Development of Functionalised Hydrogels to Enhance Stem Cell Delivery and Integration into the Ischaemic Myocardium

Laura Gallagher
Royal College of Surgeons in Ireland, lauragallagher@rcsi.ie

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Development of Functionalised Hydrogels to Enhance Stem Cell Delivery and Integration into the Ischaemic Myocardium

A thesis submitted to the Royal College of Surgeons in Ireland in partial fulfilment of the requirements for the degree of

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Laura Bridget Gallagher, B.Sc.
Department of Anatomy

Supervisors
Prof. Garry Duffy, Prof. Fergal O’Brien and Dr Tom Farrell
I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Philosophy, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Student Number

Date
Abstract
Heart failure is a progressive, debilitating disease commonly caused by the irreversible loss of functional cardiac tissue after myocardial infarction. Aside from heart transplantation, current treatment options are considered palliative as they may prolong survival but fail to address the underlying damage to cardiac tissue. The delivery of multipotent stem cells to the damaged heart has been under extensive investigation for almost two decades. This treatment strategy offers a reparative approach, with the potential to salvage damaged heart muscle in the diseased organs of patients. Although safety and feasibility has been demonstrated using multiple cell types, only a minimal recovery of left ventricular function has been achieved. The curative potential of stem cells is hindered by poor retention and survival of transplanted cells in the harsh microenvironment of the infarcted heart. The overall aim of this thesis was to develop functionalised hydrogels to enhance stem cell delivery and integration into the ischaemic myocardium.

Natural biomaterials such as hyaluronic acid (HyA) are commonly used as cell delivery vehicles due to their intrinsic ability to generate hydrogels that are analogous to native tissue. However, single-factor extracellular matrix (ECM) scaffolds over-simplify the multicomponent ECM of the stem cell niche. The aim of this research was to determine if functionalisation of HyA hydrogel could improve stem cell viability and function under ischaemic culture conditions, compared to non-functionalised HyA hydrogel. As the optimal cell source for cardiac repair is unknown, two of the leading candidates, cardiac stem cells (CSCs) and mesenchymal stem cells (hMSCs), were examined.

HyA is known to resist cellular attachment, therefore the adhesive peptide RGD was incorporated to facilitate cell adhesion. In chapter 2, the mechanical properties of HyA and HyA-RGD hydrogel were compared. Both hydrogels were reported to gelate rapidly, exhibit minimal swelling and achieve appropriate mechanical properties. Next, the effect of RGD on hMSC viability, morphology and function in HyA hydrogel was investigated under standard culture conditions. Our results show that while hMSC viability was maintained in both HyA and HyA-RGD hydrogel, RGD
significantly increased hMSC spreading and paracrine factor release, compared to HyA hydrogel.

In order to assess the effect of RGD on hMSC survival under ischaemic culture conditions, we sought to design a controlled experimental system using the well-known anti-apoptotic factor IGF-1 as a positive control. IGF-1 is known to enhance the growth and survival of multiple cell types, however, its effect on hMSCs is poorly understood. In Chapter 3, IGF-1 treatment was found to have no effect on hMSC proliferation or survival. The bioactivity of our IGF-1 protein was confirmed using CSCs, which demonstrated significant proliferation in response to IGF-1 stimulation.

Having demonstrated the beneficial effects of RGD under standard culture conditions, Chapter 4 investigated effect of RGD on hMSCs survival, morphology and function under ischaemic culture conditions. RGD was reported to improve encapsulated hMSC survival, but only if cells were allowed to adhere to the RGD before exposure to ischaemic culture conditions. In addition, the ECM protein nidogen-1 was successfully incorporated into HyA hydrogel and its effect on hMSC viability, morphology and function was investigated. Our results show that while nidogen-1 promoted hMSC viability and function under standard culture conditions, it was unable to enhance hMSC survival under ischaemic culture conditions. In Chapter 5, HyA-RGD hydrogel was found to promote CSC viability under both standard and ischaemic culture conditions, compared to unmodified HyA. IGF-1 was also shown to enhance rCSC viability in HyA hydrogel under ischaemic culture conditions.

Collectively, the results presented in this thesis provide evidence that the functionalisation of HyA hydrogel has the potential to enhance stem cell delivery under ischaemic conditions and to improve the efficacy of cell products already under clinical investigation.
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Publications, Presentations and Prizes

Publications

Presentations


Prizes
2017 Graduate Research Advances in Delivery Science (GRADS) award sponsored by MERCK for poster “Development of Functionalised Hydrogels to Enhance Stem Cell Therapy for Cardiac Regeneration” at Controlled Release Society (CRS) conference in Boston.

Second Prize for oral Presentation “An injectable, hyaluronic acid hydrogel modified with RGD to enhance stem cell delivery to the infarcted heart” at the Anatomical Society and American Association of Anatomists “Anatomists on the Edge” conference (2017).

Joint runner-up for oral presentation “Development of Functionalised Hydrogels to Enhance Stem Cell Delivery and Integration into the Ischaemic Myocardium” at the Matrix Biology Ireland (MBI) meeting (2016).
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Nomenclature

°C – Degree Celsius
2D – Two Dimensional
3D – Three Dimensional
ADSC – Adipose-Derived Stem Cell
ANOVA – Analysis of Variance
bFGF – basic Fibroblast Growth Factor
BM – Basement Membrane
BMMNC – Bone Marrow Mononuclear Cell
BSA – Bovine Serum Albumin
CABG – Coronary Artery Bypass Grafting
CCK8 – Cell Counting Kit-8
CDC – Cardiosphere-Derived Cell
CEM – Complete Explant Medium
CGM – Complete Growth Medium
CO₂ – Carbon Dioxide
CPC – Cardiac Progenitor Cell
CSC – Cardiac Stem Cell
CT – Computerised Tomography
CVD – Cardiovascular Disease
DAPI – 4’, 6- diamino-2-phenylindole
DMEM – Dulbecco’s Modified Eagle’s Medium
DNA – Deoxyribonucleic Acid
DS – Degree of Substitution
dsDNA – double stranded Deoxyribonucleic Acid
ECG – Electrocardiogram
ECM – Extracellular Matrix
EGF – Epidermal Growth Factor
EGFR – Epidermal Growth Factor Receptor
ELISA – Enzyme Linked Immunosorbent Assay
EPO – Erythropoietin
ERK – Extracellular Signal-Regulated Kinase
ESC – Embryonic Stem Cells
FACs – Fluorescence-Activated Cell sorting
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<th>Acronym</th>
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<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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</tr>
<tr>
<td>PCI</td>
<td>Percutaneous Coronary Intervention</td>
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<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
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<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
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<td>Polytetrafluoroethylene</td>
<td></td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl Alcohol</td>
<td></td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
<td></td>
</tr>
<tr>
<td>RGD</td>
<td>Arginylglycylaspartic acid</td>
<td></td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised Controlled Trial</td>
<td></td>
</tr>
<tr>
<td>RHAMM</td>
<td>Receptor for Hyaluronan-Mediated Motility</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
<td></td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal-Derived Factor-1</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>Skeletal Myoblasts</td>
<td></td>
</tr>
<tr>
<td>STEMI</td>
<td>ST-Elevation Myocardial Infarction</td>
<td></td>
</tr>
<tr>
<td>TA</td>
<td>Tyramine</td>
<td></td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming Growth Factor-alpha</td>
<td></td>
</tr>
<tr>
<td>™</td>
<td>Trade mark</td>
<td></td>
</tr>
<tr>
<td>VAD</td>
<td>Ventricular Assist Device</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
<td></td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
<td></td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre (x10^-9)</td>
<td></td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre (x10^-6)</td>
<td></td>
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<tr>
<td>mm</td>
<td>Millimetre (x10^-3)</td>
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<td>mM</td>
<td>Millimolar (x10^-3)</td>
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<td>μM</td>
<td>Micromolar (x10^-6)</td>
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<td>μL</td>
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<td>mL</td>
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<td>L</td>
<td>Litre</td>
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Chapter 1: Introduction and Literature Review
**Overview**

A typical myocardial infarction (MI) results in the death of up to 1 billion cardiomyocytes. As the heart has a limited regenerative potential, dead cells are replaced with non-contractile scar tissue, which leads to additional stress on the remaining cardiac muscle and compromises heart function. Depending on the size of the infarct, MI survivors may develop chronic heart failure (HF). While pharmacological treatments and mechanical assist devices have reduced adverse cardiac remodelling and improved mortality rates, these therapies are unable to restore damaged cardiac tissue and HF patients face a 5-year mortality rate of over 50%. Organ transplantation produces the best patient outcomes but this treatment is limited by donor availability.

Stem cells have the potential to achieve the elusive goal of cardiac repair; however, transplanted cells display low retention and survival which affects the success of cardiac cell therapy. The research described in this thesis aims to address this challenge through the combination of cells and biomaterial carriers. We focus on the development of an injectable hydrogel delivery system, functionalised hyaluronic acid (HyA), to enhance the longevity of cells under conditions of hypoxia and reduced nutrients, as present in ischaemic injury sites. The aim is to improve the efficacy of cell products already under clinical investigation in order to further progress towards the successful clinical translation of stem cell therapy and to improve treatment outcomes for patients with MI.
1.1 Cardiovascular disease
Cardiovascular disease (CVD) is defined as a pathophysiological condition that causes impaired function of the heart or blood vessels of the circulatory system. CVD is the leading cause of death worldwide, responsible for 17.3 million deaths per year, or 31.5% of all global deaths. The number of fatalities per year is expected to grow to more than 22.2 million in 2030. Accordingly, while the current global cost of CVD is estimated at around US $863 billion, this number is projected to rise to US $1044 billion by 2030 (1–5). In Europe, CVD causes more than 4 million deaths each year, accounting for 45% of all deaths (6). The majority of CVD related deaths are attributable to ischaemic heart disease including MI and ischaemic cardiomyopathy (7,8). MI, commonly known as a heart attack, is responsible for significant morbidity and mortality, causing an estimated 7.3 million deaths annually (9).

1.2 Myocardial infarction
MI occurs following occlusion of a coronary vessel, typically due to rupture of an atherosclerotic plaque followed by thrombus formation. The sudden inhibition of blood flow to the heart muscle creates an area of ischaemia, where mass death of cardiac myocytes ensues almost immediately. As the myocytes die by both apoptotic and necrotic processes, they initiate an inflammatory response, recruiting a host of white blood cells including neutrophils, macrophages and lymphocytes to the site. Following removal of the debris, cardiac fibroblasts secrete an abundance of extracellular matrix (ECM) proteins, replacing the dead cells with a dense collagen scar (Fig. 1.1). While the fibrotic scar helps to protect the heart wall from rupturing, it also encourages contractile dysfunction and rhythm disorders. Strong correlations have been found between scar size and an increased risk of arrhythmias and sudden cardiac death (10).
Figure 1.1 A schematic diagram showing the key events in MI development. The black arrow indicates the progression of the pathology. Following occlusion of a coronary artery, the heart undergoes remodelling in response to ischaemia. This leads to an increased incidence of heart failure due to the presence of a fibrotic scar in the left ventricle. Note: CMC refers to cardiomyocyte (11).

Patients presenting with MI can be subdivided into two categories, those with ST-segment elevation MI (STEMI) and those with non-ST-segment elevation MI (NSTEMI). ST-segment elevation is an abnormality detected on an electrocardiogram (ECG) that indicates full thickness or transmural injury of heart muscle. STEMI is typically caused by complete blockage of a major coronary artery, whereas NSTEMI is caused by occlusion of a minor coronary artery or partial blockage of a major coronary artery (12). A coronary angiogram can be performed to corroborate the ECG findings and identify the site of obstruction (13). Rapid reperfusion of the ischaemic tissue is a crucial strategy used to reduce scar size, improve long-term myocardial function and decrease mortality (14). Although reperfusion is known to inflict additional damage on the myocardium as a result of ischaemia/reperfusion injury, it is the most effective remedy against ischaemic damage during MI (14). Reperfusion methods include anti-thrombotic therapy,
percutaneous coronary intervention (PCI) and coronary-artery bypass-graft surgery (CABG) (15).

PCI at the earliest stage of STEMI aims to reduce infarct size, thereby decreasing the risk of a substantial reduction in left ventricular ejection fraction (LVEF) with subsequent development of HF (16). This non-surgical technique uses a catheter to inflate a balloon within the blocked coronary artery to eradicate the plaque and restore blood flow. Concurrently, a stent may be deployed to act as a scaffold to maintain vessel integrity and prevent closure (15). Coronary angiographic images in Fig. 1.2 below, demonstrate coronary blood flow before (A) and after (B) successful PCI with implantation of two stents in a 55-year-old patient with STEMI caused by proximal left anterior descending coronary artery occlusion (13).

