smith RR, Zhang Y, Sun B, et al. Direct comparison of different stem cell types and subpopulations reveals superior paracrine potency and myocardial repair efficacy with cardiosphere-derived cells. J Am Coll Cardiol. Journal of the American College of Cardiology; 2012;59: 942–953. doi:10.1016/j.jacc.2011.11.029
30. Wendel JS, Ye L, Tao R, Zhang J, Zhang J, Kamp TJ, et al. Functional Effects of a Tissue-Engineered Cardiac Patch From Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes in a Rat Infarct Model. Stem Cells Transl Med. AlphaMed Press; 2015;4: 1324–32. doi:10.5966/sctm.2015-0044
31. Sanganalmath SK, Bolli R. Cell therapy for heart failure: A comprehensive

overview of experimental and clinical studies, current challenges, and future directions. Circ Res. 2013;113: 810–834. doi:10.1161/CIRCRESAHA.113.300219
32. Silva G V., Litovsky S, Assad JAR, Sousa ALS, Martin BJ, Vela D, et al. Mesenchymal Stem Cells Differentiate into an Endothelial Phenotype, Enhance Vascular Density, and Improve Heart Function in a Canine Chronic Ischemia Model. Circulation. 2005;111: 150–156. doi:10.1161/01.CIR.0000151812.86142.45
33. Cai L, Johnstone BH, Cook TG, Tan J, Fishbein MC, Chen P-S, et al. IFATS Collection: Human Adipose Tissue-Derived Stem Cells Induce Angiogenesis and Nerve Sprouting Following Myocardial Infarction, in Conjunction with Potent Preservation of Cardiac Function. Stem Cells. 2009;27: 230–237. doi:10.1634/stemcells.2008-0273
34. Botta R, Gao E, Stassi G, Bonci D, Pelosi E, Zwas D, et al. Heart infarct in NOD-SCID mice: Therapeutic vasculogenesis by transplantation of human CD34+ cells and low dose CD34+KDR+ cells. FASEB J. 2004;18: 1392–4. doi:10.1096/fj.03-0879fje
35. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, et al. Adult Cardiac Stem Cells Are Multipotent and Support Myocardial Regeneration. Cell. 2003;114: 763–776. doi:10.1016/S0092-8674(03)00687-1
36. Bearzi C, Leri A, Lo Monaco F, Rota M, Gonzalez A, Hosoda T, et al. Identification of a coronary vascular progenitor cell in the human heart. Proc Natl Acad Sci U S A. 2009;106: 15885–15890. doi:10.1073/pnas.0907622106
37. Hong KU, Guo Y, Li Q-H, Cao P, Al-Maqtari T, Vajravelu BN, et al. c-kit+ Cardiac stem cells alleviate post-myocardial infarction left ventricular dysfunction despite poor engraftment and negligible retention in the recipient heart. Hare JM, editor. PLoS One. 2014;9: e96725. doi:10.1371/journal.pone.0096725
38. Tang X-L, Li Q, Rokosh G, Sanganalmath SK, Chen N, Ou Q, et al. Long-Term Outcome of Administration of c-kit ^{POS} Cardiac Progenitor Cells After Acute Myocardial InfarctionNovelty and Significance. Circ Res. 2016;118: 1091–1105. doi:10.1161/CIRCRESAHA.115.307647
39. Chimenti I, Smith RR, Li T-S, Gerstenblith G, Messina E, Giacomello A, et al. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. Circ Res. 2010;106: 971–980. doi:10.1161/CIRCRESAHA.109.210682
40. Yang D, Wang W, Li L, Peng Y, Chen P, Huang H, et al. The Relative Contribution of Paracine Effect versus Direct Differentiation on Adipose-Derived Stem Cell Transplantation Mediated Cardiac Repair. Qin G, editor. PLoS One. 2013;8: e59020. doi:10.1371/journal.pone.0059020
41. Cheng K, Shen D, Smith J, Galang G, Sun B, Zhang J, et al. Transplantation of platelet gel spiked with cardiosphere-derived cells boosts structural and functional benefits relative to gel transplantation alone in rats with myocardial infarction. Biomaterials. 2012;33: 2872–2879. doi:10.1016/j.biomaterials.2011.12.040
42. Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med. Nature Publishing Group; 2005;11: 367–368. doi:10.1038/nm0405-367
43. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, et al. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J. Federation of American Societies for Experimental Biology; 2006;20: 661–9. doi:10.1096/fj.05-

5211com
44. Timmers L, Lim SK, Arslan F, Armstrong JS, Hoefer IE, Doevendans PA, et al. Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. Stem Cell Res. 2008;1: 129–137. doi:10.1016/j.scr.2008.02.002
45. Sharma S, Mishra R, Bigham GE, Wehman B, Khan MM, Xu H, et al. A Deep Proteome Analysis Identifies the Complete Secretome as the Functional Unit of Human Cardiac Progenitor Cells. Circ Res. 2016; CIRCRESAHA.116.309782. doi:10.1161/CIRCRESAHA.116.309782
46. Nguyen PK, Neofytou E, Rhee J-W, Wu JC. Potential Strategies to Address the Major Clinical Barriers Facing Stem Cell Regenerative Therapy for Cardiovascular Disease. JAMA Cardiol. 2016;1: 953. doi:10.1001/jamacardio.2016.2750
47. Banovic M, Loncar Z, Behfar A, Vanderheyden M, Beleslin B, Zeiher A, et al. Endpoints in stem cell trials in ischemic heart failure. Stem Cell Res Ther. BioMed Central; 2015;6: 159. doi:10.1186/s13287-015-0143-9
48. Hare JM, Bolli R, Cooke JP, Gordon DJ, Henry TD, Perin EC, et al. Phase II clinical research design in cardiology: learning the right lessons too well: observations and recommendations from the Cardiovascular Cell Therapy Research Network (CCTRN). Circulation. NIH Public Access; 2013;127: 1630–5. doi:10.1161/CIRCULATIONAHA.112.000779
49. Dey D, Han L, Bauer M, Sanada F, Oikonomopoulos A, Hosoda T, et al. Dissecting the molecular relationship among various cardiogenic progenitor cells. Circ Res. 2013;112: 1253–1262. doi:10.1161/CIRCRESAHA.112.300779
50. Linke A, Müller P, Nurzynska D, Casarsa C, Torella D, Nascimbene A, et al. Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. Proc Natl Acad Sci U S A. National Academy of Sciences; 2005;102: 8966–71. doi:10.1073/pnas.0502678102
51. Tang X-L, Rokosh G, Sanganalmath SK, Yuan F, Sato H, Mu J, et al. Intracoronary administration of cardiac progenitor cells alleviates left ventricular dysfunction in rats with a 30-day-old infarction. Circulation. 2011;121: 293–305. doi:10.1161/CIRCULATIONAHA.109.871905.INTRACORONARY
52. Bolli R, Tang X-L, Sanganalmath SK, Rimoldi O, Mosna F, Abdel-Latif A, et al. Intracoronary Delivery of Autologous Cardiac Stem Cells Improves Cardiac Function in a Porcine Model of Chronic Ischemic CardiomyopathyClinical Perspective. Circulation. 2013;128.
53. Itzhaki-Alfia A, Leor J, Raanani E, Sternik L, Spiegelstein D, Netser S, et al. Patient Characteristics and Cell Source Determine the Number of Isolated Human Cardiac Progenitor Cells. Circulation. 2009;120: 2559–2566. doi:10.1161/CIRCULATIONAHA.109.849588
54. Chugh AR, Beache GM, Loughran JH, Mewton N, Elmore JB, Kajstura J, et al. Administration of cardiac stem cells in patients with ischemic cardiomyopathy: the SCIPIO trial: surgical aspects and interim analysis of myocardial function and viability by magnetic resonance. Circulation. 2012;126: S54-64. doi:10.1161/CIRCULATIONAHA.112.092627
55. Crisostomo V, Baez-Diaz C, Maestre J, Garcia-Lindo M, Sun F, Casado JG, et al. Delayed administration of allogeneic cardiac stem cell therapy for acute myocardial infarction could ameliorate adverse remodeling: experimental study in swine. J Transl Med. BioMed Central; 2015;13: 156. doi:10.1186/s12967-015-0512-2

56. Lauden L, Boukouaci W, Borlado LR, López IP, Sepúlveda P, Tamouza R, et al. Allogenicity of Human Cardiac Stem/Progenitor Cells Orchestrated by Programmed Death Ligand 1Novelty and Significance. Circ Res. 2013;112: 451–464. doi:10.1161/CIRCRESAHA.112.276501
57. Williams AR, Hatzistergos KE, Addicott B, McCall F, Carvalho D, Suncion V, et al. Enhanced Effect of Combining Human Cardiac Stem Cells and Bone Marrow Mesenchymal Stem Cells to Reduce Infarct Size and to Restore Cardiac Function After Myocardial InfarctionClinical Perspective. Circulation. 2013;127.
58. Quijada P, Salunga HT, Hariharan N, Cubillo JD, El-Sayed FG, Moshref M, et al. Cardiac Stem Cell Hybrids Enhance Myocardial RepairNovelty and Significance. Circ Res. 2015;117.
59. Mishra R, Vijayan K, Colletti EJ, Harrington DA, Matthiesen TS, Simpson D, et al. Characterization and functionality of cardiac progenitor cells in congenital heart patients. Circulation. 2011;123: 364–373. doi:10.1161/CIRCULATIONAHA.110.971622
60. He J-Q, Vu DM, Hunt G, Chugh A, Bhatnagar A, Bolli R, et al. Human Cardiac Stem Cells Isolated from Atrial Appendages Stably Express c-kit. Rota M, editor. PLoS One. Public Library of Science; 2011;6: e27719. doi:10.1371/journal.pone.0027719
61. Wehman B, Sharma S, Mishra R, Guo Y, Colletti EJ, Kon ZN, et al. Pediatric End-Stage Failing Hearts Demonstrate Increased Cardiac Stem Cells. Ann Thorac Surg. 2015;100: 615–622. doi:10.1016/j.athoracsur.2015.04.088
62. Genead R, Fischer H, Hussain A, Jaksch M, Andersson AB, Ljung K, et al. Ischemia-reperfusion injury and pregnancy initiate time-dependent and robust signs of up-regulation of cardiac progenitor cells. PLoS One. Public Library of Science; 2012;7: e36804. doi:10.1371/journal.pone.0036804
63. Leite CF, Lopes CS, Alves AC, Fuzaro CSC, Silva MV, Oliveira LF de, et al. Endogenous resident c-Kit cardiac stem cells increase in mice with an exercise-induced, physiologically hypertrophied heart. Stem Cell Res. 2015;15: 151–164. doi:10.1016/j.scr.2015.05.011
64. Agarwal U, Smith AW, French KM, Boopathy A V., George A, Trac D, et al. Age-Dependent Effect of Pediatric Cardiac Progenitor Cells After Juvenile Heart Failure. Stem Cells Transl Med. 2016;5: 883–892. doi:10.5966/sctm.2015-0241
65. Leonardini A, Avogaro A. Abnormalities of the cardiac stem and progenitor cell compartment in experimental and human diabetes. Arch Physiol Biochem. 2013;119: 179–187. doi:10.3109/13813455.2013.798334
66. Mohsin S, Khan M, Toko H, Bailey B, Cottage CT, Wallach K, et al. Human Cardiac Progenitor Cells Engineered With Pim-I Kinase Enhance Myocardial Repair. J Am Coll Cardiol. 2012;60: 1278–1287. doi:10.1016/j.jacc.2012.04.047
67. Zakharova L, Nural-Guvener H, Feehery L, Popovic-Sljukic S, Gaballa MA. Transplantation of Epigenetically Modified Adult Cardiac c-Kit+ Cells Retards Remodeling and Improves Cardiac Function in Ischemic Heart Failure Model. Stem Cells Transl Med. AlphaMed Press; 2015;4: 1086–96. doi:10.5966/sctm.2014-0290
68. Malliaras K, Li T-S, Luthringer D, Terrovitis J, Cheng K, Chakravarty T, et al. Safety and Efficacy of Allogeneic Cell Therapy in Infarcted Rats Transplanted With Mismatched Cardiosphere-Derived Cells. Circulation. 2012;125: 100–112. doi:10.1161/CIRCULATIONAHA.111.042598
69. Malliaras K, Smith RR, Kanazawa H, Yee K, Seinfeld J, Tseliou E, et al. Validation of Contrast-Enhanced Magnetic Resonance Imaging to Monitor Regenerative

