

# DEVELOPMENT OF DELIVERY STRATEGY FOR ADIPOSE- DERIVED STEM CELLS IN THE TREATMENT OF MYOCARDIAL INFARCTION

by

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## Abstract

Cell-based therapies involving adipose-derived stem cells (ASCs) have shown promise in stimulating cardiovascular regeneration, including in the treatment of myocardial infarction (MI) and ischemic heart disease. However, previous studies involving the delivery of ASCs following MI have indicated that therapeutic efficacy has been limited by low survival and/or poor retention of the transplanted cells at the site of injury. To address these limitations, the goal of this thesis was to develop a more effective delivery strategy incorporating an injectable biomaterial combined with chemotactic growth factor delivery to enhance ASC retention within the gel. Working towards future *in vivo* analysis in a rat model, multilineage characterization studies confirmed that ASCs isolated from the epididymal fat pad of male Wistar rats could differentiate *in vitro* along the adipogenic, osteogenic, and chondrogenic lineages. Subsequently, the chemotactic response of the rat ASCs (rASCs) to varying concentrations of stromal derived factor-1  $\alpha$  (SDF-1 $\alpha$ ) and hepatocyte growth factor (HGF) was analyzed using a modified Boyden chamber assay. The results demonstrated that SDF-1 $\alpha$  and HGF, at 20, 50, and 100 ng/mL elicited significant migratory responses under normoxic (21%) and hypoxic (5%) culture conditions. RT-PCR analysis was conducted to assess the expression of the two chemotactic growth factors and their associated receptors in the rASCs, and secreted SDF-1 $\alpha$  protein expression was quantified by ELISA. Moving towards the development of the biomaterials-based delivery approach, the viability of rASCs encapsulated by photopolymerization in methacrylated glycol chitosan (MGC) hydrogels modified with various degrees of arginine-glycine-aspartic acid (RGD)-peptide modification was examined. More

specifically, rASCs were encapsulated in MGC hydrogels with 0%, 4%, and 7% RGD modification and cultured for up to 14 days. Viability staining results indicated that rASC viability was enhanced in the 4% and 7% RGD-modified MGC hydrogels in comparison to the MGC hydrogels with no peptide modification. Pre-loading the gels with 50 ng/mL of SDF-1 $\alpha$  had no significant effects on cell viability over 14 days. Overall, the results demonstrate that peptide modification to promote cell adhesion within the MGC hydrogels is key to improving cell viability and thereby improving the therapeutic potential of ASCs.

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## List of Abbreviations

ALD	Aldehyde dehydrogenase	HGF	Hepatocyte growth factor
ALP	Alkaline phosphatase	HIF-1	Hypoxia-inducible factor-1
ASC	Adipose-derived stem cell	IBMX	Isobutylmethylxanthine
BMSC	Bone marrow-derived stem cell	IGF-1	Insulin-like growth factor-1
BSA	Bovine serum albumin	JNK	C-Jun N-terminal kinases
cAMP	Cyclic adenosine monophosphate	LVEF	Left ventricular ejection fraction
CHF	Congestive heart failure	MAPK	Mitogen activated protein kinase
CXCR4	Chemokine (C-X-C motif) receptor 4	MGC	<i>N</i> -Methacrylate glycol chitosan
DEX	Dexamethasone	MI	Myocardial infarction
DMEM	Dulbecco's Modified Eagle's Medium	MSC	Mesenchymal stem cell
DMMB	Dimethylmethylene blue	PBS	Phosphate buffered saline
ECM	Extracellular matrix	PDL-LA	Poly (D,L-lactic acid)
eGFP	Enhanced-green fluorescent protein	PEG	Poly (ethylene glycol)
ELISA	Enzyme-linked immunosorbent assay	PKA	Protein kinase A
EPC	Endothelial progenitor cell	PLGA	Poly (lactic-co-glycolic acid)
ERK	Extracellular signal-regulated kinases	PPAR	Peroxisome proliferator activated receptor
ESC	Embryonic stem cell	PVA	Poly (vinyl alcohol)
FAS	Fatty acid synthase	rASC	Rat adipose-derived stem cell
FBS	Fetal bovine serum	RGD	Arginine-glycine-aspartic acid
Glut-4	Glucose transporter type-4	RT-PCR	Reverse-transcription polymerase chain reaction
GPDH	Glycerol-3-phosphate dehydrogenase	SDF-1 $\alpha$	Stromal cell-derived factor-1 $\alpha$
HA	Hyaluronic acid	TGF- $\beta$ 1	Transforming growth factor- $\beta$ 1
		VEGF	Vascular endothelial growth fact

# **Chapter 1**

## **Introduction**

### **1.1 Clinical Motivation**

Cardiovascular disease has been identified as a leading cause of morbidity and mortality in the Western world [1]. In 2008, cardiovascular diseases were the cause of 69,648 deaths in Canada, approximately 30% of all Canadian deaths, and almost equal to the mortality rate due to cancer [2]. Of these cardiovascular disease-related deaths, approximately 23% were the result of acute myocardial infarction [2]. The prevalence of cardiovascular disease places a substantial economic burden on the Canadian healthcare system as the cost of hospitalizations and treatments can be quite high.

Following acute myocardial infarction (MI), more commonly known as a heart attack, cardiomyocytes (cardiac muscle cells) are irreversibly damaged or undergo cell death due to an interruption in the blood flow, and consequently oxygen and nutrient delivery, to the cardiac muscle tissue. The reduction in blood flow during MI is typically caused by the occlusion of one or more of the coronary arteries, which are the blood vessels that supply the myocardium. Cardiomyocyte damage or death is a significant cause for concern because the mature heart is unable to substantially repair damaged tissues as mature cardiomyocytes do not proliferate within the necrotic zone, creating an irreversible region of cell death [1]. Within this zone, fibrotic scar tissue, which does not possess the contractile, mechanical, and electrical capabilities seen in normal myocardium, replaces the damaged cardiac muscle tissue [1]. As a result, the scar tissue compromises the contractility of the heart and pumping efficiency of the ventricles.

Although certain compensatory mechanisms such as systemic vasoconstriction and the Frank Starling mechanism initiated by an increased left ventricular end-diastolic volume can help to maintain cardiac output initially, these mechanisms place further stress on the heart, which is already in a weakened state. Eventually, these compensatory mechanisms will fail, leading to the development of heart failure, where the heart is unable to pump sufficient amounts of blood to meet the metabolic demands of the body.

In end-stage heart failure, the only therapeutic options are ventricular assist devices (VADs) or heart transplantation [1, 3]. However, VAD therapy is cost prohibitive and donor organs are often in short supply [1, 4]. Therefore, there is a significant need for alternative therapies to enhance regeneration following MI to improve individual health outcomes and also to reduce the economic burden of treating MIs and the progression of cardiovascular disease.

## **1.2 Strategies for Myocardial Repair**

Regenerative medicine offers promising new approaches for treating cardiovascular disease [5]. The current standard methods of treatment include balloon angioplasty, coronary artery bypass grafting, and the use of various cardiac medications such as nitrates, angiotensin converting enzyme (ACE) inhibitors, and beta-blockers among others. Certain cardiac medications can reduce mortality rates, but require chronic use, which can lead to other adverse health side effects and may be a financial burden. Surgical treatment options can improve blood flow and help to prevent the further progression of cardiovascular damage, but do not substantially improve lost heart

function or structure. Regenerative medicine, however, works to address these limitations by stimulating the healing of tissues and organs [6].

Repair of the injured myocardium may potentially be facilitated through several regenerative mechanisms including: (i) cardiomyocyte grafting, (ii) increasing the blood flow to the ischemic myocardium, (iii) reducing cardiomyocyte apoptosis, (iv) regulating inflammation to inhibit negative remodeling of the myocardium, and (v) stimulating endogenous stem cells to regenerate the damaged myocardium [7]. In this context, stem cell transplantation has emerged as a key strategy to enable these repair mechanisms [8, 9, 10]. The three main approaches in stem cell transplantation that have been investigated in the context of myocardial repair include the direct injection of stem cells to regenerate functional myocardium, the seeding of stem cells on three-dimensional scaffolds for transplantation, or stimulating endogenous stem cells to secrete paracrine growth factors and/or differentiate into cardiomyocytes at the site of damage [7, 8, 11, 12].

### **1.3 Regenerative Medicine in Myocardial Infarction Therapy – Stem Cell Transplantation**

To be considered for potential use in regenerative therapeutic applications, stem cells must meet the following important criteria: the cell source must yield an abundant population of stem cells (million to billions), cells should be able to be collected using minimally invasive techniques, ideally the cells should be able to differentiate along multiple lineages demonstrating plasticity, and the cells must be able to be safely transplanted into autologous or allogenic hosts [13].

Several different types of regenerative cell populations have been investigated in the treatment of MI. For example, embryonic stem cells (ESCs), skeletal myoblasts, and bone marrow-derived mesenchymal stem cells (BMSCs) have been used with varying degrees of success. Human ESCs have been tested in animal models and can induce functional and structural improvement in the heart [14, 15, 16]. However, limiting factors with ESCs include difficulty in maintaining the cells in an undifferentiated state, potential oncogenicity, and perhaps most importantly, the ethical concerns regarding ESC harvest and use [17]. BMSCs have also been studied for the treatment of MI, including in human clinical trials, with positive results [18, 19, 20]. However, the use of BMSCs is not ideal for wide-scale clinical practice due to the invasive procedure required to extract the cells from the bone marrow, associated donor site morbidities, and also the low yield of stem cells in the bone marrow aspirates [9, 17, 21].

Adipose-derived stem cells (ASCs) have several key advantages over other types of stem cells and are gaining interest in the field. Some of the advantages of ASCs include the ability to extract large volumes of lipoaspirate tissue in minimally invasive procedures with little donor site morbidity and that there is a 100 – 1000 x higher yield of stem cells in adipose tissue as compared to bone marrow [7, 22, 23]. These advantages make ASCs a more attractive candidate population for potential translation into widespread clinical use. Several studies have shown the beneficial effects of ASC transplantation in the treatment of MI in animal models [24, 25, 26]. ASCs have been shown to elicit improvements in left ventricular ejection fraction (LVEF), limit ventricular wall thinning, and increase capillary density in peri-infarct zones, as well as decreasing the size of the infarcted region [24, 25, 26].

## 1.4 Thesis Overview

Regenerative medicine and stem cell transplantation are promising approaches for treating MIs and cardiovascular disease. In particular, ASC transplantation has been demonstrated in previous studies to elicit both functional and structural improvements in the myocardium [24, 25, 26]. However, many studies have also highlighted common limitations with ASC transplantation therapy. More specifically, there are consistent trends in terms of poor ASC viability and engraftment soon after transplantation, noted as early as one week after cell transplantation [26, 27, 28].

This thesis focused on the initial characterization of a delivery vehicle for ASCs to improve ASC retention and survival post-transplantation. The first part of this thesis focused on establishing protocols for isolating and culturing rat ASCs, and demonstrating their multilineage differentiation capacity (adipogenic, osteogenic, chondrogenic) *in vitro*. Subsequent studies assessed the rat ASC chemotactic response to two specific growth factors under both normoxic and hypoxic culture conditions. In addition, the expression of specific growth factor-related genes and proteins under varying oxygen tensions were analyzed. These characteristics were examined to characterize how rat ASC biology differs under hypoxic conditions compared to normoxia and to develop an understanding as to how they may react in the hypoxic conditions of infarcted myocardium. Finally, the viability of ASCs encapsulated in an RGD-modified, photo-crosslinkable methacrylated glycol chitosan (MGC) hydrogel delivery vehicle was characterized by confocal microscopy with and without growth factor loading. RGD is the cell adhesion sequence found in fibronectin, an extracellular matrix (ECM) protein, among others [29]. MGC hydrogels were modified with RGD to promote cell viability

via cell attachment because ASCs are anchorage-dependent cells [30].

## **1.5 Research Objectives**

The long-term objective of this research is to develop injectable polymer delivery vehicles for ASC delivery in the context of cardiovascular repair, to promote long-term cell viability and retention. Moving towards this goal, this project investigated the potential of MGC hydrogels modified with peptides containing the RGD sequence and used in conjunction with specific chemotactic growth factors to enhance rat adipose-derived stem cell (rASC) viability within the hydrogels.

The specific research objectives of this project were as follows:

### **i. Isolation and Differentiation of rASCs**

- Isolate and culture rASCs from the epididymal fat pads of male Wistar rats.
- Establish whether the rASCs can be differentiated along the adipogenic, osteogenic, and chondrogenic lineages *in vitro* to confirm the presence of multilineage cells within the extracted populations.

### **ii. Determine the effect of growth factors and oxygen tension on rASC migration**

- Determine the capacity of the growth factors stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) and hepatocyte growth factor-1 (HGF-1) to elicit a chemotactic migratory response in rASCs.
- Characterize the migratory response of rASCs to increasing concentrations of both SDF-1 $\alpha$  and HGF-1 (20, 50, 100 ng/mL).

- Examine the influence of normoxic (21% O<sub>2</sub>) and hypoxic (5% O<sub>2</sub>) culture conditions on the migratory response of rASCs in conjunction with exposure to growth factors.

**iii. Characterize the effects of growth factors and oxygen tension on rASC growth factor-related gene and protein expression**

- Evaluate the effects of the growth factors and oxygen tension on the expression of specific growth factor-related genes in the rASCs.
- Determine the effects of normoxic and hypoxic culture conditions on SDF-1 $\alpha$  and HGF-1 production by rASCs.

**iv. Assess rASC viability after encapsulation in MGC hydrogels**

- Examine the effect of increasing concentrations of RGD-peptide modification of MGC hydrogels on rASC viability over a 14-day period.
- Determine if SDF-1 $\alpha$ -loading of the MGC hydrogels has an effect on rASC viability over a 14-day period.



## **Chapter 2**

### **Literature Review**

#### **2.1 Overview of Cardiovascular Biology**

The heart is the muscular organ responsible for circulating blood and nutrients throughout the body. Spatially, it is located in the thoracic cavity situated between the lungs and sitting slightly to the left of the midline of the body. Macroscopically, the heart consists of four major chambers, two atria and two ventricles. The right and left atria are the chambers that collect deoxygenated blood from the body and oxygenated blood from the lungs respectively. The right and left ventricles are the highly muscular compartments responsible for pushing the blood into the pulmonary and systemic circulations respectively. Microscopically, the myocardium is composed of functional cells called cardiomyocytes, which are striated muscle cells found exclusively in the heart [31]. The heart is referred to as a functional syncytium because the cardiomyocytes are mechanically and electrically coupled, allowing the cardiac muscle to contract and relax synchronously [31]. This property greatly increases the heart's efficiency at circulating the blood throughout the body.

In order to function, the heart requires a steady supply of oxygen because cardiomyocytes obtain the necessary energy for contraction almost entirely from aerobic respiration [31]. The coronary circulation is the system responsible for maintaining the blood supply to the cardiomyocytes [31]. The major coronary arteries include the left and right coronary arteries and their associated branches [31]. Coronary veins shuttle the

deoxygenated blood away from the cardiomyocytes. Any disruption of the blood supply to the heart can have dire consequences.

One of the most serious consequences of disrupting the blood supply to the heart is myocardial infarction (MI) – an event in which the rupture of a coronary artery plaque forms an occlusive thrombus that results in the cessation of adequate blood supply to the heart and leads to myocardiocyte necrosis. The aftermath of an MI – electrical instability, decreased cardiac contractility, and tissue necrosis can be life threatening [32].

Electrical instability after an MI manifests as abnormal heart rhythms, also known as arrhythmias. Arrhythmias may be the result of disruption of blood supply to the structures of the conduction system, abnormal transcellular ion concentrations due to leaky cardiomyocyte membranes, and autonomic stimulation [32]. Ventricular fibrillation (VF) is the rapid and disorganized electrical activity of the ventricles and is often responsible for sudden cardiac death in the peri-infarct period. VF is caused by reentrant circuits, which may be formed by the necrotic myocardium, or the increased automaticity of ventricular cardiomyocytes [32]. Other arrhythmias that are seen in MIs include supraventricular arrhythmias such as sinus bradycardia and tachycardia, atrial premature beats, and atrial fibrillation.

Myocardial dysfunction as a result of MI can result in congestive heart failure (CHF). Ischemic damage to the myocardium can result in systolic dysfunction, which manifests as impaired contractility, and diastolic dysfunction, which leads to impaired filling of the ventricles during diastole [32]. Both systolic and diastolic dysfunctions contribute to CHF, the condition in which the heart is unable produce sufficient cardiac output to meet the metabolic demands of the body. Cardiogenic shock is a condition in

which the cardiac output is severely reduced resulting in abnormally low blood pressures that develops when more than 40% of the left ventricle is infarcted. Cardiogenic shock may develop in up to 10% of post-MI patients and has mortality rate that is greater than 70% [32].

An MI may also result in structural damage to the heart due to ischemia and necrosis of the myocardium. Post-MI mechanical complications include papillary muscle rupture, ventricular free wall rupture, and ventricular septal rupture, all of which have the ability to significantly impair cardiac output and may be life-threatening [32].

## **2.2 Regenerative Medicine in Cardiovascular Disease**

Regenerative medicine holds promise for the development of new treatments for myocardial infarctions, as well as many other diseases. This interdisciplinary field focuses on the repair, replacement, or regeneration of cells, tissues, or organs to restore function that has been impaired by congenital defects, traumatic injury, disease, or aging [5]. Cell-based therapies hold the potential of restoring damaged tissues and organs by stimulating previously irreparable organs to heal [6].

Regenerative medicine works to repair the damage to the heart caused by myocardial infarctions by employing one or more potential mechanisms of repair. These may include regeneration of injured myocardium, increasing blood flow to ischemic areas of the myocardium, reducing cardiomyocyte apoptosis, regulating inflammation to inhibit negative remodeling, or stimulating endogenous stem cells to help regenerate the myocardium [7]. All of these different repair mechanisms focus on improving the overall structural integrity and function of the heart. Cellular transplantation has emerged as the

chief strategy used to induce these different mechanisms in the context of myocardial repair [8, 9, 10]. In particular, the inherent properties of stem cells have made them an attractive cell type for use in regenerative approaches. Stem cells from multiple sources have been investigated for the treatment of myocardial infarctions.

### **2.3 Stem Cell Treatment for Myocardial Infarctions**

Stem cells are characterized by their ability to self-renew and the capacity to differentiate along multiple lineages [33]. These properties make stem cells an invaluable resource in regenerative medicine applications because they have the potential to restore damaged cell populations, including within the ischemic heart.

There are three major strategies for the application of stem cells in the treatment of myocardial infarctions: (i) stem cells and their derivatives can be transplanted into the heart to regenerate myocardium *in vivo*; (ii) stem cells can be used in conjunction with three-dimensional scaffolds to create bioartificial myocardium *ex vivo* that can be implanted into the body; and (iii) resident *in vivo* stem cells in the heart can be stimulated to proliferate and differentiate at the site of damage [11]. The ultimate goal of all three approaches is to repair the structural and functional damage caused by a myocardial infarction.

Regardless of their potential, all facets of stem cell use must be practical and sustainable if they are to be used in the clinic. In order to be considered for regenerative medicine applications, stem cells should meet the following criteria: (a) found in abundance (sources yielding millions to billions of cells); (b) collected through minimally invasive procedures; (c) can be differentiated along multiple lineages in a

reproducible manner; (d) can be safely and effectively transplanted to either autologous or allogenic hosts [13]. At this point, stem cells from multiple different sources have been investigated to repair the damage caused by myocardial infarctions.

### **2.3.1 Embryonic Stem Cells**

Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst that develops five days after fertilization [10]. These stem cells are of great interest because they are pluripotent, capable of differentiating into all cell types of the body, and therefore having great breadth for potential applications in regenerative medicine [10].

It has been shown that ESCs can be guided to differentiate into cardiomyocytes *in vitro* [34]. This makes them an attractive option for cell transplantation to treat myocardial infarctions. Studies have investigated rat ESCs transplanted into adult rat hearts post-myocardial infarction [14, 15]. The results of these studies have shown that a percentage of transplanted ESCs can differentiate into cardiomyocytes *in vivo* and induce structural improvements, such as normalized ventricular architecture and reductions in scar size and myocardial necrosis [14]. The rat hearts also demonstrated functional improvements, as global cardiac function and myocardial contractility were significantly enhanced compared to control groups and remained that way for up to 3 months after transplantation [15].

Laflamme *et al.* (2005) transplanted cardiomyocytes differentiated from human embryonic stem cells (hESCs) into infarcted rat hearts [16]. This study found that the hESC-derived cardiomyocytes formed stable grafts that grew larger over time due to the

proliferation of the engrafted cardiomyocytes [16]. They also found evidence of considerable amount of angiogenesis occurring in the hESC-derived graft, noted by the incorporation of both rat and human-derived cell populations within new blood vessels [16].

Despite the numerous benefits of ESCs, their widespread clinical use is limited by several factors. Concerns have been raised over embryonic cell stability (the difficulties of maintaining and proliferating these cells in an undifferentiated state,) potential oncogenicity, and ethical concerns over cell harvest and use [17]. The ethical concerns may be the most significant of all the concerns surrounding the use of hESCs. Blastocysts must be sacrificed to extract the cells [10], and therefore, the use of hESCs remains highly controversial due to the ethical and philosophical implications of such sacrifices. Until these concerns can be addressed, the use of hESCs in regenerative medicine will remain highly restricted.

### **2.3.2 Adult Stem Cells**

Due to the controversial issues surrounding ESCs, recent research in regenerative medicine has focused on the use of adult stem cells. Although the degree of plasticity of adult stem cells is less than that of embryonic stem cells, their multipotentiality and paracrine factor secretion capacity holds promise for the treatment of myocardial infarctions.

### 2.3.2.1 Bone Marrow-derived Stem Cells

Bone marrow includes populations of multipotent mesenchymal stem cells (MSCs), which have been characterized extensively. Bone marrow-derived mesenchymal stem cells (BMSCs) have been shown to differentiate into bone, cartilage, fat, tendon, and muscle [35]. Furthermore, these stem cells have the capacity to differentiate into cardiomyocytes, *in vitro* and *in vivo*, making them a candidate source for the cell-based treatment of myocardial infarctions [36, 37].

After skeletal myoblasts, BMSCs were the second candidates for cell-based therapy of myocardial infarction to have reached the clinical testing phase. A study by the Strauer group examined the effects of autologous intracoronary injections of BMSCs into ten patients approximately seven days after myocardial infarction [18]. Three months after treatment, several improvements were noted in the treatment group. More specifically, hypokinesia and dyskinesia of the myocardium were less pronounced in the experimental group compared to the control group; the infarct size had also decreased significantly in comparison to the control group and relative to its size prior to treatment [18]. The myocardium also showed increased uptake of thallium after cell treatment, an indication of improved metabolic function [18]. Another clinical trial by Assmus *et al.* (2002) also showed that BMSC transplantation led to significant functional and structural improvements in the heart after myocardial infarction [19]. In this study, treatment with BMSCs resulted in significant improvements in global left ventricular ejection fraction (LVEF), regional wall motion in the infarct zone, and reduced end-systolic left ventricular volumes four months after treatment [19]. Conversely, such improvements were not found in the control group.

Larger-scale randomized, controlled clinical studies have also been conducted to examine the ability of BMSCs to heal myocardial infarctions. The BOOST study by Wollert *et al.* (2004) involved 60 patients, 30 of whom received autologous intracoronary injections of BMSCs approximately six days after myocardial infarction [20]. Six months after treatment, patients that had received the cell therapy experienced a significant increase in the LVEF [20]. A large multicentre trial conducted by Schachinger *et al.* (2006) involving 204 patients, demonstrated that intracoronary infusion of BMSCs could promote healing in the heart after myocardial infarction [184]. In this clinical trial, four months after receiving an autologous injection of BMSCs, global LVEF was significantly higher in the treatment group than in the control group [184]. Overall, the clinical trials have consistently shown that intracoronary injections of BMSCs can benefit the heart by improving perfusion, tissue viability, and/or function [8].

Although these clinical trials show a promising and consistent trend of beneficial effects there are several hurdles that may potentially limit BMSC transplantation from being implemented as a large-scale treatment for myocardial infarctions. The rarity of the mesenchymal stem cell population in the bone marrow is arguably the most significant problem limiting the use of these cells in widespread treatment of myocardial infarctions [9, 17]. Many of the studies using BMSCs to treat myocardial infarctions have required millions to billions of cells [18, 19, 20]. Therefore, the low yield of BMSCs necessitates *ex vivo* expansion in order to reach clinically significant numbers, which can be time consuming, expensive, and increases the risk of cell contamination and loss [21]. Other problems such as invasive and painful extraction procedures, which often require general anesthesia and result in donor site morbidity, limit the volume of bone marrow aspirates



that can be extracted from patients [17, 21]. Furthermore, the proliferative capacity and abundance of BMSCs, as well as their ability to differentiate, decrease with increasing age [37]. Since the majority of people affected by myocardial infarctions are over the age of 65, the effectiveness of autologous BMSC therapy may be reduced.

#### **2.3.2.2 Adipose-Derived Stem Cells**

Multipotent MSCs have also been found in adipose tissue [21, 38]. These adipose-derived stem cells (ASCs) have been shown to differentiate into fat, bone, cartilage, and muscle cells, including cardiomyocytes, as well as other lineages [21, 38, 39]. Planat-Benard *et al.* (2004) demonstrated that ASCs could develop cardiomyocyte-like characteristics such as contractile activity, cardiac-specific genes, and morphology in culture without the addition of cardiac differentiation factors such as 5-azacytidine [40]. Naturally, these characteristics make ASCs candidates for cell-based treatment of myocardial infarctions.