![Figure 1.2](image)

**Figure 1.2** Coronary angiogram confirming (A) occlusion of the proximal left anterior descending coronary artery and (B) reperfusion of the tissue following implantation of stents. Arrow indicates the site of obstruction (13).

While reperfusion after STEMI has significantly reduced patient mortality, it has led to an increased incidence of chronic HF, as patients who would have died in the past, now survive and live with a significantly damaged heart (14). As illustrated in Fig. 1.3, cardiac remodelling continues after injury in response to abnormal wall stress, leading to cardiomyocyte hypertrophy at the peri-infarct zone, wall thinning
and chamber dilation. Dilation of the left ventricle (LV) leads to an increase in both end-diastolic and end-systolic volumes and reduced ejection fraction (10).

![Image of heart showing normal heart vs infarcted heart]

**Figure 1.3** Adverse remodeling in the LV after myocardial infarction (17).

### 1.3 Heart failure

HF is a clinical syndrome characterised by symptoms of effort intolerance (e.g. breathlessness and fatigue) which may be accompanied by signs of fluid retention (peripheral oedema and pulmonary congestion) caused by structural and/or functional cardiac abnormality, resulting in reduced cardiac output and/or elevated intracardiac pressures at rest or during stress (16). This dysfunction is typically due to the irreversible loss of functional cardiac tissue following MI (18). HF encompasses a wide range of patients, including those with normal LVEF, preserved LVEF and those with reduced LVEF (considered as <40%). Diagnosis of HF can be challenging, as typical symptoms are not specific to the condition and so do not help to distinguish between HF and other disorders. The New York Heart Association (NYHA) functional classification can be used to standardise the terminology used to describe the severity of symptoms and exercise intolerance (Table 1.1) (16).
Table 1.1 NYHA Functional Classification System. Patients in class I have no symptoms, while those in II, III or IV exhibit mild, moderate or severe symptoms, respectively (19).

<table>
<thead>
<tr>
<th>Class</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>No limitation of physical activity. Ordinary physical activity does not cause undue breathlessness, fatigue, or palpitations.</td>
</tr>
<tr>
<td>II</td>
<td>Slight limitation of physical activity. Comfortable at rest, but ordinary physical activity results in undue breathlessness, fatigue, or palpitations.</td>
</tr>
<tr>
<td>III</td>
<td>Marked limitation of physical activity. Comfortable at rest, but less than ordinary physical activity results in undue breathlessness, fatigue, or palpitations.</td>
</tr>
<tr>
<td>IV</td>
<td>Unable to carry on any physical activity without discomfort. Symptoms at rest can be present. If any physical activity is undertaken, discomfort is increased.</td>
</tr>
</tbody>
</table>

Despite advancements in medical therapies to prevent and treat HF, survival rates are poor with >50% mortality within 5 years (20). Heart transplantation is the most effective treatment for end-stage HF patients and the only therapy to address the loss of cardiomyocytes. Transplantation has the potential to extend life by an average of 10 years, but this option is limited by donor supply (21). While patients wait for a suitable donor, ventricular assist devices (VAD) may be implanted to prolong survival in a practice known as Bridge-to-Transplantation. VADs may also be used to give lifelong support to patients not considered suitable for transplantation due to age or other diseases (22). Alternative and complementary strategies employed in the treatment of HF include pharmacological interventions and exercise (23).

The first generation of VADs were large, pulsatile and contained artificial valves. These initial pumps had many disadvantages including a short battery life (provided 3-5 hours of charge), multiple moving parts and a high risk of thrombus formation due to large blood-contacting surfaces. Second generation VADs were designed to be smaller and to facilitate continuous flow using a valveless axial pump with an internal motor. The third generation of VADs further refined these designs by incorporating contactless bearings and magnetic levitation technology to allow rotation in the pump without friction or wear (24).
As illustrated in Fig. 1.4, modern VADs have three main features:

1. The inflow cannula, a tube that drains blood from the heart into the pump
2. The pump
3. The outflow cannula, a tube that returns blood to either the aorta (left VAD) or pulmonary artery (right VAD) (25).

Figure 1.4 Fundamental design of a typical VAD. A pump is connected to both the heart and aorta via an inflow and outflow cannula. A driveline exits the skin and a system controller is worn on a belt. Both the pump and controller are powered by external batteries (25).

While VADs have revolutionised advanced HF care, adverse events such as bleeding, infection, arrhythmia, respiratory failure and stroke are common within the first year of implantation (26). In addition, contact between blood and artificial surfaces still poses a problem, necessitating long-term anti-coagulation treatment for patients with VADs.

1.4 Stem cell therapy
Stem cells are defined as unspecialised cells that can give rise to both differentiated daughter cells and cells that maintain their stemness by self-renewal. Pluripotent
stem cells, those isolated from the inner cell mass of a blastocyst (embryonic stem cells) or reprogrammed adult cells (induced-pluripotent stem cells), have the ability to generate all cell types. Multipotent or adult stem cells may only differentiate into certain cell lineages. The principal biological functions of stem cells are to regulate the repair of diseased or damaged tissues and to maintain normal cell turnover in renewable tissues (27). For decades, the heart was considered to be a post-mitotic organ with no stem and/or progenitor cells to potentially differentiate into functional cardiac myocytes (28). This concept was primarily based upon clinical observations: the absence of regeneration following pathologies and the rarity of primary tumours arising from the myocardium (29). However, the long-held view of the adult heart as a terminally differentiated organ without regenerative capacity was discredited by the identification of mitotic nuclei in both normal and pathological myocardial tissue (30,31). As published in a recent consensus statement by leaders in the field of cardiac regeneration, it is now generally accepted that cardiomyocytes renew throughout life (32). In the healthy, uninjured adult heart, the total number of cardiomyocytes remains stable over the human lifespan, with an estimated turnover rate of ~1% per year (33). Interestingly, studies using adult mice indicate that cardiomyocyte renewal rates may be higher after injury (34). The source of the new cardiomyocytes has been attributed to both proliferation of existing myocytes (35) and to stem/progenitor cells residing within the heart or in exogenous niches such as bone marrow (32,36,37).

The concept that stem cells could generate de novo cardiomyocytes raised hope of revolutionising the treatment of heart failure and led to the extensive investigation of numerous cell types based on their ability to generate new cardiac tissue. As depicted in Fig 1.5, initial clinical studies focused on the delivery of ‘first-generation’ cell types, such as bone marrow mononuclear cells (BMMNCs), skeletal myoblasts (SMs) and mesenchymal stem cells (MSCs). Following heterogeneous results, the field partially shifted towards the use of “second-generation” cells including cardiac stem/progenitor cells and lineage-selected cardiopoietic cells in order to accomplish a greater regenerative potential (38–40).
Figure 1.5 Cell types for cardiac cell therapy. Graphic depiction of the progression of cardiac cell therapy trials towards purified cell populations, trials listed, with cell type underneath (41).

Although stem cells were initially envisaged to promote cardiac regeneration by differentiation into functional cardiomyocytes that could integrate mechanically and electrically with the myocardium, this theory has been refuted (42). Today, it is widely accepted that paracrine mechanisms play the primary role in the reparative process observed following cell injection into infarcted hearts (43). The release of paracrine substances including soluble factors, exosomes and non-coding RNAs, enhance cardiac repair by stimulating endogenous progenitor cells, promoting neovascularisation, regulating extracellular matrix, cytoprotection and inhibiting apoptosis, fibrosis and inflammation (44). Stem cells are ideal candidates for reparative therapies as they allow for the delivery of thousands of proteins alongside an entire genome of nucleic acids. This unique property gives stem cells the potential to elicit a multifaceted biological response, as opposed to protein and gene delivery, which are better suited to inducing an intended targeted response. Another advantage of stem cells is their ability to sense and respond to their environment, making them a dynamic therapeutic with the ability to adjust their function according to their surroundings. However, with such advantages comes considerable challenge, as the complexity of cells makes them an intriguing yet
arduous entity to utilise and study (45). The advantages and disadvantages of different cell sources for cardiac cell therapy are discussed in the following sections.

1.4.1 Skeletal myoblasts

Skeletal myoblasts (SMs) were among the first cell types to be considered for transplantation into the damaged heart (46). SMs can be readily obtained by muscle biopsy allowing for autologous cell transplantation and avoiding the risk of graft rejection. Early clinical trials in patients with ischaemic cardiomyopathy illustrated the feasibility of myoblast transplantation as well as their ability to survive and differentiate in the damaged myocardium (47). The MAGIC (Myoblast Autologous Grafting in Ischaemic Cardiomyopathy) trial was the first randomised controlled study on patients suffering from ischaemic heart disease. Results revealed the reversal of left ventricular remodelling but no improvement in left ventricular function and an increased risk of ventricular arrhythmias (48). The inability to improve cardiac function may be due to the inability of SMs to electrically couple with native cardiac tissue or to differentiate into cardiomyocytes (49).

1.4.2 Bone marrow mononuclear cells

The term bone marrow mononuclear cells (BMMNCs) is used to collectively describe the mixed population of mononuclear cells in the bone marrow including monocytes, lymphocytes and three types of stem cells; haematopoietic stem cells, mesenchymal stem cells and endothelial progenitor cells. BMMNCs have been predominantly used in clinical trials for MI to date (50). This heterogeneous mixture of regenerative cell types is an ideal source for autologous cell-based therapy as it can be easily obtained by aspiration of the iliac crest (51). In 2001, preclinical studies in rodents provided the first evidence that BMMNCs could be used to generate de novo myocardium. The injection of bone marrow cells into the infarcted LV of mice was reported to result in the formation of new myocytes and vasculature (52). Implanted BMMNCs were also reported to migrate to the site of injury and to promote neovascularisation around the infarct region (53). Large animal studies have exhibited promising but mixed results in terms of efficacy
In a swine model of MI, the transplantation of haematopoietic stem cells generated no evidence of myocardial differentiation, but an increase in angiogenesis, vasculogenesis and cardiac function was reported (56). Around this time an autopsy study of gender mismatched bone marrow transplantation patients identified bone marrow derived cells as extracardiac cardiopoietic progenitor cells in humans, following the discovery of Y-chromosome-positive cardiomyocytes in the hearts of female subjects (57).

These primary observations formed the basis for large randomised clinical trials in which BMMNCs were transplanted into the heart via intracoronary infusion shortly after acute MI (39). As with the large animal studies, heterogeneous outcomes were observed. While the BOOST (58), REPAIR AMI (59), BALANCE (60) and FINCELL (61) trials showed significant but modest improvement of LVEF in BMMNC treated groups compared to controls, TOPCARE-AMI (62), Leuven-AMI (63) and ASTRAMI (64) reported no significant functional benefits. In their most recent recommendations, the Task Force of the European Society of Cardiology identified the BAMI phase-III randomised controlled trial (RCT) as the only clinical trial with the potential to definitively conclude if bone marrow cells can significantly enhance cardiac function following acute MI (65). The ongoing BAMI trial (NCT01569178) aims to determine the safety of intracoronary-delivered autologous BMMNCs and to investigate their effect on all-cause mortality in patients with LVEF <45% following MI. The results of this trial are eagerly anticipated.

1.4.3 Mesenchymal stem cell/multipotent stromal cell

Mesenchymal stem cells (MSCs) are multipotent stromal cells derived from the mesoderm. MSCs can be easily isolated from many tissues in the body including bone marrow, adipose tissue and the umbilical cord (66). Characterisation of MSCs has proven challenging as no specific marker has been identified. In 2006, the International Society for Cellular Therapy (ISCT) proposed minimum criteria to define human MSCs and standardise cell preparations. Criteria include: adherence to plastic, specific surface antigen expression and multipotent differentiation potential, as outlined in Table 1.2 (67).
Table 1.2 ISCT’s minimum criteria to define human MSC (67).