Efficacy After Cell Therapy in a Porcine Model of Convalescent Myocardial Infarction. Circulation. 2013;128: 2764–2775. doi:10.1161/CIRCULATIONAHA.113.002863
70. Malliaras K, Ibrahim A, Tseliou E, Liu W, Sun B, Middleton RC, et al. Stimulation of endogenous cardioblasts by exogenous cell therapy after myocardial infarction. EMBO Mol Med. 2014;6: 760–777. doi:10.1002/emmm.201303626
71. Marbán E. Breakthroughs in cell therapy for heart disease: focus on cardiosphere-derived cells. Mayo Clin Proc. NIH Public Access; 2014;89: 850–8. doi:10.1016/j.mayocp.2014.02.014
72. Leite CF, Almeida TR, Lopes CS, Dias da Silva VJ. Multipotent stem cells of the heartâ€"do they have therapeutic promise? Front Physiol. Frontiers; 2015;6: 123. doi:10.3389/fphys.2015.00123
73. Simari RD, Pepine CJ, Traverse JH, Henry TD, Bolli R, Spoon DB, et al. Bone Marrow Mononuclear Cell Therapy for Acute Myocardial Infarction: A Perspective From the Cardiovascular Cell Therapy Research Network. Circ Res. 2014;114: 1564–1568. doi:10.1161/CIRCRESAHA.114.303720
74. Chong JJH, Yang X, Don CW, Minami E, Liu Y-W, Weyers JJ, et al. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. Nature. Nature Research; 2014;510: 273–277. doi:10.1038/nature13233
75. Menasché P, Vanneaux V, Hagège A, Bel A, Cholley B, Cacciapuoti I, et al. Human embryonic stem cell-derived cardiac progenitors for severe heart failure treatment: first clinical case report. Eur Heart J. 2015;
76. Youssef AA, Ross EG, Bolli R, Pepine CJ, Leeper NJ, Yang PC. The Promise and Challenge of Induced Pluripotent Stem Cells for Cardiovascular Applications. JACC Basic to Transl Sci. 2016;1: 510–523. doi:10.1016/j.jacbts.2016.06.010
77. Tan Y, Ooi S, Wang L. Immunogenicity and tumorigenicity of pluripotent stem cells and their derivatives: genetic and epigenetic perspectives. Curr Stem Cell Res Ther. Bentham Science Publishers; 2014;9: 63–72. doi:10.2174/1574888x113086660068
78. Golpanian S, Schulman IH, Ebert RF, Heldman AW, DiFede DL, Yang PC, et al. Concise Review: Review and Perspective of Cell Dosage and Routes of Administration From Preclinical and Clinical Studies of Stem Cell Therapy for Heart Disease. Stem Cells Transl Med. AlphaMed Press; 2016;5: 186–91. doi:10.5966/sctm.2015-0101
79. Quyyumi AA, Waller EK, Murrow J, Esteves F, Galt J, Oshinski J, et al. CD34+ cell infusion after ST elevation myocardial infarction is associated with improved perfusion and is dose dependent. Am Heart J. 2011;161: 98–105. doi:10.1016/j.ahj.2010.09.025
80. Hare JM, Fishman JE, Gerstenblith G, DiFede Velazquez DL, Zambrano JP, Suncion VY, et al. Comparison of Allogeneic vs Autologous Bone Marrow–Derived Mesenchymal Stem Cells Delivered by Transendocardial Injection in Patients With Ischemic Cardiomyopathy. JAMA. 2012;308: 2369. doi:10.1001/jama.2012.25321
81. Tang X-L, Rokosh G, Sanganalmath SK, Tokita Y, Keith MCL, Shirk G, et al. Effects of Intracoronary Infusion of Escalating Doses of Cardiac Stem Cells in Rats With Acute Myocardial InfarctionCLINICAL PERSPECTIVE. Circ Hear Fail. 2015;8.
82. Barbash IM, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, et al. Systemic Delivery of Bone Marrow–Derived Mesenchymal Stem Cells to the Infarcted Myocardium. Circulation. 2003;108.

83. Grimaldi V, Mancini FP, Casamassimi A, Al-Omran M, Zullo A, Infante T, et al. Potential benefits of cell therapy in coronary heart disease. J Cardiol. Japanese College of Cardiology; 2013;62: 267–276. doi:10.1016/j.jjcc.2013.05.017
84. Hou D, Youssef EA-S, Brinton TJ, Zhang P, Rogers P, Price ET, et al. Radiolabeled cell distribution after intramyocardial, intracoronary, and interstitial retrograde coronary venous delivery: implications for current clinical trials. Circulation. 2005;112: I150-6. doi:10.1161/CIRCULATIONAHA.104.526749
85. Tokita Y, Tang X-L, Li Q, Wysoczynski M, Hong KU, Nakamura S, et al. Repeated Administrations of Cardiac Progenitor Cells Are Markedly More Effective Than a Single AdministrationNovelty and Significance. Circ Res. 2016;119.
86. Yao K, Huang R, Sun A, Qian J, Liu X, Ge L, et al. Repeated autologous bone marrow mononuclear cell therapy in patients with large myocardial infarction. Eur J Heart Fail. 2009;11: 691–698. doi:10.1093/eurjhf/hfp062
87. Mann I, Rodrigo SF, van Ramshorst J, Beeres SL, Dibbets-Schneider P, de Roos A, et al. Repeated Intramyocardial Bone Marrow Cell Injection in Previously Responding Patients With Refractory Angina Again Improves Myocardial Perfusion, Anginal Complaints, and Quality of Life. Circ Cardiovasc Interv. 2015;8.
88. Gavira JJ, Nasarre E, Abizanda G, Pérez-Ilzarbe M, de Martino-Rodriguez A, García de Jalón JA, et al. Repeated implantation of skeletal myoblast in a swine model of chronic myocardial infarction. Eur Heart J. 2010;31.
89. Zhang Y, Sievers RE, Prasad M, Mirsky R, Shih H, Wong ML, et al. Timing of bone marrow cell therapy is more important than repeated injections after myocardial infarction. Cardiovasc Pathol. 2011;20: 204–212. doi:10.1016/j.carpath.2010.06.007
90. Behfar A, Crespo-Diaz R, Terzic A, Gersh BJ. Cell therapy for cardiac repair--lessons from clinical trials. Nat Rev Cardiol. Nature Publishing Group; 2014;11: 232–46. doi:10.1038/nrcardio.2014.9
91. Hu X, Wang J, Chen J, Luo R, He A, Xie X, et al. Optimal temporal delivery of bone marrow mesenchymal stem cells in rats with myocardial infarction. Eur J Cardio-Thoracic Surg. 2007;31: 438–443. doi:10.1016/j.ejcts.2006.11.057
92. Follin B, Juhl M, Cohen S, Perdersen AE, Kastrup J, Ekblond A. Increased Paracrine Immunomodulatory Potential of Mesenchymal Stromal Cells in Three-Dimensional Culture. Tissue Eng Part B Rev. Mary Ann Liebert, Inc.; 2016;22: 322–329. doi:10.1089/ten.teb.2015.0532
93. Shachar M, Tsur-Gang O, Dvir T, Leor J, Cohen S. The effect of immobilized RGD peptide in alginate scaffolds on cardiac tissue engineering. Acta Biomater. 2011;7: 152–162. doi:10.1016/j.actbio.2010.07.034
94. Ma B, Wang X, Wu C, Chang J. Crosslinking strategies for preparation of extracellular matrix-derived cardiovascular scaffolds. Regen Biomater. Oxford University Press; 2014;1: 81–9. doi:10.1093/rb/rbu009
95. Davidenko N, Schuster CF, Bax D V, Raynal N, Farndale RW, Best SM, et al. Control of crosslinking for tailoring collagen-based scaffolds stability and mechanics. Acta Biomater. Elsevier; 2015;25: 131–42. doi:10.1016/j.actbio.2015.07.034
96. Bellis SL. Advantages of RGD peptides for directing cell association with biomaterials. Biomaterials. NIH Public Access; 2011;32: 4205–10. doi:10.1016/j.biomaterials.2011.02.029
97. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix Elasticity Directs Stem Cell Lineage Specification. Cell. 2006;126: 677–689. doi:10.1016/j.cell.2006.06.044
98. Swierczewska M, Lee KC, Lee S. What is the future of PEGylated therapies?

Expert Opin Emerg Drugs. Taylor & Francis; 2015;20: 531–536. doi:10.1517/14728214.2015.1113254
99. Lin C-C. Recent advances in crosslinking chemistry of biomimetic poly(ethylene glycol) hydrogels. RSC Adv. Royal Society of Chemistry; 2015;5: 39844–39853. doi:10.1039/C5RA05734E
100. Phelps EA, Enemchukwu NO, Fiore VF, Sy JC, Murthy N, Sulchek TA, et al. Maleimide cross-linked bioactive PEG hydrogel exhibits improved reaction kinetics and cross-linking for cell encapsulation and in situ delivery. Adv Mater. 2012;24: 64–70, 2. doi:10.1002/adma.201103574
101. Zhang M, Desai T, Ferrari M. Proteins and cells on PEG immobilized silicon surfaces. Biomaterials. 1998;19: 953–60. Available: http://www.ncbi.nlm.nih.gov/pubmed/9690837
102. Terrovitis J V, Smith RR, Marbán E. Assessment and optimization of cell engraftment after transplantation into the heart. Circ Res. 2010;106: 479–94. doi:10.1161/CIRCRESAHA.109.208991
103. Roche ET, Hastings CL, Lewin SA, Shvartsman DE, Brudno Y, Vasilyev N V., et al. Comparison of biomaterial delivery vehicles for improving acute retention of stem cells in the infarcted heart. Biomaterials. NIH Public Access; 2014;35: 6850–6858. doi:10.1016/j.biomaterials.2014.04.114
104. Mayfield AE, Tilokee EL, Latham N, McNeill B, Lam B-K, Ruel M, et al. The effect of encapsulation of cardiac stem cells within matrix-enriched hydrogel capsules on cell survival, post-ischemic cell retention and cardiac function. Biomaterials. PMC Canada manuscript submission; 2014;35: 133–42. doi:10.1016/j.biomaterials.2013.09.085
105. Lai CY, Wu PJ, Roffler SR, Lee ST, Hwang SM, Wang SS, et al. Clearance kinetics of biomaterials affects stem cell retention and therapeutic efficacy. Biomacromolecules. American Chemical Society; 2014;15: 564–573. doi:10.1021/bm401583b
106. Davis ME, Motion JPM, Narmoneva DA, Takahashi T, Hakuno D, Kamm RD, et al. Injectable self-assembling peptide nanofibers create intramyocardial microenvironments for endothelial cells. Circulation. NIH Public Access; 2005;111: 442–50. doi:10.1161/01.CIR.0000153847.47301.80
107. Kshitiz, Park J, Kim P, Helen W, Engler AJ, Levchenko A, et al. Control of stem cell fate and function by engineering physical microenvironments. Integr Biol (Camb). 2012;4: 1008–18. Available: http://www.ncbi.nlm.nih.gov/pubmed/23077731
108. Nam J, Huang Y, Agarwal S, Lannutti J. Improved cellular infiltration in electrospun fiber via engineered porosity. Tissue Eng. NIH Public Access; 2007;13: 2249–57. doi:10.1089/ten.2006.0306
109. Phelps EA, Landázuri N, Thulé PM, Taylor WR, García AJ. Bioartificial matrices for therapeutic vascularization. Proc Natl Acad Sci U S A. National Academy of Sciences; 2010;107: 3323–8. doi:10.1073/pnas.0905447107
110. Li Z, Guo X, Palmer AF, Das H, Guan J. High-efficiency matrix modulus-induced cardiac differentiation of human mesenchymal stem cells inside a thermosensitive hydrogel. Acta Biomater. 2012;8: 3586–3895. doi:10.1016/j.actbio.2012.06.024
111. Engler AJ, Griffin MA, Sen S, Bönnemann CG, Sweeney HL, Discher DE. Myotubes differentiate optimally on substrates with tissue-like stiffness. J Cell Biol. 2004;166.
112. Khetan S, Guvendiren M, Legant WR, Cohen DM, Chen CS, Burdick JA. Degradation-mediated cellular traction directs stem cell fate in covalently