Extensive efforts to characterize ASCs have revealed several important advantages over BMSCs. Unlike bone marrow aspirate extractions, adipose tissue has been shown to be easily extractable through minimally invasive procedures with little risk of donor site morbidity [22, 23]. The most significant advantages that ASCs have over BMSCs are the amount of tissue that can be extracted and the yield of stem cells. Strem *et al.* (2005) have suggested that approximately 1-2% of nucleated cells found in adipose tissue are stem cells [7]. Furthermore, compared to BMSCs, ASCs have a significantly higher rate of proliferation [7, 41]. Due to the risk of donor site morbidity, the volume of bone marrow aspirate extracted under local anesthesia is limited to

approximately 40 mL, yielding approximately  $2.4 \times 10^4$  stem cells [7]. Conversely, under local anesthesia, the volume of adipose tissue extracted can easily exceed 200 mL, yielding more than  $1 \times 10^6$  stem cells [7]. Therefore, under local anesthesia, adipose tissue may yield up to approximately 40-fold more stem cells than bone marrow aspirate, although stem cell numbers may be donor dependent [7]. For procedures such as liposuctions with general anesthesia, the volume of adipose tissue extracted can range from 100 mL to in excess of 3 L depending on the patient [42]. With approximately 400,000 liposuction procedures being performed in the United States annually, ASCs as a stem cell source are available in significantly greater abundance than BMSCs [42]. Unlike BMSCs, the relatively large number of ASCs that can be obtained from a single tissue extraction may negate the need for extensive *ex vivo* expansion. In short, the availability, abundance and easy extractability of ASCs are significant advantages over bone marrow stem cells with respect to clinical applicability.

ASCs have other beneficial properties that make them an excellent candidate for cell-based therapies. It has been suggested that only approximately 1% of ASCs express major histocompatibility complexes I or II or that they may not even express MHC II at all [43]. Therefore, ASCs are unlikely to elicit a deleterious immune response after transplantation in an allogenic application. Additionally, ASCs have been shown to release angiogenic factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and angiopoietin-1 [7, 22]. The release of these factors can promote reperfusion of the infarct zone, survival of transplanted stem cells, cardiomyocyte regeneration, and prevention of ischemic events that can lead to a subsequent myocardial infarction [7, 22].

Extensive *in vitro* characterization has demonstrated that ASCs are an excellent candidate for use in cell-based repair of myocardial infarctions. As such, many groups have rapidly progressed to conducting *in vivo* studies with ASCs in this application. Li *et al.* (2007) examined the effects of ASC transplantation on heart function in a rat model of acute myocardial infarction [24]. The results of this study showed that after four weeks, ASC-treated rats had significantly improved left ventricular function, as measured by ejection fraction and fractional shortening, compared to control rats [24]. Capillary density in the peri-infarct area was also significantly higher in ASC-treated rats than in control rats [24].

The Valina group examined the effects of ASC and BMSC treatment compared to control treatment in a porcine model of acute myocardial infarction and reperfusion [25]. This study found that both ASC and BMSC treatment led to functional benefits, seen by significantly improved left ventricular ejection fraction and significantly decreased absolute and relative perfusion defects [25]. Structural benefits to the heart were also apparent as stem cell treatment resulted in significantly thicker ventricular walls in the infarcted area compared to the control animals; vascular density around the infarct zone was also increased [25].

A study by Mazo *et al.* (2008) compared the effects of ASC, BMSC, and adipose-derived cardiomyocyte (AD-CMG) transplantation in a rat model of chronic myocardial infarction [26]. One month after transplantation, only the ASC-treated rats demonstrated significantly improved LVEF compared to control rats [26]. Tissue metabolism, measured by the uptake of <sup>18</sup>fluorine-fluorodeoxyglucose, was also significantly improved only in the ASC-treated rats when compared to control rats [26]. An increase

in capillary density in the heart was seen with all three types of cells; however, ASC-treated rats demonstrated the most significant increase [26]. Likewise, a significant reduction in infarct size was seen with ASCs, BMSCs, and AD-CMGs, but this effect was most profound in ASC-treated rats [26]. These results in particular are very important because these three cell types were directly compared to each under the same conditions in one study. By doing so, concerns of potential experimental variations that would arise when comparing the results of different studies are eliminated.

Schenke-Layland *et al.* (2009) investigated the potential of ASCs to improve left ventricular function in a rat model of myocardial infarction [27]. After ligating the left anterior descending coronary artery, ASCs were injected into the chamber of the left ventricle. Twelve weeks after ASC treatment, no significant left ventricular dilation was seen in the treatment rats, unlike the control rats that received no treatment [27]. This demonstrates that ASCs are capable of mediating the remodeling that occurs after a myocardial infarction. Increased angiogenesis was also associated with ASC treatment [27]. Rats treated with ASCs showed significantly more capillaries per unit area in the infarct border zone and healthy myocardium [27]. Increased arteriolar density was also seen in the infarct border zones and in the infarct zone itself in ASC-treated rats compared to control rats [27]. In terms of functional end-points, ASC-treated rats displayed significantly higher cardiac outputs, ejection fractions and stroke volumes than control rats, suggesting improved left ventricular contractility [27].

Many studies have consistently shown the positive effects on heart structure and function that can be derived from using a stem cell-based regenerative medicine approach to treat myocardial infarctions. Specifically, ASCs appear to be an ideal cell

type for use in this regard due to the fact they have significant advantages over BMSCs with respect to accessibility, availability and most importantly, stem cell yield.

As consistent as the positive results have been, many studies have also demonstrated problems with poor cell survival and engraftment when using ASCs to treat myocardial infarctions. Mazo *et al.* (2008) implanted ASCs from male rats expressing enhanced-green fluorescent protein (eGFP) into female rats [26]. Only a small number of ASCs were detected one week after transplantation and at four weeks no eGFP-positive signals or y-chromosome gene expression could be detected in any of the rats treated with ASCs [26]. These findings suggest that there was a high level of cell leakage and/or death soon after transplantation [25]. Schenke-Layland *et al.* (2009) also found that 12 weeks after GFP-ASC transplantation, GFP-positive cells could be detected in only two out of the eleven treated rats [27]. Similarly, van der Bogt *et al.* (2009) found that robust signals from GFP-ASCs could be detected from the infarct region two days after treatment, confirming that transplantation had been successful [28]. However, no signals could be detected at 6 weeks, suggesting that all transplanted cells had migrated or died by that time [28].

The problem of poor long-term survival of transplanted cells has also been shown in several studies transplanting BMSCs for myocardial infarction treatment [44, 45, 46]. These results suggest that the problem is not specific to ASCs; rather, it is an issue applying to all transplanted cells having to adjust to a chemically and physically different *in vivo* environment. For example, the presence of the immune response and shear forces of blood flow, which are absent in the *in vitro* setting but present *in vivo*, may contribute to the poor long-term survival of transplanted ASCs and other cell types. If transplanted

stem cells have poor survival and engraftment rates, there must be other mechanisms of action to support the improvements in cardiac structure and function observed after cell transplantation.

Although the mechanism by which the benefits derived from ASC transplantation in treating myocardial infarctions remains unclear, there is strong evidence to support that they are the result of paracrine effects induced by the transplanted cells [7, 8, 26, 47]. This paracrine mechanism has been suggested to be the reason why structural and functional benefits to the heart can be seen even after the transplanted cells are no longer detected within the tissues. As previously mentioned, ASCs are capable of releasing growth factors and cytokines such as VEGF, HGF, and angiopoietin-1 [7, 22]. These factors are potent inducers of angiogenesis, which can be therapeutic by increasing the perfusion of oxygenated blood and nutrients to the infarcted myocardium. The secreted factors also include chemoattractants that can recruit endogenous stem cells to the site of injury to promote healing and functional recovery. Additionally, the growth factors and cytokines released by ASCs may stimulate endogenous stem cells to differentiate into cardiomyocytes resulting in reduced infarct size and the regeneration of functional myocardium with improved cardiac contractility [47]. ASCs may also elicit therapeutic effects by releasing antioxidants, free radical scavengers and heat shock proteins at the infarct site to remove toxic compounds released by the damaged cardiomyocytes [47]. Removal of such toxins from the local environment could promote the recovery of surviving cardiomyocytes, as well as the development of new cells to replace the lost populations [47].

## 2.4. ASC Immunophenotype

Identifying the surface immunophenotype of ASCs has been important in helping to differentiate the ASCs within the heterogeneous cell population found in the stromal vascular fraction (SVF) of adipose tissue [48, 49, 50]. Multiple groups have established the profile of cell surface markers expressed by ASCs with a high degree of consistency [33, 47]. ASCs express the adhesion molecules CD9 (tetraspan), CD29 ( $\beta_1$  integrin), CD49 days (  $\alpha_4$  integrin), CD54 (intracellular adhesion molecule 1 (ICAM-1)), CD105 (endoglin), and activated lymphocyte cell adhesion molecule (ALCAM; CD166). The receptor molecules for hyaluronate (CD44) and transferrin (CD71) and the surface enzymes common acute lymphocytic leukemia antigen (CALLA; CD10), aminopeptidase (CD13), and ecto 5' nucleotidase (CD73) have also been identified on the surface of ASCs.

Though multiple independent groups have reported ASC immunophenotype profiles with a high degree of consistency, some discrepancies do remain with regard to particular surface molecules. CD34, CD106 (VCAM-1), and Stro-1 have been identified on ASCs by some groups but not others [51, 52]. It has been suggested that these differences may be the result of variations in cell isolation techniques, length of time in culture, and sensitivity differences between immunohistochemical and flow cytometric detection methods [51, 52, 53].

The immunophenotypic profile of ASCs and BMSCs are highly similar, with some comparisons showing the two cell types to be up to 90% identical in their surface profiles [47]. There are some differences in the types of protein markers expressed on the surface of ASCs and BMSCs. For example, ASCs express VLA-4 (CD49d) but not

VCAM-1 (CD106), while the opposite is true for BMSCs [7]. Some studies have also indicated that ASCs express the stem cell marker CD34 in early passages, but no such expression has been noted for BMSCs to date [35, 54].

The immunophenotype of ASCs is dynamic and has been shown to change with adherence and passaging [55]. The expression of stromal-cell associated markers CD13, CD29, CD44, CD63, CD73, CD90, and CD166 significantly increases with serial passaging [60]. Conversely, the stem cell-associated marker, CD34, is most highly expressed in SVF cells and early passage ASCs and decreases with successive passages [55]. Aldehyde dehydrogenase (ALDH) and ATP-binding cassette subfamily G member 2 (ABCG2) are also expressed by ASCs. The surface markers CD31, CD144 (VE-cadherin), vascular endothelial growth factor receptor 2 (Flk-1), and von Willebrand factor are also expressed on subpopulations of the primary isolates and expression levels remain relatively constant over time [55].

## **2.5 Multilineage Differentiation of ASCs**

### **2.5.1 Adipogenic Differentiation**

In order to induce adipogenic differentiation, ASCs are cultured in medium containing specific supplements that target the cellular pathways involved in lipogenesis. Although specific medium formulations, chemical composition and concentrations may vary depending on the source from which the ASCs are isolated, many studies have implemented similar supplements. ASCs derived from human adipose tissue are typically cultured in medium containing insulin, isobutylmethylxanthine (IBMX), hydrocortisone or dexamethasone, indomethacin or a thiazolidinedione, pantothenate, biotin, and



triiodothyronine [33]. Adipogenic differentiation of ASCs from rat fat pads has also been investigated and media formulations are similar to what is used for the induction of human ASCs. Differentiation media for rASCs have included supplements such as dexamethasone, insulin, biotin, IBMX, pantothenate, and troglitazone [56].

IBMX is an inhibitor of the phosphodiesterase enzyme, which results in elevated intracellular levels of cyclic AMP (cAMP), creating a favourable environment for adipogenesis [33, 57, 58]. Also, thiazolidinediones are synthetic ligands for peroxisome proliferator activated receptor- $\gamma$  (PPAR  $\gamma$ ) [33]. PPAR $\gamma$  is one of the master regulators of adipogenesis, and the inclusion of a supplement such as thiazolidinedione can be a strong inducer of *in vitro* adipogenesis [59]. PPAR $\gamma$  and CCAATT-enhancer-binding proteins (C/EBPs) are the major transcriptional regulators of adipogenesis. During adipogenic differentiation, C/EBP $\beta$  and C/EBP $\delta$  bind to the promoter region of the PPAR $\gamma$  gene increasing the expression of PPAR $\gamma$  thus increasing the expression of C/EBP $\alpha$ , which functions to maintain differentiated state of adipocytes [59]. Another transcriptional regulator, ADD-1/SREBP-1c, regulates adipogenic differentiation by increasing the expression of PPAR $\gamma$  and also by upregulating the expression of other genes expressed adipocytes such as glycerol-3-phosphate (GPDH), lipoprotein lipase (LPL), fatty acid synthase (FAS), glucose transporter type-4 (Glut-4), and adipocyte-specific fatty acid binding protein 2 (aP2) [59].

Successful adipogenic differentiation of ASCs is often assessed in terms of morphological changes by microscopy, as well as adipogenic gene and protein expression. Oil Red O staining can be used to detect the accumulation of intracellular lipid characteristic of adipogenic differentiation [21]. Another common assay involves

measuring the level of GPDH enzyme activity (involved in triglyceride synthesis) as a determinant of adipogenic differentiation, as differentiating adipocytes have significantly higher GPDH activity compared to undifferentiated ASCs [60].

### **2.5.2 Osteogenic Differentiation**

Many groups have demonstrated that ASCs can be stimulated in culture to differentiate into osteoblasts when cultured in differentiation media containing the appropriate supplements [21, 61]. ASCs have also been shown to carry the same capacity for osteogenic differentiation as BMSCs and are able to retain this ability with increasing donor age [62].

To stimulate osteogenesis, ASCs are cultured in medium containing supplements such as dexamethasone (DEX), ascorbate-2-phosphate, and  $\beta$ -glycerophosphate [21, 61]. DEX plays a role in the regulation of the mitogen-activated protein kinase (MAPK) signaling pathways [63]. The activation of the MAPKs, extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK), are critical events at the molecular level that trigger the osteogenic differentiation of ASCs, while inhibiting adipogenic differentiation [64]. Ascorbate-2-phosphate is a source of ascorbate acid, which regulates alkaline phosphatase (ALP) activity, an enzyme characteristic of osteoblasts that is involved in collagen type I synthesis, a component of the extracellular matrix (ECM) of bone tissue [65].  $\beta$ -glycerophosphate acts a supplier of inorganic phosphate that is released by ALP and used for the production of calcium phosphate, which is deposited in the ECM of maturing bone tissue [65]. From a genetic perspective, transcription factors *Menin*, *Shh*, and *Notch-1* are believed to play roles in the acquisition of the osteoblast-like phenotype [66].

When cultured in a pro-osteogenic environment, ASCs express genes and proteins such as alkaline phosphatase, osteopontin, osteonectin, osteocalcin, bone sialo protein, RunX-1, BMP-2, BMP-4, BMP receptors I and II, and the PTH-receptor, which are characteristic of the osteoblast phenotype [59, 67, 68, 69]. Furthermore, ASCs differentiated along the osteogenic lineage are able to form mineralized matrix in 2D and 3D *in vitro* cultures and display specific responses to mechanical loading (such as fluid shear stress) [7, 68, 69].

Several methods have been used to determine the osteogenic differentiation of ASCs. Von Kossa staining is used to detect the presence of calcified matrix (deposits of calcium phosphate) [61, 70]. ALP enzyme activity can also be used as a determinant of osteogenesis because it is upregulated during differentiation as it plays a role in the production and maturation of bone tissue [68, 70].

### **2.5.3 Chondrogenic Differentiation**

ASCs have also been shown to be capable of differentiation along the chondrogenic lineage [21, 70]. For chondrogenic differentiation, the culturing technique plays an important role in helping ASCs to obtain the cartilage-like phenotype. Methods such as micromass culturing and centrifugation can be used to produce 3D high-density cell masses that mimic the natural morphology of chondrocytes and aid in chondrogenic differentiation. When cultured in a pro-chondrogenic environment, ASCs can express cartilage-specific genes such as aggrecan and type II collagen and synthesize cartilage matrix molecules such as collagen type II, type IX, and chondroitin-4-sulfate [69, 71].

ASCs must also be cultured in specific medium formulations designed to stimulate chondrogenic differentiation. Supplements most commonly used in chondrogenic differentiation media are ascorbate-2-phosphate, dexamethasone, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), and insulin [21, 61]. Ascorbate-2-phosphate is used because it helps to stimulate collagen synthesis, a major component of cartilage tissue, and is more stable in aqueous media than ascorbic acid, which readily decomposes via oxidation in aqueous environments [72]. TGF- $\beta$ 1 is another important factor required for the chondrogenic differentiation of ASCs, which has been shown to increase proteoglycan and sulfated-glycosaminoglycan (S-GAG) synthesis [73]. Furthermore, TGF- $\beta$ 1 has also been demonstrated to increase the production of collagen type II, the principle type of collagen in the ECM of cartilage tissue [74].

Sox9, a high-mobility group (HMG)-box transcription factor, plays a significant role in the regulation of chondrogenic differentiation and is expressed in all chondrogenic progenitor cells and differentiated non-hypertrophic chondrocytes [75]. Sox9 is required for the transition of undifferentiated mesenchymal cells to osteochondroprogenitor cells and subsequently for cellular condensation [75]. Ultimately, Sox9 also regulates the overt differentiation into chondrocytes, proliferation, formation of chondrocyte columns in growth plates, and inhibits the transition into hypertrophic chondrocytes [75]. Sox9 is also required for the expression of other chondrogenic regulators, Sox5 and Sox6, which act to upregulate the activity of Sox9 and the expression of collagen IX, aggrecan, and cartilage link protein, which are components of the extracellular matrix of cartilage tissue [75, 76].

Histologically, chondrogenic differentiation of ASCs can be visually assessed by alcian blue or toluidine blue staining, both of which stain for sulfated proteoglycans, a characteristic component of cartilaginous ECM [21, 61, 77]. Chondrogenic differentiation can also be characterized through the immunohistochemical staining and ELISAs for collagens type I and II, hydroxyproline assay, and dimethylmethylene blue (DMMB) assay [61].

## **2.6 Growth Factor-Induced Mobilization of Progenitor/Stem Cells**

Chemokines are pro-inflammatory chemoattractant cytokines that together with their appropriate receptors play an important role in progenitor/stem cell trafficking [78]. Chemokines can be divided into four families, which are CXC (alpha), CC (beta), C (gamma), and CX3C (delta), based on the organization of the first two conserved cysteine residues [162]. Stromal cell derived-factor 1 (SDF-1) belongs to the CXC (alpha) family of chemokines, which are defined by the separation of the first two cysteine residues by one amino acid [162]. The SDF-1 structure is composed of three anti-parallel  $\beta$  strands and an  $\alpha$ -helix, and its amino acid sequence is well known with the exception of N-terminal residues 1-8 and C-terminal residues 66-67 [162]. SDF-1 has two major isoforms, SDF-1 $\alpha$  and SDF-1 $\beta$ . SDF-1 $\alpha$  consists of 68 amino acids and is the predominant isoform, whereas the non-dominant SDF-1 $\beta$  has four additional C-terminal residues (arginine-leucine-lysine-methionine) [185]. Chemokines such as SDF-1 mediate their effects by binding to their specific receptors, which belong to the seven-transmembrane receptor superfamily, that activate signal transduction pathways via G proteins [162].

SDF-1 $\alpha$  and its receptor CXCR4 have been shown to regulate the homing of stem cells to its location by inducing chemotaxis [79]. Moepps *et al.* (2000) ‘knocked-out’ the SDF-1 $\alpha$ -CXCR4 axis in murine models and showed that SDF-1 $\alpha$  plays a critical role in the colonization of the bone marrow by hematopoietic stem cells during embryogenesis [80]. Studies have also shown that this axis is active in adult life in the homing and retention of stem cells into the bone marrow normally and also after bone marrow transplantation [81, 82]. Also, animals with the SDF-1-CXCR4 axis knocked out have been shown to have developmental defects of the heart, large vessel, and brain suggesting that this chemokine and its receptor may also have a role in organogenesis [79]. The ability of SDF-1 $\alpha$  to mediate stem cell homing is an important therapeutic mechanism in the event of an MI. The heart expresses SDF-1 $\alpha$  immediately after an acute MI, which would act to attract ASCs, endothelial progenitor cells, and hematopoietic stem cells to promote neovascularization and myocardial tissue repair, all of which express SDF-1 $\alpha$ ’s cognate receptor, CXCR4 [83, 84]. Furthermore, Askari *et al.* (2003) demonstrated that re-establishing the expression of SDF-1 up to 8 weeks after the occurrence of an MI resulted in successful homing of stem cells to injured myocardium and improvement in cardiac function [83]. Therefore, the ability of SDF-1 $\alpha$  to mobilize cells to sites of injury is a critical aspect of stem cell therapy for MI.

Hepatocyte growth factor (HGF) is a heterodimer cytokine that is composed of  $\alpha$  and  $\beta$  subunits that are connected by a disulfide bridge and bears resemblance to plasminogen [186]. The heavy  $\alpha$  subunit contains four different domains known as kringles and a hairpin loop at the N terminus [186]. Kringles are composed of two polypeptide loops, with the smaller loop held within a larger loop by disulfide bonds

[187]. The light  $\beta$  subunit has a portion that resembles a serine protease, though HGF has no serine protease activity [186]. HGF exerts its effects by combining with its receptor, c-Met, a transmembrane tyrosine kinase cell surface receptor [186].

HGF is secreted by ASCs and has been shown to have an important role in the mobilization and homing of stem/progenitor cells from different tissue sources within the body [85, 86, 87]. Multiple studies have demonstrated that HGF is capable of inducing a significant chemotactic migratory response of human MSCs *in vitro* [88, 89]. The ability of HGF to mobilize different types of cells may be an important way that it is able to exert its therapeutic angiogenic, antifibrotic, and anti-inflammatory properties [90, 91]. For example, the expression of HGF by ASCs has been shown to be critical for mediating the migration of endothelial progenitor cells (EPCs) and endothelial cells (ECs), which are responsible for promoting revascularization of ischemic tissue environments [85, 92]. Cai *et al.* (2007) demonstrated that silencing HGF expression or pre-incubating ASCs with an HGF-inactivating body diminished the migration of EPCs and that this effect could be reversed by restoring HGF [85]. HGF has also been shown to mobilize cardiac stem cells, which express c-Met, and promote migration to sites of injury where they can proliferate and differentiate and by doing so exert some therapeutic effects [93]. Therefore, the ability of ASCs to secrete HGF and thereby promote the mobilization of various different stem/progenitor cells and home them to sites of injury may be an important process by which the benefits of ASCs in the treatment of MI may be effected.

## **2.7 Effect of Oxygen Tension on ASC Proliferation, Chemotaxis, and Phenotype**

Oxygen tension can significantly influence many aspects of ASC biology. The hypoxic environment is a key mediator of promoting neovascularization of ischemic sites by influencing ASCs to adopt pro-angiogenic characteristics [94]. The cellular response to hypoxic conditions is controlled by hypoxia inducible factor-1 (HIF-1), a transcription factor [95]. ASC expression of HIF-1 is significantly increased in ASCs cultured under hypoxic atmospheric conditions (5% O<sub>2</sub>) as compared normoxic cultures (21% O<sub>2</sub>) [96].

The HIF-1 $\alpha$  subunit is stabilized under hypoxic conditions and promotes the expression of pro-angiogenic genes, particularly vascular endothelial growth factor (VEGF), an important promoter of neovascularization [95, 96]. VEGF expression by ASCs has been shown to increase as an inverse function of oxygen-tension, with expression increasing significantly as oxygen tension decreases from 21% to 1% [94, 96]. Other angiogenic factors such as insulin-like growth factor-1 (IGF-1), secreted frizzled-related protein-2 (Sfrp-2), stromal cell-derived factor-1 $\alpha$ , and hepatocyte growth factor (HGF) also demonstrate increased expression under low oxygen tension [83, 94, 97, 98, 99].

SDF-1 $\alpha$  and HGF are important mediators of stem cell homing and migration [83, 88, 89, 99, 100]. Under normoxic conditions, the presence of SDF-1 $\alpha$  mediates an increased migratory response in ASCs [96]. Preconditioning of ASCs under hypoxic conditions has been shown to significantly increase the migratory response of the cells to SDF-1 $\alpha$  by increasing the expression of CXCR4, the receptor for SDF-1 $\alpha$ , on ASC surfaces [96]. Similarly, HGF has been shown to be a chemoattractant for ASCs. ASCs



have also been demonstrated to upregulate the expression of c-met, the receptor for HGF, on their surfaces under hypoxic conditions thereby making them more sensitive to the presence of HGF [88, 99].

Oxygen tension also affects ASC proliferation, with hypoxic conditions stimulating significant proliferation [96, 99]. However, there appears to be a threshold where extreme hypoxic conditions begin to negatively impact the cultured cells. Generally, proliferation of ASCs is significantly increased under low atmospheric oxygen tension levels (5%) as compared to normoxic conditions (21%). However, under extreme hypoxic conditions (1% O<sub>2</sub>) ASC proliferation has been reported to be lower than that observed at 5% O<sub>2</sub> [94].