<table>
<thead>
<tr>
<th></th>
<th>Adherence to plastic in standard culture conditions</th>
<th>Phenotype</th>
<th>In vitro differentiation: osteoblasts, adipocytes, chondroblasts (demonstrated by staining of in vitro cell culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Adherent (≥95% +)</td>
<td>CD105</td>
<td>BM MSCs consist of a single phenotypic population (95% and 98% homogenous at passages 1 and 2, respectively) (68).</td>
</tr>
<tr>
<td>2</td>
<td>Phenotype</td>
<td>CD45</td>
<td>Isolated BM MSCs make up a very small subdivision, 0.0001-0.01%, of nucleated cells in the marrow, where they play a role in maintaining the haematopoietic niche (68).</td>
</tr>
<tr>
<td></td>
<td>Positive (≥95% +)</td>
<td>CD73</td>
<td>As depicted in Fig. 1.6, ex vivo expansion of BM MSCs requires three steps; 1) separation of nucleated cells from nonnucleated cells by Percoll or Ficoll density gradient centrifugation, 2) adherence of cells to plastic tissue culture flasks, and 3) passaging of adhered BM MSCs via trypsinisation (69).</td>
</tr>
<tr>
<td></td>
<td>Negative (≤2% +)</td>
<td>CD90</td>
<td>Isolated BM MSCs consist of a single phenotypic population (95% and 98% homogenous at passages 1 and 2, respectively) (68). Typically, a 10mL bone marrow aspirate sample can generate 50-400 million BM MSCs, allowing for scalable delivery following expansion in vitro (66).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD34</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD14 or CD11b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD79α or CD19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLA-DR</td>
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</tr>
</tbody>
</table>

Bone marrow-derived mesenchymal stem cells (BMMSCs) have been extensively characterised and commonly used in clinical trials for cardiac repair to date (39). BM MSCs make up a very small subdivision, 0.0001-0.01%, of nucleated cells in the marrow, where they play a role in maintaining the haematopoietic niche (68). As depicted in Fig. 1.6, ex vivo expansion of BM MSCs requires three steps; 1) separation of nucleated cells from nonnucleated cells by Percoll or Ficoll density gradient centrifugation, 2) adherence of cells to plastic tissue culture flasks, and 3) passaging of adhered BM MSCs via trypsinisation (69). Isolated BM MSCs consist of a single phenotypic population (95% and 98% homogenous at passages 1 and 2, respectively) (68). Typically, a 10mL bone marrow aspirate sample can generate 50-400 million BM MSCs, allowing for scalable delivery following expansion in vitro (66).
Figure 1.6 Cell transplantation technique using BMMSCs. Bone marrow is harvested before mononuclear cell enrichment using Ficoll gradient. Cells are expanded for 3-4 weeks in tissue culture flasks and then cryopreserved until they are ready to be implanted. Representative fluorescence-activated cell sorting (FACS) analysis of CD105+ expression of MSCs isolated from BM. CD105 expression >90% (69).

BMMSCs are ideal for cell-based therapy in inflammatory diseases due to their low immunogenicity and immuno suppressive properties. They play an important role as regulators of the immune system by suppressing white blood cells and stimulating anti-inflammatory pathways (66). The POSEIDON trial provided evidence for the safety of both allogeneic and autologous BMMSC transplantation in 30 patients with chronic ischaemic left ventricular dysfunction secondary to MI. These findings were of great significance as allogeneic BMMSCs may provide a valuable “off-the-shelf” therapeutic option for patients with impaired endogenous cells due to aging or comorbidity (70).

BMMSCs are known to produce and secrete a wide range of factors that have been shown to contribute to cardiac functional recovery by stimulating endogenous repair mechanisms. Soluble factors released by BMMSCs can stimulate angiogenesis, as well as induce anti-remodelling, anti-apoptotic and anti-
inflammatory effects (69). Several growth factors have been identified in the conditioned medium of BMMSCs including vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), placental growth factor (PGF) and basic fibroblast growth factor (bFGF) (71). More recently, BMMSCs have been shown to release extracellular vesicles and exosomes, which play a role in intracellular signalling. Exosomes are specialised nanosized membrane vesicles, which may contain functional proteins, mRNAs, microRNAs (miRNA) and ribosomal RNAs (72). The delivery of BMMSC-derived exosomes has been shown to reduce inflammation, inhibit fibrosis and improve cardiac function in a rat MI model (73). MSCs may also communicate with other cells by mitochondrial transfer. Co-culture of human MSCs and rat cardiomyocytes led to transfer of mitochondria within nanotubes, illustrating a possible role in cardiac repair (74).

A key disadvantage of BMMSCs is their heterogeneity at multiple levels, including among donors, tissues, clonal subpopulations and single cells. Donor health may affect both the availability and functional potential of MSCs. Likewise, a reduction in MSC availability, self-renewal capacity and differentiation capacity is observed with increasing donor age. Notably, even MSCs from young, healthy donors demonstrate differences in their proliferation rate, differentiation potential and sequentially their clinical utility (75). In addition, MSCs fate may be unpredictable when implanted. A small number of studies found that implanted BMMSCs differentiated into osteoblasts inside ventricular tissue (76). This must be fully assessed prior to full-scale therapy.

1.4.4 Cardiac stem cells

In 2003, Beltrami et al. described the existence of a cardiac stem cell population in the heart based on the presence of stem cell factor receptor CD117 or c-kit. These cells were reported to be self-renewing, clonogenic and multipotent, generating cardiomyocytes, endothelial cells and vascular smooth muscle cells (77). Since then, multiple resident heart populations have been identified based on their expression of different surface markers (Table 1.3). Given the considerable overlap of the main
and secondary markers used for their characterisation, it is likely that these different populations are closely related (78).

Table 1.3 Summary of endogenous CSC populations (78).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Markers</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Kit+ CSCs</td>
<td>CD34+, CD45-, Sca-1-, Abcg2-, CD105*, CD166+, GATA4+, NKK2-S-+/−, MEF2C*</td>
<td>Mouse, rat, dog, pig, human</td>
</tr>
<tr>
<td>Sca-1+ CSCs</td>
<td>CD34+, CD45-, FLK1+, c-Kit+, Sca-1+, MEF2C*</td>
<td>Mouse, human</td>
</tr>
<tr>
<td>Side population cells</td>
<td>CD34+, CD45-, Abcg2-, Sca-1-, c-Kit+, NKK2-S-, GATA4*</td>
<td>Mouse</td>
</tr>
<tr>
<td>Cardiosphere-derived cells</td>
<td>CD105+, CD45-, CD166-, Abcg2+, Sca-1-, c-Kit*</td>
<td>Mouse, rat, dog, pig, human</td>
</tr>
<tr>
<td>Colony-forming unit fibroblasts (CFUs)</td>
<td>CD31+, CD45-, CD44+, CD45+, CD105*</td>
<td>Mouse</td>
</tr>
<tr>
<td>Cardiac mesangioblasts</td>
<td>CD31+, CD45-, CD44+, c-Kit+, Sca-1-, c-Kit*</td>
<td>Mouse, human</td>
</tr>
<tr>
<td>Isl1+ CPCs</td>
<td>CD31+, Sca-1+, c-Kit+, GATA4+, NKK2-S-</td>
<td>Mouse, rat, human</td>
</tr>
</tbody>
</table>

In the mammalian heart, clusters of CSCs cells are found predominantly in the atrial and apical myocardium at a very low density (1 cell per every 10,000 myocytes) (79). Given the scarcity of CSCs, an optimised protocol has been developed to isolate and characterise these cells (78). CSCs can be expanded over long-term culture and maintained in a self-renewing, undifferentiated state, without demonstrating chromosomal or growth abnormalities (80).

The predominant view is that the positive effects of CSC therapy are mediated by paracrine release of anti-apoptotic, pro-angiogenic and immunomodulatory effects that modulate the function of cells in the target tissue (81). While c-kit+ cardiac cells are the most extensively characterised and studied to date, this heterogeneous mixture of is poorly understood and is mainly composed of c-kit+ mast and endothelial/progenitor cells (>90%). This heterogeneity has generated confusion and controversy about the existence and function of c-kit+ CSCs in the adult heart. A recent study concluded that true CSCs represent just 1-2% of the cardiac c-kit+ cell population. Future work should consider additional markers when identifying and isolation endogenous CSCs in order to enhance the therapeutic effect of c-kit+ CSC therapy (82).

1.4.5 Embryonic stem cells

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass of a blastocyst. These cells have huge potential in the field of regenerative medicine
due to their ability to self-renew in culture while retaining the capacity to differentiate into any cell type, including cardiomyocytes (83). The first ESC lines were derived in 1981 from the inner cell mass of mouse blastocysts (84). ESC lines were not successfully derived from humans until much later in 1998 (85). In animal models, the delivery of human ESC-derived cardiomyocytes to the heart has demonstrated the ability of these cells to integrate and mature in the myocardium and to electrically couple with the host tissue (86,87). However, the clinical use of ESCs has been hindered by reports of teratoma formation, severe immunological rejection and ethical considerations related to the origin of these cells (88,89).

### 1.4.6 Induced pluripotent stem cells

The genetic reprogramming of differentiated human somatic cells back to a pluripotent state by the retroviral induction of transcription factors Oct3/4, Sox2, c-Myc and Klf4 has led to the development of induced pluripotent stem cells (iPSCs) with ESC properties. iPSCs can be generated using various adult cell types and are indistinguishable from ESCs in terms of morphology, gene expression, proliferation and teratoma formation (90). iPSCs are a suitable cell source for cardiac regeneration due to their ability to create unlimited autologous cells while also overcoming the ethical and immunological issues surrounding ESCs. However, safety issues including a risk of tumorigenesis currently limit their use in clinical trials (91). Recent advances have uncovered methods of reprogramming differentiated cells without exogenous DNA integration in the hope of advancing iPSC technology towards clinical applications (92).

### 1.5 Critical factors affecting the efficacy of cardiac cell therapy

Over the past two decades, thousands of patients with heart conditions have undergone trials to test different cell types based on their ability to repair injured myocardium. While the clinical safety profile has been satisfactory, over-all efficacy has been modest and unconvincing. Given the lack of conclusive evidence, lead researchers in the field are faced with justifying the continued analysis of stem cells in humans as a treatment for ischaemic heart disease (93,94). Amidst rising scepticism, it is important to emphasise that cardiac cell therapy cannot be deemed
ineffective based on results to date, as many critical limitations have come to light (95). Clinical translation has been hindered by the lack of consensus regarding clinical trial methodologies, poor knowledge surrounding the reparative mechanisms of stem cells and uncertainty in identifying the ideal cell source, delivery method and timing. Regardless of cell type, the efficacy of cell therapy is limited by poor engraftment as a result of low cell retention and a high degree of cell death in the harsh ischaemic microenvironment of the infarcted heart. Until these problems are addressed, it is unlikely the full therapeutic potential of cell therapy will ever be realised (96). Critical factors affecting cell therapy are discussed in further detail below.

1.5.1 Delivery route

In most clinical trials, stem cells are delivered to the heart suspended in saline, which cannot efficiently retain cells at the injection site due to its low viscosity, nor can it provide a matrix for cell interactions (58,97). Unsurprisingly, both preclinical (98,99) and clinical studies (100,101) have demonstrated that retention does not exceed 10% of the volume delivered in the heart 24 hours post-delivery. Further analysis in large animal models of MI have shown that regardless of delivery modality, the majority of transplanted cells egress from the heart and become distributed to non-target organs including the lungs, liver, kidneys and spleen (98). This phenomenon can be attributed to leakage from the injection site, mechanical washout due to contraction of the heart muscle as well as dispersion through the vasculature (102).

Three principal delivery routes have been utilised in clinical studies to date:

1. Intravenous (IV) injection, where cells are delivered to a peripheral vein (103).
2. Intracoronary (IC) infusion, where cells are administered to the coronary artery (58).
3. Intramyocardial (IM) injection, where a cell suspension is injected directly into the myocardium (104).
The principal delivery routes are depicted in Fig 1.7.

**Figure 1.7 Stem cell delivery to the heart** (98).

The delivery of cells by IV injection is the least invasive route, whereby cells are infused into the venous blood supply and anticipated to home to the injured myocardium. However, this technique demonstrates low accumulation in the heart, with most cells becoming sequestered in the lungs (105,106). The majority of clinical studies to date have delivered cells via IC infusion (107). Given the familiarity of cardiologists with the procedure, this approach can be easily incorporated into clinical practice. Cells may be administered directly to the site of injury either while maintaining coronary blood flow or following balloon occlusion to interrupt blood flow and minimise cell washout (108). As with IV delivery, cells implanted by IC infusion can be rapidly lost through the circulatory system as retention is reliant on transendothelial passage and migration of cells into the myocardium (95). IM injection on the other hand is a highly invasive technique but it allows for the precise targeting of the infarct and border regions (98,109). IM delivery of cells of can be performed by three routes: epicardial, endocardial and transcoronary (95). Cells are generally injected into the peri-infarct area to ensure they have access to an adequate blood supply to aid their survival, however, if cells are to replace the dead tissue they will need to migrate to the site of injury (110).
While there is no consensus regarding the optimal delivery route, numerous preclinical trials have indicated IM injection is the most efficient. In an ischaemic swine model, significantly more cells were retained within the myocardium 1 hour after IM injection (11%) compared with IC delivery (2.6%). A recent preclinical study examined the retention of fluorescently labelled CSCs 4 hours after injection using state of the art positron emission tomography (PET) and computerised tomography (CT) imaging. While no significant difference was observed in the retention of cells by either IM (13.4%) or IC delivery (17.4%), cell engraftment, measured 3 days post-injection, was only evident following IM injection (111).