crosslinked three-dimensional hydrogels. Nat Mater. Nature Research; 2013;12: 458–465. doi:10.1038/nmat3586
113. Kraehenbuehl TP, Zammaretti P, Van der Vlies AJ, Schoenmakers RG, Lutolf MP, Jaconi ME, et al. Three-dimensional extracellular matrix-directed cardioprogenitor differentiation: systematic modulation of a synthetic cell-responsive PEG-hydrogel. Biomaterials. 2008;29: 2757–2766. doi:10.1016/j.biomaterials.2008.03.016
114. Follin B, Juhl M, Cohen S, Pedersen AE, Gad M, Kastrup J, et al. Human adipose-derived stromal cells in a clinically applicable injectable alginate hydrogel: Phenotypic and immunomodulatory evaluation. Cytotherapy. 2015;17: 1104–1118. doi:10.1016/j.jcyt.2015.04.008
115. Abdeen AA, Weiss JB, Lee J, Kilian KA. Matrix composition and mechanics direct proangiogenic signaling from mesenchymal stem cells. Tissue Eng Part A. Mary Ann Liebert, Inc.; 2014;20: 2737–45. doi:10.1089/ten.TEA.2013.0661
116. Breitbach M, Bostani T, Roell W, Xia Y, Dewald O, Nygren JM, et al. Potential risks of bone marrow cell transplantation into infarcted hearts. Blood. 2007;110: 1362–1369. doi:10.1182/blood-2006-12-063412
117. Engler AJ, Carag-Krieger C, Johnson CP, Raab M, Tang H-Y, Speicher DW, et al. Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. J Cell Sci. NIH Public Access; 2008;121: 3794–802. doi:10.1242/jcs.029678
118. Young JL, Engler AJ. Hydrogels with time-dependent material properties enhance cardiomyocyte differentiation in vitro. Biomaterials. NIH Public Access; 2011;32: 1002–9. doi:10.1016/j.biomaterials.2010.10.020
119. Jackson BM, Gorman JH, Salgo IS, Moainie SL, Plappert T, St. John-Sutton M, et al. Border zone geometry increases wall stress after myocardial infarction: contrast echocardiographic assessment. Am J Physiol - Hear Circ Physiol. 2003;284: H475–H479. doi:10.1152/ajpheart.00360.2002
120. Wall ST, Walker JC, Healy KE, Ratcliffe MB, Guccione JM. Theoretical Impact of the Injection of Material Into the Myocardium: A Finite Element Model Simulation. Circulation. 2006;114: 2627–2635. doi:10.1161/CIRCULATIONAHA.106.657270
121. Ifkovits JL, Tous E, Minakawa M, Morita M, Robb JD, Koomalsingh KJ, et al. Injectable hydrogel properties influence infarct expansion and extent of postinfarction left ventricular remodeling in an ovine model. Proc Natl Acad Sci U S A. National Academy of Sciences; 2010;107: 11507–12. doi:10.1073/pnas.1004097107
122. Rane AA, Chuang JS, Shah A, Hu DP, Dalton ND, Gu Y, et al. Increased Infarct Wall Thickness by a Bio-Inert Material Is Insufficient to Prevent Negative Left Ventricular Remodeling after Myocardial Infarction. Chin W-C, editor. PLoS One. Public Library of Science; 2011;6: e21571. doi:10.1371/journal.pone.0021571
123. Dobner S, Bezuidenhout D, Govender P, Zilla P, Davies N. A Synthetic Non-degradable Polyethylene Glycol Hydrogel Retards Adverse Post-infarct Left Ventricular Remodeling. J Card Fail. 2009;15: 629–636. doi:10.1016/j.cardfail.2009.03.003
124. Martorell L, Gentile M, Rius J, Rodríguez C, Crespo J, Badimon L, et al. The hypoxia-inducible factor 1/NOR-1 axis regulates the survival response of endothelial cells to hypoxia. Mol Cell Biol. American Society for Microbiology; 2009;29: 5828–42. doi:10.1128/MCB.00945-09
125. Hu X, Yu SP, Fraser JL, Lu Z, Ogle ME, Wang J-A, et al. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function

via enhanced survival of implanted cells and angiogenesis. J Thorac Cardiovasc Surg. 2008;135: 799–808. doi:10.1016/j.jtcvs.2007.07.071
126. Feng Y, Huang W, Meng W, Jegga AG, Wang Y, Cai W, et al. Heat Shock Improves Sca-1 + Stem Cell Survival and Directs Ischemic Cardiomyocytes Toward a Prosurvival Phenotype Via Exosomal Transfer: A Critical Role for HSF1/miR-34a/HSP70 Pathway. Stem Cells. 2014;32: 462–472. doi:10.1002/stem.1571
127. Suzuki K, Smolenski RT, Jayakumar J, Murtuza B, Brand NJ, Yacoub MH. Heat shock treatment enhances graft cell survival in skeletal myoblast transplantation to the heart. Circulation. 2000;102: III216-21. Available: http://www.ncbi.nlm.nih.gov/pubmed/11082390
128. Feyen D, Gaetani R, Liu J, Noort W, Martens A, den Ouden K, et al. Increasing short-term cardiomyocyte progenitor cell (CMPC) survival by necrostatin-1 did not further preserve cardiac function. Cardiovasc Res. 2013;99: 83–91. doi:10.1093/cvr/cvt078
129. Madonna R, Rinaldi L, Rossi C, Geng Y-J, De Caterina R. Prostacyclin improves transcoronary myocardial delivery of adipose tissue-derived stromal cells. Eur Heart J. 2006;27.
130. Pons J, Huang Y, Arakawa-Hoyt J, Washko D, Takagawa J, Ye J, et al. VEGF improves survival of mesenchymal stem cells in infarcted hearts. Biochemical and Biophysical Research Communications. 2008. doi:10.1016/j.bbrc.2008.09.003
131. Davis ME, Hsieh PCH, Takahashi T, Song Q, Zhang S, Kamm RD, et al. Local myocardial insulin-like growth factor 1 (IGF-1) delivery with biotinylated peptide nanofibers improves cell therapy for myocardial infarction. Proc Natl Acad Sci U S A. National Academy of Sciences; 2006;103: 8155–60. doi:10.1073/pnas.0602877103
132. Liu J, Wang H, Wang Y, Yin Y, Wang L, Liu Z, et al. Exendin-4 Pretreated Adipose Derived Stem Cells Are Resistant to Oxidative Stress and Improve Cardiac Performance via Enhanced Adhesion in the Infarcted Heart. Tang YL, editor. PLoS One. 2014;9: e99756. doi:10.1371/journal.pone.0099756
133. Matsumoto R, Omura T, Yoshiyama M, Hayashi T, Inamoto S, Koh K-R, et al. Vascular endothelial growth factor-expressing mesenchymal stem cell transplantation for the treatment of acute myocardial infarction. Arterioscler Thromb Vasc Biol. 2005;25: 1168–73. doi:10.1161/01.ATV.0000165696.25680.ce
134. Lee RJ, Springer ML, Blanco-Bose WE, Shaw R, Ursell PC, Blau HM. VEGF Gene Delivery to Myocardium. Circulation. 2000;102.
135. Mohsin S, Khan M, Nguyen J, Alkatib M, Siddiqi S, Hariharan N, et al. Rejuvenation of human cardiac progenitor cells with Pim-1 kinase. Circ Res. NIH Public Access; 2013;113: 1169–79. doi:10.1161/CIRCRESAHA.113.302302
136. Kim HW, Jiang S, Ashraf M, Haider KH. Stem cell-based delivery of Hypoxamir-210 to the infarcted heart: implications on stem cell survival and preservation of infarcted heart function. J Mol Med. 2012;90: 997–1010. doi:10.1007/s00109-012-0920-1
137. Chavakis E, Koyanagi M, Dimmeler S. Enhancing the outcome of cell therapy for cardiac repair: progress from bench to bedside and back. Circulation. 2010;121: 325–35. doi:10.1161/CIRCULATIONAHA.109.901405
138. Mao Q, Lin C, Gao J, Liang X, Gao W, Shen L, et al. Mesenchymal stem cells overexpressing integrin-linked kinase attenuate left ventricular remodeling and improve cardiac function after myocardial infarction. Mol Cell Biochem. 2014;397: 203–214. doi:10.1007/s11010-014-2188-y