Cell survival is also influenced by oxygen tension. Generally, ASCs appear to be very resistant of hypoxic conditions as evidenced by the fact that the relative proportion of apoptotic cells under low oxygen tension cultures (5% O<sub>2</sub>) has been reported to be significantly lower than the proportion in normoxic cultures (21% O<sub>2</sub>) [94]. However, under extreme hypoxia (1% O<sub>2</sub>) the proportion of apoptotic cells was significantly higher as compared to ASCs cultured in 5% O<sub>2</sub> or normoxic conditions [94].

Oxygen tension may also play a role in influencing the phenotype of ASCs. Under hypoxic conditions, ASCs have been shown to partially adopt certain characteristics of endothelial cells [96]. Under hypoxic conditions (1% O<sub>2</sub>) and in the presence of VEGF, a significantly larger percentage of the ASC population expresses the endothelial markers Flk-1 and CD31 [96]. The ASCs also demonstrate an enhanced ability to form tubules, a characteristic of endothelial cells, when co-cultured with bEND.3 endothelial cells from mice under hypoxic conditions compared to normoxic

conditions [96]. bEND.3 cells alone demonstrate a poor ability to form tubules under both normoxic and hypoxic conditions [96, 101]. This indicates that the enhanced capacity for tubule formation is a product of both cells acting together and that this ability is enhanced by hypoxia.

## **2.8 Polymeric Biomaterials in Myocardial Regenerative Medicine**

The method of delivery is an important aspect of cellular therapy for myocardial infarctions that has significant implications on the success of a developed strategy. To date, the primary methods of cell delivery have been injections of cell suspension directly into the area of damaged myocardium or into the coronary vasculature [102, 103].

However, both of these methods have drawbacks that limit the efficacy of this treatment.

Direct injection of cells into the area of damaged myocardium is done through open chest surgery, which is highly invasive, or via injection through a catheter inserted into the interior surface of the heart. Myocardial injection is beneficial because cells are delivered directly into the infarct region, however, there is poor cell localization and rapid loss at the site of injury, expedited by the mechanical forces associated with the beating heart [1].

Cells can also be introduced indirectly to the infarcted myocardium via injection into the coronary circulation, which requires subsequent cell migration to the site of injury. This method has also been shown to be inefficient, with less than five percent of injected cells being found in the heart 50-75 minutes after injection [104]. With direct intramyocardial injection, GFP-ASCs have been found to be abundant and give off strong signals two days after injection [28].

Multiple studies have shown that transplanted stem cells are not retained long-term in the infarct site after transplantation, with little to no viable stem cells found at the site of injury approximately four to six weeks after transplantation [8, 9]. Poor localization and premature cell death may be attributed to the fact that cells are injected in suspension into a hostile environment. Long-term cell retention and viability have been suggested to be the major factors limiting the efficacy of stem cell transplantation as a treatment for myocardial infarction [8, 9]. However, these problems may potentially be overcome by selecting an appropriate delivery vehicle for the stem cells, which can play an important role in promoting cell localization and survival.

Polymers, a large class of natural and synthetic biomaterials, have been used extensively in constructing scaffolds for use as cell delivery vehicles, and they have been used effectively in similar therapies for soft tissue, orthopedic, and dental implants [1]. Polymer scaffolds of varying architecture and material composition have been modified and used as delivery vehicles for various types of cells, including stem cells, and can be engineered to enhance cell survival.

### **2.8.1 Hydrogel Scaffolds for Stem Cell Transplantation**

Scaffolds are temporary structures that initially support cell survival, growth, and tissue formation [105]. Choosing the appropriate scaffold architecture and subsequent materials for construction can significantly influence cell survival. Two main approaches exist for cell-seeded scaffold fabrication: (i) Cells can be seeded onto a pre-made, porous scaffold, or (ii) cells can be encapsulated within the scaffold during its formation [105]. Cell encapsulation within hydrogel scaffolds has several advantages that make it an

attractive strategy for stem cell delivery.

Hydrogels are three-dimensional, hydrophilic polymer networks that swell in water but are insoluble and have many properties that are conducive to cell survival [106]. Intrinsic properties such as high water content, tissue-like elasticity, similar mechanical properties to soft tissues, high permeability for oxygen, nutrients, water-soluble metabolites, and biocompatibility make hydrogels an extremely attractive scaffold option for stem cell delivery [106, 107, 108]. These properties allow hydrogels to provide encapsulated cells with a three-dimensional environment similar to what the cells would experience *in vivo* [109].

Hydrogels can be formed under mild conditions that cause minimal harm to the cells being encapsulated. Temperature-sensitive hydrogels that form at body temperature and photopolymerizable hydrogels that undergo gelation after short exposure to low-intensity UV light are two ways that hydrogels can be formed *in situ* [107, 110]. An *in situ* formation capacity is also advantageous because the cells can be delivered through minimally invasive injections rather than through open surgical implantation.

Although the properties of hydrogels make them an attractive delivery vehicle, cell survival in unmodified hydrogels is often very poor [108]. Many studies indicate that hydrogels need to be modified to support better cell attachment and viability. However, the nature of the polymeric materials can allow for chemical modifications with a range of different factors that can significantly enhance the ability of hydrogels to sustain the survival of encapsulated cell populations.

Hydrogels can be broadly classified as natural or synthetic based on the type of polymer used in its construction [105]. The application of hydrogels as delivery vehicles

for stem cells in the treatment of myocardial infarction is a relatively new strategy [111]. Therefore, not all of the following hydrogels have been used specifically for the delivery of stem cells in cardiovascular applications. However, they are presented because they have been investigated for cell delivery in tissue engineering applications and have the potential to be used in the context of the ischemic heart.

## **2.9 Hydrogels from Naturally-Derived Polymers**

Natural hydrogels are made from polymers originating from biological sources [105], and may exhibit low toxicity, biocompatibility, and biodegradability, mediated through cell-secreted enzymes [112]. Also, biological polymers used to make natural hydrogels can have different biologically recognizable moieties that can help regulate cell adhesion, proliferation, phenotype, and enzyme activity [113, 114]. Chitosan, alginate and hyaluronic acid are three naturally derived polymers that have been extensively used in the construction of hydrogels for cell delivery.

### **2.9.1 Chitosan**

Chitosan is a cationic polysaccharide consisting of the monomers  $\beta(1\rightarrow4)$ -glucosamine and *N*-acetyl-D-glucosamine [115]. Chitosan is formed from the complete or partial deacetylation of chitin, a natural polysaccharide found in several biological sources including crustacean shells, insect cuticles and the cell walls of fungi [115]. Chitosan has many suitable properties that make it a desirable base material for hydrogel scaffolds to be used for cell delivery, such as being non-antigenic, non-toxic and biodegradable (degraded by lysozymes) [116].

Zhu *et al.* (2002) compared the adhesion of cells to unmodified poly (D,L-lactic acid) (PDL-LA) surfaces to PDL-LA surfaces modified with the attachment of chitosan polymer chains and chitosan chains modified with amino acids [117]. Cell-extracellular matrix interactions can have significant influence over cell survival, differentiation, and tissue formation [118]. Without these interactions, cells undergo anoikis, programmed cell death initiated by detachment from the ECM [119]. Being anchorage-dependent, ASC interactions with the ECM are vital for cell survival [118]. They demonstrated that PDL-LA surfaces modified with chitosan chains promoted better cell adhesion, proliferation, and activity compared to unmodified PDL-LA surfaces [117]. Also, by attaching alkaline amino acids such as lysine and arginine to the chitosan chains, cell adhesion, proliferation, and activity was further improved [117]. The hydroxyl groups located along the chitosan polymer backbone allow for the attachment of biological moieties such as amino acids that can also further enhance its cell-adhesive properties. Growth factors that can enhance cell survival may also be incorporated into chitosan hydrogels using the amino and hydroxyl groups on the polymer backbone [115].

Lu *et al.* (2009) demonstrated the retention and survival of ESCs encapsulated in temperature-sensitive chitosan hydrogels injected into the heart of a Sprague-Dawley rat to treat myocardial infarction [120]. Also, stem cells encapsulated in the chitosan hydrogel resulted in significantly larger graft sizes *in vivo* four weeks after implantation compared to stem cells injected in phosphate buffered saline [120]. The results demonstrated that cell survival and retention were better when the chitosan delivery vehicle was used.

There are several possible reasons why the chitosan hydrogels had a favorable outcome. The injection of chitosan alone was found to induce significant neovascularization in the infarct area as compared to the injection of PBS alone [120]. The induction of neovascularization can substantially improve the microenvironment of the encapsulated stem cells by supplying them with oxygen and nutrients, thereby sustaining their survival [120]. In addition, the degradation rate of the hydrogel can influence the survival of stem cells. Lu *et al.* (2009) demonstrated that a chitosan hydrogel could completely degrade *in vivo* approximately four to six weeks after injection into the heart [120]. The degradation products of chitosan are chitosan oligosaccharides that vary in length [120]. This length of time may be long enough to allow the encapsulated stem cells to secrete their own extracellular matrix and generate new tissue to sustain their own attachment and survival [120]. Also, if growth factors were incorporated into the hydrogel, this degradation time may be long enough to allow encapsulated stem cells to be exposed to the growth factors for an adequate length of time to augment their survival, proliferation, and differentiation *in vivo* [120].

The degradation rate can influence the level of immune response over time. The degree of deacetylation of chitosan has been shown to influence the degradation rate of chitosan hydrogels [121]. The degradation rate increases as the degree of deacetylation decreases due to the presence of more acetyl groups in the polymer chain that can be acted on by lysozymes [121]. Molinaro *et al.* (2002) demonstrated that chitosan hydrogels with higher degrees of deacetylation elicited smaller immune responses *in vivo* compared to hydrogels with low degrees of deacetylation because their slower degradation rate produced less fragments [121].

Finally, the ability of temperature-responsive chitosan hydrogels to form *in situ* may also be advantageous to stem cell survival. Due to the fact that they can gel *in situ* at body temperature, the gelation process is very mild and allows for the delivery of the chitosan hydrogel through minimally invasive procedures [111, 120]. Removing the need for surgical implantation may significantly reduce the immune response to the implantation of chitosan hydrogels and consequently promote the survival of the encapsulated stem cells.

### **2.9.2 Alginate**

Alginate is a naturally occurring polysaccharide found in brown algae that is a block copolymer formed from  $\beta$ -D-mannuronic acid and  $\alpha$ -L-guluronic acid monomers [115]. Exposing polymer chains to divalent cations forms alginate hydrogels and the physical and mechanical properties can be adjusted by controlling the number of guluronate monomers present in the polymer chain [115]. Alginate hydrogels are attractive for cell encapsulation due to their ease of fabrication, biocompatibility, and mild gelation process [115]. A number of different modifications to alginate hydrogels have been considered to better sustain the survival and viability of encapsulated cells.

The degradation of alginate hydrogels has been shown to affect the survival and proliferation of encapsulated stem cells [122]. Ashton *et al.* (2007) demonstrated that cell proliferation could be enhanced by adding increasing concentrations of alginate lyase incorporated in poly lactic-co-glycolic acid (PLGA) microspheres (PLGA-AL) into the hydrogel to increase the rate of degradation [122]. Cell proliferation was significantly affected by the concentration of PLGA-AL and the rate of hydrogel degradation. A



thirty-fold increase in the proliferation of stem cells was found in alginate hydrogels containing 10 µg/mg of PLGA-AL microspheres while only a two-fold increase in cell proliferation was found in alginate hydrogels devoid of alginate lyase [122]. Similarly, Alsberg *et al.* (2003) demonstrated that osteoblast survival and tissue deposition was improved by increasing the rate of degradation of the alginate hydrogel [123]. However, alginate hydrogels that degraded too rapidly (in less than six days) led to minimal bone tissue formation [124]. Enhanced cell survival, proliferation, and tissue formation in response to increased hydrogel degradation may be attributed to several factors. Accelerated hydrogel degradation may result in more space for cell spreading, matrix deposition and reduced physical constraints on cell growth [122]. However, as previous work demonstrates, degradation that is too fast can result in the premature loss of structural and protective support for the encapsulated cells that may negatively affect cell survival [125].

The degradation rate should be adjusted to maintain an optimal balance between providing cell support and space for cell spreading, proliferation, and tissue formation to best support cell survival. For alginate hydrogels, the degradation rate can also be manipulated by the molecular weight distribution of the polymer chains and through partial oxidation, in addition to controlling the exposure to degradative enzymes [125]. Partial oxidation makes alginate polymer chains susceptible to degradation by hydrolysis with no observable harmful effects on the viability of the encapsulated cells [125]. Hydrogels formed from alginate polymers with a bimodal molecular weight distribution with one polymer being partially oxidized degrade faster than using a single molecular weight distribution [126].

The monomer composition of alginate hydrogels and the addition of cell adhesion ligands can also influence cell survival. The proliferation of myoblasts encapsulated in alginate hydrogels has been demonstrated to be influenced by controlling the mannuronic acid to guluronic acid ratio (M:G) and the density of RGD cell-adhesion ligands bound to the hydrogel [127]. RGD (arginine-glycine-aspartic acid) is the cell binding sequence found on fibronectin, an extracellular matrix protein, which promotes cell anchoring [128]. At a constant density of RGD ligands, alginate hydrogels containing a large proportion of guluronic acid monomers (40:60) promoted better myoblast proliferation than alginate hydrogels of equal monomer composition (50:50) or high mannuronic acid composition (70:30) [127]. Also, myoblast proliferation was shown to increase by increasing the density of RGD ligands on alginate hydrogels with high guluronic acid composition [127]. Wang *et al.* (2003) demonstrated that the proliferation of bone marrow-derived stem cells was significantly higher on alginate hydrogels with a high concentration of guluronic acid monomers (M:G = 29:71) compared to hydrogels with lower guluronic acid monomer ratios (expressed as M:G) of 61:39 and 40:60, respectively [129]. Bone marrow-derived stem cell proliferation was noted to have ceased after 12 days *in vitro* in the latter two hydrogels but continued to increase with time in the hydrogel with the highest guluronic acid concentration [129].

Enhanced cell survival in alginate hydrogels with higher guluronic acid monomer content may be the result of mechanical influences. The tensile strength of alginate hydrogels increases as the concentration of guluronic acid increases [129]. This results in a more stable surface onto which the cells can migrate and spread, resulting in better overall cell survival and proliferation [129]. Also, because alginate is not inherently cell

adhesive, the incorporation of cell binding sequences like RGD ligands can promote cell anchorage, which is critical to cell survival [128, 129].

### 2.9.3 Hyaluronic Acid

Hyaluronic acid (HA) is a naturally occurring non-sulfated glycosaminoglycan composed of (1- $\beta$ -4) D-glucuronic acid and (1- $\beta$ -3) *N*-acetyl-D-glucosamine [130]. It is a major component of the extracellular matrix (ECM) of connective tissues such as cartilage, vitreous from the human eye, and umbilical cord and synovial fluid [115]. HA can be used in the construction of hydrogels by being modified with photoreactive groups and be used for stem cell encapsulation in tissue engineering applications [130]. The use of HA for hydrogel construction can be advantageous for several reasons. HA content is abundant in embryonic tissues and regenerating tissues, and so its use in the construction of hydrogels may be of great benefit to stem cell viability due its ability to mimic the native environment of the cell [130]. It is also a biocompatible and biodegradable material that can be degraded *in vivo* by naturally occurring enzymes, hyaluronidases. Furthermore, the chemistry of hyaluronic acid hydrogels can be controlled with relative ease by manipulating the reaction conditions [131]. Though the mechanism of action is not clearly understood, the fact that HA plays a role in cell proliferation, morphogenesis, and wound repair suggests that its intrinsic properties can promote cell survival [131].

Stem cells have been shown to maintain their viability after encapsulation in HA hydrogels. Gerecht *et al.* (2007) demonstrated that the majority of the human ESCs encapsulated in a HA hydrogel expressed the proliferation marker protein Ki-67 after 20

days in culture [130]. Degradation studies by Gerecht *et al.* (2007) demonstrated that high levels of stem cell viability (> 70%) could be maintained after exposure to 2000 units/mL hyaluronidase, which is sufficient to completely degrade a HA hydrogel [130]. Karyotype analysis revealed no genetic abnormalities in the stem cells released from the HA hydrogels via hyaluronidase-mediated degradation [130]. This demonstrated that the encapsulation in the hydrogel by exposure to UV light and exposure to hyaluronidase did not affect the genetic stability of the stem cells [130]. Furthermore, HA hydrogels can also be modified with the addition of the RGD cell adhesion peptide sequence to improve cell attachment and proliferation [130].

Burdick *et al.* (2005) examined the effects of macromolecular concentration and the resulting degree of HA hydrogel crosslinking on the viability of encapsulated cells [132]. For hydrogels constructed from 50 kDa macromers, fibroblast viability significantly decreased as the concentration of macromer increased [132]. For both 50 kDa and 100 kDa macromers, fibroblast viability was shown to be highest and sustained the longest at 2 wt% after one week in culture [132]. Cell viability in these hydrogels was greater than 95% using fluorescent live/dead staining [132]. It has been suggested that increasing the crosslink density within the hydrogel can decrease the mesh size of the polymer network chain and restrict the diffusion of nutrients and wastes to and from the encapsulated cells and the external environment, which can significantly influence cell viability [132].

## **2.10 Hydrogels from Synthetic Polymers**

Hydrogels constructed from synthetic polymers offer unique advantages for use in stem cell delivery. The use of synthetic hydrogels offers more comprehensive control over multiple aspects of scaffold design [114]. Specific properties such as crosslinking density, degradation rates, mechanical strength and chemical modification can be modified with greater ease resulting in hydrogels of greater consistency with less batch-to-batch variability [112, 114]. The aforementioned properties can therefore be tailored to create an environment that best supports the survival of the encapsulated stem cells.

### **2.10.1 Poly(ethylene glycol) (PEG)**

Poly(ethylene glycol) (PEG) is one of the most widely used synthetic polymers in hydrogel construction. It is a useful polymer because it is nontoxic and highly inert to most proteins, making it a biocompatible material potentially suitable for stem cell delivery [112]. Despite these beneficial properties, cell viability in unmodified PEG hydrogels has been shown to be poor [128]. The hydrophilic nature of PEG makes it inherently resistant to the adsorption of integrin-binding ligands [128]. Anchorage-dependent cells like MSCs require substrates to attach to and demonstrate poor viability after encapsulation in unmodified PEG hydrogels [128]. Different modifications to PEG hydrogels to enhance cell survival have been examined.

The addition of pendant RGD groups to PEG hydrogels has been shown to improve to viability of encapsulated MSCs by supporting native cell-matrix interactions [128]. Salinas *et al.* (2008) demonstrated the effects of different physical presentations of the RGD binding motif in PEG hydrogels on cell viability. The RGD motif was bound to

the PEG backbone by two links (forming a loop structure), a single short link, or by a single link incorporating a glycine spacer arm [128]. RGD-modified PEG hydrogels maintained approximately 70% viable stem cells after two weeks compared to the only approximately 7% viable cells maintained in unmodified PEG hydrogels [128]. Specifically, PEG hydrogels modified with the RGD sequence bound to a glycine spacer arm maintained the highest percentage of surviving MSCs, approximately 84% [128]. Short, single-tethered RGD sequences and RGD loop PEG hydrogels demonstrated cell survival rates of approximately 79% and 61% respectively [128]. The ability to modify PEG hydrogels with cell adhesion components such as the RGD sequence appear to be a significant way in which stem cell survival may be enhanced.

The degradation characteristics of synthetic hydrogels can also have significant effects on the behaviour and viability of encapsulated stem cells [133]. The rate of degradation affects the mesh size of the polymer network, subsequently influencing the diffusion of nutrients and wastes to and from the stem cells encapsulated in the hydrogel [133]. Also, degradation of the hydrogel can affect the cells' abilities to migrate, form junctions, and secrete extracellular matrix all of which affects cell survival and new tissue formation [133]. Hudalla *et al.* (2008) examined the effects hydrolytically susceptible PEG hydrogels on the viability of human MSCs [133]. PEG-diacrylate (PEG-DA) polymer chains were reacted with dithiothreitol (DTT) to form polymer chains that were acrylate-terminated to allow for photo-crosslinking and contained DTT bridges susceptible to hydrolytic degradation [133]. The viability of MSCs in PEG hydrogels containing 2.5, 5, or 10 mM DTT was compared to cell viability in DTT-free hydrogels. The results showed that stem cells survived in greater numbers in degradable PEG

hydrogels compared to the non-degradable networks [133]. Based on metabolic activity, it was found that the viability of stem cells was significantly increased in PEG hydrogels containing 2.5, 5, or 10 mM of DTT compared to DTT-free, non-degradable PEG hydrogels [133]. Cell aggregate sizes and cell spreading were also increased as the degradability of the hydrogel increased, suggesting that cell-to-cell adhesion and spreading are heavily reliant on the degradability of the polymer network [133]. Even when the RGD cell-binding motif was incorporated into the hydrogels, stem cell viability was higher in degradable hydrogels compared to non-degradable [133].

It has been demonstrated that the simultaneous optimization of both cell attachment and hydrogel degradation is more effective at sustaining cell survival than just the improvement of one of these factors alone [134]. Patel *et al.* (2005) examined MSC survival in PEG hydrogels under the following four different modification conditions: no modification, incorporation of the laminin-binding peptide sequence YIGSR, incorporation of the collagenase-susceptible LGPA peptide sequence, and the incorporating both YIGSR and LGPA sequences [134]. Unmodified PEG hydrogels did not sustain cell survival as most cells died within one week of encapsulation. The integration of YIGSR to promote cell adhesion, resulted in better cell survival but no proliferation. The LGPA peptide sequence was incorporated to allow the degradation of the PEG hydrogel and this resulted in initial cell proliferation followed by cell death [134]. Finally, cells encapsulated in the PEG hydrogel modified with both YIGSR and LGPA sequences to allow for cell attachment and hydrogel degradation was most effective at sustaining cell survival and proliferation [134]. This strongly suggests that

both the cell attachment and degradation properties of a hydrogel need to be optimized in order to effectively sustain cell survival.

### **2.10.2 Poly(vinyl alcohol)**

Poly(vinyl alcohol) (PVA) hydrogels have several properties that make them an appropriate material to be used for stem cell encapsulation. These properties include having high water content, tissue-like elasticity, simple fabrication and sterilization processes, and an abundance of hydroxyl groups on the polymer backbone that can be modified to incorporate different biological molecules [135]. In particular, the large number of hydroxyl groups along the PVA polymer chains provides many more sites for attachment of biological molecules such as cell adhesion peptides and growth factors that can support the survival of encapsulated cells [108]. The availability of all of these binding sites for the attachment of biological molecules gives PVA hydrogels a distinct advantage over PEG and many other polymers used in hydrogel construction [108].

As previously mentioned, cell adhesion is critical for cell survival and without it, encapsulated cells are prone to undergo apoptosis [119]. Like PEG hydrogels, the hydrophilic nature of PVA hydrogels does not support the adsorption of cell adhesion proteins vital for cell attachment [135]. In a study by Nuttelman *et al.* (2001), fibroblast attachment and proliferation were compared between unmodified PVA hydrogels and PVA hydrogels modified with the covalent attachment of fibronectin to the polymer chain network [135]. Fibroblast attachment was significantly enhanced on fibronectin-modified PVA hydrogels with attachment occurring after one hour, spreading by eight hours, and almost complete confluence by 16 hours [135]. Unmodified control PVA



hydrogels showed significantly less cell attachment as the cells appeared to attach to each other rather than the hydrogel surface and consequently did not spread effectively [135]. Fibroblast survival was enhanced on fibronectin-modified PVA hydrogels because the cells were able to form attachments to the polymer network leading to increased proliferation and cell migration [135].

PVA hydrogels have also been modified with the addition of peptide sequences to promote the attachment of encapsulated cells to the hydrogel and enhance cell survival [108]. Schmedlen *et al.* (2002) covalently attached arginine-glycine-aspartic acid-serine (RGDS) or arginine-glycine-glutamic acid-serine (RGES) sequences to PVA hydrogels to determine their effects on cell attachment [108]. Fibroblasts seeded on RGDS-modified PVA hydrogels showed increased cell attachment and spreading in a positive dose-dependent manner [108]. Fibroblasts seeded on RGES-modified or unmodified PVA hydrogels showed poor attachment as most were washed away during a rinsing step and those remaining displayed a rounded morphology, indicative of apoptosis [108, 119].

Nuttelman *et al.* (2002) demonstrated improvements in cell attachment by decreasing the hydrophilicity of PVA hydrogels [136]. Hydrophobic poly(lactic acid) (PLA) side chains were grafted onto the pendant hydroxyl groups to increase hydrophobicity of PVA hydrogels [136]. Cell attachment to PVA hydrogels was shown to improve as the number of PLA side chains grafted to the PVA hydrogel increased [136]. It has been suggested that by increasing the hydrophobicity of the hydrogel, the adhesion of cell adhesion proteins can be improved resulting in better cell attachment to the hydrogel [136].

## **Chapter 3**

### **Materials and Methods**

#### **3.1 Materials**

Unless otherwise indicated, all materials used in the experimentation were purchased from Sigma-Aldrich Ltd. (Oakville, ON), and were used as received.