In addition to promising preclinical results, multiple trials have demonstrated the safety and feasibility of IM cell administration in patients with ischaemic HF (112–115), but the efficacy has not been established. In a phase-I trial, Krause et al. transplanted 200 million BMMNCs in 20 patients by 20 small-volume injections (100µl) 10.5 days after PCI. Although patients achieved a significant increase in LVEF after 6 months, strong conclusions in terms of efficacy could not be drawn due to the lack of a randomised blinded control group (112). Efficacy was reported in the C-CURE trial (phase II) in which patients received autologous cardiopoietic cells by transendocardial IM delivery. Treated patients demonstrated a statistically significant improvement in LVEF (27.5±1.0 to 34.5±1.1%) compared to standard care alone (27.8±2.0 to 28.0±1.8%, p<0.0001), as well as a statistically significant improvement in the 6-minute walk distance (114).

Following these positive results, the CHART-1 (Phase III) clinical trial was initiated. Eligible patients were either administered cardiopoietic cells by IM delivery (n=120) or underwent a sham control procedure (n=151). IM injections were performed using a cell retention-enhanced injection catheter (C-Cath™, Celyad, Mont-Saint-Guibert, Belgium). Although safety was demonstrated across the cohort, the trial failed to meet its primary endpoint: a statistically significant improvement of outcome for patients with chronic advanced ischaemic HF. The CHART-1 trial did claim to identify a clinically relevant patient population that appear to obtain
consistent benefit from the stem cell treatment as regards the primary endpoint (115).

1.5.2 Timing

The timing of cell administration may be a critical factor in the efficacy of cell therapy due to the adaptive environment in the myocardium in the days following ischaemic insult. Given that the optimal timing of cell delivery following MI has not yet been established, several investigations have focused on this issue to date. The MYSTAR trial combined IC and IM delivery in a bid to improve outcomes for BMMNC therapy. In addition, this study assessed the influence of timing on efficacy of cell therapy after MI. This Phase II trial demonstrated both early (3-6 weeks after MI) and late (3 months after AMI) combined cell delivery improved infarct size and global ejection fraction, but did not report a significant difference between early and late treatment (113). In contrast to the positive functional effects observed in the MYSTAR trial, multiple clinical studies have failed to show an improvement in LV function regardless of delivery timing. SWISS-AMI reported no functional benefits at 1-year follow up following the IC delivery of autologous BMMNCs either 5-7 days (early) or 3-4 weeks (late) after MI, compared to control (116). However, these results may have been limited by an important dropout rate (117). The TIME and LateTIME trials reported no significant improvement following the IC delivery of BMMNCs at either 3-7 days and 2-3 weeks post AMI, compared to placebo (118,119).

Interestingly, the REGENERATE-AMI trial demonstrated that early IC delivery of BMMNCs (within 24 hours of primary PCI) increased myocardial salvage and reduced infarct size in patients with AMI. Treatment also resulted in a small non-significant improvement in LVEF at 1 year compared to control. This study is of great importance as it demonstrates the safety and feasibility of BMMNC cell therapy within the timeframe of standard AMI hospitalisation (48 hours) (120). In light of this small yet significant progress, further trials are warranted to elucidate some of the conflicting results achieved to date.
1.5.3 Cell survival

Cell injection protocols generally result in a reduced number of live cells, with post-transplantation viability as low as 1-32% (121). A lack of matrix support is the first stress that cells face during the engraftment procedure and this occurs even before cells are injected. Adherent cells are kept in suspension, leading to loss of cell-matrix interactions which induces anoikis, a form of programmed cell death due to a lack of ECM support (122). The mechanical disruption of cells during the injection process is also a significant cause of transplanted cell death as the expulsion of cells through a needle exposes them to extensional stretching forces and mechanical shear forces, which may rupture the cell membrane (123). Furthermore, the hostile environment of the ischaemic heart contributes towards low transplanted cell viability as once cells reach the host tissue they are immediately faced with harsh conditions including the deprivation of nutrients and oxygen, acidosis, inflammation and reactive oxygen species. The number of viable cells is further reduced by high mechanical forces during systole and phagocytosis by immune cells removing debris during the repair process (95).

1.6 Strategies to enhance cardiac cell therapy

As illustrated in Fig. 1.8, several methods have been explored to enhance the longevity of stem cells in the ischaemic myocardium. Strategies include the preconditioning of cells before transplantation, the genetic manipulation of cells, combined administration with another cell type or pharmaceutical compound and tissue engineering approaches (124).
1.6.1 Preconditioning

Preconditioning of stem cells has been proposed as a method to improve the adaptability and viability of cells in the harsh environment of the ischaemic heart. Evidence of the protective effect of preconditioning was first reported in 1986,
when researchers discovered that brief ischaemic episodes could protect the heart from subsequent ischaemic injury in a canine model of MI (125). Since then, several strategies have been explored including exposing cells to hypoxia, cytokines or heat shock (126). Hypoxic preconditioning of MSCs (0.5% oxygen for 24h) was demonstrated to upregulate several pro-survival factors including Bcl-2, Bcl-xL and hypoxia-inducible factor 1 (HIF-1), as well as pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO). When transplanted in to a rat model of MI, hypoxic preconditioned MSCs displayed enhanced survival and increased angiogenesis in the heart (127). Anoxic preconditioning has also been shown to enhance the cardioprotective effect of MSCs and increase their capacity to promote functional recovery through the release of paracrine factors (128).

The pre-exposure of cells to cytokines has also been successful in enhancing transplanted cell viability. Insulin like growth factor 1 (IGF-1) is a well-known activator of Akt, which is a serine-threonine kinase in the PI3-kinase signalling pathway and a key mediator of cell survival. Akt is upregulated by hypoxia and reduces cell death by acting on apoptotic molecules Bcl-2 and caspases. Akt can also increase cellular glucose metabolism and promote energy production during hypoxia. The multifaceted effects of Akt make it an attractive target to enhance cell therapy (129). The survival of IGF-1 preconditioned Sca-1+ cells was found to be 5.5-fold higher than non-preconditioned cells, 7 days after transplantation in a rat model of MI (130). Preconditioning with the chemokine stromal-derived factor 1 alpha (SDF-1), a chemotactic factor for lymphocytes, has also been shown to significantly increase the longevity of cells through the Akt signalling pathway (131). SDF-1 treated MSCs were found to enhance angiogenesis, and reduce LV remodelling and infarct size (131). Preconditioning of MSCs with transforming growth factor-alpha (TGF-α) (132) and platelet-derived growth factor-BB (PDGF)-BB (133) has been shown to induce MSC VEGF production and improve MSC-mediated cardioprotection following ischaemic injury.
Heat shock treatment is an attractive method due to its low cost, simplicity and potency. Heat shock is typically induced by exposing cells to 43°C for 30-60 minutes, one day before transplantation. This episode of non-lethal thermal stress increases the expression of anti-apoptotic heat shock proteins (HSPs), which may play a role in enhanced cell survival (129). Following exposure to elevated temperature, MSCs have been shown to increase their expression of HSPs 27 and 70 (134). HSP 70 is well-known for its ability to limit cell death by inhibiting pro-apoptotic signalling (135). Upregulation of HSP 70 by heat shock was found to promote the survival of Sca-1+ stem cells after oxygen glucose deprivation. Transplantation of these cells in vivo was shown to significantly reduce apoptosis, ameliorate cardiac fibrosis and improve heart function (136). Lastly, as oxidative stress plays a central role in transplanted cell death, strategies to directly counteract this stressor are under analysis (137). Pretreatment of cells with low-concentrations of H2O2 has been demonstrated to protect human umbilical cord-derived MSCs and prevent their damage when exposed to high-concentrations of H2O2 (138). However, further studies are required to elucidate the underlying process involved in this protection.

While the preconditioning of stem cells for therapy is a simple method to adopt and has achieved desirable outcomes it has some limitations. For example, the exposure of cells to hypoxia must be carefully regulated as oxygen deprivation can be detrimental. Hypoxia preconditioning must be performed in a well-established chamber system to avoid discrepancies in oxygen concentrations between different laboratories. Preconditioning with chemical factors is also challenging as cytokine treatment of MSCs may promote tumorigenesis (139).

1.6.2 Genetic modification

Although gene modification is more labour intensive than preconditioning, it has the potential to influence stem cell viability, function, contractility, proliferative capacity and differentiation (139). By uncovering the signalling cascades involved in cell survival, strategies to exploit these pathways can be developed (140). As highlighted in the previous section, Akt is a principal mediator of cell survival. Transplantation of Akt-upregulated MSCs has been shown to prevent myocardial
remodelling and repair infarcted myocardium (141). Zhang et al. demonstrated that genetic engineering of MSCs to overexpress SDF-1 could enhance MSC survival in hypoxia and serum deprived conditions. They also found SDF-1 transfected MSCs exhibited greater retention in the heart, compared to non-engineered MSCs in vivo (142). In a study by Tang et al., vector modification of MSCs with the anti-apoptotic and anti-oxidant enzyme heme oxygenase-1 (HO-1) was shown to increase the survival of MSCs in the infarcted heart by 5-fold. The increased tolerance of engrafted MSCs led to enhanced functional recovery of the heart and improved LV remodelling two weeks after MI (143).

Although gene therapy has shown promising results, clinical progression faces several problems. These include developing methods to promote transfection efficiency, limiting the mutagenic potential, reducing cytotoxicity and controlling gene targeting. While both viral and non-viral vectors may be employed as gene transfection agents, viral vectors including adenoviruses and retroviruses are superior due to their high transfection efficiency and long term-regulation of gene expression. However, the utilisation of viral vectors is surrounded by safety concerns due to their immunogenicity (139). To overcome these limitations, research has focused on the bioengineering of stem cells with microRNAs (miRNAs), which can influence cell behaviour without jeopardising genomic stability (45). MiRNAs are small noncoding RNAs that act as negative regulators of protein coding genes. The transfection of cardiac progenitor cells (CPCs) with a combination of 3 miRNAs was found to significantly increase CPC engraftment through inhibition of pro-apoptotic pathways, resulting in significant improvement in heart function in a mouse model of MI (144).

1.6.3 Co-administration: cells and small molecules
As previously described, both CSCs and BMMSCs are good candidates for stem cell therapy to treat HF. Following reports that BMMSCs can interact with CSCs to influence their proliferation and differentiation, preclinical studies have been performed to assess the effect of co-delivery of BMMSCs with CSCs to the infarcted heart (145). Williams et al. demonstrated that IM injection of BMMSCs with c-kit+...
CSCs resulted in a 2-fold greater reduction in scar size compared to either cell type administered alone in a large animal model of MI (146). Further investigations to unveil the mechanisms responsible for this synergistic effect discovered that the co-injection of CSC and BMMSCs maximally enhances cell survival and proliferation in the myocardium post-transplantation via the Akt signalling pathway. The increased number of cells increased the secretion of pro-angiogenic factors compared to transplantation of CSCs or BMMSCs alone, leading to superior cardiac function and reduced scar size after MI (147). The combined administration of MSCs with haematopoietic stem cells (CD34+ cells) has also been shown to amplify the therapeutic effect of transplanted cells, with co-transplantation resulting in significantly decreased scar tissue and increased vascular density in a small animal model of MI, compared with transplantation of MSCs or CD34+ cells separately (148).