139. Song S-W, Chang W, Song B-W, Song H, Lim S, Kim H-J, et al. Integrin-Linked Kinase Is Required in Hypoxic Mesenchymal Stem Cells for Strengthening Cell Adhesion to Ischemic Myocardium. Stem Cells. 2009;27: 1358–1365. doi:10.1002/stem.47
140. Ling L, Bai J, Gu R, Jiang C, Li R, Kang L, et al. Sca-1+ Cardiac Progenitor Cell Therapy With Cells Overexpressing Integrin-Linked Kinase Improves Cardiac Function After Myocardial Infarction. Transplant J. 2013;95: 1187–1196. doi:10.1097/TP.0b013e31828a9423
141. Bartosh TJ, Wang Z, Rosales AA, Dimitrijevich SD, Roque RS. 3D-model of adult cardiac stem cells promotes cardiac differentiation and resistance to oxidative stress. J Cell Biochem. 2008;105: 612–623. doi:10.1002/jcb.21862
142. Bauer M, Kang L, Qiu Y, Wu J, Peng M, Chen HH, et al. Adult Cardiac Progenitor Cell Aggregates Exhibit Survival Benefit Both In Vitro and In Vivo. Wold LE, editor. PLoS One. 2012;7: e50491. doi:10.1371/journal.pone.0050491
143. Sarig U, Sarig H, de-Berardinis E, Chaw S-Y, Nguyen EBV, Ramanujam VS, et al. Natural myocardial ECM patch drives cardiac progenitor based restoration even after scarring. Acta Biomater. 2016;44: 209–220. doi:10.1016/j.actbio.2016.08.031
144. Boopathy A V, Che PL, Somasuntharam I, Fiore VF, Cabigas EB, Ban K, et al. The modulation of cardiac progenitor cell function by hydrogel-dependent Notch1 activation. Biomaterials. NIH Public Access; 2014;35: 8103–12. doi:10.1016/j.biomaterials.2014.05.082
145. Simón-Yarza T, Formiga FR, Tamayo E, Pelacho B, Prosper F, Blanco-Prieto MJ. Vascular endothelial growth factor-delivery systems for cardiac repair: an overview. Theranostics. Ivyspring International Publisher; 2012;2: 541–52. doi:10.7150/thno.3682
146. Salimath AS, Phelps EA, Boopathy A V, Che P, Brown M, García AJ, et al. Dual delivery of hepatocyte and vascular endothelial growth factors via a protease-degradable hydrogel improves cardiac function in rats. PLoS One. 2012;7: e50980. doi:10.1371/journal.pone.0050980
147. Gray WD, French KM, Ghosh-Choudhary S, Maxwell JT, Brown ME, Platt MO, et al. Identification of Therapeutic Covariant MicroRNA Clusters in Hypoxia-Treated Cardiac Progenitor Cell Exosomes Using Systems BiologyNovelty and Significance. Circ Res. 2015;116.
148. Agarwal U, George A, Bhutani S, Ghosh-Choudhary S, Maxwell JT, Brown ME, et al. Experimental, Systems and Computational Approaches to Understanding the MicroRNA-Mediated Reparative Potential of Cardiac Progenitor Cell-Derived Exosomes From Pediatric Patients. Circ Res. 2016;
149. Swynghedauw B. Molecular Mechanisms of Myocardial Remodeling. Physiol Rev. 1999;79: 215–262. doi:S0022282802920882 [pii]
150. Burchfield JS, Xie M, Hill JA. Pathological Ventricular Remodeling: Mechanisms: Part 1 of 2. Circulation. 2013;128: 388–400. doi:10.1161/CIRCULATIONAHA.113.001878
151. Gambini E, Pompilio G, Biondi A, Alamanni F, Capogrossi MC, Agrifoglio M, et al. C-kit+ cardiac progenitors exhibit mesenchymal markers and preferential cardiovascular commitment. Cardiovasc Res. 2011;89: 362–73. doi:10.1093/cvr/cvq292
152. Davis ME, Hsieh PCH, Grodzinsky AJ, Lee RT. Custom design of the cardiac microenvironment with biomaterials. Circ Res. NIH Public Access; 2005;97: 8–15. doi:10.1161/01.RES.0000173376.39447.01
153. Kaminsky SM, Rosengart TK, Rosenberg J, Chiuchiolo MJ, Van de Graaf B,

Sondhi D, et al. Gene therapy to stimulate angiogenesis to treat diffuse coronary artery disease. Hum Gene Ther. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA; 2013;24: 948–63. doi:10.1089/hum.2013.2516

154. Kawasuji M, Nagamine H, Ikeda M, Sakakibara N, Takemura H, Fujii S, et al. Therapeutic angiogenesis with intramyocardial administration of basic fibroblast growth factor. Ann Thorac Surg. 2000;69: 1155–61. Available: http://www.ncbi.nlm.nih.gov/pubmed/10800811
155. Shah AM, Mann DL. In search of new therapeutic targets and strategies for heart failure: recent advances in basic science. Lancet. 2011;378: 704–12. doi:10.1016/S0140-6736(11)60894-5
156. Tirziu D, Chorianopoulos E, Moodie KL, Palac RT, Zhuang ZW, Tjwa M, et al. Myocardial hypertrophy in the absence of external stimuli is induced by angiogenesis in mice. J Clin Invest. 2007;117: 3188–97. doi:10.1172/JCI32024
157. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med. 2001;7: 430–6. doi:10.1038/86498
158. van der Spoel TIG, Jansen of Lorkeers SJ, Agostoni P, van Belle E, Gyöngyösi M, Sluijter JPG, et al. Human relevance of pre-clinical studies in stem cell therapy: systematic review and meta-analysis of large animal models of ischaemic heart disease. Cardiovasc Res. 2011;91: 649–58. doi:10.1093/cvr/cvr113
159. Segers VFM, Lee RT. Biomaterials to enhance stem cell function in the heart. Circ Res. 2011;109: 910–22. doi:10.1161/CIRCRESAHA.111.249052
160. Dobaczewski M, Gonzalez-Quesada C, Frangogiannis NG. The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction. J Mol Cell Cardiol. 2010;48: 504–11. doi:10.1016/j.yjmcc.2009.07.015
161. Saif J, Schwarz TM, Chau DYS, Henstock J, Sami P, Leicht SF, et al. Combination of injectable multiple growth factor-releasing scaffolds and cell therapy as an advanced modality to enhance tissue neovascularization. Arterioscler Thromb Vasc Biol. 2010;30: 1897–904. doi:10.1161/ATVBAHA.110.207928
162. Singh S, Wu BM, Dunn JCY. Accelerating vascularization in polycaprolactone scaffolds by endothelial progenitor cells. Tissue Eng Part A. 2011;17: 1819–30. doi:10.1089/ten.TEA.2010.0708
163. Chan S, Li H, Hsueh Y, Lee D. Fibroblast growth factor-10 promotes cardiomyocyte differentiation from embryonic and induced pluripotent stem cells. PLoS One. 2010; Available: http://dx.plos.org/10.1371/journal.pone.0014414
164. Bolli R, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, et al. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet. 2011;378: 1847–57. doi:10.1016/S0140-6736(11)61590-0
165. D'Amario D, Fiorini C, Campbell PM, Goichberg P, Sanada F, Zheng H, et al. Functionally competent cardiac stem cells can be isolated from endomyocardial biopsies of patients with advanced cardiomyopathies. Circ Res. 2011;108: 857–61. doi:10.1161/CIRCRESAHA.111.241380
166. Ellison GM, Vicinanza C, Smith AJ, Aquila I, Leone A, Waring CD, et al. Adult c-kit(pos) cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair. Cell. Elsevier; 2013;154: 827–42.

doi:10.1016/j.cell.2013.07.039
167. van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin S-CJ, et al. c-kit+ cells minimally contribute cardiomyocytes to the heart. Nature. 2014;509: 337–41. doi:10.1038/nature13309
168. Eliceiri BP, Cheresh DA. The role of alphav integrins during angiogenesis: insights into potential mechanisms of action and clinical development. J Clin Invest. American Society for Clinical Investigation; 1999;103: 1227–30. doi:10.1172/JCI6869
169. Ferrara N, Gerber H-P, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9: 669–76. doi:10.1038/nm0603-669
170. Schwarz ER, Speakman MT, Patterson M, Hale SS, Isner JM, Kedes LH, et al. Evaluation of the effects of intramyocardial injection of DNA expressing vascular endothelial growth factor (VEGF) in a myocardial infarction model in the rat--angiogenesis and angioma formation. J Am Coll Cardiol. 2000;35: 1323–30. Available: http://www.ncbi.nlm.nih.gov/pubmed/10758976
171. Pearlman JD, Hibberd MG, Chuang ML, Harada K, Lopez JJ, Gladstone SR, et al. Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. Nat Med. 1995;1: 1085–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/7489368
172. Nourse MB, Halpin DE, Scatena M, Mortisen DJ, Tulloch NL, Hauch KD, et al. VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering. Arterioscler Thromb Vasc Biol. 2010;30: 80–89. doi:10.1161/ATVBAHA.109.194233
173. Ikuno T, Masumoto H, Marui A, Ikeda T, Sakata R, Yamashita JK. Efficient endothelial cell differentiation protocol from human induced pluripotent stem cells based on monolayer and serum-free culture for realization of vascular regenerative medicine. Eur Hear J. 2013;34: 4371-. doi:10.1093/eurheartj/eht310.4371
174. Wang N, Zhang R, Wang S-J, Zhang C-L, Mao L-B, Zhuang C-Y, et al. Vascular endothelial growth factor stimulates endothelial differentiation from mesenchymal stem cells via Rho/myocardin-related transcription factor--a signaling pathway. Int J Biochem Cell Biol. 2013;45: 1447–56. doi:10.1016/j.biocel.2013.04.021
175. Benavides OM, Petsche JJ, Moise KJ, Johnson A, Jacot JG. Evaluation of endothelial cells differentiated from amniotic fluid-derived stem cells. Tissue Eng Part A. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA; 2012;18: 1123–31. doi:10.1089/ten.TEA.2011.0392
176. Vajravelu BN, Hong KU, Al-Maqtari T, Cao P, Keith MCL, Wysoczynski M, et al. C-Kit Promotes Growth and Migration of Human Cardiac Progenitor Cells via the PI3K-AKT and MEK-ERK Pathways. Qin G, editor. PLoS One. Public Library of Science; 2015;10: e0140798. doi:10.1371/journal.pone.0140798
177. Tang J, Wang J, Kong X, Yang J, Guo L, Zheng F, et al. Vascular endothelial growth factor promotes cardiac stem cell migration via the PI3K/Akt pathway. Exp Cell Res. 2009;315: 3521–31. doi:10.1016/j.yexcr.2009.09.026
178. Tang J-M, Luo B, Xiao J, Lv Y, Li X, Zhao J, et al. VEGF-A promotes cardiac stem cell engraftment and myocardial repair in the infarcted heart. Int J Cardiol. 2015;183: 221–231. doi:10.1016/j.ijcard.2015.01.050
179. Zachary I, Mathur A, Yla-Herttuala S, Martin J. Vascular protection: A novel nonangiogenic cardiovascular role for vascular endothelial growth factor. Arterioscler Thromb Vasc Biol. 2000;20: 1512–1520. doi:10.1161/01.ATV.20.6.1512