#### **3.2 Rat Adipose-Derived Stem Cell Isolation and Culture**

Rat adipose-derived stem cells (rASCs) were extracted from the epididymal fat pads of 12-week-old male Wistar rats (Charles River), located adjacent to the testes using established methods [137]. Under sterile conditions, the extracted adipose tissue was finely minced with sharp scissors and digested through agitation in a solution comprised of Kreb's Ringer bicarbonate buffer, 2 mg/mL collagenase type VIII, 3 mM glucose, 25 mM HEPES, and 20 mg/mL bovine serum albumin at 120 rpm and 37 °C for 45 minutes. The digest solution was then filtered through a 250 µm pore stainless steel filter to remove undigested adipose tissue. The filtrate was allowed to sit for 5 minutes to allow mature adipocytes to float to the top, after which they were removed by aspiration. An equal volume of complete growth medium, comprised of Dulbecco's Modified Eagle's Medium and Ham's F-12 nutrient mixture (DMEM:Ham's F12) supplemented with 10% fetal bovine serum (FBS) (Hyclone Characterized FBS, Fisher Scientific, Toronto, ON), 100 U/mL penicillin and 0.1 mg/mL streptomycin, was added to the filtrate to inactivate the collagenase. The resulting solution was centrifuged at 1200 x g for 5 minutes to obtain a rASC-rich pellet. The cells were subsequently re-suspended and gently agitated

in erythrocyte lysing buffer (0.154 M  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KHCO}_3$ , 0.1 mM ethylenediaminetetraacetic acid (EDTA)) for 10 minutes at room temperature to lyse contaminating red blood cells [138]. After a second centrifugation stage, the rASC pellet was re-suspended in complete growth medium and filtered through a 100  $\mu\text{m}$  pore nylon filter, centrifuged again and re-suspended in complete growth medium. The isolated rASCs were plated at a density of 100,000 cells/ $\text{cm}^2$  in 75  $\text{cm}^2$  flasks (Corning, New York, USA) and incubated in a humid environment at 37 °C with 5%  $\text{CO}_2$ . The growth medium was changed 24 hours after cell isolation and initial plating and subsequently every 2-3 days. At 80% confluence, the cells were passaged by rinsing with cation-free phosphate buffered saline (PBS) (Hyclone, Fisher Scientific, Toronto, ON) and released with trypsin (0.25% trypsin/0.1% EDTA, Gibco, Burlington, ON). The number of viable cells was then counted using a hemacytometer and trypan blue viability staining, and re-plated in new flasks at a density of 100,000 cells/ $\text{cm}^2$ . Cells at passage 2 or 3 were used for all cellular assays.

### **3.3 Multilineage Differentiation Potential of Rat Epididymal ASC**

At passage 2 (P2), rASCs were plated in 6-well plates and differentiated along the adipogenic, osteogenic, and chondrogenic lineages, as described in detail below. Differentiation was assessed at 7 and 14 days after the induction of differentiation. Triplicate negative control samples of the same cell source cultured in complete growth medium were included in every trial.

### **3.3.1 Adipogenic Differentiation**

rASCs were trypsinized from the tissue culture flasks and plated at a density of 50,000 cells/well in tissue culture-treated 6-well plates (Becton-Dickinson, Mississauga, ON), and cultured in complete growth medium for 48 hours to achieve a high density of adherent cells [139]. After 48 hours, the cells were rinsed in PBS and transferred into adipogenic differentiation medium (10% FBS, 0.5 mM isobutyl-methylxanthine (IBMX), 0.50  $\mu$ M dexamethasone (DEX), 100 U/mL penicillin, 0.1 mg/mL streptomycin in DMEM:Ham's F-12) [56], and the medium was changed every 2-3 days for the remainder of the culture period. Successful adipogenic differentiation was determined through oil red O staining (n=3) and glycerol-3-phosphate dehydrogenase (GPDH) activity (n=3).

#### **3.3.1.1 Oil Red O Staining**

Oil red O staining was used to assess intracellular lipid accumulation at 7 and 14 days after the induction of differentiation [139]. To prepare the cells, the differentiation medium was removed by aspiration and the cultures were rinsed with PBS and fixed for 30 minutes in 10% neutral buffered formalin. For the staining, an oil red O stock solution (3 g/L in isopropanol) was diluted 3:2 in deionized water to form the working solution. 1 mL of the oil red O working solution was placed into each well and allowed to incubate at room temperature for 5 minutes, after which each well was thoroughly rinsed with distilled water, and counterstained with hematoxylin for 2 minutes.

### 3.3.1.2 GPDH Activity

GPDH enzyme activity was measured using a GPDH assay kit (Kamiya Biomedical, Cat #: KT-010, Seattle, Washington, USA) [60]. At 7 and 14 days after the induction of adipogenic differentiation, triplicate induced and control wells were rinsed with PBS and filled with 2 mL of an enzyme extraction buffer provided with the kit. The cells were then lysed in the tissue culture plates via multiple 5-second bursts of sonication and intracellular protein content was then extracted with cooling on ice. All samples were subsequently centrifuged at 13,000 x g for 10 minutes at 4 °C, and the resulting supernatant was assayed for GPDH activity according to the manufacturer's instructions.

Briefly, GPDH in combination with the coenzyme nicotinamide adenine dinucleotide (NADH), can reduce the substrate dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate. The decrease in the concentration of NADH as a result of the enzymatic activity of the GPDH in the samples can be measured spectrophotometrically over time to determine the GPDH activity. To perform this assay, the DHAP/NADH substrate reagent (provided with the GPDH kit) was first dissolved in 2 mL of deionized water. 50 µL of each sample supernatant were added in duplicate to a 96-well microplate and 100 µL of the dissolved substrate reagent was subsequently added to all samples simultaneously using a multi-channel pipettor. Absorbance of the samples was immediately measured every 15 seconds for 10 minutes at 25 °C and 340 nm with a Synergy™ HT multi-detection microplate reader and KC4™ software (Bio-Tek Instruments Inc., Winooski, VT, USA). GPDH activity was then determined from the

change in absorbance over time from the linear portion of the kinetic curve for each sample.

The GPDH activity data was normalized to the total intracellular protein concentration, which was measured using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a bovine serum albumin (BSA) standard. The BSA standard curve was created by serial dilution of a stock solution to produce standard concentrations of 0, 20, 40, 60, and 80  $\mu\text{g/mL}$ . 160  $\mu\text{L}$  of each standard or sample were transferred into a 96-well microplate and mixed with 40  $\mu\text{L}$  of Bio-Rad Coomassie® Brilliant Blue G-250 dye. Standards were assayed in duplicate and samples in triplicate. After incubating the microplate for 5 minutes at room temperature, absorbance was measured with a Synergy™ HT multi-detection microplate reader at 595 nm and analyzed with KC4™ software (Bio-Tek Instruments, Inc., Winooski, VT, USA). Total intracellular protein content was determined using a standard curve derived from the absorbance values of the BSA standards. One unit of GPDH activity was defined as the amount of enzyme required to consume 1  $\mu\text{mol}$  of NADH in 1 minute.

### **3.3.2 Osteogenic Differentiation**

rASCs were seeded at a density of 20,000 cells/cm<sup>2</sup> in 6-well plates and allowed to adhere and proliferate for 48 hours in complete growth medium [61]. After 48 hours, the medium was removed and the ASCs were cultured in osteogenic differentiation medium (10% FBS, 50  $\mu\text{M}$  ascorbate-2-phosphate, 10 mM  $\beta$ -glycerophosphate, 100 nM DEX, 0.01  $\mu\text{M}$  1,25-dihydroxy vitamin D<sub>3</sub>, 100 U/mL penicillin, 0.1 mg/mL streptomycin in DMEM:Ham's F-12) for 7 or 14 days, with medium changes every 2-3

days [61]. Osteogenic differentiation was assessed by demonstrating calcium deposition through von Kossa staining (n=3, N=3) and by measuring alkaline phosphatase (ALP) enzyme activity (n=3).

#### **3.3.2.1 von Kossa Staining**

After 7 days or 14 days, control and osteo-induced cells were fixed in 10% neutral buffered formalin for 30 minutes, after which the formalin was removed and all of the cultures were rinsed carefully 3 times with PBS. 1 mL of silver nitrate solution was added to each well, and the plates were exposed to UV light for 30 minutes to stain the cells [61]. The silver nitrate solution was then removed, and all cultures were rinsed at least 3 times with PBS. 1 mL of sodium thiosulfate solution (5% in distilled water (dH<sub>2</sub>O)) was placed in each well and incubated for 5 minutes at room temperature to dissolve any unbound silver nitrate precipitate, after which it was removed and the cultures were rinsed with PBS. All cultures were subsequently counterstained with hematoxylin for 2 minutes.

#### **3.3.2.2 Alkaline Phosphatase Assay**

The complete medium or osteogenic differentiation medium was removed from triplicate wells from the control and osteogenic-induced cultures, which were then rinsed with PBS. The cells were then lysed in PBS with 3, 5-second bursts of sonication, with cooling on ice, then the total intracellular protein content was extracted using a pipette. All samples were subsequently centrifuged at 13,000 x g for 10 minutes at 4 °C. The Bio-Rad protein assay was used to quantify the amount of total intracellular protein, as

described in the methods for the GPDH assay. A p-nitrophenol phosphate liquid substrate system was used to measure the ALP activity in the control and induced samples [61]. 100  $\mu$ L of the p-nitrophenol phosphate liquid substrate was added to triplicate wells in a 96-well plate that contained either (i) 100  $\mu$ L of the supernatant samples containing the extracted intracellular proteins or (ii) p-nitrophenol standards (0-200  $\mu$ M). The plate was then incubated at 37 °C for 15 minutes, after which the reactions were stopped by adding 100  $\mu$ L of NaOH (1 M) to each well. Absorbance readings of the plate were taken using a Synergy™ HT multi-detection microplate reader at a wavelength of 405 nm and analyzed with KC4™ software (Bio-Tek Instruments, Inc., Winooski, VT, USA). The absorbance values obtained were normalized to the amount of total intracellular protein. One unit of ALP activity was defined as the quantity of ALP required for the catalysis of 1  $\mu$ mol of p-nitrophenol per minute.

### **3.3.3 Chondrogenic Differentiation**

At passage 2, rASCs were trypsinized and centrifuged at 1200 x g for 5 minutes. The cells in the pellet were counted with a hemocytometer with trypan blue staining, and re-suspended in complete medium at a concentration of  $1 \times 10^6$  cells/mL. 2 mL aliquots of this cell suspension were added to separate 15 mL conical tubes and centrifuged at 300 x g for 10 minutes to form three-dimensional pellet cultures, each containing  $2 \times 10^6$  cells [77]. The pellets were cultured in complete medium for 48 hours, after which the medium was changed to a chondrogenic differentiation medium containing 10 ng/mL transforming growth factor-beta 1 (TGF- $\beta$ 1), 50  $\mu$ g/mL ascorbate-2-phosphate, 6.25  $\mu$ g/mL human insulin, 100 nM DEX, and 1% FBS in DMEM [61]. The differentiation



medium was changed every 2-3 days for the duration of the induction period.

Chondrogenic differentiation was qualitatively assessed through staining the pellets with toluidine blue, to detect the presence of glycosaminoglycans, characteristic of chondrogenesis, within the extracellular matrix surrounding the cells (n=3, N=3) [77].

#### **3.3.3.1 Toluidine Blue Staining**

At 7 and 14 days after the induction of chondrogenic differentiation, the pellets were fixed in 4% paraformaldehyde overnight at 4 °C. After fixation, the pellets were embedded in paraffin wax and sectioned into 5 µm sections (John L. DaCosta, Department of Pathology and Molecular Medicine). The paraffin-embedded sections were incubated at 65 °C overnight to remove the paraffin, and then rehydrated in dH<sub>2</sub>O in preparation for staining with the toluidine blue solution (0.1% solution for 2-3 minutes) [77].

### **3.4 rASC Migration Studies**

#### **3.4.1 Growth Factor-Induced rASC Migration Under Normoxic Conditions**

At passage 3, rASCs were released from the flasks with trypsin and centrifuged at 1200 x g for 5 minutes to form a pellet. The cells were counted using a hemocytometer with trypan blue viability staining, and the pellet was re-suspended in a low-serum control medium (97% DMEM:Ham's F-12, 2% FBS, 1% Penicillin-Streptomycin) to achieve a concentration of  $5 \times 10^5$  cells/mL [88]. This cell suspension was maintained in an incubator (37 °C, 5% CO<sub>2</sub>), with agitation every 15 minutes to prevent the cells from settling, while the transwell filters were prepared, as described in detail below.

Polyethylene transwell filters with 8  $\mu\text{m}$  pores (Greiner Bio-One, Frickenhausen, Germany) were used to assess the migratory response of rASCs exposed to either stromal cell derived factor (SDF)-1 $\alpha$  or hepatocyte growth factor (HGF) under normoxic (21% O<sub>2</sub>) conditions, using a modified Boyden Chamber assay [88]. To prepare the inserts, both sides of the transwell filter membranes were coated with 0.25% gelatin (Type B, bovine) in DMEM:Ham's F-12 medium, to enhance cell attachment to the membrane. The inserts were then allowed to dry for approximately 1 hour at 37 °C. After 1 hour, any excess gelatin solution was gently removed and the transwell filters were transferred into 12-well plates containing control medium. The upper chamber of each transwell filter was seeded with  $5 \times 10^5$  rASCs in 1 mL of control medium, and incubated for 1 hour at 37 °C, 5% CO<sub>2</sub> to allow for cell adherence.

To assess the growth factor-induced migratory response, chemotactic factor solutions were prepared containing ranging concentrations of either SDF-1 $\alpha$  (20 ng/mL, 50 ng/mL, 100 ng/mL) or HGF-1 (20 ng/mL, 50 ng/mL, 100 ng/mL). In a 12-well plate, 3 wells were filled with 1.5 mL of control medium, 3 wells with control medium containing 20 ng/mL of SDF-1 $\alpha$  (Peprotech, New Jersey, USA), 3 wells with medium containing 50 ng/mL of SDF-1 $\alpha$ , and 3 wells with medium supplemented with 100 ng/mL of SDF-1 $\alpha$  [140]. A second 12-well plate was similarly filled with medium containing 0 ng/mL, 20 ng/mL, 50 ng/mL, and 100 ng/mL of HGF (Peprotech, New Jersey, USA) [88]. The chemotactic factor solutions were prepared during the cell adherence period, and were stored in an incubator (37 °C, 5% CO<sub>2</sub>) until ready for use. After 1 hour had passed to allow for cell adherence, the rASC-seeded transwell filters were transferred into the 12-well plates containing the chemotactic factor solutions and

subsequently placed in an incubator (37 °C, 5% CO<sub>2</sub>) for 48 hours to allow for cell migration across the membrane.

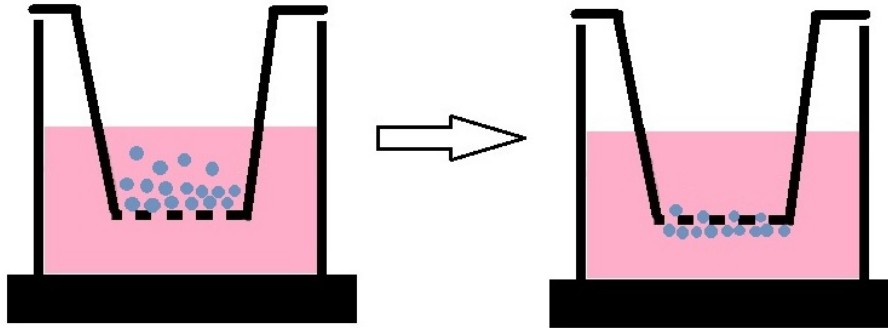


Figure 3.1 The set-up of the modified Boyden Chamber used for rASC migration. The lower compartment contains the chemoattractant. The blue dots represent the rASCs.

After 48 hours, the transwell filters were removed from the chemotactic solutions and the filter membranes were fixed in 10% neutral buffered formalin overnight at 4 °C. After fixation, the transwell filters were stained with hematoxylin for approximately 2.5 hours, and subsequently rinsed 2-3 times with PBS to remove excess staining solution. 500 µL of PBS were also transferred into each upper chamber, and any cells remaining on the upper face of the membrane were removed gently with a cotton swab. The entire transwell filter unit was then placed on a microscope slide, and viewed under a light microscope (Zeiss Invertoskop 40C; Carl Zeiss, Germany) at 100 x magnification. Digital images were taken of three random fields on each membrane using an Infinity1 camera (Lumenera Corp., Ottawa, ON), and the number of cells that had migrated through the membrane in each field was counted using the ImageJ software program. For this experiment, the migration for all growth factor concentrations was assayed in triplicate (n=3).

#### **3.4.2 Influence of Oxygen Tension on Growth Factor-Induced rASC Migration:**

The influence of oxygen tension (normoxic versus hypoxic conditions) on rASC migration in the presence of the chemotactic factors (SDF-1 $\alpha$ , HGF) was investigated. This experiment was performed using the same methods and concentrations as described in the normoxic migration experiment, with the only difference being that one set of 12-well plates (one for SDF-1 $\alpha$  and one for HGF) was cultured in a hypoxic incubator (5% O<sub>2</sub>) and another was incubated in a normoxic (21% O<sub>2</sub>) incubator. Migration in the controls, as well as for each concentration of SDF-1 $\alpha$  and HGF, was assayed in triplicate samples for each condition (n=3).

#### **3.5 Influence of Oxygen Tension on Growth Factor-Related Gene Expression**

The influence of oxygen tension in the culture environment, as well as the supplementation of the growth factors of interest (SDF-1 $\alpha$ , HGF), on rASC gene expression of SDF-1 $\alpha$ , HGF and their respective receptors, CXCR4 and c-MET, were examined using end-point reverse transcriptase-polymerase chain reaction (RT-PCR).

At passage 2, rASCs were released with trypsin and seeded onto 6-well plates at a density of  $5 \times 10^5$  cells/well. Following seeding, the rASCs were cultured in low-serum DMEM:Ham's F12 medium (2% FBS) supplemented with either 50 ng/mL HGF, 50 ng/mL SDF-1 $\alpha$ , or no growth factor. For each condition, one set was cultured under normoxic (21% O<sub>2</sub>) conditions and another under hypoxic (5% O<sub>2</sub>) conditions for 72 h, 7 days, or 14 days. All samples were assayed in triplicate (n=3) for each time point.

At each time point, the total ribonucleic acid (RNA) from the rASCs was extracted using TRIzol® Reagent (Invitrogen Canada Ltd., Burlington, ON) according to

the manufacturer's instructions. A NanoDrop spectrophotometer (Fisher Scientific, Toronto, ON) was used to determine the concentration of total RNA, as well as the purity of the samples, with the 260/280 ratio typically ranging from 1.9 – 2.1. Random primers (Invitrogen Canada Ltd., Burlington, ON) and SuperScript™ II Reverse Transcriptase (Invitrogen Canada Ltd., Burlington, ON) were used to synthesize cDNA from 1 µg of total RNA. Reverse transcription was performed in a 20 µL solution comprised of first strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>), 10 mM DTT, 0.09 OD<sub>260</sub> units of random primers (Invitrogen Canada Ltd., Burlington, ON), 0.5 mM of each dNTP (Invitrogen Canada Ltd.), and 200 units of SuperScript™ II RT. Primer3 software was used to design the primers for rat SDF-1 $\alpha$ , CXCR4, HGF, and c-Met, which are shown in Table 3.1. The melting temperature for the gene-specific primers was 60 °C, and all primers were designed to span an intron-exon boundary.

Table 3.1 Rat-specific primers used for RT-PCR analysis.

Gene	Primer Sequence (5'-3')	Fragment Size (bp)	Accession No.
SDF-1 $\alpha$	Forward: GCTCTGCATCAGTGACGGTA Reverse: TTCAGCCTTGCAACAATCTG	147	NM022177
CXCR4	Forward: GCAGGTAGCAGTGACCCTCT Reverse: CCAGGATTACCAACCCATTG	245	NM022205
HGF	Forward: CCCAAATGTGACGTGTCAAG Reverse: ATCCCAAGGAACGAGAGGAT	246	NM017017.2
cMet	Forward: CGAGTACCGGACGGAGTTTA Reverse: GAAATGTGCTGTGCGAGAGA	178	NM031517.1

PCR reactions were performed in a 50  $\mu$ L reaction volume comprised of 2.5  $\mu$ L of diluted cDNA (with 50 ng of input RNA), 1X Taq buffer (10mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40), 250 nM of forward primer, 250 nM of reverse primer, 250 nM of dNTP, 2.5 mM of  $MgCl_2$  and 0.375 units of recombinant Taq DNA Polymerase (Fermentas International Inc., Burlington, ON). The Bio-Rad C1000 thermal cycler was used to carry out the PCR runs. PCR was carried out for 40 cycles with each cycle consisting of denaturation for 30s at 95  $^{\circ}C$ , annealing for 30 s at 58  $^{\circ}C$ , elongation for 1 min at 72  $^{\circ}C$ , with a final elongation for 5 min at 72  $^{\circ}C$ . The expression of the specific gene products was visualized via electrophoresis using a 5% agarose gel with ethidium bromide staining (G:box Chemi HR16, Syngene, Cambridge, UK). The expression of each gene product of interest was determined based on amplicon size, determined by comparison to an Ultra Low Range ladder (Fermentas, Burlington, ON).

### **3.6 Influence of Oxygen Tension on Growth Factor Protein Expression**

The effect of normoxic and hypoxic oxygen tensions on SDF-1 $\alpha$  and HGF expression by rASCs was investigated. At passage 2,  $2 \times 10^5$  rASCs were seeded into 6-well plates and cultured in low-serum complete medium (2% FBS) under normoxic (21%  $O_2$ ) or hypoxic (5%  $O_2$ ) conditions. The medium was changed 24 hours prior to collection where applicable. At 24 hours and 7 days after seeding, the medium was collected and flash frozen in liquid nitrogen and stored in a – 80  $^{\circ}C$  freezer until ready to be used for an enzyme-linked immunosorbent assay (ELISA). ELISA analysis was used to compare the expression SDF-1 $\alpha$  and HGF by rASCs cultured under normoxic and hypoxic conditions. ELISA kits for rat SDF-1 $\alpha$  (assay range: 156 pg/mL – 5000 pg/mL)

and HGF (assay range: 62.5-1000 ng/mL) were purchased from TSZ ELISA (Framingham, MA, USA).

Briefly, 50  $\mu$ L of each standard solution (0, 312.5, 625, 1250, 2500, 5000 pg/mL) and each sample were added in duplicate to a 96-well microplate pre-coated with the appropriate antibody. The microplate was then incubated at 37 °C for 30 minutes, after which the solutions were discarded from the wells. After allowing the plate to dry, washing buffer was added to each well for 30 seconds, and then drained, and this washing procedure was repeated 5 times. After rinsing the wells, 50  $\mu$ L of HRP-conjugated SDF-1 $\alpha$ /HGF antibody was added to each well and incubated at 37 °C for 30 minutes. The plate was washed again in the same manner as previously described using the washing buffer. While protecting the plate from light, 50  $\mu$ L of both Chromogen substrate A and B were added to each well and incubated at 37 °C for 15 minutes. The reactions were then stopped by the addition of 50  $\mu$ L of stop solution into each well. The microplate was then read using a Synergy™ HT multi-detection microplate reader at a wavelength of 540 nm and analyzed with KC4™ software (Bio-Tek Instruments, Inc., Winooski, VT, USA). The level of SDF-1 $\alpha$  and HGF were normalized to the level of total protein, which determined in the same manner as previously described in § 3.3.2.

### **3.7 rASC Viability in Peptide-Modified *N*-Methacrylated Glycol Chitosan (MGC) Hydrogels**

#### **3.7.1 Preparation of RGD-Modified MGC**

The RGD-modified MGC (RGD-MGC) was prepared according to an established protocol in the laboratory of Dr. B. Amsden and provided by Fraz Anjum [175]. The

MGC used had a degree of N-methacrylation of 13%, and was modified so as to contain 4%, or 7% RGD peptides grafted to its backbone through reaction with pendant amine groups.

### **3.7.2 Photo-Encapsulation of rASCs in MGC Hydrogels**

Lyophilized RGD-MGC was sterilized by exposure to ultraviolet light for 15 minutes. Once sterilized, the RGD-MGC was dissolved overnight in low-serum (2% FBS) DMEM:Ham's F12 medium to produce a 6% w/v solution.

Immediately prior to encapsulation, rASCs at passage 3 were released with trypsin and centrifuged at 1200 x g for 5 minutes. The resulting pellet was re-suspended in low-serum complete medium (2% FBS) at a concentration of  $1 \times 10^6$  cells/mL. This cell suspension was incubated (37 °C, 5% CO<sub>2</sub>), with gentle mixing every 10 minutes, until it was ready to be used.

To prepare for the crosslinking procedure, 5 mg of the photoinitiator, 2-hydroxy-4'-(2-hydroxy-ethoxy)-2-methyl-propiophenone (Sigma Aldrich Ltd, Oakville, ON), was dissolved in 1 mL of deionized water and sterile filtered to produce a 5 w/v % solution. In a separate vial, 500 µL of the dissolved RGD-MGC solution was mixed with 50 µL of the dissolved photoinitiator solution to obtain a final concentration of 10 w/v%.