Statins, a family of drugs used to lower cholesterol, have been used to enhance the therapeutic effects of stem cell therapy. Aside from their lipid lowering abilities, statins have many cardioprotective effects. In a large animal study by Yang et al., Atorvastatin/stem cell combined therapy was shown to significantly reduce oxidative stress, suppress inflammatory cytokines and inhibit cardiac cell apoptosis in the post-infarct myocardium. This led to the increased survival of implanted MSCs and enhanced their therapeutic benefit (149). In another study, Yang et al. demonstrated that the combination of Simvastatin (0.25mg/kg/day) and MSCs resulted in approximately 4-fold higher MSC survival rate compared with MSC alone. This was attributed to the fact that Simvastatin significantly reduced inflammation and oxidative stress in the infarcted myocardium (149). These results were further supported in a study showing that treatment with Rosuvastatin could enhance adipose-derived stem cell (ADSC) survival 1.3-fold compared to MSCs alone in infarcted hearts (150).
1.6.4 *Tissue engineering strategies*

Although the preconditioning, genetic manipulation and co-transplantation of cells has been reported to extend the survival of transplanted cells, these approaches are unable to address the physical factors affecting cellular retention including dispersion from the injection site and anoikis due to a lack of attachment sites. Tissue engineering strategies aim to overcome these issues. To date, two biomaterial approaches have been utilised to enhance transplanted cell retention: injectable hydrogels, which may be administered directly into the myocardial wall and patches, which may be affixed to the epicardial surface. In the case of injectable hydrogels, cells are suspended in the biomaterial prior to transplantation and injected through a needle into the ventricular wall (epicardial injection) or a percutaneous catheter (transendocardial injection). Ideally, the biomaterial carrier should remain a liquid during the injection process, before rapidly forming a gel *in situ* to avoid cell washout (45). Another injectable method is the use of microspheres; cells may adhere to the microspheres or be encapsulated within them for delivery to the infarcted heart (151).

An advantage of patch-based strategies is that these tissue-like structures are fabricated *in vitro* before transplantation, allowing cell proliferation, differentiation and tissue structure to be manipulated under controlled culture conditions before delivery (45). However, patches may be subjected to poor nutrient diffusion that restricts the thickness of the material and long term culture *in vitro* (152). Three-dimensional (3D) bioprinting can be used to overcome this limitation through the generation of porous scaffolds. Studies have demonstrated CSCs can be successfully printed in both gelatin/hyaluronic acid and alginate scaffolds (152,153). CSCs printed in porous alginate scaffolds demonstrated preserved viability (88% at 1 week) compared with a non-porous structures (62% at 1 week) (153). As with other patch methods, 3D-printed scaffolds can be implanted *in vivo* to enhance cell survival and ameliorate adverse remodelling (152). The advantages of both injectable and patch-based approaches are outlined in Fig 1.9. As patch implantation requires an invasive surgical procedure, it is believed that the use of injectable biomaterials, which can be delivered in a minimally invasive manner, will
facilitate more rapid clinical translation. The initial fluid-like properties of injectable materials allow for IM delivery using a procedure and delivery route similar to PCI, enabling clinicians to easily master this approach and minimise technical challenges.

![Diagram of injectable material and patch-based material applications]

**Figure 1.9** Tissue engineering strategies to enhance cell delivery to the infarcted myocardium.

Injectable carriers and patches are the two major engineering approaches currently under investigation to improve cardiac cell therapy. For injectable approaches, either (A) cross-linking materials or (B) microcarrier system can be used to deliver stem cells directly into the myocardium. Patches can be fabricated by (C) direct combination of cells and material, (D) cell seeding of pre-formed scaffolds, (E) 3D-printing of bioink containing cells or (F) generation of material-free cell sheets which can subsequently be transplanted epicardially on the surface of the injured heart (45).

1.7 Hydrogels for cell delivery

Hydrogels are three-dimensional, highly hydrated, polymer networks (154). They can be injected as a liquid and then crosslinked to a gel phase *in situ* or may
undergo shear thinning during injection to permit flow (155). Gel formation after injection permits cell delivery in a minimally invasive manner. In situ gelation may occur by photo-crosslinking, ionic crosslinking or pH-dependent crosslinking, Alternatively, thermo-sensitive hydrogels can be designed to undergo gelation at body temperature (156). Hydrogels can be derived from either natural or synthetic sources (155). Natural hydrogel materials refer to those isolated from biological sources including collagen (157), gelatin (158), alginate (159), hyaluronic acid (159) and dextran (159). Typical synthetic hydrogels include polyethylene glycol (PEG) (160), polyvinyl alcohol (PVA) (161) and polyvinylpyrrolidone (PVP) (162).

For cell delivery applications, natural biomaterials are commonly used due to their inherent bioactivity, biocompatibility and biodegradability. Many natural hydrogels have the ability to facilitate cell adhesion and proliferation through intrinsic peptide sequences. However, their production may be inconsistent, with batch-to-batch variations in their composition and physical features. Furthermore, naturally derived hydrogels generally have weak mechanical properties (163). In contrast, synthetic biomaterials have a precise manufacturing process which permits control of their composition and physical properties. However, a significant shortcoming in the use of synthetic hydrogels is the lack of recognition sites which may interact with cells to initiate a desirable response (164). For this reason, natural hydrogels are preferential for tissue engineering applications due to their intrinsic ability to generate scaffolds that are analogous to native tissue (165). The advantages and disadvantages of natural and synthetic hydrogels are outlined in Table 1.4.

Table 1.4 Summary of advantages and disadvantages of natural and synthetic materials (166).
In 2004, the first reports on using injectable biomaterials as a delivery vehicles to increase the retention and survival of cells for the treatment of MI were published (167,168). Since then, a wide variety of biomaterials have demonstrated the ability to increase cellular retention in the heart compared to saline controls (Table 1.5).

Table 1.5 Fold-increase in cell retention over saline delivery reported with various injectable hydrogels.

<table>
<thead>
<tr>
<th>Study</th>
<th>Hydrogel</th>
<th>Time of analysis</th>
<th>Fold increase in retention compared to saline control</th>
</tr>
</thead>
<tbody>
<tr>
<td>(169)</td>
<td>Hyaluronic acid</td>
<td>48hr</td>
<td>~3.5</td>
</tr>
<tr>
<td>(170)</td>
<td>Chitosan</td>
<td>24hr</td>
<td>~1.5</td>
</tr>
<tr>
<td>(171)</td>
<td>Hyaluronic acid-Gelatin</td>
<td>24hr</td>
<td>~7</td>
</tr>
<tr>
<td>(172)</td>
<td>Chitosan</td>
<td>24hr</td>
<td>~4.7</td>
</tr>
<tr>
<td>(173)</td>
<td>Fibrin</td>
<td>90min</td>
<td>~1.76</td>
</tr>
<tr>
<td>(174)</td>
<td>PEG-based</td>
<td>4 weeks</td>
<td>~2.5</td>
</tr>
<tr>
<td>(175)</td>
<td>Hyaluronic acid</td>
<td>2 months</td>
<td>~2</td>
</tr>
</tbody>
</table>

In addition to their use as cell delivery vehicles, hydrogels may be used to stabilise the heart wall and prevent adverse LV remodelling by bulking or stiffening in the infarct region (176). To date, two natural acellular biomaterials, alginate (NCT01311791 and NCT01226563) and ECM (NCT02305602), have progressed to clinical trials. Algisyl-LVR™ was the first-in-man trial to demonstrate the safety and feasibility of alginate biopolymer delivery in patients with dilated cardiomyopathy (177). This study aimed to investigate the ability of alginate to improve global function by modifying LV geometry to reduce LV size/increase wall thickness as shown in Fig 1.10.
While patients who received Algisyl-LVR achieved a statistically significant improvement in VO$_2$ at anaerobic threshold, 6-minute walk test distance and NYHA functional class, implantation required open chest surgery and full anaesthesia (179). In the interest of reducing invasiveness and achieving faster recovery times, catheter-based approaches are currently under consideration. For this purpose, Rodell et al. recently developed shear thinning, injectable hyaluronic acid (HyA) based hydrogels with clinically relevant properties for percutaneous IM delivery (180). HyA is a promising biopolymer for cardiac tissue engineering as several HyA systems have already progressed to clinical use in humans and veterinary patients as dermal fillers, intra-articular viscosupplements and for wound repair (154).

### 1.7.1 Hyaluronic acid hydrogel

HyA is a linear polysaccharide composed of repeating disaccharide units of β-1,4-D-glucuronic acid-β-1,3-N-acetyl-D-glucosamine. It is a principal component of the ECM with high concentrations typically found in the skin, cartilage and brain. Industrial manufacture of HyA is reliant on two main processes, extraction from animal sources such as rooster combs and microbial fermentation in bacterial strains such as *Escherichia coli* (181). HyA is a particularly attractive material for tissue engineering due to its ability to interact with cells *via* the CD44 receptor, its degradability *via* hyaluronidase and reactive oxygen species and its involvement in

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*Figure 1.10* Schematic of Algisyl-LVR™ implantation. Algisyl-LVR is injected at 10-15 locations in the LV (178).
the development, structure and function of normal adult tissues. Native HyA has a short half-life and poor mechanical properties, therefore its clinical use is generally limited to viscosurgery applications (182). To overcome this drawback, HyA can be covalently crosslinked to create hydrophilic matrices with improved mechanical and degradation properties. For cell encapsulation, covalent crosslinking of HyA must be non-cytotoxic and should occur without the generation of toxic by-products. Furthermore, hydrogel gelation should occur rapidly to allow for in vivo hydrogel formation following delivery of cells in a minimally invasive manner (183).

As stem cells are anchorage-dependent, they require adhesion to the matrix and a spreading morphology to prevent anoikis (184). Although HyA interacts with cells through cell surface markers including RHAMM (CD168) and CD44, it does not support integrin-mediated cell adhesion (185). Integrins are a large family of heterodimer transmembrane receptors that connect the intracellular cytoskeleton to the ECM. Integrins are considered unique as they signal bi-directionally, meaning extracellular stimuli can induce intracellular changes and vice versa. Normally integrins are expressed on the cell surface in an inactive conformation, unable to bind to the ECM. Once activated, the individual integrins may form clusters to generate tight bonds to the ECM. Receptor activation initiates intracellular changes such as the rearrangement of the cytoskeleton to allow cells to adopt their characteristic morphology and initiate migration by connections via integrins and actin filaments (F-actin). Long-term attachment activates gene expression and signalling cascades that influence the survival, growth and differentiation of cells (186). Given the importance of stem cell adhesion to the biomaterial scaffold, adhesive ligands are commonly incorporated into HyA to enable cell-matrix interactions and influence cell behaviour.

Arginylglycylaspartic acid (RGD) is a tripeptide sequence composed of L-arginine, glycine and L-aspartic acid, that is found in many natural adhesive proteins including fibronectin, vitronectin, laminin and collagen type 1 (166). This physiologically ubiquitous binding motif is one of the most commonly used in tissue engineering (166,187). Cell adhesion to RGD has been shown to be comparable to
that observed using full length proteins (188). RGD plays a role in cellular attachment and spreading as well as in both actin-skeleton and focal-adhesion formation with integrins (189). The anchorage of cells to the matrix via RGD allows cells to signal bi-directionally across their membrane to engage in a dynamic relationship with the hydrogel (190). Functionalisation of biomaterials with RGD has been shown to improve the adhesion and spreading of multiple cell types. In a two dimensional (2D) hydrogel study, fibroblast cells seeded atop RGD-modified HyA hydrogel demonstrated enhanced attachment and spreading compared to cells on unmodified HyA hydrogel, which remained rounded (187). Successful attachment to RGD was also observed in another 2D culture system using chondrocytes, whose reported adherence was 10-20 fold higher on the surface of RGD-modified alginate than on the surface of unmodified alginate (191). In a more recent study, significant cell spreading was demonstrated in both 2D and 3D culture conditions using RGD-modified alginate flat hydrogel and RGD-modified alginate microspheres, respectively. Significant spreading in the presence of the RGD peptide was shown using three different cell types; human embryonic palatal mesenchyme (HEPM) cells, BMMSCs and adipose-derived stem cells (ADSCs) (192).

Traditional 2D culture systems are generally more suited to cells that exist as multilayer sheets in vivo such as endothelial cells and epithelial cells (181,193). In order to accurately examine cellular process and behaviours in vitro there is an increasing demand for the development of 3D cell culture systems that can more accurately replicate the complexity of the native ECM. 3D cultures differ from standard 2D culture with regard to nutrient access, cell-cell interactions and cellular mechanics. Given these disparities, it is unsurprising that several researchers have reported differences in the proliferation, differentiation and gene expression of cells cultured in 2D compared to 3D (194,195).

The aim of Chapter 2 in this thesis was to investigate the viability and morphology of BMMSCs within a 3D hydrogel system; HyA hydrogel modified with the RGD peptide known for its role in cell attachment/adherence. In addition, the effect of cell seeding density on cell viability within the hydrogel was also assessed.
1.8 Growth factors to enhance stem cell survival

Another possible approach to increase cell survival under ischaemic conditions is the combination of cells with growth factors (GFs). GFs are endogenous proteins that bind to cell surface receptors to stimulate multiple cellular functions including:

- Migration - chemotactic effect
- Proliferation - mitogenic effect
- Differentiation - morphogenic effect
- Programmed cell death – anti-apoptotic/apoptotic effect
- Modulation of metabolic activity
- Combinations of any of the above

Commonly used prosurvival factors such as IGF-1 and hepatocyte growth factor (HGF) can be used to improve short-term cell survival. While angiogenic factors such as fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) can be used to enhance long-term cell survival by stimulating angiogenesis (97).