180. Sieminski AL, Was AS, Kim G, Gong H, Kamm RD. The Stiffness of Three-dimensional Ionic Self-assembling Peptide Gels Affects the Extent of Capillary-like Network Formation - Springer. Cell Biochem Biophys. 2007;49: 73–83. Available: http://link.springer.com/article/10.1007/s12013-007-0046-1/fulltext.html
181. Georges PC, Janmey PA. Cell type-specific response to growth on soft materials. J Appl Physiol. American Physiological Society; 2005;98: 1547–53. doi:10.1152/japplphysiol.01121.2004
182. Joseph P. Califano CAR-K. A Balance of Substrate Mechanics and Matrix Chemistry Regulates Endothelial Cell Network Assembly - Springer. Cell Mol Bioeng. 2008;1: 122–132. Available: http://link.springer.com/article/10.1007/s12195-008-0022-x/fulltext.html
183. Chen Y-C, Lin R-Z, Qi H, Yang Y, Bae H, Melero-Martin JM, et al. Functional Human Vascular Network Generated in Photocrosslinkable Gelatin Methacrylate Hydrogels. Adv Funct Mater. 2012;22: 2027–2039. doi:10.1002/adfm.201101662
184. Shamloo A, Heilshorn SC. Matrix density mediates polarization and lumen formation of endothelial sprouts in VEGF gradients. Lab Chip. The Royal Society of Chemistry; 2010;10: 3061–8. doi:10.1039/c005069e
185. Patterson J, Hubbell JA. Enhanced proteolytic degradation of molecularly engineered PEG hydrogels in response to MMP-1 and MMP-2. Biomaterials. 2010;31: 7836–45. doi:10.1016/j.biomaterials.2010.06.061
186. García JR, Clark AY, García AJ. Integrin-specific hydrogels functionalized with VEGF for vascularization and bone regeneration of critical-size bone defects. J Biomed Mater Res Part A. 2016;104: 889–900. doi:10.1002/jbm.a.35626
187. Koch S, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harb Perspect Med. Cold Spring Harbor Laboratory Press; 2012;2: a006502. doi:10.1101/cshperspect.a006502
188. Mayani H. Biological Differences Between Neonatal and Adult Human Hematopoietic Stem/Progenitor Cells. Stem Cells Dev. 2010;19: 285–298. doi:10.1089/scd.2009.0327
189. Chiu Y-C, Cheng M-H, Engel H, Kao S-W, Larson JC, Gupta S, et al. The role of pore size on vascularization and tissue remodeling in PEG hydrogels. Biomaterials. 2011;32: 6045–51. doi:10.1016/j.biomaterials.2011.04.066
190. Sandstedt J, Jonsson M, Dellgren G, Lindahl A, Jeppsson A, Asp J. Human C-kit+CD45− cardiac stem cells are heterogeneous and display both cardiac and endothelial commitment by single-cell qPCR analysis. Biochem Biophys Res Commun. 2014;443: 234–238. doi:10.1016/j.bbrc.2013.11.086
191. Sandstedt J, Jonsson M, Lindahl A, Jeppsson A, Asp J. C-kit+ CD45− cells found in the adult human heart represent a population of endothelial progenitor cells. Basic Res Cardiol. Springer-Verlag; 2010;105: 545–556. doi:10.1007/s00395-010-0088-1
192. Matuszczak S, Czapla J, Jarosz-Biej M, Wiśniewska E, Cichoń T, Smolarczyk R, et al. Characteristic of c-Kit+ progenitor cells in explanted human hearts. Clin Res Cardiol. Springer; 2014;103: 711–8. doi:10.1007/s00392-014-0705-3
193. Sultana N, Zhang L, Yan J, Chen J, Cai W, Razzaque S, et al. Resident c-kit+ cells in the heart are not cardiac stem cells. Nat Commun. 2015;6: 8701. doi:10.1038/ncomms9701
194. Tsur-Gang O, Ruvinov E, Landa N, Holbova R, Feinberg MS, Leor J, et al. The effects of peptide-based modification of alginate on left ventricular remodeling and function after myocardial infarction. Biomaterials. 2009;30: 189–195. doi:10.1016/j.biomaterials.2008.09.018

195. Werdich XQ, Penn JS. Src, Fyn and Yes play differential roles in VEGF-mediated endothelial cell events. Angiogenesis. 2006;8: 315–326. doi:10.1007/s10456-005-9021-x
196. Luo B-H, Carman C V., Springer TA. Structural Basis of Integrin Regulation and Signaling. Annu Rev Immunol. 2007;25: 619–647. doi:10.1146/annurev.immunol.25.022106.141618
197. Harburger DS, Calderwood DA. Integrin signalling at a glance. J Cell Sci. 2008;122.
198. Sa S, Wong L, McCloskey KE. Combinatorial fibronectin and laminin signaling promote highly efficient cardiac differentiation of human embryonic stem cells. Biores Open Access. Mary Ann Liebert, Inc.; 2014;3: 150–61. doi:10.1089/biores.2014.0018
199. Duan Y, Liu Z, O'Neill J, Wan LQ, Freytes DO, Vunjak-Novakovic G. Hybrid gel composed of native heart matrix and collagen induces cardiac differentiation of human embryonic stem cells without supplemental growth factors. J Cardiovasc Transl Res. NIH Public Access; 2011;4: 605–15. doi:10.1007/s12265-011-9304-0
200. Suhaeri M, Subbiah R, Van SY, Du P, Kim IG, Lee K, et al. Cardiomyoblast (h9c2) differentiation on tunable extracellular matrix microenvironment. Tissue Eng Part A. Mary Ann Liebert, Inc.; 2015;21: 1940–51. doi:10.1089/ten.TEA.2014.0591
201. Zeng D, Ou D-B, Wei T, Ding L, Liu X-T, Hu X-L, et al. Collagen/β1 integrin interaction is required for embryoid body formation during cardiogenesis from murine induced pluripotent stem cells. BMC Cell Biol. BioMed Central; 2013;14: 5. doi:10.1186/1471-2121-14-5
202. Cheng P, Andersen P, Hassel D, Kaynak BL, Limphong P, Juergensen L, et al. Fibronectin mediates mesendodermal cell fate decisions. Development. 2013;140: 2587–2596. doi:10.1242/dev.089052
203. García AJ, Vega MD, Boettiger D. Modulation of cell proliferation and differentiation through substrate-dependent changes in fibronectin conformation. Mol Biol Cell. American Society for Cell Biology; 1999;10: 785–98. Available: http://www.ncbi.nlm.nih.gov/pubmed/10069818
204. Ahmadi A, McNeill B, Vulesevic B, Kordos M, Mesana L, Thorn S, et al. The role of integrin α2 in cell and matrix therapy that improves perfusion, viability and function of infarcted myocardium. Biomaterials. 2014;35: 4749–4758. doi:10.1016/j.biomaterials.2014.02.028
205. Chavakis E, Aicher A, Heeschen C, Sasaki K, Kaiser R, El Makhfi N, et al. Role of β2-integrins for homing and neovascularization capacity of endothelial progenitor cells. J Exp Med. 2005;201.
206. Wu Y, Ip JE, Huang J, Zhang L, Matsushita K, Liew C-C, et al. Essential role of ICAM-1/CD18 in mediating EPC recruitment, angiogenesis, and repair to the infarcted myocardium. Circ Res. 2006;99: 315–322. Available: https://www.ncbi.nlm.nih.gov/pubmed/16825578
207. Campbell ID, Humphries MJ. Integrin structure, activation, and interactions. Cold Spring Harb Perspect Biol. Cold Spring Harbor Laboratory Press; 2011;3: a004994. doi:10.1101/cshperspect.a004994
208. Mould AP, Symonds EJH, Buckley PA, Grossmann JG, McEwan PA, Barton SJ, et al. Structure of an Integrin-Ligand Complex Deduced from Solution X-ray Scattering and Site-directed Mutagenesis. J Biol Chem. 2003;278: 39993–39999. doi:10.1074/jbc.M304627200
209. Ren D, Hou S, Wang H, Luo D, Zhang L. Evaluation of RGD Modification on Collagen Matrix. Artif Cells, Blood Substitutes, Biotechnol. Taylor & Francis;

2006;34: 293–303. doi:10.1080/10731190600683829
210. Choi WS, Bae JW, Lim HR, Joung YK, Park J-C, Kwon IK, et al. RGD peptide-immobilized electrospun matrix of polyurethane for enhanced endothelial cell affinity. Biomed Mater. 2008;3: 44104. doi:10.1088/1748-6041/3/4/044104
211. Park K-H, Kim M-H, Park S-H, Lee HJ, Kim IK, Chung H-M. Synthesis of Arg-Gly-Asp (RGD) sequence conjugated thermo-reversible gel via the PEG spacer arm as an extracellular matrix for a pheochromocytoma cell (PC12) culture. Biosci Biotechnol Biochem. 2004;68: 2224–2229. doi:10.1271/bbb.68.2224
212. Pinkse GGM, Jiawan-Lalai R, Bruijn JA, de Heer E. RGD peptides confer survival to hepatocytes via the beta1-integrin-ILK-pAkt pathway. J Hepatol. 2005;42: 87–93. doi:10.1016/j.jhep.2004.09.010
213. Pinkse GGM, Bouwman WP, Jiawan-Lalai R, Terpstra OT, Bruijn JA, de Heer E. Integrin signaling via RGD peptides and anti-beta1 antibodies confers resistance to apoptosis in islets of Langerhans. Diabetes. 2006;55: 312–7. Available: http://www.ncbi.nlm.nih.gov/pubmed/16443762
214. Le Saux G, Magenau A, Gunaratnam K, Kilian KA, Böcking T, Gooding JJ, et al. Spacing of Integrin Ligands Influences Signal Transduction in Endothelial Cells. Biophys J. 2011;101: 764–773. doi:10.1016/j.bpj.2011.06.064
215. Wang X, Ye K, Li Z, Yan C, Ding J. Adhesion, proliferation, and differentiation of mesenchymal stem cells on RGD nanopatterns of varied nanospacings. Organogenesis. Taylor & Francis; 2013;9: 280–6. doi:10.4161/org.26080
216. Frith JE, Mills RJ, Hudson JE, Cooper-White JJ. Tailored integrin-extracellular matrix interactions to direct human mesenchymal stem cell differentiation. Stem Cells Dev. Mary Ann Liebert, Inc.; 2012;21: 2442–56. doi:10.1089/scd.2011.0615
217. Salinas CN, Anseth KS. The enhancement of chondrogenic differentiation of human mesenchymal stem cells by enzymatically regulated RGD functionalities. Biomaterials. Howard Hughes Medical Institute; 2008;29: 2370–7. doi:10.1016/j.biomaterials.2008.01.035
218. Ferris DM, Moodie GD, Dimond PM, Gioranni CW, Ehrlich MG, Valentini RF. RGD-coated titanium implants stimulate increased bone formation in vivo. Biomaterials. 1999;20: 2323–31. Available: http://www.ncbi.nlm.nih.gov/pubmed/10614938
219. Eid K, Chen E, Griffith L, Glowacki J. Effect of RGD coating on osteocompatibility of PLGA-polymer disks in a rat tibial wound. J Biomed Mater Res. 2001;57: 224–31. Available: http://www.ncbi.nlm.nih.gov/pubmed/11484185
220. Yu X, Bellamkonda R V. Tissue-Engineered Scaffolds Are Effective Alternatives to Autografts for Bridging Peripheral Nerve Gaps. Tissue Eng. 2003;9: 421–430. doi:10.1089/107632703322066606
221. Barber TA, Ho JE, De Ranieri A, Virdi AS, Sumner DR, Healy KE. Peri-implant bone formation and implant integration strength of peptide-modified p(AAM-co-EG/AAC) interpenetrating polymer network-coated titanium implants. J Biomed Mater Res Part A. 2007;80A: 306–320. doi:10.1002/jbm.a.30927
222. Petrie TA, Raynor JE, Reyes CD, Burns KL, Collard DM, García AJ. The effect of integrin-specific bioactive coatings on tissue healing and implant osseointegration. Biomaterials. NIH Public Access; 2008;29: 2849–57. doi:10.1016/j.biomaterials.2008.03.036
223. Hennessy KM, Clem WC, Phipps MC, Sawyer AA, Shaikh FM, Bellis SL. The effect of RGD peptides on osseointegration of hydroxyapatite biomaterials. Biomaterials. NIH Public Access; 2008;29: 3075–83. doi:10.1016/j.biomaterials.2008.04.014