A sterilized cylindrical Teflon mold (0.8 mm x 0.8 mm) was placed on top of a glass slide, and using a 1 mL syringe with an 18 gauge needle, 50 µL of the RGD-MGC/photoinitiator solution was added to the centre of the mold. Subsequently, 50 µL of the prepared cell suspension was pipetted into the mold, and the entire mixture was gently pipetted up and down twice to ensure mixing. This final solution was exposed to



ultraviolet light (320-390 nm) with an intensity ranging from 10.5-10.8 mW/cm<sup>2</sup> from an EXFO lite source (EFOS Corporation, Mississauga, ON) for 3 minutes. After photo-crosslinking, the Teflon mold was gently removed to extract a hydrogel (750  $\mu$  m (D) x 500  $\mu$  m (H)) with  $5 \times 10^5$  rASCs encapsulated within each gel. This process was repeated to create the desired number gels with encapsulated rASCs.

### **3.7.3 Effect of SDF-1 $\alpha$ -Loading and RGD Grafting on rASC Viability in RGD-MGC Hydrogels**

The viability of rASCs encapsulated in RGD-MGC hydrogels loaded with 50 ng/mL of SDF-1 $\alpha$  or growth factor-free RGD-MGC hydrogels was investigated to determine if SDF-1 $\alpha$ -loading or the degree of RGD-substitution had an effect on cell viability. For these experiments, the degree of RGD substitution in the MGC hydrogels was 4% and 7%, with the control gels remaining unmodified. A total of 72 gels were constructed for this study, 36 gels were SDF-1 $\alpha$ -free and 36 gels were loaded with 50 ng/mL of SDF-1 $\alpha$ . Subsequently, rASC viability was assessed by confocal analysis with LIVE/DEAD® viability staining (Invitrogen, Burlington, ON) at 24 h, 72 h, 7 days and 14 days. For each time point and degree of RGD substitution per growth factor condition, the experiment was performed with triplicate samples (n=3).

RGD-MGC hydrogels containing  $5 \times 10^5$  rASCs were soaked for 24 hours in low-serum medium (97% DMEM:Ham's F12, 2% FBS, 1% Pencillin-Streptomycin) supplemented with 50 ng/mL of SDF-1 $\alpha$  or kept in control media if they were to be free of growth factor. After 24 hours, all of the gels were transferred to 12-well plates containing low-serum medium without SDF-1 $\alpha$ , and cultured under hypoxic conditions

(5% O<sub>2</sub>) for the remainder of the experiment, with medium changes every 2-3 days. Cell viability was determined at 24 h, 72 h, 7 and 14 days via the LIVE/DEAD® cell viability assay (Invitrogen, Burlington, ON). At each time point, each hydrogel was placed in a 1.5 mL Eppendorf tube containing 500 µL of LIVE/DEAD® staining solution for 30 minutes at room temperature. The staining solution was comprised of calcein AM (2 µM), which stains live cells green, and ethidium homodimer-1 (4 µM), which stains dead cells red. After 30 minutes, the cells within the gel constructs were visualized with an Olympus FV 1000 confocal scanning laser microscope. The excitation/emission wavelengths for calcein AM and ethidium homodimer-1 are 494/517 nm and 528/617 nm, respectively. Images were taken of 5-10 different horizontal planes separated by 70-150 µm throughout the construct at 100x magnification. Due to the fact that an entire plane through the gel could not be captured within a single image, multiple pictures of the construct were taken and stitched together using Fluoview software, to produce a mosaic image showing the entire plane as a single picture. This process was repeated for every plane captured throughout the gel.

Using ImageJ, the total live and dead cells within each gel were counted and cell viability was calculated using the following formula:

$$\% \text{ cell viability} = \{[(\text{live} + \text{dead}) - \text{dead}]/[\text{live} + \text{dead}]\} \cdot 100$$

### **3.8 Statistical Analyses**

Where appropriate, statistical analyses of the data were performed using the OriginPro 8.0 software program (OriginLab Corp, Northhampton, MA, USA). Unless otherwise indicated, data is represented as the mean ± standard deviation (SD). For

relevant experiments, data from the control and experimental groups were compared by one-way ANOVA with a Tukey's post-hoc test. For all analyses, data were considered to be statistically significant at  $p < 0.05$ .

## **Chapter 4**

### **Results**

#### **4.1 Culture and Differentiation of Rat Adipose-Derived Stem Cells**

The initial experiments were designed to confirm the presence of multipotent stem cells within the cell population extracted through the enzymatic digestion of epididymal fat tissue from male Wistar rats. The harvested cells were cultured in specific differentiation media formulations (adipogenic, osteogenic, chondrogenic) containing supplements known to induce differentiation along a specific lineage, and compared to control cells cultured in complete proliferation medium (DMEM:Ham's F12, 10% FBS, 1% pen-strep). Overall, the cell population demonstrated the capacity to differentiate along all three lineages, confirming that adipose-derived stem cells (ASCs) were successfully extracted from the epididymal fat pads.

##### **4.1.1 Adipogenic Differentiation**

Adipogenic differentiation was successfully demonstrated using the differentiation medium formulation described by the Lopez group for rat white adipose tissue, comprised of complete medium supplemented with the phosphodiesterase inhibitor isobutyl-methylxanthine (IBMX) and the synthetic glucocorticoid dexamethasone (DEX) [61].

Control cells cultured in proliferation medium displayed a spindle-like fibrous morphology throughout the entire 2-week culture period, with an increasing cell density over time. Rat ASCs cultured in the adipogenic differentiation medium initially

displayed similar morphology to the control cells after seeding. However, 7 days after seeding, there were evident morphological differences between the control and induced cultures. Clusters of differentiating cells accumulating intracellular lipid were clearly visible in the induced cells, but not in the control cultures (Figure 4.1).

#### 4.1.1.1 Oil Red O Staining

Adipogenic differentiation was also confirmed using Oil Red O staining, which stains lipid as a red colour. The induced cells displayed obvious staining of intracellular lipid droplets indicative of adipogenic differentiation (Figure 4.1), with many of the cells having a unilocular morphology consistent with a mature adipocyte phenotype by 14 days in culture.

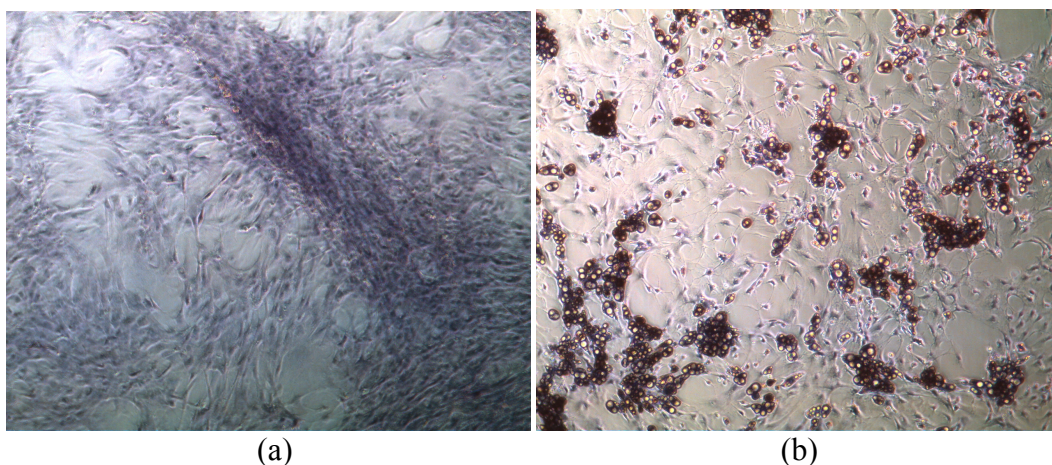


Figure 4.1. Oil Red O staining of rASCs cultured in control medium (left) and adipogenic differentiation medium (right) at 14 days, showing the staining of intracellular lipid droplets (red) in the induced cells. Magnification = 100x.

#### 4.1.1.2 GPDH Enzyme Activity

The level of GPDH enzyme activity was used as a measure of adipogenic differentiation. GPDH activity in the control and induced cells were measured at 3, 7, and 14 days after the induction of differentiation. No significant differences in GPDH activity between control and induced cells were noted at Day 3. By Day 7, the induced cells demonstrated higher GPDH activity than the control cells (Figure 4.2). At Day 14, GPDH activity was significantly upregulated in the induced cells as compared to the control cells, and also higher than the level measured at Day 7 (Figure 4.2). These results indicated that the cells cultured in the differentiation medium were undergoing adipogenic differentiation, with a progression in the response over time.

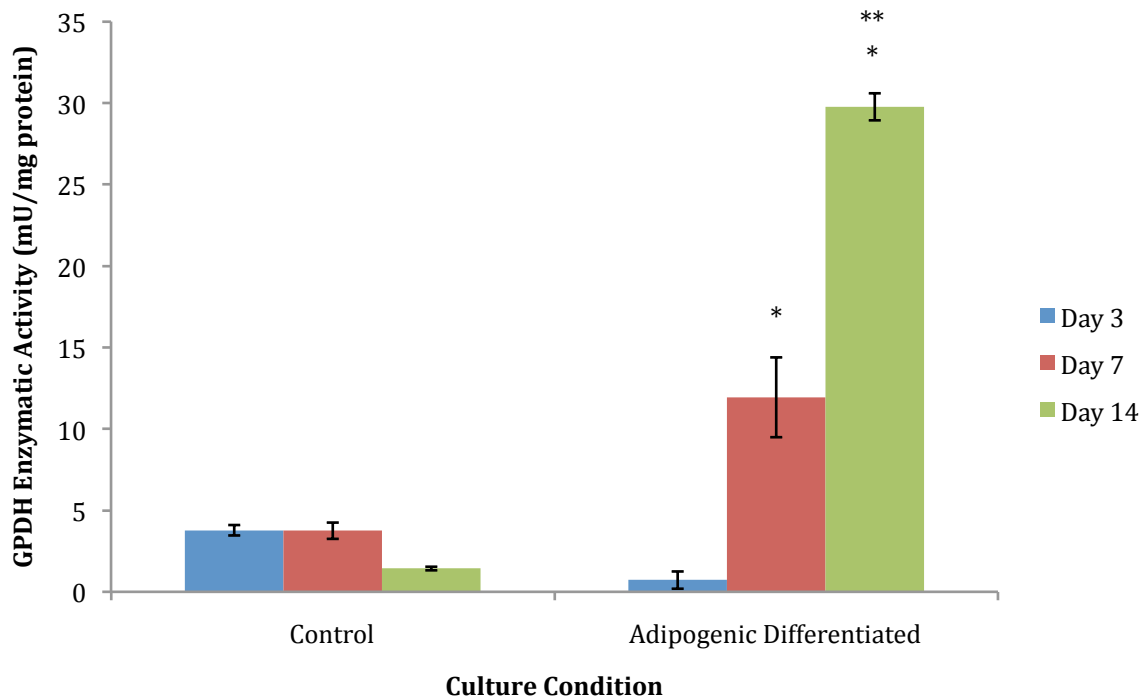


Figure 4.2. GPDH activity in induced cells and control cells after 3, 7 and 14 days in culture. All data is represented as the mean  $\pm$  SD. \* indicates significant difference between induced cultures and control and \*\* indicates significant difference between induced cultures on Day 7 and Day 14 ( $n=3$ , \* and \*\* indicate significance at  $p < 0.05$ ).

## 4.1.2 Osteogenic Differentiation

### 4.1.2.1 ALP Enzyme Activity

Osteogenic differentiation was assessed by alkaline phosphatase (ALP) enzyme activity after culturing in an osteogenic medium containing ascorbate-2-phosphate,  $\beta$ -glycerophosphate, and DEX, described in the literature for cells extracted from rat inguinal tissue. At Day 7, ALP activity was upregulated in the induced cells relative to the control cells cultured in proliferation medium (Figure 4.3). However, the addition of Vitamin D3 to the differentiation medium resulted in a significant increase in ALP activity as compared to the control cells and cells cultured in differentiation medium without Vitamin D3 (Figure 4.3).

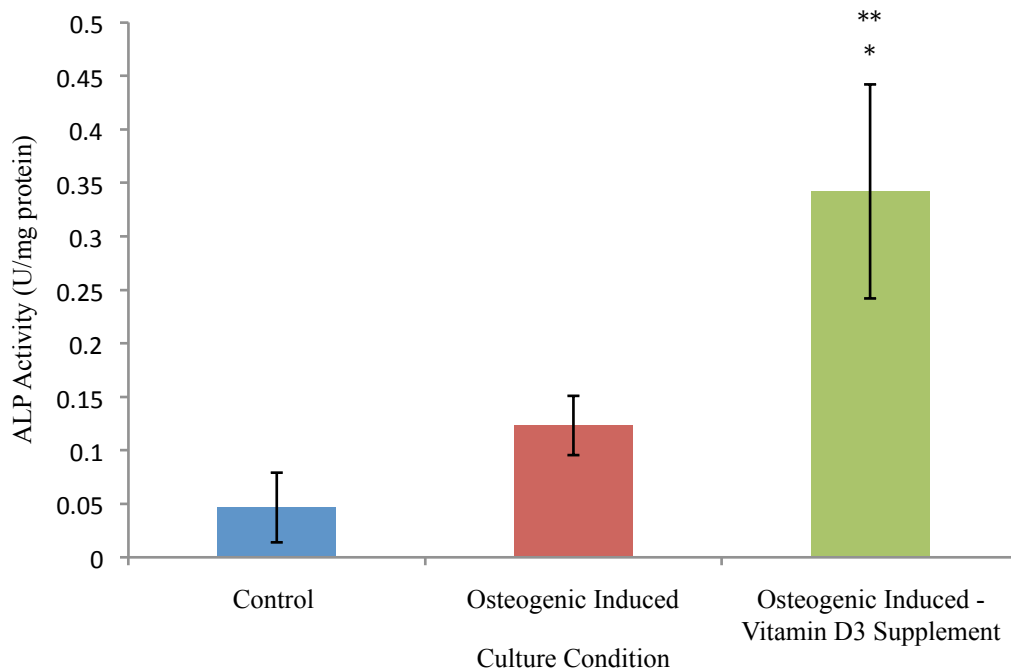


Figure 4.3. ALP enzyme activities of rASCs cultured in proliferation medium, osteogenic differentiation medium with no Vitamin D3, and differentiation medium supplemented with Vitamin D3. \* indicates significant difference between induced cultures with Vitamin D3 and control and \*\* indicates significant difference between induced cultures with Vitamin D3 and induced cultures without Vitamin D3. (n=3, \* and \*\* indicate significance at  $p < 0.05$ ).

#### 4.1.2.2 von Kossa Staining

Von Kossa staining was used to visualize calcification of the extracellular matrix. No staining indicative of mineralization was noted at 7 or 14 days after the induction of differentiation.

#### 4.1.3 Chondrogenic Differentiation

Chondrogenic differentiation was assessed with toluidine blue staining which stains for proteoglycans and glycosaminoglycans (GAG), which are characteristic components of cartilage and are secreted by chondrocytes. At 14 days, the pellets cultured in differentiation medium displayed more intense staining than the control pellets that were cultured in complete medium, indicating that the cells were undergoing chondrogenesis (Figure 4.4).

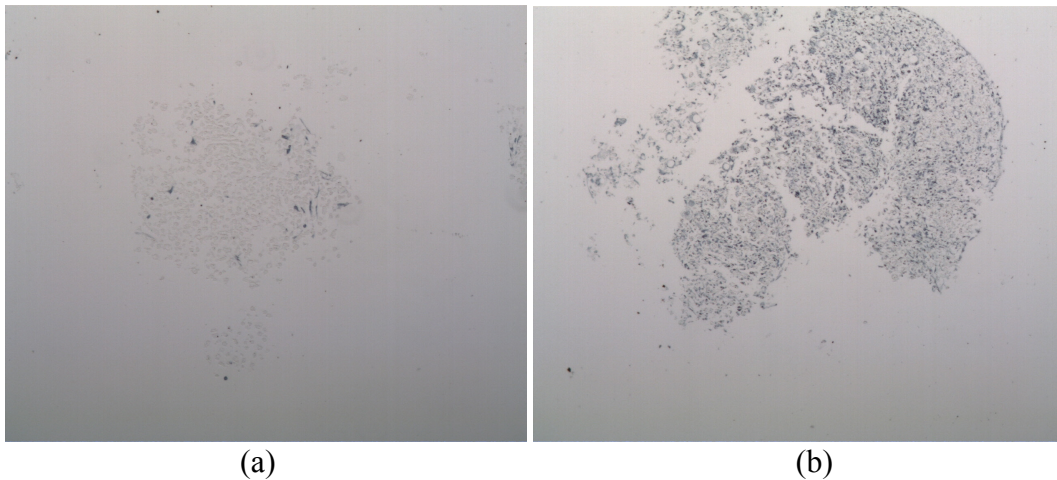


Figure 4.4. Chondrogenic differentiation of rASC pellets assessed by toluidine blue staining of proteoglycans and glycosaminoglycans in the control pellet (a) and induced pellet cultured in chondrogenic differentiation medium (b). Magnification = 100x.



## 4.2 rASC Migration Studies

### 4.2.1 Growth Factor-Induced rASC Migration Under Normoxic Conditions

The migration of rASCs in response to growth factor exposure was measured under normoxic conditions to determine whether SDF-1 $\alpha$  and HGF were chemoattractants for rASCs. The results indicated that exposure to SDF-1 $\alpha$  induced a significant migratory response in rASCs at 20, 50, and 100 ng/mL compared to control conditions (Figure 4.5). Increasing the concentration of SDF-1 $\alpha$  did not result in a significant increase in rASC migration (Figure 4.5).

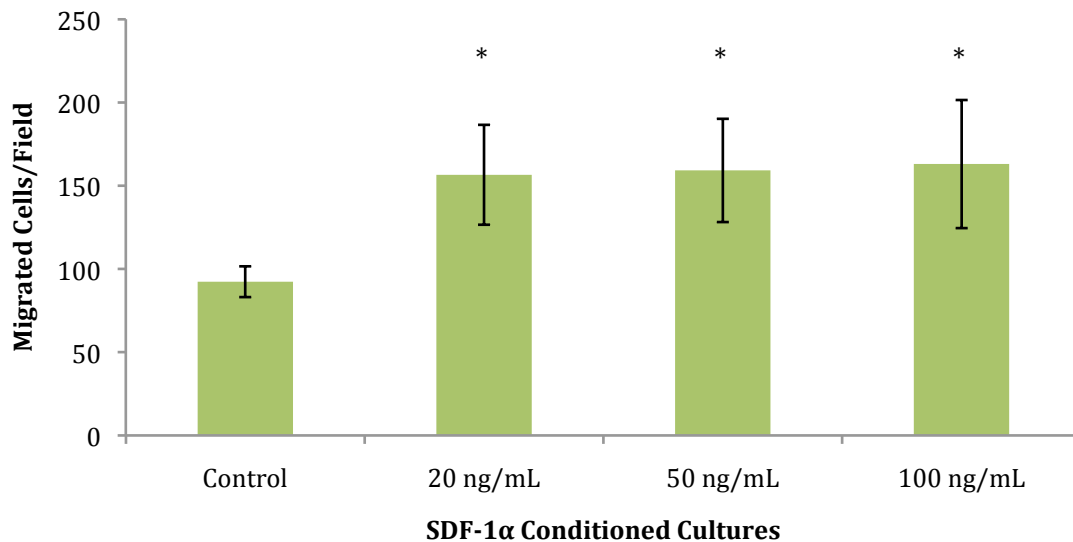


Figure 4.5. The migration of rASCs across the modified Boyden Chamber membrane in response to different concentrations of SDF-1 $\alpha$  under normoxic conditions. (n=3), \* indicates statistically significant difference compared to control at  $p < 0.05$ ).

The migratory response of rASCs to HGF was also examined under normoxic conditions. The results indicated that HGF was able to significantly increase rASC migration at 20, 50, and 100 ng/mL compared to control culture conditions (Figure 4.6). Similar to the SDF-1 $\alpha$  results, there was no significant difference in migration between the three concentrations of HGF tested. When comparing both growth factors, SDF-1 $\alpha$

induced greater cell migration at 20 and 50 ng/mL compared to control than HGF versus control at the same concentrations. However, at 100 ng/mL, cell migration under HGF was 1.95 times greater than control cultures compared to a 1.77 times increase in SDF-1 $\alpha$  cultures versus control.

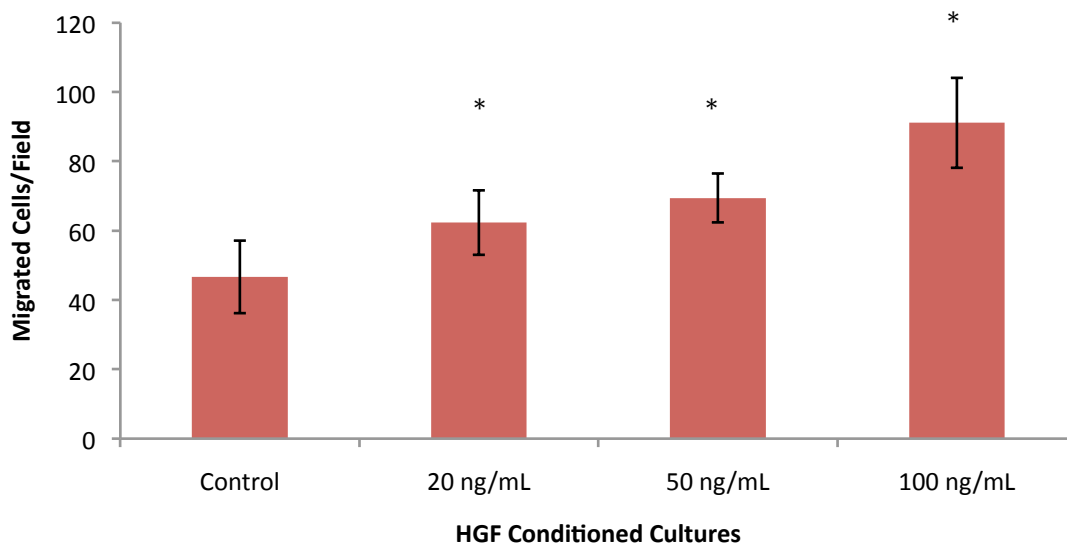


Figure 4.6. The migration of rASCs across a modified Boyden Chamber membrane in response to different concentrations of HGF under normoxic conditions. (n=3), \* indicates statistically significant difference compared to control at  $p < 0.05$ .

#### 4.2.2 Influence of Oxygen Tension on Growth Factor-Induced rASC Migration

The effect of oxygen tension on rASC migration in the presence of the chemoattractive growth factors was also examined. The cell migration studies were performed under hypoxic (5% O<sub>2</sub>) and normoxic (21% O<sub>2</sub>) conditions.

The migratory response under normoxic conditions with SDF-1 $\alpha$  was consistent with the results of the previous experiment examining rASC migration in response to SDF-1 $\alpha$ . Under the normoxic conditions, cell migration was significantly increased at 20, 50, and 100 ng/mL SDF-1 $\alpha$  compared to control conditions (Figure 4.7). No significant difference in migration was noted between the different concentrations of SDF-1 $\alpha$ . Under

hypoxia, cell migration was only significantly increased at 50 ng/mL SDF-1 $\alpha$  and also demonstrated a plateau response to the increasing growth factor concentration. However, the overall migratory response was enhanced under hypoxic conditions.

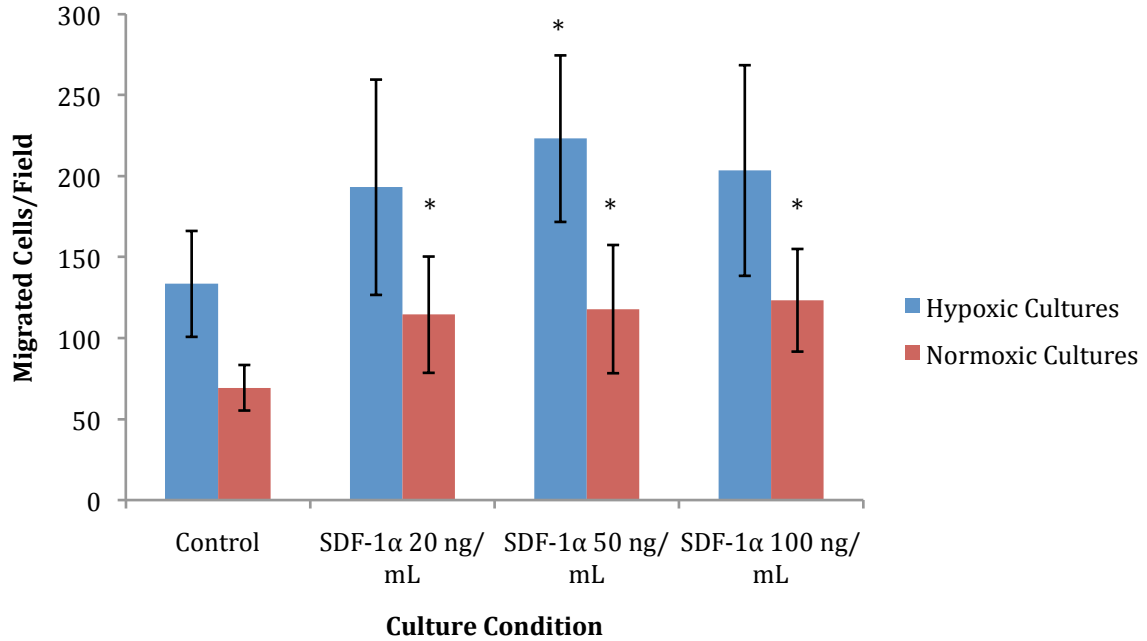


Figure 4.7. The migratory response of rASCs to SDF-1 $\alpha$  under hypoxic (5%) and normoxic (21%) culture conditions. (n=3), \* indicates statistically significant difference compared to control at  $p < 0.05$ .