1.8.1 IGF-1

IGF-1 is natural anabolic peptide hormone that is primarily synthesised by hepatocytes in the liver in response to hypothalamic growth hormone (GH), but also produced locally in the heart (Fig. 1.11). It is a small peptide consisting of 70 amino acids and shares 50% homology with insulin. IGF-1 acts on the tetrameric tyrosine kinase receptor IGF-1R, which is found on the surface of most mammalian cells. This receptor has two extracellular α-subunits and two transmembrane β-subunits (196). IGF-1R can form a heterodimer with insulin receptors (IR) and also epidermal growth factor receptor (EGFR) (197,198). The binding of IGF-1 to the α-subunit initiates autophosphorylation of the β-subunit, which activates multiple pathways associated with cell proliferation, differentiation, metabolism and apoptosis (199). Two main pathways have been identified, the phosphatidyl
inositol-3 kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK)/Erk branches (200).

**Figure 1.11** Physiological and biological effects of IGF-1. IGF-1 is synthesised primarily in the liver following stimulation by hypothalamic growth hormone (GH). It is also produced locally in the heart. IGF-1 binds to IGF-1R and some other hybrid receptors. IGF-1 activation stimulates cell survival and growth, protein synthesis and contractility. It also inhibits fibrosis, apoptosis and oxidative stress (196).

Numerous *in vitro* studies have shown that treatment with IGF-1 can successfully increase the survival of various cell types including CSCs (201), muscle cells (202) and neurons (203). However, conflicting reports have been published surrounding its effects on BMMSCs (204–206). GFs typically have multifaceted effects, eliciting a different response depending on the concentration and/or exposure time as well as the target cell phenotype (207).
The aim of Chapter 3 in this thesis was to assess the ability of IGF-1 to increase the proliferation of BMMSCs under standard conditions and to determine if IGF-1 promotes the survival of BMMSCs cultured under adverse culture conditions representative of the infarcted heart.

1.9 The extracellular matrix

Stem cells are regulated by both intrinsic and extrinsic signals. Intrinsic mechanisms include transcription factors produced by the cells, whereas extrinsic factors refer to stimuli from the ECM, growth factors, local microenvironment (niche) and contact with other cells. (208). All cell types are in contact with the ECM, a cell secreted, complex 3D network of extracellular proteins that provides cells with structural and biochemical support (209). Both the composition and structural integrity of the ECM differs between tissues. Molecules in the ECM can be divided into two categories; structural proteins such as collagen, laminin and fibronectin, and proteoglycans, including glycosaminoglycans (GAGs) such as HyA (27). Collagen type I is the most abundant protein in the heart, making up more than 75% of the matrix (27). Other components include collagens III, IV and VI, fibronectin, and the collagen/fibronectin binding glycoproteins nidogen-1 and thrombospondin-1 (210).

Over the last few years, naturally derived ECM proteins such as HyA have been employed as delivery vehicles to enhance transplanted cell viability. However, these single-factor ECM scaffolds over-simplify the complex ECMs found within biological tissues. Few studies have examined the effect of multi-component ECM scaffolds on stem cell survival and function under the condition of hypoxia and reduced nutrients as is present in the infarcted heart (211).

The aim of Chapter 4 was to investigate the ability of RGD–modified HyA hydrogel to increase the survival, spreading and function of BMMSCs under adverse culture conditions representative of the infarcted heart. The pro-survival effect of Nidogen-1-modified HyA hydrogel was also assessed.
1.10 Cardiac stem cell therapy

While many different types of progenitor cells have been isolated and characterised from the adult heart, cardiac stem cells expressing the c-kit\(^+\) receptor have been the most heavily investigated for heart regeneration (212). C-kit\(^+\) CSCs are self-renewing, clonogenic and multipotent, with the ability to form myocytes, smooth muscle cells and endothelial cells (77). Despite their inherent differentiation potential, it is widely accepted that CSCs exert their actions primarily through paracrine effects (212). Consistent with other ex vivo expanded cell products, long-term engraftment in the injured myocardium has been modest. Yet despite low retention rates, treatment with CSCs has demonstrated significant functional benefits (81). Encouraging results from multiple preclinical trials (77,213,214) led to the first in-human trial, SCIPIO, which demonstrated the safety and feasibility of using autologous c-kit\(^+\) CSCs for the treatment of HF resulting from ischaemic heart disease. IC infusion of CSCs was found to improve LV function, decrease infarct size and increase viable tissue (215). Yet although preclinical and a phase I clinical trial have yielded encouraging results, overall the results have been disappointing or modest and their therapeutic benefits have been called into question (93,95).

These comments have ignited research to explore methods to enhance the therapeutic efficiency of CSCs. Roberto Bolli and his team have focused their efforts on establishing the viability of repeated cell doses (94). So far this approach has proven successful using two different cell types, c-kit\(^+\) CSCs and cardiac mesenchymal cells (CMCs) (216,217). When rats with chronic ischaemic cardiomyopathy were infused with 3 doses of CSCs 35 days apart, each dose induced a similar increase in LV function, so that the total cumulative improvement was roughly three times that observed after one dose. As shown in Fig 1.12, the multidose group also exhibited more viable tissue and less scar tissue (216).
Repeated cell administrations are markedly more effective than a single administration. Rats with an old MI received either one or three CPC infusions into the LV cavity. Representative Masson trichrome-stained myocardial sections; scar tissue and viable myocardium are stained blue and red, respectively (216).

Research has also focused on the use of biomaterials to enhance CSC engraftment. A HyA-gelatin hydrogel has demonstrated success in a mouse model of MI. The IM injection of cardiosphere-derived cells (CDCs) encapsulated in a HyA hydrogel with incorporated collagen binding sites demonstrated a 7-fold increase in cell retention compared to the control at 24h post-implantation. The increased retention of cells was associated with improved LVEF, increased angiogenesis and reduced adverse remodelling (171). Very limited results have been published on the effect of RGD immobilisation in scaffolds for cardiac tissue engineering. In an in vitro study by Shachar et al., the presence of RGD immobilised in alginate scaffolds promoted cardiac cell adherence to the matrix, decreased cell apoptosis, improved cell survival and stimulated cardiac muscle tissue organisation (218).

The aim of Chapter 5 in this thesis was to investigate the ability of an RGD-modified HyA hydrogel to increase CSC survival and function under adverse culture conditions representative of the infarcted heart. The ability of IGF-1 to increase CSC survival under adverse culture conditions was also assessed in 2D studies with an aim to including this anti-apoptotic factor as a positive control in the 3D hydrogel studies.
1.11 Aim and objectives

The overall aim of this thesis was to develop a functionalised HyA hydrogel capable of enhancing stem cell viability, attachment and function for the treatment of myocardial infarction.

The specific aims of this study were:

• To determine the suitability of an in vivo crosslinking HyA hydrogel +/- RGD to act as a surrogate matrix to maintain the viability of stem cells for the cardiac cell therapy (Chapter 2).

• To assess the ability of IGF-1 to act as a pro-survival signal to BMMSCs under conditions of hypoxia (1% oxygen) and reduced nutrients, as is present at ischaemic injury sites (Chapter 3).

• To determine if functionalisation of the HyA hydrogel with RGD or nidogen-1 can enhance encapsulated BMMSC viability, attachment and function under both standard and ischaemic culture conditions (Chapter 4).

• To determine the ability of HyA hydrogel modified with RGD to enhance the viability and function of encapsulated cardiac stem cells for cardiac cell therapy under both standard and ischaemic culture conditions (Chapter 5).
Chapter 2: Mesenchymal Stem Cell Cultivation in an Injectable \textit{in situ} Forming Hyaluronic Acid Hydrogel Modified with RGD
2.1 Introduction

Stem cell-based therapies have the potential to dramatically reform current treatment options for patients with ischaemic heart disease. As discussed in the previous chapter, many different cell types have been investigated for this purpose. Of these, mesenchymal stem cells (MSCs) are leading candidates due to their easy isolation, extensive capacity for *in vitro* expansion and multipotent differentiation potential (66). While MSCs can be isolated from almost every tissue of the body, human bone marrow-derived MSCs (hMSCs) are one of the most frequently used cell sources for cardiac regeneration (219). hMSCs are known to migrate to sites of injury, reduce inflammation and fibrosis, stimulate endogenous stem cells and contribute to tissue healing (69). In addition, hMSCs are immunomodulatory and multiple clinical studies have demonstrated that allogeneic hMSC therapy is safe, creating potential for the development of a readily available “off-the-shelf” cellular regenerative product (70,220,221). Clinical trials have reported that hMSCs improve cardiac function and structure in patients with acute MI (220) and ischaemic cardiomyopathy (70). However, the beneficial effects have been modest, with only a small increase in LV function and a minor reduction in infarct size observed (70,220,222,223). The curative potential of implanted hMSC has been hindered by multiple factors including mechanical washout, leakage from the injection site and adverse conditions including hypoxia, ischaemia, inflammation and reactive oxygen species (224,225). The rapid disappearance of cells from the heart makes it difficult to determine the efficacy of cell products (217). Successful clinical translation of hMSC therapy is reliant on the development of novel strategies to improve the survival, retention and engraftment of cells following delivery to the infarcted heart.

While preconditioning and genetic manipulation of cells prior to transplantation has been identified as a means to extend their lifespan, this approach cannot address the physical factors affecting cellular retention including dispersion from the injection site and anoikis due to a lack of attachment sites (226–229). To overcome these limitations, tissue engineering strategies using hydrogels as cell delivery
vehicles are currently under investigation. The combination of cells and hydrogel has led to enhanced cardiac repair in both large and small animal models of MI, compared to injection of cells alone (230–232). Of the synthetic and naturally derived biomaterials that have been examined, HyA-based hydrogels have attracted much interest. From a biomimetic point of view, this natural material is considered superior as its mechanical and structural properties are similar to living tissues and extracellular matrices (184). HyA is a linear polysaccharide that consists of alternating units of a repeating disaccharide, β-1,4-D-glucuronic acid-β-1,3-N-acetyl-D-glucosamine (Fig. 2.1). It is a principal component of the ECM with high concentrations typically found in the connective and neural tissues of vertebrates (233).

HyA is a linear polysaccharide that consists of alternating units of a repeating disaccharide, β-1,4-D-glucuronic acid-β-1,3-N-acetyl-D-glucosamine (Fig. 2.1). It is a principal component of the ECM with high concentrations typically found in the connective and neural tissues of vertebrates (233).

![Molecular formula of hyaluronic acid disaccharide unit (234).](image)

**Figure 2.1** Molecular formula of hyaluronic acid disaccharide unit (234).

HyA is an attractive material for the fabrication of artificial matrices due to its inherent biocompatibility, biodegradability and non-immunogenic characteristics (235). While native HyA has some clinical applications such as viscosurgery, this unmodified molecule is not very useful as a biomaterial due to poor mechanical properties and rapid degradation (182). To solve this issue, the HyA molecule can be modified to generate crosslinkable hydrogels with more robust mechanical properties and an extended biological half-life (236). This chapter reports on an enzyme-mediated hydrogel composed of tyramine substituted HyA (HyA-TA). The crosslinking reaction is triggered by horseradish peroxidase (HRP) and hydrogen peroxide (H2O2) and proceeds under mild conditions that are not toxic to encapsulated cells. To date, several small animal studies have concluded that HyA-TA does not evoke an immune response when injected subcutaneously (237–240).
As illustrated in Fig. 2.2, this gelation kinetic can be exploited to deliver cells directly into the myocardium in a minimally invasive injectable manner with subsequent sol-gel transition \textit{in situ}, trapping cells within a protective matrix and sustaining the release of paracrine factors at the target site (183).

\textbf{Figure 2.2} Diagram illustrating the minimally invasive intramyocardial delivery of stem cells using an \textit{in situ} crosslinking hydrogel. Magnified picture demonstrates tip of catheter in myocardial wall. Extruded gel retains stem cells within the injured tissue (241).