224. Schliephake H, Scharnweber D, Dard M, Rössler S, Sewing A, Meyer J, et al. Effect of RGD peptide coating of titanium implants on periimplant bone formation in the alveolar crest. An experimental pilot study in dogs. Clin Oral Implants Res. 2002;13: 312–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/12010163
225. Wojtowicz AM, Shekaran A, Oest ME, Dupont KM, Templeman KL, Hutmacher DW, et al. Coating of biomaterial scaffolds with the collagen-mimetic peptide GFOGER for bone defect repair. Biomaterials. NIH Public Access; 2010;31: 2574–82. doi:10.1016/j.biomaterials.2009.12.008
226. Zhang W-M, Kapyla J, Puranen JS, Knight CG, Tiger C-F, Pentikainen OT, et al. alpha 11beta 1 integrin recognizes the GFOGER sequence in interstitial collagens. J Biol Chem. 2003;278: 7270–7. doi:10.1074/jbc.M210313200
227. Emsley J, Knight CGG, Farndale RW, Barnes MJ, Liddington RC. Structural basis of collagen recognition by integrin alpha2beta1. Cell. 2000;101: 47–56. doi:10.1016/S0092-8674(00)80622-4
228. Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ. The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2000;275: 35–40. doi:10.1074/jbc.275.1.35
229. Reyes CD, García AJ. Engineering integrin-specific surfaces with a triple-helical collagen-mimetic peptide. J Biomed Mater Res A. Wiley Subscription Services, Inc., A Wiley Company; 2003;65: 511–523. doi:10.1002/jbm.a.10550
230. Shekaran A, García JR, Clark AY, Kavanaugh TE, Lin AS, Guldberg RE, et al. Bone regeneration using an alpha 2 beta 1 integrin-specific hydrogel as a BMP-2 delivery vehicle. Biomaterials. 2014;35: 5453–5461. doi:10.1016/j.biomaterials.2014.03.055
231. Reyes CD, García AJ. Alpha2beta1 integrin-specific collagen-mimetic surfaces supporting osteoblastic differentiation. J Biomed Mater Res A. 2004;69: 591–600. doi:10.1002/jbm.a.30034
232. Mhanna R, Öztürk E, Vallmajo-Martin Q, Millan C, Müller M, Zenobi-Wong M. GFOGER-modified MMP-sensitive polyethylene glycol hydrogels induce chondrogenic differentiation of human mesenchymal stem cells. Tissue Eng Part A. Mary Ann Liebert, Inc.; 2014;20: 1165–74. doi:10.1089/ten.TEA.2013.0519
233. Kutschka I, Chen IY, Kofidis T, Arai T, von Degenfeld G, Sheikh AY, et al. Collagen Matrices Enhance Survival of Transplanted Cardiomyoblasts and Contribute to Functional Improvement of Ischemic Rat Hearts. Circulation. 2006;114: I-167-I-173. doi:10.1161/CIRCULATIONAHA.105.001297
234. Zhang Y, Thorn S, DaSilva JN, Lamoureux M, deKemp RA, Beanlands RS, et al. Collagen-Based Matrices Improve the Delivery of Transplanted Circulating Progenitor Cells: Development and Demonstration by Ex Vivo Radionuclide Cell Labeling and In Vivo Tracking With Positron-Emission Tomography. Circ Cardiovasc Imaging. 2008;1: 197–204. doi:10.1161/CIRCIMAGING.108.781120
235. Dai W, Wold LE, Dow JS, Kloner RA. Thickening of the Infarcted Wall by Collagen Injection Improves Left Ventricular Function in Rats. J Am Coll Cardiol. 2005;46: 714–719. doi:10.1016/j.jacc.2005.04.056
236. Blackburn NJR, Sofrenovic T, Kuraitis D, Ahmadi A, McNeill B, Deng C, et al. Timing underpins the benefits associated with injectable collagen biomaterial therapy for the treatment of myocardial infarction. Biomaterials. 2015;39: 182–192. doi:10.1016/j.biomaterials.2014.11.004
237. Sato H, Takahashi M, Ise H, Yamada A, Hirose S, Tagawa Y, et al. Collagen

synthesis is required for ascorbic acid-enhanced differentiation of mouse embryonic stem cells into cardiomyocytes. Biochem Biophys Res Commun. 2006;342: 107–112. doi:10.1016/j.bbrc.2006.01.116
238. French KM, Maxwell JT, Bhutani S, Ghosh-Choudhary S, Fierro MJ, Johnson TD, et al. Fibronectin and Cyclic Strain Improve Cardiac Progenitor Cell Regenerative Potential *In Vitro*. Stem Cells Int. Hindawi Publishing Corporation; 2016;2016: 1–11. doi:10.1155/2016/8364382
239. Konstandin MH, Toko H, Gastelum GM, Quijada P, De La Torre A, Quintana M, et al. Fibronectin Is Essential for Reparative Cardiac Progenitor Cell Response After Myocardial Infarction. Circ Res. 2013;113: 115–125. doi:10.1161/CIRCRESAHA.113.301152
240. Yan C, Pochan DJ. Rheological properties of peptide-based hydrogels for biomedical and other applications. Chem Soc Rev. NIH Public Access; 2010;39: 3528–40. doi:10.1039/b919449p
241. Bateman A, Martin MJ, O'Donovan C, Magrane M, Apweiler R, Alpi E, et al. UniProt: A hub for protein information. Nucleic Acids Res. Oxford University Press; 2015;43: D204–D212. doi:10.1093/nar/gku989
242. Guan K, Czyz J, Fürst DO, Wobus AM. Expression and Cellular Distribution of αvIntegrins inβ1 Integrin-deficient Embryonic Stem Cell-derived Cardiac Cells. J Mol Cell Cardiol. 2001;33: 521–532. doi:10.1006/jmcc.2000.1326
243. Czyz J, Guan K, Zeng Q, Wobus AM. Loss of beta1 integrin function results in upregulation of connexin expression in embryonic stem cell-derived cardiomyocytes. Int J Dev Biol. 2005;49: 33–41. doi:10.1387/ijdb.041835jc
244. Herron TJ, Rocha AM Da, Campbell KF, Ponce-Balbuena D, Willis BC, Guerrero-Serna G, et al. Extracellular Matrix–Mediated Maturation of Human Pluripotent Stem Cell–Derived Cardiac Monolayer Structure and Electrophysiological Function. Circ Arrhythmia Electrophysiol. 2016;9: e003638. doi:10.1161/CIRCEP.113.003638
245. Rowland TJ, Miller LM, Blaschke AJ, Doss EL, Bonham AJ, Hikita ST, et al. Roles of Integrins in Human Induced Pluripotent Stem Cell Growth on Matrigel and Vitronectin. Stem Cells Dev. 2010;19: 1231–1240. doi:10.1089/scd.2009.0328
246. Xie Y, Ibrahim A, Cheng K, Wu Z, Liang W, Malliaras K, et al. Importance of cell-cell contact in the therapeutic benefits of cardiosphere-derived cells. Stem Cells. NIH Public Access; 2014;32: 2397–406. doi:10.1002/stem.1736
247. Xiao Q, Zeng L, Zhang Z, Hu Y, Xu Q. Stem cell-derived Sca-1+ progenitors differentiate into smooth muscle cells, which is mediated by collagen IV-integrin alpha1/beta1/alphav and PDGF receptor pathways. Am J Physiol Cell Physiol. 2007;292: C342-52. doi:10.1152/ajpcell.00341.2006
248. Semon JA, Nagy LH, Llamas CB, Tucker HA, Lee RH, Prockop DJ. Integrin expression and integrin-mediated adhesion in vitro of human multipotent stromal cells (MSCs) to endothelial cells from various blood vessels. Cell Tissue Res. 2010;341: 147–158. doi:10.1007/s00441-010-0994-4
249. Ip JE, Wu Y, Huang J, Zhang L, Pratt RE, Dzau VJ. Mesenchymal stem cells use integrin beta1 not CXC chemokine receptor 4 for myocardial migration and engraftment. Mol Biol Cell. American Society for Cell Biology; 2007;18: 2873–82. doi:10.1091/mbc.E07-02-0166
250. Steingen C, Brenig F, Baumgartner L, Schmidt J, Schmidt A, Bloch W. Characterization of key mechanisms in transmigration and invasion of mesenchymal stem cells. J Mol Cell Cardiol. 2008;44: 1072–1084. doi:10.1016/j.yjmcc.2008.03.010

251. Jing D, Fonseca A V., Alakel N, Fierro FA, Muller K, Bornhauser M, et al. Hematopoietic stem cells in co-culture with mesenchymal stromal cells - modeling the niche compartments in vitro. Haematologica. 2010;95: 542–550. doi:10.3324/haematol.2009.010736
252. Koyanagi M, Urbich C, Chavakis E, Hoffmann J, Rupp S, Badorff C, et al. Differentiation of circulating endothelial progenitor cells to a cardiomyogenic phenotype depends on E-cadherin. FEBS Lett. 2005;579: 6060–6066. doi:10.1016/j.febslet.2005.09.071
253. Prowse ABJ, Chong F, Gray PP, Munro TP. Stem cell integrins: Implications for ex-vivo culture and cellular therapies. Stem Cell Res. 2011;6: 1–12. doi:10.1016/j.scr.2010.09.005
254. Tan G, Shim W, Gu Y, Qian L, Ying Chung Y, Yun Lim S, et al. Differential effect of myocardial matrix and integrins on cardiac differentiation of human mesenchymal stem cells. Differentiation. 2010;79: 260–271. doi:10.1016/j.diff.2010.02.005
255. Schiller HB, Hermann M-R, Polleux J, Vignaud T, Zanivan S, Friedel CC, et al. β1- and αv-class integrins cooperate to regulate myosin II during rigidity sensing of fibronectin-based microenvironments. Nat Cell Biol. Nature Research; 2013;15: 625–636. doi:10.1038/ncb2747
256. Kalcioglu ZI, Mrozek RA, Mahmoodian R, VanLandingham MR, Lenhart JL, Van Vliet KJ. Tunable mechanical behavior of synthetic organogels as biofidelic tissue simulants. J Biomech. 2013;46: 1583–1591. doi:10.1016/j.jbiomech.2013.03.011
257. Tallawi M, Rai R, Boccaccini AR, Aifantis KE. Effect of substrate mechanics on cardiomyocyte maturation and growth. Tissue Eng Part B Rev. Mary Ann Liebert, Inc.; 2015;21: 157–65. doi:10.1089/ten.TEB.2014.0383
258. Kshitiz, Hubbi ME, Ahn EH, Downey J, Afzal J, Kim D-H, et al. Matrix Rigidity Controls Endothelial Differentiation and Morphogenesis of Cardiac Precursors. Sci Signal. 2012;5.
259. Chatterjee K, Lin-Gibson S, Wallace WE, Parekh SH, Lee YJ, Cicerone MT, et al. The effect of 3D hydrogel scaffold modulus on osteoblast differentiation and mineralization revealed by combinatorial screening. Biomaterials. NIH Public Access; 2010;31: 5051–62. doi:10.1016/j.biomaterials.2010.03.024
260. Williams C, Budina E, Stoppel WL, Sullivan KE, Emani S, Emani SM, et al. Cardiac extracellular matrix-fibrin hybrid scaffolds with tunable properties for cardiovascular tissue engineering. Acta Biomater. NIH Public Access; 2015;14: 84–95. doi:10.1016/j.actbio.2014.11.035
261. Kan L, Thayer P, Fan H, Ledford B, Chen M, Goldstein A, et al. Polymer microfiber meshes facilitate cardiac differentiation of c-kit+ human cardiac stem cells. Exp Cell Res. 2016;347: 143–152. doi:10.1016/j.yexcr.2016.07.024
262. Caliari SR, Burdick JA. A practical guide to hydrogels for cell culture. Nat Methods. Nature Research; 2016;13: 405–414. doi:10.1038/nmeth.3839
263. Schwartz MA. Integrins and extracellular matrix in mechanotransduction. Cold Spring Harb Perspect Biol. Cold Spring Harbor Laboratory Press; 2010;2: a005066. doi:10.1101/cshperspect.a005066
264. Deng S, Zhao Q, Zhou X, Zhang L, Bao L, Zhen L, et al. Neonatal Heart-Enriched miR-708 Promotes Differentiation of Cardiac Progenitor Cells in Rats. Int J Mol Sci. Multidisciplinary Digital Publishing Institute (MDPI); 2016;17. doi:10.3390/ijms17060875
265. Zhang LX, DeNicola M, Qin X, Du J, Ma J, Tina Zhao Y, et al. Specific inhibition of HDAC4 in cardiac progenitor cells enhances myocardial repairs. Am J Physiol