Cell migration under varying oxygen tensions and HGF concentrations was also investigated. Under normoxic conditions, the rASCs demonstrated a general pattern of increased migration as the concentration of HGF increased, although this trend was not statistically significant (Figure 4.8). Under hypoxic conditions, the rASCs demonstrated a trend of enhanced migration when exposed to HGF, as compared to the control cultures. However, the migratory response appeared to plateau as the concentration of HGF was increased (Figure 4.8). Interestingly, in contrast to the SDF-1 $\alpha$  migration experiments, rASC migration was reduced under hypoxic conditions, including within

the controls. This experiment was only performed once and therefore further trials would help to elucidate these trends.

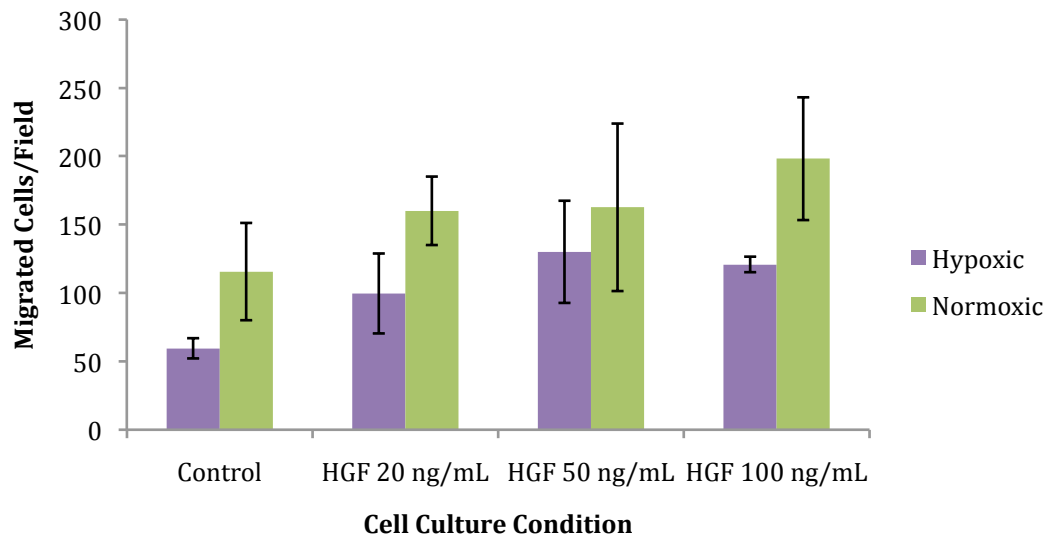


Figure 4.8. The migratory response of rASCs to HGF supplementation under hypoxic and normoxic culture conditions. There were no statistically significant differences in migratory responses between hypoxic and normoxic cultures at  $p < 0.05$ , ( $n=3$ ).

### 4.3 Growth Factor-Related Gene Expression

The influence of oxygen tension and chemoattractive growth factor supplementation on rASC gene expression of SDF-1 $\alpha$ , HGF and their respective receptors, CXCR4 and c-MET, were examined using end-point reverse transcriptase-polymerase chain reaction (RT-PCR).

In general, growth factor and receptor expression was upregulated in the hypoxic cultures under most conditions (Figure 4.9). When no exogenous growth factors were supplemented into the culture medium, SDF-1 $\alpha$  was expressed at similar levels between the normoxic and hypoxic conditions. Interestingly, cMET expression was upregulated under hypoxic conditions. Oxygen tension did not appear to induce the expression of

CXCR4 or HGF when exogenous growth factors were not added to the cultures (Figure 4.9).

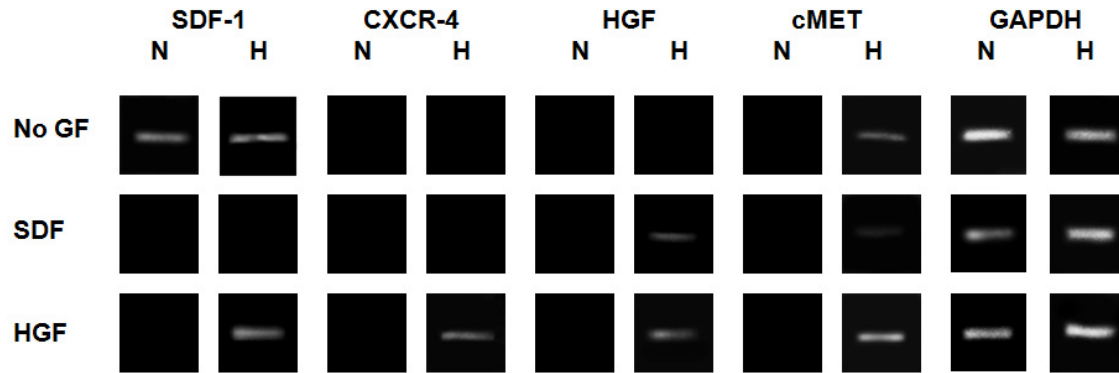


Figure 4.9. Gene expression of SDF-1 $\alpha$ , CXCR4, HGF, and cMET at Day 7 under normoxic (N) and hypoxic (H) conditions, with GAPDH as the housekeeping gene.

In the cultures supplemented with SDF-1 $\alpha$  (50 ng/mL), HGF was detected under hypoxic conditions and cMET was weakly expressed (Figure 4.9), but not under normoxic conditions. The expression of SDF-1 $\alpha$  and its receptor CXCR4 were not detected in either oxygen tension condition, potentially indicative of a feedback control mechanism due to the presence of exogenous SDF-1 $\alpha$ . In contrast, the addition of exogenous HGF upregulated the expression of SDF-1 $\alpha$ , CXCR4, HGF, and cMET under hypoxic conditions (Figure 4.9). These results suggest that under hypoxic conditions the presence of HGF in the medium enhanced ASC sensitivity to both SDF-1 $\alpha$  and HGF.

Overall, oxygen tension and the presence of each of the growth factors appear to influence the expression of these four genes. Generally, there was much higher expression of the genes under hypoxic conditions as compared to normoxic conditions, with the exception of SDF-1 $\alpha$  expression in the control medium that did not contain any exogenous growth factors (Figure 4.9).

#### 4.4 Growth Factor-Related Protein Expression

The effect of oxygen tension on rASC secretion of SDF-1 $\alpha$  was examined by ELISA analysis of the supernatant media. All data was normalized to the total intracellular protein content found within the rASCs in culture. After one day, there was no significant difference in the relative amounts of SDF-1 $\alpha$  between the normoxic and hypoxic cultures. After seven days in culture, SDF-1 $\alpha$  secretion was enhanced under hypoxic conditions relative to the normoxic cultures, however, the difference was not statistically significant (Figure 4.10).

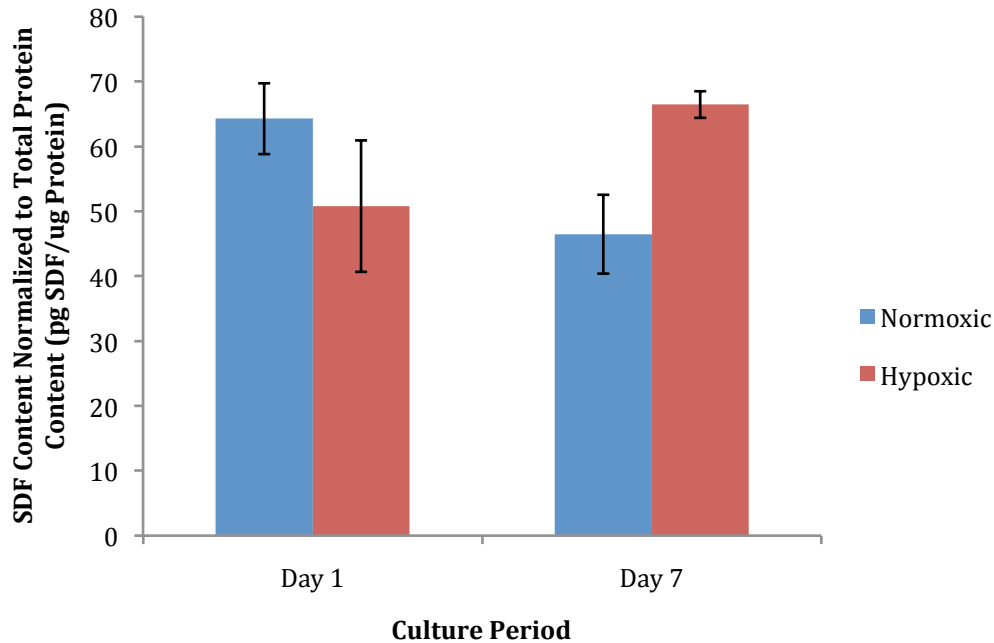


Figure 4.10. Secreted SDF-1 $\alpha$  at 1 and 7 days in culture normalized to the total intracellular protein content for the rASCs. (n=3)

#### 4.5 rASC Viability in MGC Hydrogels

The effect of hydrogel encapsulation on rASC viability was examined. rASCs were encapsulated in methacrylated glycol chitosan (MGC) hydrogels that were

unmodified or modified with 4% or 7% RGD. One set of hydrogels was loaded with 50 ng/mL SDF-1 $\alpha$ . rASC viability was examined at 1, 3, 7, and 14 days after encapsulation.

In general, rASC viability in the SDF-1 $\alpha$ -free hydrogels was lowest at 24 h after encapsulation but rebounded in all three types of hydrogels over the next 14 days. On day 3, rASC viability was significantly higher in the 4% and 7% RGD-modified hydrogels as compared to the control hydrogels (RGD 0%). After day 7, there was no significant difference in rASC viability between any of the hydrogel groups. Between 7 and 14 days, cell viability within the hydrogels remained relatively constant, however, rASC viabilities in the 4% and 7% RGD-modified hydrogels were significantly higher than the unmodified control hydrogels at 14 days (Figure 4.11). Cell viability was determined from confocal images taken at the designated time points (Figure 4.12).

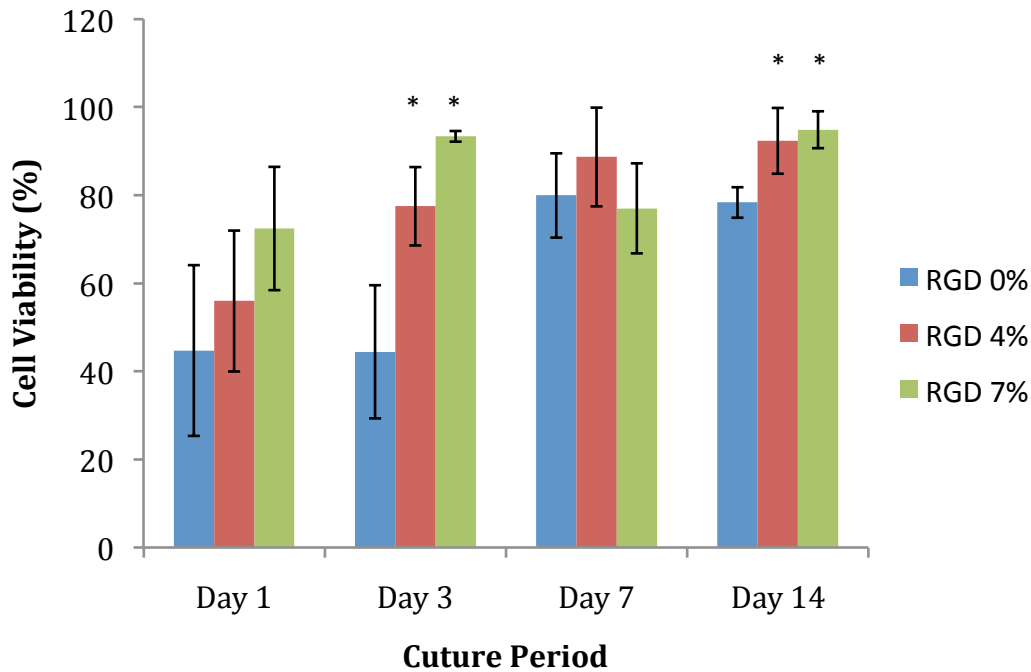


Figure 4.11. The viability of rASCs encapsulated in SDF-1 $\alpha$ -free MGC hydrogels over a 2-week period. (n=3, \* indicates statistically significant difference compared to RGD 0% at  $p < 0.05$  within the same timepoint ).

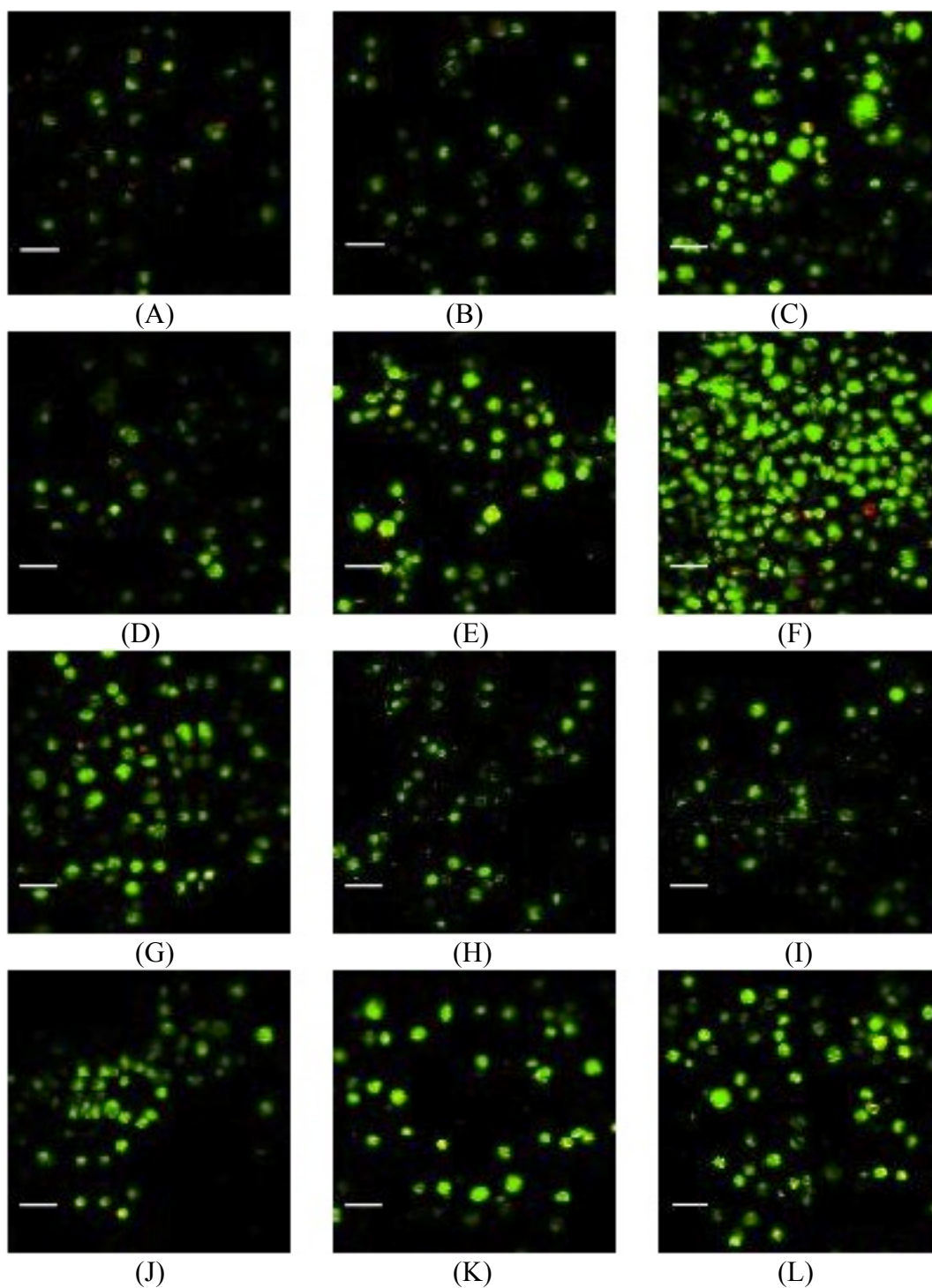


Figure 4.12. Confocal images of rASCs encapsulated in MGC hydrogels free of growth factor and stained with the LIVE/DEAD<sup>®</sup> stain. (A, B, C = 0%, 4%, 7% RGD-modified hydrogels on Day 1; D, E, F = 0%, 4%, 7% gels on Day 3; G, H, I = Day 7; J, K, L = Day 14). Green – Live, Red – Dead. Magnification = 100x, scale bars = 150  $\mu\text{m}$ .



rASC viability was also examined in unmodified, 4% and 7% RGD-modified hydrogels that were loaded with 50 ng/mL SDF-1 $\alpha$ . One day after loading with SDF-1 $\alpha$ , there were no significant differences in cell viability between the three types of hydrogels. On Day 3, the cell viabilities in the 4% and 7% RGD-modified hydrogels were significantly higher than that of rASCs encapsulated in the unmodified hydrogels (Figure 4.13). By Day 7, cell viability was significantly higher in the 7% RGD-modified hydrogels relative to the 4% RGD-modified and control hydrogels. The cell viabilities appeared to plateau at 14 days, with no significant difference amongst the rASCs encapsulated in the three types of hydrogels (Figure 4.13). Incorporating SDF-1 did not appear to affect cell viability within the various gels. Cell viability was determined from confocal images taken at the designated time points (Figure 4.14).

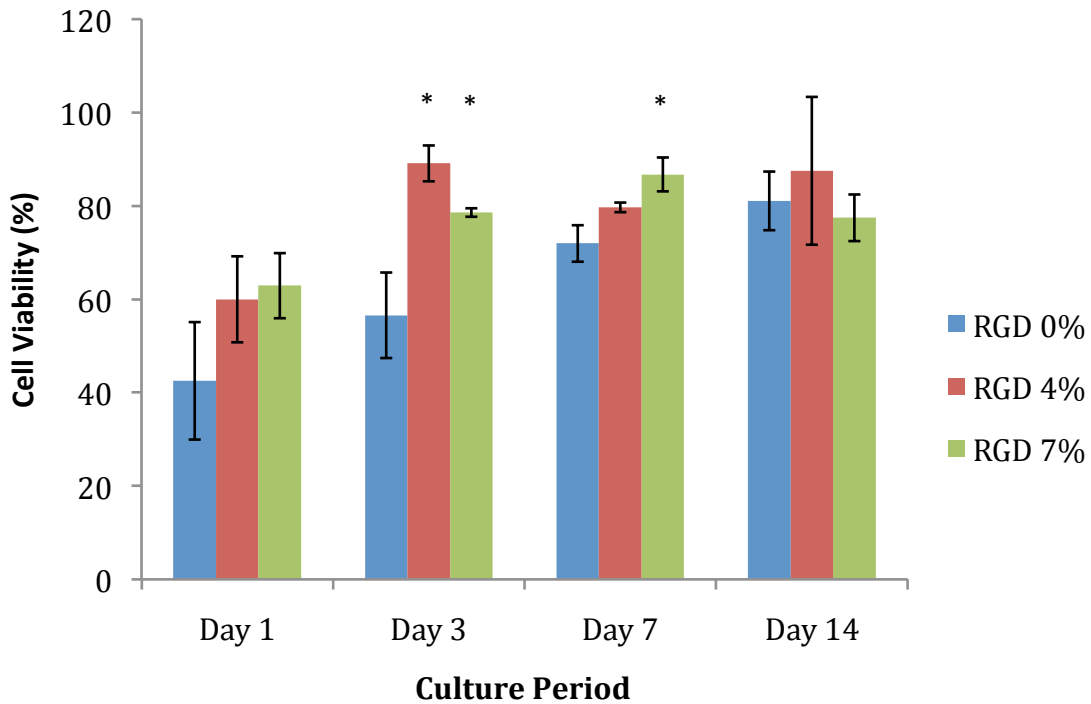


Figure 4.13. The viability of rASCs encapsulated in MGC hydrogels loaded with 50 ng/mL SDF-1 $\alpha$  over a 2- week period. (n=3, \* indicates statistically significant difference compared to RGD 0% at  $p < 0.05$  within the same timepoint).

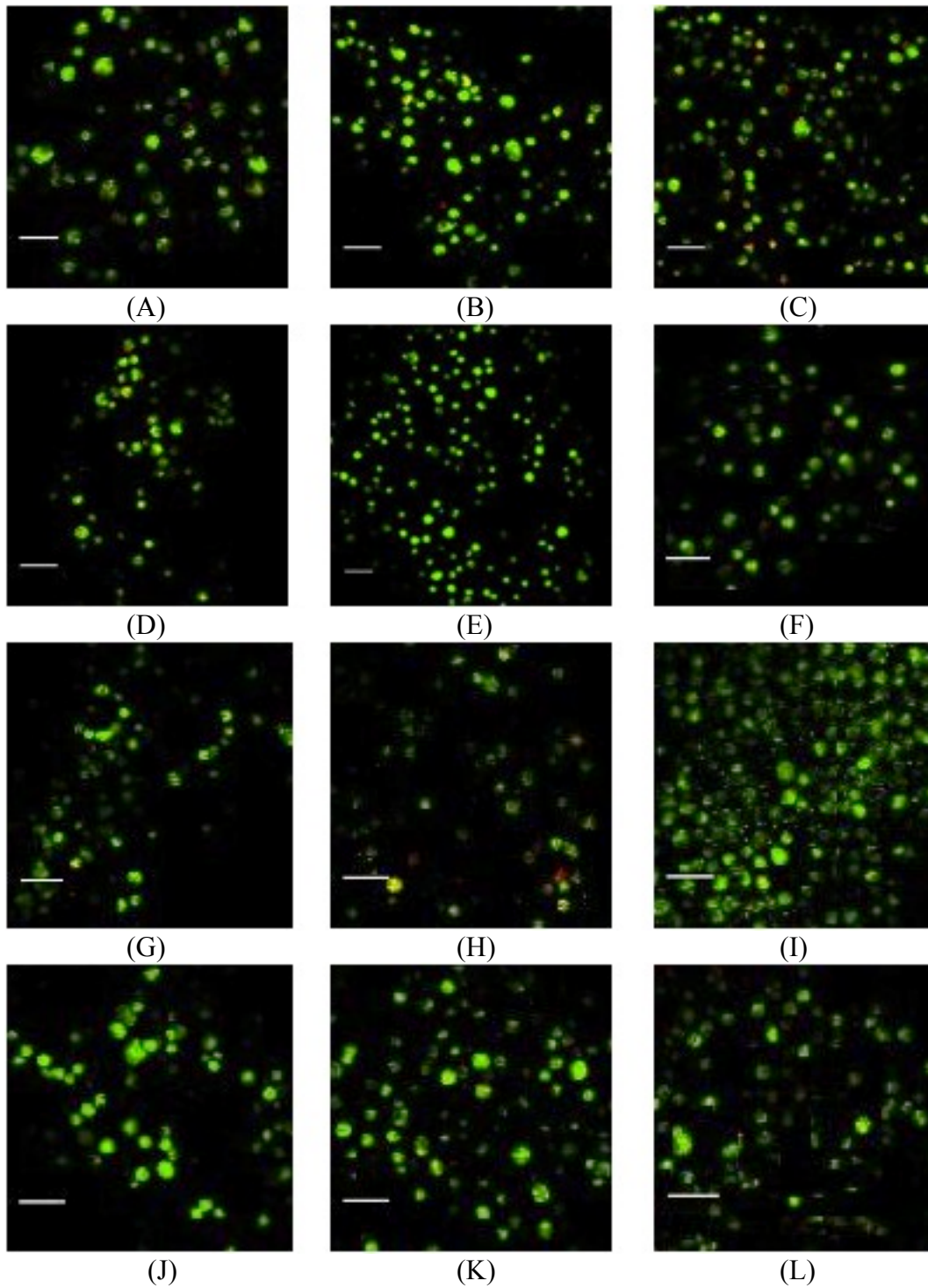


Figure 4.14. Confocal images of rASCs encapsulated in MGC hydrogels loaded with 50 ng/mL SDF-1 $\alpha$  and stained with the LIVE/DEAD<sup>®</sup> stain. (A, B, C = 0%, 4%, 7% RGD-modified hydrogels on Day 1; D, E, F = 0%, 4%, 7% gels on Day 3; G, H, I = Day 7; J, K, L = Day 14). Green – Live, Red – Dead. Magnification = 100x, scale bars = 150  $\mu$ m.

## **Chapter 5**

### **Discussion**

#### **5.1 Differentiation of Rat Adipose-Derived Stem Cells (rASCs)**

The purpose of this specific study was to confirm the pluripotency of the extracted adipose-derived stem cell (ASC) populations, as a new ASC source of interest in the lab for assessing cell-based therapies in rat models [61, 70, 139]. In this study, the ASCs derived from the epididymal fat pads of male Wistar rats were successfully differentiated along the adipogenic, osteogenic, and chondrogenic lineages.