When designing hydrogels for cell encapsulation it is important to consider that stem cells depend upon both biophysical and biochemical environmental signals for their cellular activities (242). Within hydrogels, stem cells are responsive to matrix degradation, topography, mechanical strength and the presentation of growth factors and adherence sites (243,244). Of these stimuli, cell adhesion is of critical importance as many cells, including hMSCs, are anchorage-dependent and require adhesion to the matrix in order to survive. Although cells can bind to HyA \textit{via} several surface receptors, such as CD44 and RHAMM, HyA is unable to interact with key adhesion receptors such as integrins (245). To overcome this, adhesive ligands such as the tripeptide sequence Arg-Gly-Asp (RGD) are typically incorporated into non-adhesive hydrogels to enable cell-matrix interactions and influence cell behaviour (246).
The overall aim of this thesis is to develop a hydrogel delivery system capable of enhancing stem cell survival under conditions representative of the infarcted heart. By incorporating RGD into HyA-TA hydrogels, we aim to provide encapsulated cells with adhesion points within the biomaterial carrier as a means of protecting them from anoikis. To develop this system, it is first necessary to determine the biocompatibility of the HyA-TA-based hydrogels with hMSCs and to explore the effect of RGD on encapsulated cell behaviour under standard culture conditions.

**Objectives**

The overall objective of this chapter was to investigate the potential of an RGD-modified hyaluronic acid hydrogel to maintain hMSC viability and to function as a supportive cell matrix under standard culture conditions

- The first objective was to assess the physical properties of HyA and HyA-RGD hydrogels.

- The second objective was to confirm the biocompatibility of HyA and HyA-RGD hydrogels with hMSCs.

- The third objective was to assess the effect of the RGD adhesive peptide on encapsulated hMSC viability, spreading and function.

- The fourth objective was to explore the effect of cell seeding density on cell viability within the hydrogel.
2.2 Materials and Methods

2.2.1 Materials

Lyophilised HyA-TA and a mixture of HyA-TA/HyA-PH-RGD derivatives with a molecular weight ranging between 240-500kDa were supplied by Contipro (Czech Republic). The degree of substitution (number of tyramine molecules per 100 repeating units of HyA) ranged between 2-4%. The degree of substitution of RGD ranged between 1-1.5%. Contipro also supplied HRP and all hydrogel equipment. 30% H$_2$O$_2$ was purchased from Sigma-Aldrich.

2.2.2 Modification of hyaluronic acid

2.2.2.1 Hyaluronic acid-tyramine (HyA-TA)

This process involves several synthetic steps. Firstly, HyA is partially oxidised at position 6 of N-acetylglucosamine by a TEMPO mediated process. This results in a polyaldehyde of HyA (HyA-CHO) with degree of substitution 3-10%. Degree of substitution is defined as the ratio of modified disaccharide units of HyA to total amount of units. Concomitantly, 6-amino-N-[2-(4-hydroxyphenyl)ethyl]hexanamide (Ahx-TA) is synthesised by acylation of tyramine via 6-Boc-aminohexanoic acid (6-Boc-Ahx) followed by removal of the Boc protective group. The final step of HyA-TA synthesis is conjugation of Ahx-TA with HyA-CHO that is accomplished by reductive amination. Characterisation of crucial intermediates (HyA-CHO, Ahx-TA) and final product (HyA-TA) involves chemical identification (nuclear magnetic resonance spectroscopy, Fourier transform infrared spectroscopy), determination of chemical residues and product purity (high-performance liquid chromatography) and control of microbiological parameters. A general description of HyA-TA conjugate synthesis is depicted in Fig 2.3.
Figure 2.3 Schematic diagram of HyA-TA synthesis.
2.2.2.2 HyA-PH-RGD

The N-terminus of the well-known RGD adhesive peptide was modified by attachment of a linker composed of lysine, 6-aminohexanoic acid (Ahx) and glycine. Furthermore, α-NH2 of lysine was acylated by 3-(4-hydroxyphenyl)propionic acid (HPA). This peptide sequence (PH-RGD) was synthesised according to the Fmoc-SPPS protocol. The product was purified by reverse phase cartridge (RPC) and characterised by proton nuclear magnetic resonance spectroscopy (1H NMR) and mass spectrometry. HyA-CHO was prepared as previously described in section 2.2.2.1. PH-RGD was conjugated with HyA-CHO via reductive amination (Fig. 2.4). The confirmation of structure and determination of degree of substitution was performed by 1H NMR and 2D DOSY NMR. Molecular weight was determined by SEC/MALLS.

![HyA-PH-RGD Synthesis Diagram](image)

**Figure 2.4** Schematic diagram of HyA-PH-RGD synthesis.
The final HyA-TA and HyA-PH-RGD derivatives are shown in Fig 2.5. The studies in this chapter compare:

A) pure HyA-TA, herein referred to as HyA
B) mixtures of HyA-TA and HyA-PH-RGD at a ratio of 1:1, herein referred to as HyA-RGD

![Structures of HyA derivatives](image)

**Figure 2.5** Structures of HyA derivatives.

### 2.2.3 Hydrogel synthesis

To prepare 2% (w/v) hydrogel, lyophilised precursor powder was added to phosphate buffer saline (PBS, Sigma Aldrich) at a concentration of 20mg/mL and stirred overnight for complete dissolution. The precursor solution was subsequently divided into two equal volumes and HRP was added to one solution while H$_2$O$_2$ was added to the other solution. Equal volumes of the HRP and H$_2$O$_2$ precursor solutions were drawn into separate Luer lock syringes before being expelled synchronously through a gel rig containing a static mixer. This was done in a rapid, continuous motion to prevent blockage of the outflow nozzle. The final concentration of crosslinking agents was 0.12 units/mL HRP and 0.0015%/mL (0.495µmol/mL) H$_2$O$_2$. 
Hydrogel formation is based on the enzyme-mediated crosslink reaction of HyA-TA derivative in aqueous medium (Fig. 2.6). The tyramine moiety of HyA-TA molecule is a substrate for HRP-mediated oxidation, which leads to the formation of tyramine dimers. These dityramine bridges crosslink the linear HyA-TA chains to create a polymer network within the whole volume of the sample. In the case of HyA-PH-RGD, the phenolic crosslinkable group (PH) works in the same way as TA. The final concentration of RGD in formed hydrogels ranged between 0.5-0.75%.

*Figure 2.6 Crosslinking of HyA-TA conjugates by enzymatic coupling reaction*
2.2.4 Measurement of physical properties of HyA hydrogels

For physical characterisation, all hydrogels were prepared as cylindrical blocks, 8mm in diameter with a total volume of 200µl unless stated otherwise. Mechanical and physical properties of HyA hydrogels were characterised by measuring gelation time, swelling ratio and compressive modulus.

2.2.4.1 Gelation time analysis

Using a 2-200µl pipetman, HyA-H₂O₂ and HyA-HRP were mixed and pipetted up and down until the solutions could no longer be pipetted. The time at which this happened was designated as the gelation time. This was repeated for HyA-RGD hydrogel.

2.2.4.2 Equilibrium swelling ratio analysis

Hydrogel samples were incubated in PBS for 24h in order to measure their wet weight at maximum saturation. They were subsequently transferred to centrifuge tubes and freeze-dried (Labcono freeze dryer) under a vacuum at -55°C for 48h in order to measure dry weight. The ratio of wet to dry weight was determined as the swelling ratio of the hydrogels.

2.2.4.3 Compressive (Young’s) modulus analysis

Hydrogels were mechanically tested in unconfined compression between impermeable platens using a standard materials testing machine with a 5N load cell with an accuracy of ± 1% (Zwick Z005, Roell, Germany). A preload of 0.01N was applied to ensure that the hydrogel surfaces were in direct contact with the impermeable loading platens and also to determine the height of the samples (distance between the loading platens). Samples were loaded to 20% compressive strain at 0.01mm strain per second. The compressive modulus was determined as the slope between 10% and 20% strain of the resulting stress-strain curve (247).
2.2.5 Human mesenchymal stem cell culture
Human bone marrow-derived mesenchymal stem cells (hMSCs) were isolated from bone marrow aspirates obtained from human volunteers, at REMEDI, the National University of Ireland, Galway. All procedures were carried out with informed consent and ethically approved by the Clinical Research Ethical Committee at University College Hospital, Galway. hMSCs were isolated using standard protocols and stringent analysis of cell phenotype as published in Duffy et al. (248). hMSCs were cultured in T175 tissue culture flasks (Sarstedt, Ireland) using low glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (D6046, Sigma) supplemented with 10% fetal bovine serum (FBS), 100U/mL Penicillin and 100µg/mL Streptomycin (Sigma Aldrich, Ireland) at 37°C in a 5% carbon dioxide (CO₂) environment. Cells were passaged at 80-90% confluency, and were not used beyond passage 5.

2.2.6 hMSC encapsulation in HyA/HyA-RGD hydrogel
A solution of HyA-TA derivative was prepared as before in PBS and filter sterilised using a 0.2µm filter. The precursor solution was divided into equal volumes before HRP was added to one solution and H₂O₂ was added to the other solution. hMSCs were trypsinised and suspended in the HRP precursor solution. Cells were encapsulated as illustrated in Fig 2.7.

![Figure 2.7 Schematic diagram of cell encapsulation within HyA hydrogel.](image-url)
Moulds capable of generating large-volume hydrogels (500µl) were supplied by Contipro. As demonstrated in Fig. 2.8, these were used to form cylindrical constructs.

![Photographs demonstrating hydrogel fabrication using mould which contains loosely fitted caps for easy hydrogel removal.](image)

The final concentration of cells was $1 \times 10^6$/mL. Cell-seeded scaffolds were transferred to 24-well plates and incubated in 2mL of hMSC medium (as described in Section 2.2.5) at 21% oxygen. Full medium changes were performed at regular intervals.

### 2.2.7 Cellular distribution in large and small volumes of HyA hydrogel

To obtain small-volume hydrogel samples, the large-volume (500µl) hMSC-seeded scaffolds were cut perpendicular to the cylindrical base using a sterile blade, generating four fragments of 125µL each. Following culture under standard conditions, hMSC-seeded scaffolds were washed with PBS and stained using LIVE/DEAD™ Viability/Cytotoxicity kit (Life Technologies) according to the manufacturer’s protocol. Afterwards, stained scaffolds were rinsed in PBS to remove unbound stains. Live cells were stained green (Calcein-AM) and dead cells
were stained red (Ethidium homodimer-1). Samples were observed using a Leica DMIL fluorescence microscope (Leica Microsystems, Switzerland).

2.2.8 Effect of RGD on encapsulated hMSC viability, morphology and protein release
In order to yield an adequate number of cells for hydrogel studies, hMSC medium was supplemented with 5ng/mL fibroblast growth factor (FGF, R&D Systems) during hMSC isolation and expansion. FGF-treated hMSCs were harvested and encapsulated in hydrogel (200µl in volume). Samples were cultured under standard conditions for a period of 11 days, with full medium changes on day 1, 4 and 7. hMSC viability, morphology and protein release was assessed following 1, 4, 7 and 11 days in culture.

2.2.8.1 Cell counting kit-8 (CCK8) assay
Encapsulated cell viability was determined using the Cell Counting Kit-8 (CCK8, NBS Biological, EU). CCK8 is a colorimetric method to determine the number of viable cells in proliferation or cytotoxicity assays. In the CCK8 assay, a tetrazolium salt, WST-8, is reduced by dehydrogenases in cells to produce a coloured formazan which is soluble in tissue culture medium. The amount of formazan dye generated is directly proportional to the number of living cells. 100µL of the CCK8 solution was added to each gel containing well (1 gel/mL medium) and the plates were incubated at 37°C. Following an incubation period of 3 hours, 100µL of each sample was transferred to a 96-well plate and optical density (O.D) at 450nm was determined using a plate reader (Varioskan Flash, ThermoScientific, Ireland).

2.2.8.2 Lactate dehydrogenase (LDH) assay
Quantitative measurement of lactate dehydrogenase (LDH, Bio Sciences) released into the media from damaged cells was carried out as a biomarker for cellular cytotoxicity. At each timepoint, 50µl of cell supernatant was transferred to a new 96-well plate for LDH activity analysis. 50µl of LDH reaction buffer was added to each well, followed by incubation at room temperature for 30min. 50µl of stop solution was added and absorbance at 490nm and 680nm was measured using a
plate reader (Varioskan Flash, ThermoScientific, Ireland). Results are expressed as percentage cytotoxicity, whereby LDH release from cells treated with the lysis buffer, Triton X-100, for 24h are taken as 100% dead/positive control.