Cell Physiol. American Physiological Society; 2014;307: C358-72. doi:10.1152/ajpcell.00187.2013
266. Choi SH, Jung SY, Asahara T, Suh W, Kwon S-M, Baek SH. Direct comparison of distinct cardiomyogenic induction methodologies in human cardiac-derived c-kit positive progenitor cells. Tissue Eng Regen Med. Springer Netherlands; 2012;9: 311–319. doi:10.1007/s13770-012-0336-6
267. Zhang J, Huang C, Wu P, Yang J, Song T, Chen Y, et al. Differentiation induction of cardiac c-kit positive cells from rat heart into sinus node-like cells by 5-azacytidine. Tissue Cell. 2011;43: 67–74. doi:10.1016/j.tice.2010.11.005
268. Fischer KM, Cottage CT, Wu W, Din S, Gude NA, Avitabile D, et al. Enhancement of myocardial regeneration through genetic engineering of cardiac progenitor cells expressing Pim-1 kinase. Circulation. NIH Public Access; 2009;120: 2077–87. doi:10.1161/CIRCULATIONAHA.109.884403
269. Toko H, Konstandin MH, Doroudgar S, Ormachea L, Joyo E, Joyo AY, et al. Regulation of cardiac hypertrophic signaling by prolyl isomerase Pin1. Circ Res. NIH Public Access; 2013;112: 1244–52. doi:10.1161/CIRCRESAHA.113.301084
270. Kurazumi H, Kubo M, Ohshima M, Yamamoto Y, Takemoto Y, Suzuki R, et al. The Effects of Mechanical Stress on the Growth, Differentiation, and Paracrine Factor Production of Cardiac Stem Cells. McNeil P, editor. PLoS One. 2011;6: e28890. doi:10.1371/journal.pone.0028890
271. Hausenloy DJ, Yellon DM. Cardioprotective growth factors. Cardiovasc Res. 2009;83.
272. Taghavi S, George JC. Homing of stem cells to ischemic myocardium. Am J Transl Res. e-Century Publishing Corporation; 2013;5: 404–11. Available: http://www.ncbi.nlm.nih.gov/pubmed/23724164
273. Ko IK, Lee SJ, Atala A, Yoo JJ. In situ tissue regeneration through host stem cell recruitment. Exp Mol Med. KSBMB.; 2013;45: e57. doi:10.1038/emm.2013.118
274. Gao Q, Guo M, Zeng W, Wang Y, Yang L, Pang X, et al. Matrix metalloproteinase 9 secreted by hypoxia cardiac fibroblasts triggers cardiac stem cell migration in vitro. Stem Cells Int. Hindawi Publishing Corporation; 2015;2015: 836390. doi:10.1155/2015/836390
275. Cross MJ, Claesson-Welsh L, Carmeliet P, Yancopoulos G., al. et, Hanahan D, et al. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. Trends Pharmacol Sci. Elsevier; 2001;22: 201–7. doi:10.1016/S0165-6147(00)01676-X
276. Zhang G-W, Gu T-X, Guan X-Y, Sun X-J, Jiang D-Q, Tang R, et al. Delayed Enrichment for c-kit and Inducing Cardiac Differentiation Attenuated Protective Effects of BMSCs' Transplantation in Pig Model of Acute Myocardial Ischemia. Cardiovasc Ther. 2015;33: 184–192. doi:10.1111/1755-5922.12131
277. Uemura A, Ogawa M, Hirashima M, Fujiwara T, Koyama S, Takagi H, et al. Recombinant angiopoietin-1 restores higher-order architecture of growing blood vessels in mice in the absence of mural cells. J Clin Invest. 2002;110: 1619–28. doi:10.1172/JCI15621
278. Ngangan A V, Waring JC, Cooke MT, Mandrycky CJ, McDevitt TC. Soluble factors secreted by differentiating embryonic stem cells stimulate exogenous cell proliferation and migration. Stem Cell Res Ther. BioMed Central; 2014;5: 26. doi:10.1186/scrt415
279. Oshim H, Payne TR, Urish KL, Sakai T, Ling Y, Gharaibeh B, et al. Differential myocardial infarct repair with muscle stem cells compared to myoblasts. Mol Ther. Nature Publishing Group; 2005;12: 1130–1141. doi:10.1016/j.ymthe.2005.07.686

280. Funakoshi S, Miki K, Takaki T, Okubo C, Hatani T, Chonabayashi K, et al. Enhanced engraftment, proliferation, and therapeutic potential in heart using optimized human iPSC-derived cardiomyocytes. Sci Rep. Nature Publishing Group; 2016;6: 19111. doi:10.1038/srep19111
281. Ernst C, Christie BR. Isolectin-IB4 as a vascular stain for the study of adult neurogenesis. J Neurosci Methods. 2006;150: 138–142. doi:10.1016/j.jneumeth.2005.06.018
282. Song H, Cha M-J, Song B-W, Kim I-K, Chang W, Lim S, et al. Reactive Oxygen Species Inhibit Adhesion of Mesenchymal Stem Cells Implanted into Ischemic Myocardium via Interference of Focal Adhesion Complex. Stem Cells. 2010;28: N/A-N/A. doi:10.1002/stem.302
283. Cheng K, Li T-S, Malliaras K, Davis DR, Zhang Y, Marbán E. Magnetic Targeting Enhances Engraftment and Functional Benefit of Iron-Labeled Cardiosphere-Derived Cells in Myocardial Infarction. Circ Res. 2010;106.
284. Terrovitis J, Lautamäki R, Bonios M, Fox J, Engles JM, Yu J, et al. Noninvasive Quantification and Optimization of Acute Cell Retention by In Vivo Positron Emission Tomography After Intramyocardial Cardiac-Derived Stem Cell Delivery. J Am Coll Cardiol. 2009;54: 1619–1626. doi:10.1016/j.jacc.2009.04.097
285. Guarnieri D, De Capua A, Ventre M, Borzacchiello A, Pedone C, Marasco D, et al. Covalently immobilized RGD gradient on PEG hydrogel scaffold influences cell migration parameters. Acta Biomater. 2010;6: 2532–2539. doi:10.1016/j.actbio.2009.12.050
286. Wen JH, Choi O, Taylor-Weiner H, Fuhrmann A, Karpiak J V, Almutairi A, et al. Haptotaxis is cell type specific and limited by substrate adhesiveness. Cell Mol Bioeng. NIH Public Access; 2015;8: 530–542. doi:10.1007/s12195-015-0398-3
287. Umar S, van der Valk EJM, Schalij MJ, van der Wall EE, Atsma DE, van der Laarse A. Integrin stimulation-induced hypertrophy in neonatal rat cardiomyocytes is NO-dependent. Mol Cell Biochem. Springer US; 2009;320: 75–84. doi:10.1007/s11010-008-9900-8
288. Ogawa E, Saito Y, Harada M, Kamitani S, Kuwahara K, Miyamoto Y, et al. Outside-in Signalling of Fibronectin Stimulates Cardiomyocyte Hypertrophy in Cultured Neonatal Rat Ventricular Myocytes. J Mol Cell Cardiol. 2000;32: 765–776. doi:10.1006/jmcc.2000.1119
289. Konstandin MH, Völkers M, Collins B, Quijada P, Quintana M, Torre A, et al. Fibronectin contributes to pathological cardiac hypertrophy but not physiological growth. Basic Res Cardiol. 2013;108: 375. doi:10.1007/s00395-013-0375-8
290. Swartzlander MD, Barnes CA, Blakney AK, Kaar JL, Kyriakides TR, Bryant SJ. Linking the foreign body response and protein adsorption to PEG-based hydrogels using proteomics. Biomaterials. NIH Public Access; 2015;41: 26–36. doi:10.1016/j.biomaterials.2014.11.026
291. Jin Z-G, Ueba H, Tanimoto T, Lungu AO, Frame MD, Berk BC. Ligand-Independent Activation of Vascular Endothelial Growth Factor Receptor 2 by Fluid Shear Stress Regulates Activation of Endothelial Nitric Oxide Synthase. Circ Res. 2003;93: 354–363. doi:10.1161/01.RES.0000089257.94002.96
292. Napione L, Pavan S, Veglio A, Picco A, Boffetta G, Celani A, et al. Unraveling the influence of endothelial cell density on VEGF-A signaling. Blood. 2012;119: 5599–607. doi:10.1182/blood-2011-11-390666
293. Leinonen J V, Emanuelov AK, Platt Y, Helman Y, Feinberg Y, Lotan C, et al. Left atrial appendages from adult hearts contain a reservoir of diverse cardiac progenitor cells. PLoS One. Public Library of Science; 2013;8: e59228.