##### **5.1.1 Adipogenic Differentiation**

Adipogenic differentiation was achieved by culturing the rASCs in media supplemented with adipogenic-specific factors such as IBMX, dexamethasone, and insulin. IBMX is a phosphodiesterase inhibitor and functions to prevent the degradation of cAMP, which is an activator of protein kinase A (PKA), which is an important stimulatory pathway of adipogenesis [141]. Serum is also an important component for rat preadipocyte differentiation as preadipocytes have been shown to exhibit a certain propensity to convert to adipocytes in its presence [142]. Also, it has been shown that physiological concentrations of insulin can promote the differentiation of stem cells to adipocytes and that glucocorticoids in conjunction with insulin encourage the differentiation of mesenchymal stem cells into adipocytes [58, 143].

Initial attempts to achieve adipogenic differentiation were unsuccessful with different media formulations. The first adipogenic medium used, which did not achieve successful adipogenic differentiation, consisted of a DMEM:Ham's F-12 base with 10% FBS, 10  $\mu$ M insulin, 0.50  $\mu$ M dexamethasone, and 100 U/mL penicillin, 0.1 mg/mL streptomycin [139]. A second media formulation that was also not successful was comprised of a DMEM:Ham's F-12 base with 10% FBS, 0.5 mM IBMX, 1  $\mu$ M dexamethasone, 10  $\mu$ M insulin, 200  $\mu$ M indomethacin, 100 U/mL penicillin, 0.1 mg/mL streptomycin [21, 67]. The medium that did achieve successful adipogenic differentiation consisted of 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 33  $\mu$ M, biotin, 17  $\mu$ M D-pantethoic acid, 1  $\mu$ M Insulin (bovine), 0.5 mM IBMX, 5  $\mu$ M troglitazone, 20 nM Dexamethasone in DMEM:Ham's F-12 [56]. Troglitazone is a thiazolidinedione that acts as a ligand for PPAR $\gamma$ , which is a major regulator of adipogenesis and thus was an important addition to the medium.

Consistent with the results of previous studies, Oil Red O staining successfully stained lipid vacuoles in the differentiated cultures but not in the control cultures [21, 33 139]. Furthermore, glycerol-3-phosphate dehydrogenase (GPDH) activity was significantly increased in the differentiated cultures compared to the control cultures confirming that successful adipogenic differentiation had been achieved. GPDH is a characteristic enzyme of mature adipocytes that is involved in the synthesis of triglycerides and a reflection of the metabolic activities consistent with adipocytes [21, 139].

### 5.1.2 Osteogenic Differentiation

Osteogenic differentiation of rASCs was successfully induced using a defined differentiation medium containing ascorbate-2-phosphate,  $\beta$ -glycerophosphate, dexamethasone and vitamin D3. Ascorbic acid is an important component that increases the production of collagen and non-collagenous bone matrix proteins [144, 145]. However, ascorbate-2-phosphate, a derivative of ascorbic acid, was used in this study because it is stable in aqueous media unlike ascorbic acid, which is readily oxidized in the same environment [72].  $\beta$ -glycerophosphate is an important factor in the calcification of the extracellular matrix (ECM) during bone formation [145]. Dexamethasone has also been shown to be important for successful *in vitro* osteogenic differentiation of stem cells and like  $\beta$ -glycerophosphate, it promotes mineralization of the ECM [21, 146].

After one week, the rASC cultures supplemented with osteogenic differentiation medium containing vitamin D3 had significantly higher levels of alkaline phosphatase (ALP) activity compared with control cultures or cultures grown in osteogenic differentiation medium without the addition of vitamin D3. The addition of vitamin D3 to the osteogenic differentiation medium and subsequent increases in ALP activity is consistent with the results seen in other studies that have investigated human ASCs [33, 67, 147]. The addition of vitamin D3 was important because it has important influences on bone formation such as regulating the transcription of bone matrix proteins and inducing the expression of osteocalcin. In general, the increase in ALP activity in the differentiated cultures is consistent with previous studies demonstrating successful differentiation of ASCs to osteoblasts [21, 37, 148]. Von Kossa staining indicated that there was not extensive matrix mineralization in either of the types of induced cultures

after seven days. This may be due to the fact that the time period of one week was not sufficient to allow for significant matrix calcification that could be detected using the staining procedures. With other cell sources, the results of previous studies have shown successful von Kossa staining in time periods ranging from 2.5 to 4 weeks [37, 61, 149]. However, based on the ALP enzyme activity data, it was confirmed that the extracted ASCs had osteogenic potential, although the cells and ECM of the developing tissues had not fully matured over the time course in the current study.

### **5.1.3 Chondrogenic Differentiation**

Chondrogenic differentiation was achieved using 3-dimensional (3D) high-density pellet cultures of rASCs formed through centrifugation. Many previous studies have employed high cell density 3D cultures for chondrogenic differentiation, as it promotes the ASCs to maintain rounded cell morphology, which is characteristic of mature chondrocytes [61, 70, 150]. A high-density pellet culture allows the cells to create the appropriate ECM microenvironment that will allow the cells to maintain their rounded morphology, which provides the appropriate signals to direct differentiation along the chondrogenic lineage [151]. Low-density cell cultures allow cells to flatten and take on a fibroblast-like morphology, which does not promote chondrogenic differentiation [151, 152].

A chondrogenic differentiation medium was used containing transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), ascorbate-2-phosphate, and dexamethasone [31, 61, 70]. TGF- $\beta$ 1 is commonly used in chondrogenic differentiation media, as it is known to stimulate glycosaminoglycan synthesis, type II and VI collagen expression, chondroitin-4-sulfate

expression, and chondrogenic maturation [71, 73, 153]. Ascorbate-2-phosphate has been shown to significantly increase proteoglycan and glycosaminoglycan synthesis and increase collagen II mRNA expression [73]. After 14 days, the cell pellets cultured in the chondrogenic differentiation medium showed higher levels of staining with toluidine blue, which is used to stain negatively charged proteoglycans normally found in the ECM of cartilage, as compared to control pellets cultured in proliferation medium [154]. Previous studies have also shown that chondrogenic differentiation of human ASCs can occur by 14 days and can be visualized using toluidine blue staining [154, 155].

## **5.2 rASC Migration**

ASCs are mesenchymal stem cells derived from fat that are capable of differentiating along multiple lineages. Additionally, ASCs are capable of secreting a variety of factors that have therapeutic properties, which make ASCs an attractive cell source for use in the treatment of MI. ASCs have been shown to secrete a variety of angiogenic and anti-apoptotic factors such as VEGF, bFGF, HGF, TGF- $\beta$ , and GM-CSF [90, 156]. Rehman *et al.* (2004) demonstrated that human ASCs cultured in hypoxic conditions had increased their secretion of VEGF, which is both angiogenic and anti-apoptotic, by 5-fold [156]. ASCs have also been known to secrete anti-fibrotic factors such as bFGF and HGF that can inhibit the formation of fibrotic scar tissue, which would be particularly useful in the application of MI treatment [90]. ASCs also secrete mobilizing factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and SDF-1, which would be helpful in attracting cells to sites of injury [157].

An important aspect of this type of cellular therapy is the ability to mobilize the ASCs to the site of injury. In the cell migration experiments, we examined how specific chemotactic factors and oxygen tension affected the migratory responses of rASCs.

### **5.2.1 Influence of Growth Factors – SDF-1 $\alpha$ and HGF – on rASC Migration**

rASC migration was significantly increased when the cells were exposed to various concentrations of (SDF-1 $\alpha$ ) under normoxic culture conditions as compared to control cultures without SDF-1 $\alpha$ . These results are consistent with the work of Son *et al.* (2006), who demonstrated that mesenchymal stem cells (MSCs) derived from bone marrow and umbilical cord blood exhibited strong migratory responses toward SDF-1 $\alpha$  without being diminished for up to 15 passages [158]. It was also shown that by blocking the receptor for SDF-1 $\alpha$  (CXCR4) using the antagonist AMD3100, the migration of the MSCs in the presence of SDF-1 $\alpha$  was significantly inhibited [158].

The SDF-1-CXCR4 axis is intimately involved in numerous physiological processes. Generally, SDF-1 and CXCR4 have important roles in the proper development of multiple visceral systems such as the vasculature of the gastrointestinal tract, structural integrity of the heart, and aspects of the central nervous system [159, 160, 161]. The expression of SDF-1 and CXCR4 also is involved in the homing of hematopoietic progenitor cells to the bone marrow and their retention within the niche [162]. Multiple groups have shown that the SDF-1-CXCR4 axis is extremely active in the mobilization and homing of many other types of cells including bone marrow-derived MSCs and endothelial progenitor cells [163, 164]. Additionally, the SDF-1-CXCR4 axis can promote angiogenesis [162]. More specifically, studies have shown that the introduction



of SDF-1 stimulates the formation of microvessels *in vitro* and the neovascularization of ischemic tissues *in vivo* by attracting endothelial progenitor cells to sites of ischemic injury [165, 166, 167].

In our migration experiments, HGF was also shown to induce rASC migration under normoxic conditions at concentrations of 20, 50, and 100 ng/mL compared to control cultures with no exposure to HGF. These results are consistent with the work of other groups that have examined the chemotactic effects of HGF on C3H/He murine and human bone marrow-derived MSCs [88, 89, 158]. Son *et al.* (2006) also demonstrated that bone marrow-derived and umbilical cord blood MSCs have a significant migratory response when exposed to an HGF concentration gradient [158]. MSCs were shown to express c-met, the cognate receptor of HGF, and that this ligand-receptor axis was active in mediating the migratory response of the cells towards the HGF. Blocking the c-met receptors with the inhibitor K-252a was shown to significantly suppress the migratory response of the MSCs to HGF, further emphasizing the importance of a functional HGF-c-met axis in controlling cell migration [158].

The stimulatory effect of HGF on cell migration is executed through the activation of the protein kinase B (Akt) signaling pathway. Akt activation is measured by the increased phosphorylation of Ser-473 on Akt when stimulated by HGF [99]. Ye *et al.* (2008) demonstrated that the HGF/c-met axis activated the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathways, both of which mediated HGF-induced cell migration via downregulation of the cell adhesion molecules E-cadherin and  $\beta$ -catenin [168]. The downregulation of these cell adhesion molecules compromises cell-to-cell

contacts, resulting in enhanced cell motility and ability to migrate in response to biological chemoattractants.

Furthermore, it has been shown that the inhibition of the Akt signaling pathway diminishes the heightened migratory response of MSCs that is induced by HGF, further implicating the Akt pathway as an important effector of HGF in terms of cell migration [99]. HGF has also been shown to activate the Erk signaling pathway in human MSCs, suggesting that this pathway may also play a role in carrying out the physiological effects of HGF, such as increased cell migration [99].

The chemotactic properties of HGF are of clinical importance because this factor has a role in attracting endothelial progenitor cells to sites of ischemia, promoting angiogenesis and reperfusion at these sites [85]. Significant reductions in HGF secretion by ischemic cells have been shown to reduce the survival, proliferation, and migration of endothelial progenitor cells, which could subsequently have suppressive effects on angiogenesis and may lead to tissue damage at the sites of ischemia [85]. Cai *et al.* (2007) demonstrated that ASCs with a silenced HGF gene had a significant impairment in their ability to stimulate reperfusion, as seen by reduced capillary density, in the ischemic hind limbs of mice. This was in contrast to reperfusion that was seen in mouse ischemic hind limbs where HGF secretion by ASCs was not impaired [85].

### **5.2.2 Influence of Oxygen Tension on rASC Migration**

The effect of oxygen concentration in conjunction with growth factor exposure on rASC migration was investigated. When cultured under hypoxic conditions (5% O<sub>2</sub>), rASC migration in the SDF-1 $\alpha$  study appeared to be increased compared to normoxic

cultures (21% O<sub>2</sub>); however, only cultures exposed to 50 ng/mL of SDF-1 $\alpha$  were significantly increased. Previous studies have shown that *in vivo* expression of SDF-1 $\alpha$  is capable of inducing stem cell homing to the ischemic environment of the infarcted myocardium [83]. Under ischemic conditions, cells have been shown to increase their expression of SDF-1 $\alpha$  and subsequently increase the migration of mesenchymal stem cells to the site of ischemia by up to approximately 80% [140]. Additionally, the addition of exogenous SDF-1 $\alpha$  two days after the occurrence of an MI resulted in a two-fold increase in MSC migration to the ischemic site of injury compared to MSC migration in MIs without the addition of exogenous SDF-1 $\alpha$  [140].

The effect of oxygen tension and HGF exposure on rASC migration was also examined. Regardless of the oxygen tension, the rASCs exposed to HGF demonstrated a general trend of increased migration in response to increasing concentrations of HGF. In this study, the overall migration of rASCs in the hypoxic cultures was not greater than the migratory response of rASCs in the normoxic cultures. These results are in contrast with what has been noted in past studies, where cell migration has been greater under hypoxic conditions compared to normoxic conditions [99, 100, 169]. However, this experiment was only performed one time and therefore the data may be limited. Further trials should be performed to help determine the effects of oxygen tension and HGF exposure on rASC cell migration more accurately.

Rosova *et al.* (2008) demonstrated that culturing human BMSCs in hypoxia (1-3% O<sub>2</sub>) for 24 hours could significantly increase cell migration [99]. It was also shown that hypoxia and HGF could independently activate the aforementioned Akt pathway and that inhibition of this pathway suppressed cell migration and secretion of angiogenic

factors [88, 171]. Additionally, MSCs cultured under hypoxic conditions were shown to have increased phosphorylation of c-met when exposed to HGF for 15 minutes compared to MSCs cultured under normoxic conditions [99]. However, longer exposure to HGF (30 minutes) reduced the degree of c-met phosphorylation down to level seen in normoxic MSCs [99]. The decrease in c-met phosphorylation as a result of prolonged HGF exposure may have been a contributing factor to the muted migratory response seen in our hypoxic cultures compared to the normoxic cultures. Our cultures were exposed to HGF for 48 hours and this prolonged exposure may have lead to a substantial down-regulation of c-met phosphorylation. A reduction in c-met phosphorylation and activation would have downstream effects such as down-regulating the activation of the Akt pathway, which, as previously mentioned, plays an important role in cell motility and migration.

### **5.3 Growth Factor-Related Gene Expression**

Generally, our results indicated that the gene expression of SDF-1 $\alpha$ , CXCR4, HGF, and c-met were upregulated under hypoxic conditions compared to normoxic conditions. The presence of SDF-1 $\alpha$  did not appear to have any discernable pattern of influence on the expression of these 4 genes, while HGF exposure did appear to mediate gene expression under hypoxic conditions. Therefore, it appears as though hypoxia may be the predominant mediator of gene expression in these cells. Similar results have been demonstrated in previous studies analyzing gene expression by progenitor cells in hypoxic conditions. The presence of hypoxia-responsive elements in the promoter sequence of the SDF-1 gene was elucidated and was discovered to contain binding sites

for HIF-1, a major transcriptional regulator of hypoxic-specific genes [95]. HIF-1 was shown to be a significant mediator of upregulating SDF-1 $\alpha$  and CXCR4 expression under hypoxic conditions [95, 100]. Another study demonstrated that CXCR4 mRNA was increased in glioma cells that were cultured in hypoxic conditions (1% O<sub>2</sub>) as soon as 8 hours after exposure to hypoxia and remained elevated through 24 hours [172]. Overall, the general pattern of increased expression of SDF-1 $\alpha$  and CXCR4 in hypoxia compared to normoxia is consistent with previously published results in the literature.

Similar to our results for SDF-1 $\alpha$  and CXCR4, our results demonstrated that oxygen tension was the main mediator of HGF and c-met expression. Both HGF and c-met genes were more strongly expressed under hypoxia compared to normal oxygen tension conditions. The mechanisms for increased expression under hypoxic conditions appear to be similar to those of SDF-1 $\alpha$  and CXCR4 expression. The c-met promoter is known to contain binding sites for HIF-1, a transcriptional regulator whose expression is greatly increased in hypoxic conditions [173]. Eckerich *et al.* (2007) found that hypoxic stimulation of c-met gene expression only occurred when HIF-1 was upregulated in multiple glioblastoma cell lines cultured in hypoxic conditions [173]. This result suggested that the activation of HIF-1 is a crucial event required for the upregulation of c-met gene expression. This is further supported by the fact that inhibiting the induction of HIF-1 with the appropriate siRNAs was shown to significantly attenuate c-met expression as well [173]. Again, these results strongly suggest that the increase in c-met gene expression under hypoxia is mediated by HIF-1.

Additionally, Forte *et al.* (2006) demonstrated that HGF and c-met expression could be upregulated by treating MSCs with HGF (20 ng/mL) [88]. These results are

similar to what we observed in our cultures treated with HGF. Although in our experiments, the upregulation of HGF and c-met expression was mainly limited to the cultures that were treated with HGF and also cultured under hypoxic conditions. This may suggest that HIF-1 still plays an important role in the upregulation of HGF and c-met expression even when exogenous HGF is present.

#### **5.4 Growth Factor-Related Protein Expression**

Oxygen tension had an effect on SDF-1 $\alpha$  protein expression by rASCs. SDF-1 $\alpha$  content as a portion of the total cellular protein content was higher in the low oxygen tension cultures compared to normoxic rASCs after seven days in culture. This observation is similar to what is seen in work by Hitchon *et al.* (2002), who also demonstrated that hypoxia was an important stimulus in significantly increasing SDF-1 $\alpha$  protein expression [174]. Similar results were seen in the work of Ceradini *et al.* (2004) where SDF-1 $\alpha$  protein expression was significantly increased under hypoxic conditions when compared to normoxic conditions [100]. One difference, however, was that in these previous works, the increase in SDF-1 $\alpha$  protein expression was observed much earlier than was seen in this current study. Both of these previous studies defined their hypoxic culture conditions as 1% O<sub>2</sub>, whereas the hypoxic environment in the current study had a 5% O<sub>2</sub> tension. Also, the other studies used different cell sources, synovial fibroblasts and endothelial progenitor cells, which may have different SDF-1 $\alpha$  secretion capacities and profiles. These factors and potentially others may have contributed to the differences between this current study and previously published results.

In this present study, HGF protein expression by hypoxic rASCs was not significantly increased when compared to normoxic rASCs at 24 hours and seven days. Unfortunately, the data for HGF protein expression on Day 7 is based on single samples and thus may not be the most accurate reflection of the full data set. However, Rehman *et al.* (2004) demonstrated results that mirrored the trend seen in this study; they found that even after 72 hours, HGF protein expression by human ASCs cultured in hypoxia was much less than that of cells cultured in normoxic conditions [156]. While the similarities between the two studies can be seen, it would be important to repeat this experiment to obtain data from an adequate number of samples before any other significant inferences or connections can be made.

## **5.5 Influence of RGD-Peptide Grafting and SDF-1 $\alpha$ -Loading on rASC**

### **Viability in MGC Hydrogels**

Thus far, several of the characteristics of rASCs have been demonstrated. These properties include the plasticity of rASCs, positive directed migratory responses to chemotactic growth factors, and enhanced gene expression of associated growth factor/receptors and growth factor proteins involved in angiogenesis *in vivo*. All of these characteristics confirm the potential benefits of the use of ASCs in the treatment of MIs. However, all of these therapeutic properties are dependent on the survival of the ASCs in the hostile ischemic environment characteristics of infarcted myocardium. Several groups have previously established that while the therapeutic benefits are apparent *in vivo*, they are limited by the significant decline in cell viability seen as early as one week after transplantation [12, 27, 28]. Therefore, in order to maximize the healing potential of

ASCs, their viability must be prolonged to give them sufficient time to carry out and amplify their restorative effects (such as angiogenesis). A practical approach to this problem would be to find a way to optimize an appropriate delivery vehicle to maximize cell viability.

The MGC hydrogel was chosen as the delivery vehicle due to its natural biocompatible properties and for its familiarity as they had been previously used in our lab with promising results [11, 15, 33, 175]. It has also been previously established that cell-extracellular matrix (ECM) interactions have significant influence over MSC survival as cells undergo programmed cell death brought about by detachment from the ECM [13, 34]. Therefore, RGD-peptides, the cell-binding sequence found in fibronectin that promotes cell-ECM interactions [19], were grafted into the MGC hydrogels.

In this study, MGC hydrogels were constructed containing RGD-peptides concentrations of 4% and 7% and compared to hydrogels lacking any RGD-grafting to determine if this modification would improve cell viability. Over a course of 14 days, rASC viability was generally better in both 4% and 7% RGD-MGC hydrogels compared to control gels lacking any modification. Three days after encapsulation, rASC viability in the RGD-modified hydrogels was already significantly greater than compared to control samples. It was also promising to see that this pattern of cell viability was maintained by the end of 14 days, with both types of RGD-modified MGC hydrogels maintaining significantly greater cell viability compared to control. These results are in accordance with what was demonstrated by Park *et al.* (2008) who showed that the viability of bovine chondrocytes seeded in RGD-modified chitosan-pluronic hydrogels was superior to that of the viability seen in the same cells seeded in unmodified alginate



hydrogels [176]. Similarly, this cell viability was maintained over a course of 14 days, with viability in the RGD-modified hydrogels being consistently greater than that of the unmodified control group [176]. It is interesting to note that while Park *et al.* (2008) used 13.5% RGD-conjugated chitosan-pluronic hydrogels, our similar results were obtained using 4% and 7% RGD-MGC hydrogels. This suggests that lower concentrations of RGD conjugation may be as effective in maintaining cell viability and this may have implications for future work. Also, Salinas *et al.* (2008) demonstrated that the viability of MSCs encapsulated in RGD-modified PEG hydrogels were approximately 70-84% after 14 days in culture, which was significantly higher than MSC viability in unmodified hydrogels and similar to cell viabilities attained in this current study [128]. The benefits of modifying hydrogels with the RGD peptide on cell viability and after encapsulation appear to be well established in the literature.

In addition to RGD-modification, this study examined if loading SDF-1 $\alpha$  (50 ng/mL) into the hydrogels would also have an effect on cell viability. This study showed that in general, cell viability was enhanced in RGD-modified hydrogels compared to control, a trend previously seen in our SDF-1 $\alpha$ -free hydrogels. On Day 3, both 4% and 7% RGD-modified hydrogels had significantly higher cell viability compared to control and this was maintained by the 7% RGD-MGC hydrogel up to Day 7. The rASC viability in SDF-1 $\alpha$ -loaded hydrogels, though generally improved over the course of 14 days, was lower in comparison to the SDF-1 $\alpha$ -free hydrogels. Previous studies have conflicting reports with regard to the effects of SDF-1 $\alpha$  on cell survival. Exposure to SDF-1 $\alpha$  has been shown to promote cell survival by activating PI-3K/MAPK signaling cascades (involved in cell motility, proliferation etc.), deactivating pro-apoptotic proteins, and

increasing the transcription of cell survival genes [177]. However, other studies have shown that SDF-1 $\alpha$  has little to no effect on cell survival or may even inhibit growth and induce apoptosis in cells [178, 179, 180]. If SDF-1 $\alpha$  does have a positive effect on cell survival, it has been suggested that this may be an indirect effect of retaining progenitor cells in their native environment to allow interactions with the environments adhesion molecules to prevent cell death caused by detachment [78].

Overall, this current study did not conclusively determine if the presence of SDF-1 $\alpha$  is beneficial for cell survival but did confirm that RGD-grafting can significantly improve cell viability within MGC hydrogels. Similarly, Sukarto *et al.* (2012) also demonstrated that RGD-grafting in MGC hydrogels could improve the viability of encapsulated ASCs, with over 90% viability over a period of 14 days [181]. By encapsulating ASCs in these hydrogels and supporting their need for survival through attachment to RGD peptides, this may be a practical way to promote and prolong ASC viability when such work is translated *in vivo* for the treatment of MIs. By improving cell viability, ASCs and other progenitor cells will have more time to mediate their restorative effects and will increase the therapeutic benefits that can be derived from this type of treatment.

## Chapter 6

### Conclusions and Recommendations

#### 6.1 Conclusions

The use of stem cells in regenerative medical therapies holds much potential and careful consideration must be paid to the choice of stem cells. Adipose-derived stem cells (ASCs) are a promising source of mesenchymal stem cells that have several advantages over other sources of stem cells (e.g. BMSCs, ESCs) with regards to issues such as accessibility, quantity, and ethical concerns [17, 23].

The ASCs extracted from the epididymal fat pads of male Wistar rats demonstrated similar multi-lineage potential as seen in ASCs extracted from humans [21]. Differentiation experiments demonstrated successful differentiation of the extracted rASCs along the adipogenic, osteogenic, and chondrogenic lineages using appropriate inductive differentiation media formulations containing key factors that targeted lineage-specific differentiation pathways.

In the transwell cell migration experiments, rASCs demonstrated a positive chemotactic response to SDF-1 $\alpha$  and HGF exposure. When exposed to 20, 50, and 100 ng/mL concentrations of SDF-1 $\alpha$  or HGF, rASC migration was significantly increased in comparison to control cultures without the growth factors. The effect of oxygen tension on rASC migration was also examined. rASCs cultured under hypoxic conditions (5% O<sub>2</sub>) in conjunction with SDF-1 $\alpha$  exposure generally demonstrated higher levels of migration compared to migration under normoxic conditions (21% O<sub>2</sub>), though the increase was not statistically significant except at 50 ng/mL of SDF-1 $\alpha$ . When cultured

under hypoxic conditions and exposed to HGF, rASC migration was not as high as what was seen under normoxic conditions, however, this difference was not statistically significant.

The effect of oxygen tension and growth factor supplementation on the gene expression of growth factor-related products was examined. In general, hypoxic culture conditions (5% O<sub>2</sub>) appeared to be an influential factor in growth factor and cognate receptor gene expression. Under hypoxic conditions, gene expression of SDF-1 $\alpha$ , CXCR4, HGF, and c-Met were generally upregulated when cultures were supplemented with HGF in comparison to their normoxic culture counterparts. SDF-1 $\alpha$  supplementation did not appear to enhance gene expression under normoxic or hypoxic conditions. With no growth addition, SDF-1 $\alpha$  expression was similar under normoxic and hypoxic conditions and c-Met expression was upregulated under hypoxia. Overall, oxygen tension appeared to have a significant influence on the gene expression of the growth factors SDF-1 $\alpha$  and HGF and their respective receptors, CXCR4 and c-Met in cultures supplemented with exogenous HGF.

The influence of oxygen tension on SDF-1 $\alpha$  protein expression was also examined. After one day, the SDF-1 $\alpha$  content (normalized to the total protein content) was lower in the hypoxic cultures as compared to the normoxic cultures, although the difference was not statistically significant. However, after 7 days in culture, the normalized SDF-1 $\alpha$  content was greater in the hypoxic cultures when compared to normoxic cultures.

The use of MGC hydrogels modified with varying degrees of RGD peptide with and without SDF-1 $\alpha$  loading was investigated as a potential delivery vehicle that could

enhance ASC viability by providing cell attachment and localization. In SDF-1 $\alpha$ -negative MGC hydrogels, rASC viability improved over a two-week period and was significantly higher in 4% and 7% RGD-peptide modified MGC hydrogels as compared to control hydrogels without peptide modification. In SDF-1 $\alpha$ -loaded MGC hydrogels, RGD-modified hydrogels showed significantly better rASC viability after 3 days in culture and also after 1 week. At the end of two weeks, the rASC viability was relatively similar among RGD-modified hydrogels and unmodified control hydrogels. When comparing rASC viability between the SDF-1 $\alpha$ -negative and positive groups, the inclusion of SDF-1 $\alpha$  did not appear to significantly impact cell viability. Overall, this experiment demonstrated that RGD-peptide modification improves initial rASC viability after crosslinking within the MGC gels, and that RGD-modification alone is sufficient to maintain cell viability. Furthermore, RGD-modification of the MGC hydrogels maintained a high level of cell viability over a course of 14 days, which is in accordance with other studies that have also demonstrated that RGD-modification of hydrogels promotes better cell viability in culture [128, 176]. This provides evidence that RGD-modified MGC hydrogels has the potential to be a suitable delivery vehicle that will help maintain ASC viability after transplantation in the ischemic environment of the infarcted myocardium.

## **6.2 Recommendations and Future Work**

Adipose tissue is a promising source of stem cells that has many potential clinical applications. ASCs hold great therapeutic potential in the treatment of cardiovascular diseases, and for MI in particular. ASCs, unlike other cell types such as BMSCs, have yet

to be widely tested in randomized controlled multi-centre trials with large patients groups. Most of the clinical trials using ASCs have been in Phase I or Phase I/II safety studies [182]. Therefore, more work must be done in order to translate ASCs into widespread clinical therapeutic use.

It is clear that oxygen tension, particularly hypoxic conditions, plays a significant role in several different aspects of ASC physiology. In this thesis, hypoxic conditions were represented by 5% O<sub>2</sub> concentration, while oxygen tensions as low as 0.5-1% have been seen in the literature [99, 172]. Future experiments should focus on determining growth factor-related gene expression and protein/receptor expressions at lower oxygen tensions. This may be very useful in providing a comparison of how varying degrees of hypoxia will affect the growth factor-related gene and protein expression, which is important because these growth factors are thought to be involved in the paracrine mechanisms through which healing of the infarcted myocardium takes place *in vivo*.

RGD peptide modification has been demonstrated to be a key element in enhancing ASC viability. An in-depth study looking at different modifiable parameters, such as concentration and presentation, of RGD modification in the MGC hydrogels may be useful in determining the optimal conditions that best support cell viability. For example, it may be useful to determine the lowest possible concentration of RGD modification that protects the cells to prevent the wasting of costly materials for no added benefit. It would also be interesting to test higher concentrations of RGD modification to determine if cell viability plateaus at a particular concentration or if there continues to be added benefit with greater degrees of peptide modification. It may also be useful to examine modification of MGC hydrogels with other adhesive peptides, such as

YIGSR or IKVAV, and compare their effects on ASC viability to that of RGD to determine if one peptide or perhaps combinations of different peptides best supports cell viability. Additionally, determining whether or not the incorporation of growth factors in the MGC hydrogels has an effect on cell retention will be important to assess. It would be worthwhile to compare cell retention in unmodified MGC hydrogels that have been loaded with growth and those that have not and to do the same comparison for RGD-modified MGC hydrogels. In this way, it can determine if growth factor loading can enhance the delivery vehicle's ability to retain ASCs and if a combination of growth factor loading and peptide modification will have a synergistic effect on ASC retention within ischemic myocardium. Also, future experiments should also examine ASC viability in other delivery vehicles, such as chondroitin sulphate hydrogels. This will provide a useful comparison and help determine the extent to which the biomaterial itself plays a role in cell retention and viability and which is best suited for *in vivo* applications with regards to its architecture, cell and tissue compatibility, bioactivity, and mechanical properties [183].

Finally, the next step in the larger picture is to test this optimized delivery vehicle *in vivo*. Transplantation of ASCs encapsulated in RGD-modified MGC hydrogels into animal models will provide a better understanding of how the ASCs will fare in infarcted myocardium rather than a simulated hypoxic environment and help determine the physiological benefits that can be derived from this therapy.

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## Appendix A

### rASC Migration in a Modified Boyden Chamber

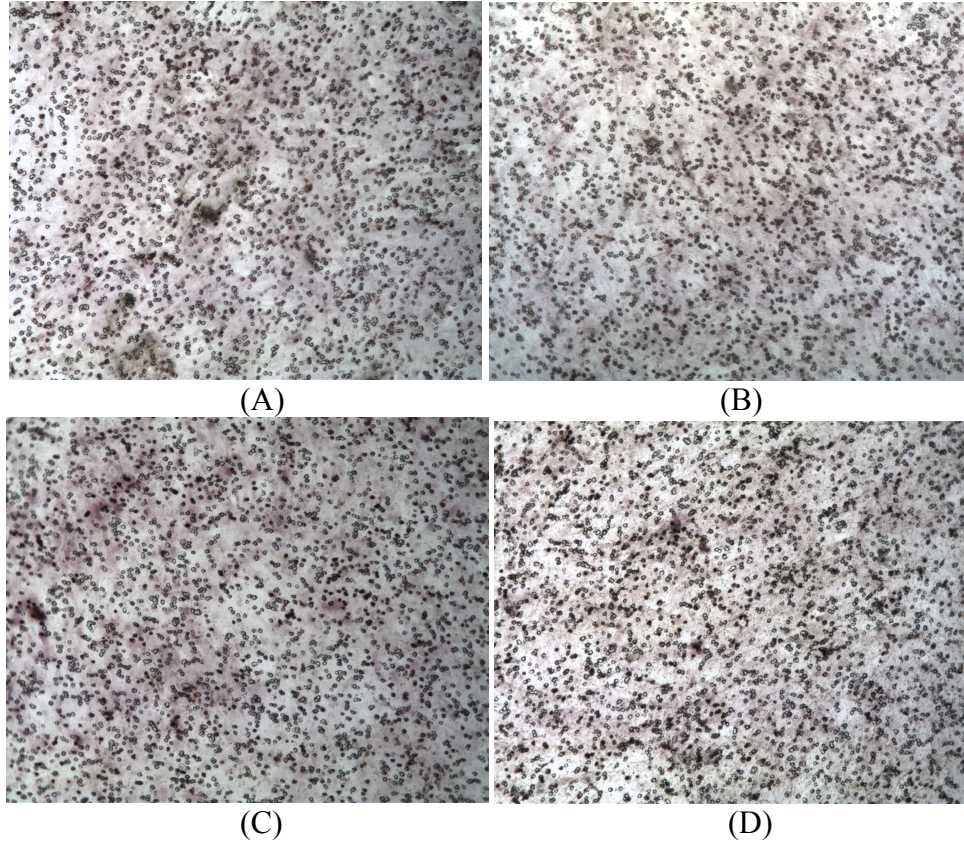


Figure A.1 Hematoxylin staining of rASCs that have migrated through the semi-porous membrane of the transwell unit in four different conditions: (A) control, (B) 20 ng/mL SDF-1 $\alpha$ , (C) 50 ng/mL SDF-1 $\alpha$ , (D) 100 ng/mL SDF-1 $\alpha$ . Magnification 100 x.



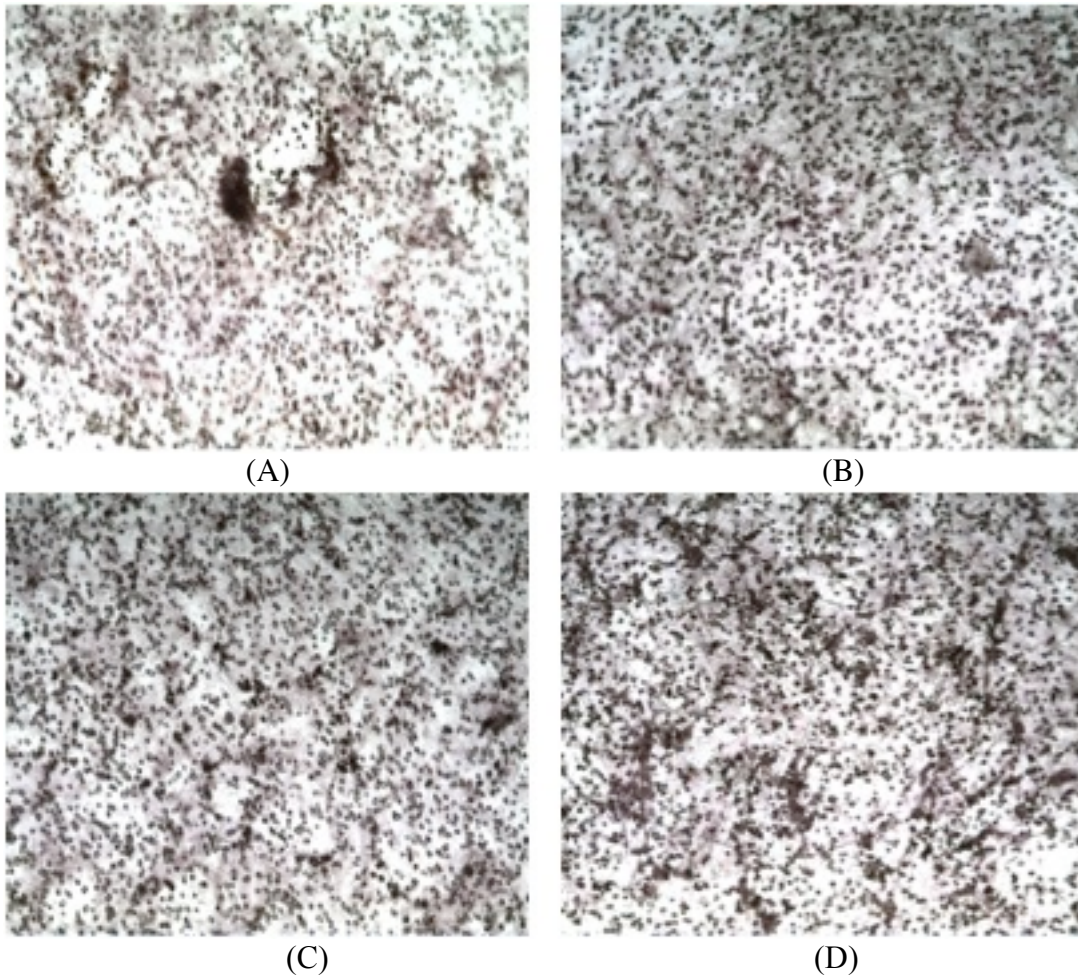


Figure A.2 Hematoxylin staining of rASCs that have migrated through the semi-porous membrane of the transwell unit in four different conditions: (A) control, (B) 20 ng/mL HGF, (C) 50 ng/mL HGF, (D) 100 ng/mL HGF. Magnification 100 x.

## Appendix B

### Influence of Oxygen Tension and Growth Factor Supplementation on rASC Migration

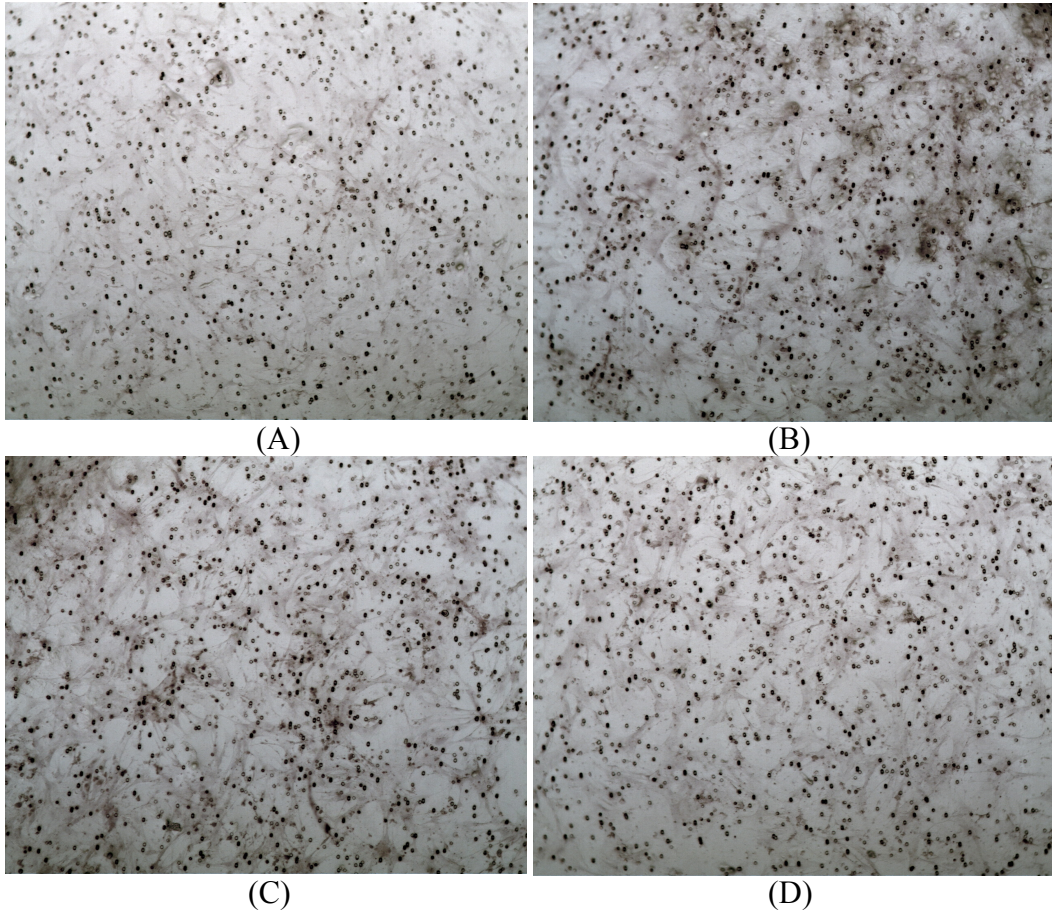


Figure B.1. The migration of rASCs under hypoxia (5% O<sub>2</sub>) and exposure to SDF-1 $\alpha$  for four culture conditions: (A) control, (B) 20 ng/mL, (C) 50 ng/mL, (D) 100 ng/mL. Magnification = 100x.



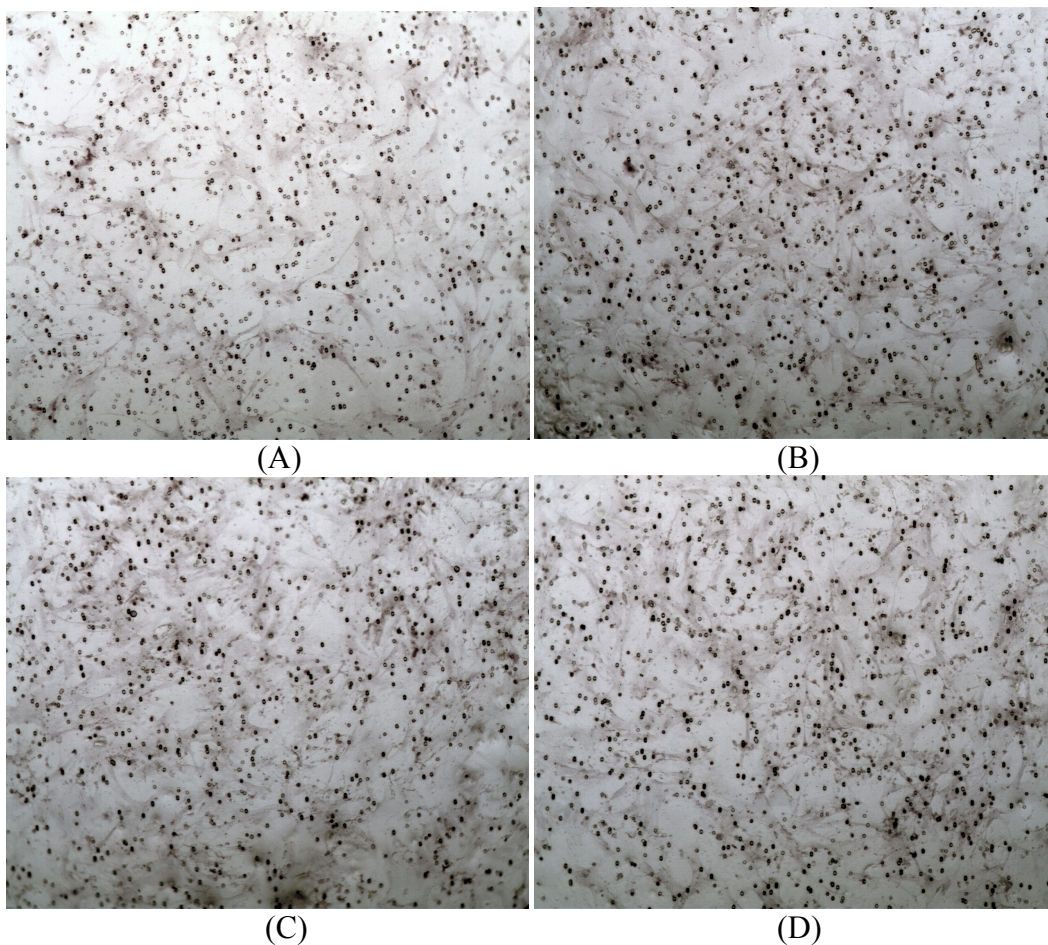


Figure B.2. The migration of rASCs under normoxia (21% O<sub>2</sub>) and exposure to SDF-1 $\alpha$  for four culture conditions: (A) control, (B) 20 ng/mL, (C) 50 ng/mL, (D) 100 ng/mL. Magnification = 100x.

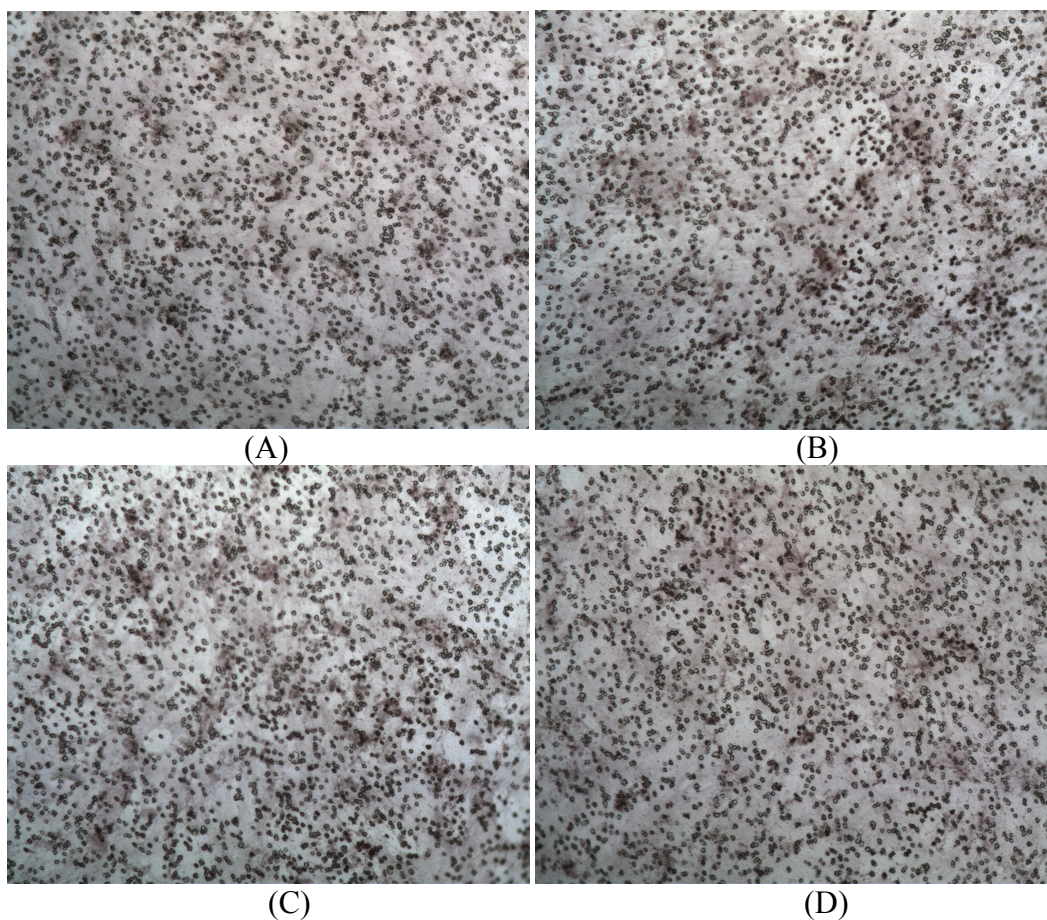


Figure B.3 The migration of rASCs under hypoxia (5% O<sub>2</sub>) and exposure to HGF for four culture conditions: (A) control, (B) 20 ng/mL, (C) 50 ng/mL, (D) 100 ng/mL. Magnification = 100x.



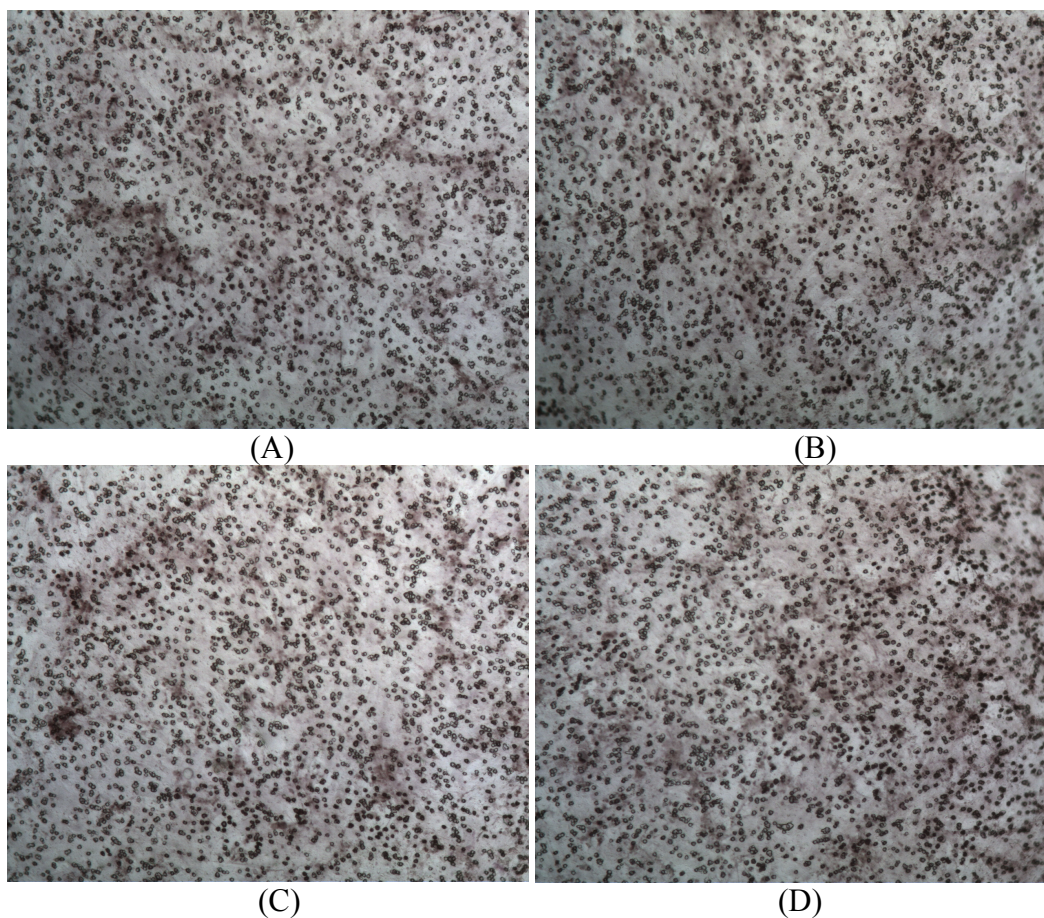


Figure B.4 The migration of rASCs under normoxia (21% O<sub>2</sub>) and exposure to HGF for four culture conditions: (A) control, (B) 20 ng/mL, (C) 50 ng/mL, (D) 100 ng/mL. Magnification = 100x.