doi:10.1371/journal.pone.0059228
294. Miyamoto S, Kawaguchi N, Ellison GM, Matsuoka R, Shin'oka T, Kurosawa H. Characterization of Long-Term Cultured c-kit + Cardiac Stem Cells Derived From Adult Rat Hearts. Stem Cells Dev. Mary Ann Liebert, Inc. publishers 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801-5215 USA ; 2010;19: 105–116. doi:10.1089/scd.2009.0041
295. Hodgkiss-Geere HM, Argyle DJ, Corcoran BM, Whitelaw B, Milne E, Bennett D, et al. Characterisation and cardiac directed differentiation of canine adult cardiac stem cells. Vet J. 2012;191: 176–182. doi:10.1016/j.tvjl.2010.12.033
296. French KM, Boopathy A V, DeQuach JA, Chingozha L, Lu H, Christman KL, et al. A naturally derived cardiac extracellular matrix enhances cardiac progenitor cell behavior in vitro. Acta Biomater. NIH Public Access; 2012;8: 4357–64. doi:10.1016/j.actbio.2012.07.033
297. Choi SH, Jung SY, Suh W, Baek SH, Kwon S-M. Establishment of Isolation and Expansion Protocols for Human Cardiac C-kit-Positive Progenitor Cells for Stem Cell Therapy. Transplant Proc. 2013;45: 420–426. doi:10.1016/j.transproceed.2012.08.017
298. Cui J, Zhang F, Wang Y, Liu J, Ming X, Hou J, et al. Macrophage migration inhibitory factor promotes cardiac stem cell proliferation and endothelial differentiation through the activation of the PI3K/Akt/mTOR and AMPK pathways. Int J Mol Med. Spandidos Publications; 2016;37: 1299–309. doi:10.3892/ijmm.2016.2542
299. Hatzistergos KE, Hare JM. Murine Models Demonstrate Distinct Vasculogenic and Cardiomyogenic cKit+ Lineages in the Heart. Circ Res. 2016;118.
300. Fioret BA, Heimfeld JD, Paik DT, Hatzopoulos AK. Endothelial Cells Contribute to Generation of Adult Ventricular Myocytes during Cardiac Homeostasis. Cell Rep. 2014;8: 229–241. doi:10.1016/j.celrep.2014.06.004
301. Yang C, Tibbitt MW, Basta L, Anseth KS. Mechanical memory and dosing influence stem cell fate. Nat Mater. NIH Public Access; 2014;13: 645–52. doi:10.1038/nmat3889
302. Donovan D, Brown NJ, Bishop ET, Lewis CE. Comparison of three in vitro human "angiogenesis" assays with capillaries formed in vivo. Angiogenesis. 2001;4: 113–21. Available: http://www.ncbi.nlm.nih.gov/pubmed/11806243
303. Tillmanns J, Rota M, Hosoda T, Misao Y, Esposito G, Gonzalez A, et al. Formation of large coronary arteries by cardiac progenitor cells. Proc Natl Acad Sci U S A. National Academy of Sciences; 2008;105: 1668–73. doi:10.1073/pnas.0706315105
304. Somanath PR, Malinin NL, Byzova T V. Cooperation between integrin alphavbeta3 and VEGFR2 in angiogenesis. Angiogenesis. NIH Public Access; 2009;12: 177–85. doi:10.1007/s10456-009-9141-9
305. West XZ, Meller N, Malinin NL, Deshmukh L, Meller J, Mahabeleshwar GH, et al. Integrin β3 crosstalk with VEGFR accommodating tyrosine phosphorylation as a regulatory switch. Ruhrberg C, editor. PLoS One. Public Library of Science; 2012;7: e31071. doi:10.1371/journal.pone.0031071
306. Mahabeleshwar GH, Feng W, Reddy K, Plow EF, Byzova T V. Mechanisms of integrin-vascular endothelial growth factor receptor cross-activation in angiogenesis. Circ Res. NIH Public Access; 2007;101: 570–80. doi:10.1161/CIRCRESAHA.107.155655
307. Reynolds LE, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, et al. Enhanced pathological angiogenesis in mice lacking β3 integrin or β3 and β5

integrins. Nat Med. 2002;8: 27–34. doi:10.1038/nm0102-27
308. Reynolds AR, Reynolds LE, Nagel TE, Lively JC, Robinson SD, Hicklin DJ, et al. Elevated Flk1 (Vascular Endothelial Growth Factor Receptor 2) Signaling Mediates Enhanced Angiogenesis in β3-Integrin–Deficient Mice. Cancer Res. 2004;64.
309. Wijelath ES, Rahman S, Murray J, Patel Y, Savidge G, Sobel M. Fibronectin promotes VEGF-induced CD34+ cell differentiation into endothelial cells. J Vasc Surg. 2004;39: 655–660. doi:10.1016/j.jvs.2003.10.042
310. Wijelath ES, Murray J, Rahman S, Patel Y, Ishida A, Strand K, et al. Novel vascular endothelial growth factor binding domains of fibronectin enhance vascular endothelial growth factor biological activity. Circ Res. 2002;91: 25–31. Available: http://www.ncbi.nlm.nih.gov/pubmed/12114318
311. Pendergrass KD, Boopathy A V, Seshadri G, Maiellaro-Rafferty K, Che PL, Brown ME, et al. Acute preconditioning of cardiac progenitor cells with hydrogen peroxide enhances angiogenic pathways following ischemia-reperfusion injury. Stem Cells Dev. 2013;22: 2414–24. doi:10.1089/scd.2012.0673
312. Campagnolo P, Tsai T-N, Hong X, Kirton JP, So P-W, Margariti A, et al. c-Kit+ progenitors generate vascular cells for tissue-engineered grafts through modulation of the Wnt/Klf4 pathway. Biomaterials. 2015;60: 53–61. doi:10.1016/j.biomaterials.2015.04.055
313. Lian X, Bao X, Al-Ahmad A, Liu J, Wu Y, Dong W, et al. Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. Stem cell reports. Elsevier; 2014;3: 804–16. doi:10.1016/j.stemcr.2014.09.005
314. Zhang Z, Nör F, Oh M, Cucco C, Shi S, Nör JE. Wnt/β-Catenin Signaling Determines the Vasculogenic Fate of Postnatal Mesenchymal Stem Cells. Stem Cells. 2016;34: 1576–1587. doi:10.1002/stem.2334
315. Zhang P, Baxter J, Vinod K, Tulenko TN, Di Muzio PJ. Endothelial Differentiation of Amniotic Fluid–Derived Stem Cells: Synergism of Biochemical and Shear Force Stimuli. Stem Cells Dev. 2009;18: 1299–1308. doi:10.1089/scd.2008.0331
316. Yamamoto K, Sokabe T, Watabe T, Miyazono K, Yamashita JK, Obi S, et al. Fluid shear stress induces differentiation of Flk-1-positive embryonic stem cells into vascular endothelial cells in vitro. Am J Physiol Heart Circ Physiol. 2005;288: H1915-24. doi:10.1152/ajpheart.00956.2004
317. Ye C, Bai L, Yan Z-Q, Wang Y-H, Jiang Z-L. Shear stress and vascular smooth muscle cells promote endothelial differentiation of endothelial progenitor cells via activation of Akt. Clin Biomech. 2008;23: S118–S124. doi:10.1016/j.clinbiomech.2007.08.018
318. Bai K, Huang Y, Jia X, Fan Y, Wang W. Endothelium oriented differentiation of bone marrow mesenchymal stem cells under chemical and mechanical stimulations. J Biomech. 2010;43: 1176–1181. doi:10.1016/j.jbiomech.2009.11.030
319. Zeng L, Xiao Q, Margariti A, Zhang Z, Zampetaki A, Patel S, et al. HDAC3 is crucial in shear- and VEGF-induced stem cell differentiation toward endothelial cells. J Cell Biol. 2006;174.
320. Kane NM, Meloni M, Spencer HL, Craig MA, Strehl R, Milligan G, et al. Derivation of Endothelial Cells From Human Embryonic Stem Cells by Directed Differentiation: Analysis of MicroRNA and Angiogenesis In Vitro and In Vivo. Arterioscler Thromb Vasc Biol. 2010;30: 1389–1397. doi:10.1161/ATVBAHA.110.204800

321. Mahpatra S, Firpo MT, Bacanamwo M. Inhibition of DNA methyltransferases and histone deacetylases induces bone marrow-derived multipotent adult progenitor cells to differentiate into endothelial cells. Ethn Dis. 2010;20: S1-60–4. Available: http://www.ncbi.nlm.nih.gov/pubmed/20521387
322. Chen CS, Tan J, Tien J. Mechanotransduction at Cell-Matrix and Cell-Cell Contacts. Annu Rev Biomed Eng. Annual Reviews; 2004;6: 275–302. doi:10.1146/annurev.bioeng.6.040803.140040
323. Zhu J, Tang C, Kottke-Marchant K, Marchant RE. Design and Synthesis of Biomimetic Hydrogel Scaffolds with Controlled Organization of Cyclic RGD Peptides. Bioconjug Chem. 2009;20: 333–339. doi:10.1021/bc800441v
324. de Jong WH, Steerenberg PA, Ursem PS, Osterhaus ADME, Vos JG, Ruitenberg EJ. The athymic nude rat. Clin Immunol Immunopathol. 1980;17: 163–172. doi:10.1016/0090-1229(80)90084-7
325. Cheers C, Waller R. Activated macrophages in congenitally athymic "nude mice" and in lethally irradiate mice. J Immunol. 1975;115: 844–7. Available: http://www.ncbi.nlm.nih.gov/pubmed/807647
326. Charles River Laboratoires International I. Charles River Immunodeficient Models Xenograft Data Catalog. Vol 1. 2014;1: 33.
327. Conradi L, Schmidt S, Neofytou E, Deuse T, Peters L, Eder A, et al. Immunobiology of fibrin-based engineered heart tissue. Stem Cells Transl Med. AlphaMed Press; 2015;4: 625–31. doi:10.5966/sctm.2013-0202
328. Liu Y, Wang L, Kikuiri T, Akiyama K, Chen C, Xu X, et al. Mesenchymal stem cell–based tissue regeneration is governed by recipient T lymphocytes via IFN-γ and TNF-α. Nat Med. 2011;17: 1594–1601. doi:10.1038/nm.2542
329. Zhang Y, Li X, Chihara T, Mizoguchi T, Hori A, Udagawa N, et al. Comparing immunocompetent and immunodeficient mice as animal models for bone tissue engineering. Oral Dis. 2015;21: 583–592. doi:10.1111/odi.12319
330. Bruin JE, Asadi A, Fox JK, Erener S, Rezania A, Kieffer TJ. Accelerated Maturation of Human Stem Cell-Derived Pancreatic Progenitor Cells into Insulin-Secreting Cells in Immunodeficient Rats Relative to Mice. Stem cell reports. Elsevier; 2015;5: 1081–96. doi:10.1016/j.stemcr.2015.10.013
331. Epelman S, Liu PP, Mann DL. Role of innate and adaptive immune mechanisms in cardiac injury and repair. Nat Rev Immunol. NIH Public Access; 2015;15: 117–29. doi:10.1038/nri3800
332. Hofmann U, Frantz S. Role of Lymphocytes in Myocardial Injury, Healing, and Remodeling After Myocardial Infarction. Circ Res. 2015;116.
333. Hofmann U, Beyersdorf N, Weirather J, Podolskaya A, Bauersachs J, Ertl G, et al. Activation of CD4+ T Lymphocytes Improves Wound Healing and Survival After Experimental Myocardial Infarction in Mice. Circulation. 2012;125: 1652–1663. doi:10.1161/CIRCULATIONAHA.111.044164
334. Zouggari Y, Ait-Oufella H, Waeckel L, Vilar J, Loinard C, Cochain C, et al. Regulatory T Cells Modulate Postischemic Neovascularization. Circulation. 2009;120.
335. Lassailly F, Griessinger E, Bonnet D. “Microenvironmental contaminations” induced by fluorescent lipophilic dyes used for noninvasive in vitro and in vivo cell tracking. Blood. 2010;115: 5347–54. doi:10.1182/blood-2009-05-224030
336. Laboratories CR, Wilmington BS. Myocardial Infarct in the CD Rat. 2011;296: 2011.
337. Perazzolo Marra M, Lima JAC, Iliceto S. MRI in acute myocardial infarction. Eur Heart J. 2011;32.

338. Hashizume R, Hong Y, Takanari K, Fujimoto KL, Tobita K, Wagner WR. The effect of polymer degradation time on functional outcomes of temporary elastic patch support in ischemic cardiomyopathy. Biomaterials. 2013;34: 7353–63. doi:10.1016/j.biomaterials.2013.06.020
339. Hassan W, Dong Y, Wang W. Encapsulation and 3D culture of human adipose-derived stem cells in an in-situ crosslinked hybrid hydrogel composed of PEG-based hyperbranched copolymer and hyaluronic acid. Stem Cell Res Ther. BioMed Central; 2013;4: 32. doi:10.1186/scrt182