2.2.8.3 Confocal Microscopy-Live/Dead imaging

hMSC-seeded scaffolds were washed with PBS and stained using LIVE/DEAD™ Viability/Cytotoxicity kit (Life Technologies) according to the manufacturer’s protocol. Afterwards, stained scaffolds were rinsed in PBS to remove unbound stains and a tiled image of the entire cross-section of the hydrogel was captured by a Carl Zeiss LSM 710 confocal microscope using Zen® 2008 software. Two depths were acquired, one at the surface of the hydrogel construct, 100µm below the surface to exclude any inconsistencies in the surface topography and one at the centre, 700µm below the surface of the hydrogel construct. The ‘Analyze Particles’ tool found in FIJI was then used to count the number of live and dead cells found within the thresholded overview images (249). The ratio of live to dead cells was generated based on the total number of cells counted for the combined cross sections.

\[
\text{% live cells} = \frac{\text{Total number of live cells}}{\text{Total live cells} + \text{total dead cells}}
\]

2.2.8.4 Circularity

Cells were imaged as described in Section 2.2.8.3 and the degree of cell spreading and attachment was quantified by measuring cell circularity. Circularity is an index of the compactness of an object, circularity would be one for a circular cell and would decrease as the cell elongated or formed extensions.

2.2.8.5 Dapi/phalloidin staining

Fluorescent staining of nuclei and F-actin was performed as follows: Medium was removed from the well-plates and hydrogels were washed with PBS before fixing for 1h using 1mL of 4% formaldehyde (Sigma). Samples were washed with blocking
solution containing 3% bovine serum albumin (BSA) and 0.5% (w/v) Tween in PBS. Permeabilisation solution consisting of 0.25% Triton X (w/v) in blocking solution was added for 20min. Hydrogels were subsequently stained phalloidin–tetramethylrhodamine B isothiocyanate (Sigma) at 250ng/mL for 30min and DAPI (4’,6-Diamidino-2-Phenylindole, Dihydrochloride, Invitrogen) at 100ng/ml for 10min. Hydrogels were washed in blocking buffer to remove unbound stains before mounting onto glass slides for analysis by confocal laser scanning.

2.2.8.6 VEGF and MCP-1 detection using enzyme linked immunosorbent assay (ELISA)

After 1, 4, 7 and 11 days in culture, spent medium was collected for enzyme-linked immunosorbent assay (ELISA) assessment. VEGF and monocyte chemoattractant protein (MCP-1) ELISAs were carried out according to the manufacturer’s instructions (R&D Systems). Using a microtiter plate reader (Varioskan Flash, ThermoScientific, Ireland) the well plate was then read at an absorbance of 450nm with correction at 570nm. The readings were converted to a concentration using a standard curve generated.

2.2.9 Effect of high seeding density on cellular viability and hydrogel formation

In order to generate a high number of cells, rat bone marrow-derived mesenchymal stem cells (rMSCs) were expanded. rMSCs previously isolated in the RCSI with the approval of the Research Ethics Committee of the Royal College of Surgeons in Ireland were cultured in DMEM (D6546, Sigma) supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine (Sigma Aldrich, Ireland), 1% glutamax and 1% non-essential amino acids (Biosciences, Ireland). The medium was replaced at 2-3 day intervals and cells were cultured until 80-90% confluence was reached. rMSCs were then trypsinised and encapsulated in HyA-RGD hydrogel using the same protocol as described previously for human MSCs at a low (1 x 10^6 cells/ml) or high (20 x 10^6 cells/ml) seeding density. After 14 days, 200µl samples were stained with LIVE/DEAD (n=1) and cut transversely along the midline in order to expose the entire centre of the gel. A tiled image covering the cross section of the gel was then
captured using confocal microscopy a set distance below the surface to exclude any inconsistencies in the surface topography.

2.2.10 Statistical Analysis

Statistical analysis of results was carried out using GraphPad Prism software, version 5.01. Student t-tests (data sets with only two groups) or two-way analysis of variance (ANOVA) followed by Bonferroni post-tests (data sets with more than two groups) were performed. Results are expressed as mean±standard deviation (SD) and significance was determined using a probability value of P<0.05.
2.3 Results

2.3.1 Physical properties of HyA and HyA-RGD hydrogel

The gelation time of HyA and HyA-RGD hydrogels was ~11s (Fig. 2.9, A & B). The swelling ratio of HyA-RGD hydrogels was significantly greater than the swelling ratio of HyA hydrogels (6.9±0.4 for HyA-RGD vs 3.5±0.6 for HyA, p=0.007) (Fig. 2.9, C). Young’s (compressive) modulus of HyA-RGD hydrogel was significantly lower than HyA hydrogel (5.5±0.16kPa for HyA-RGD vs 7±0.07kPa for HyA, p=0.001) (Fig. 2.9, D).

**Figure 2.9 Physical properties of HyA and HyA-RGD hydrogels.** (A) Gelation time of HyA and HyA-RGD hydrogels. Results are expressed as the mean ± SD (n=4). (B) Representative photographs of HyA hydrogel (i) before and (ii) after gelation. (C) Equilibrium swelling ratio of HyA and HyA-RGD hydrogels. (D) Young’s (compressive) modulus of HyA and HyA-RGD hydrogels. Results are expressed as the mean ± SD (n=3). **p<0.01.
2.3.2 Evaluation of cell viability within large and small volume hydrogel scaffolds

The effect of construct size on encapsulated hMSC viability was compared using large-volume scaffolds (500µl) and small-volume scaffolds (125µl). In the larger volume of hydrogel, live hMSCs were observed only at the periphery of the hydrogel, demonstrating poor nutrient diffusion to the centre of the large construct. (Fig. 2.10, a-d). Visualisation of hMSCs in small hydrogel fragments showed that hMSCs remained viable throughout the entire construct over a period of 9 days (Fig. 2.10, e-h).

Figure 2.10 hMSC viability in large and small-volume hydrogels. Representative live/dead images as observed under a fluorescence microscope of hMSCs encapsulated in (a-d) large-volume hydrogels of 500µl and (e-h) small-volume hydrogels of 125µl. Figures in columns from different locations of the hydrogel construct: edge and centre. Scale bar=100µm.
Following these results, polytetrafluoroethylene (PTFE) moulds were designed as shown in Fig. 2.11, A. These moulds were used to generate cylindrical hydrogel constructs of 200µl (Fig. 2.11, B).

Figure 2.11 (A) The 3D printed mould (shown) was placed on a sterile metal tray (not shown) and clamped to prevent leakage. Each well holds a volume of 200µL of hydrogel. Dimensions in millimetres (mm). (B) Photograph of 200µl hydrogel slab.
Visualisation of hMSCs within 200µl cylinders of HyA-RGD hydrogel was carried out by confocal microscopy after live/dead staining. hMSCs were found to be distributed homogenously throughout the entire hydrogel construct (Fig. 2.12). No dead cells were identified within the HyA-RGD hydrogel following 1 day in culture, and very few dead cells were observed even after 11 days in culture. The 200µl moulds were used for the remainder of the studies in this thesis.

**Figure 2.12 hMSC viability and distribution in HyA-RGD hydrogel.** Representative confocal microscopy images of live/dead stained hMSCs encapsulated in 200µl of HyA-RGD hydrogel. The images demonstrate the distribution and viability of hMSCs within the hydrogel at day 1 and day 11. Scale bar=1000µm.
2.3.3 Effect of RGD on hMSC viability

To examine the effect of the RGD peptide on cell viability, hMSCs were encapsulated in RGD-modified and unmodified HyA hydrogels and cultured under standard conditions for a period of 11 days. As determined by CCK8 assay, cells remained metabolically active in both HyA and HyA-RGD hydrogel for 11 days. RGD did not affect the metabolic activity of hMSCs (Fig. 2.13, A). Cell culture supernatants were collected at days 1, 4, 7 and 11 and analysed for levels of the cytosolic enzyme LDH. As shown in Fig. 2.13, B, cell death was less than 20% at each timepoint, indicating the hydrogel did not have a cytotoxic effect on hMSCs. RGD had no effect on the amount of LDH released by hMSCs in hydrogel.

Cell viability was also monitored by live/dead viability assay. The percentage of live cells within the HyA and HyA-RGD hydrogels post-encapsulation was 91.57% and 99.97% respectively following 1 day in culture. The percentage of live cells was consistent throughout the study, with an average of 83.9% live cells in HyA and 85.7% live cells in HyA-RGD following 11 days in culture (Fig. 2.13, C). This data is also displayed as the number of live and dead cells/mm² (Fig. 2.13, D). While RGD did not significantly influence the percentage or number of live/dead cells at days 1, 7 and 11, there was a significant reduction in live cells at day 4, compared to unmodified HyA hydrogel.

Taken together, these results demonstrate the gelation process is non-toxic to cells and both HyA and HyA-RGD hydrogels support long-term hMSC culture over an 11 day period. RGD did not influence hMSC viability under standard culture conditions.
Figure 2.13 Viability of MSCs cultured in HyA and HyA-RGD hydrogel. (A) Metabolic activity of hMSCs determined by CCK assay. (B) LDH release from hMSCs determined by LDH assay. Values are normalised to the control; cells treated with lysis buffer, taken as 100% dead. (C&D) hMSCs in hydrogels were stained with calcein AM (live-green) and EthD-1 (dead-red). Cell viability is depicted as (C) the percentage of live/dead cells and (D) the number of live/dead cells/mm². Results are expressed as mean ± SD (n≥3). **p<0.01. ‘a’ refers to a significant difference between the number of live cells, p<0.05. ‘b’ refers to a significant difference between the number of dead cells, p<0.05.
2.3.4 Effect of RGD on hMSC morphology

In addition to assessing cell viability within HyA and HyA-RGD hydrogels, the degree of cell spreading was quantified by measuring cell circularity. As shown in Fig. 2.14, A, there was a significant decrease in the circularity of cells cultured with RGD on day 4 with a further decrease in circularity observed on days 7 and 11. Cell spreading was clearly evident as shown in the representative live/dead images in Fig. 2.14, B.

Figure 2.14 Effect of RGD on hMSC morphology (live/dead staining). (A) Cell circularity was quantified. A value of 1 indicates a perfect circle and as the value approaches 0, the shape is increasingly elongated. Values expressed as mean + SD (n≥3). *p<0.05. **p<0.001. (B) Representative live/dead images of hMSCs cultured for 11 days in HyA hydrogel with or without RGD. Magnification 10x. Scale bar=100µm.
Confocal images of nuclei and F-actin stained hydrogels with or without RGD were also taken following 11 days in culture (Fig. 2.15). As shown, hMSCs cultured in HyA maintained spherical morphologies and were unable to spread. In contrast, hMSC spreading was evident in HyA-RGD hydrogels, with encapsulated hMSCs adopting elongated, spindle-like morphologies.

Figure 2.15 Effect of RGD on hMSC morphology (DAPI/phalloidin staining). DAPI (blue) and phalloidin (red) stained images of hMSCs seeded in HyA hydrogel (with or without RGD) following 11 days in culture. Magnification 20x. Scale bar =20µm.
2.3.5 Effect of RGD on VEGF and MCP-1 release under standard culture conditions

In order to examine the effect of RGD on hMSC protein release, spent medium was collected following 1, 4, 7 and 11 days in culture and analysed by ELISA. hMSC cultured within RGD-functionalised HyA hydrogels exhibited increased secretion of VEGF and MCP-1 compared to hMSCs in non-functionalised HyA hydrogels (Fig. 2.16, A & B). hMSCs cultured in HyA-RGD hydrogel exhibited significantly higher VEGF release at day 11, while MCP-1 release was significantly higher at days 7 and 11.

A

B

Figure 2.16 Effect of RGD on VEGF and MCP-1 release under standard culture conditions. Release of (A) VEGF and (B) MCP-1 from hMSCs encapsulated in HyA and HyA-RGD hydrogels at various time points determined by ELISA. Results are expressed as the mean + SD (n=4). *p<0.05, ***p<0.001.
2.3.6 Effect of seeding density on cells within hydrogels

In order to examine the effect of cell density on cell viability, rat MSCs were seeded in 200μl samples of HyA-RGD hydrogel at a density of $1 \times 10^6$ cells/mL and $20 \times 10^6$ cells/mL and cultured under standard conditions. After 14 days, viability was assessed at both the centre and surface of HyA-RGD hydrogel using confocal microscopy to capture an image of the entire cross section (Fig. 2.17, B). Live and dead cells were quantified and calculated as a percentage of live and dead cells and as cells per mm² (Fig. 2.17, C & D). Despite a difference in overall cell number, both densities experienced approximately 10% cell death following 14 days in culture.
Figure 2.17 Effect of cell seeding density on encapsulated rMSC viability. (A) Photographs of rMSCs encapsulated in HyA-RGD hydrogels at a density of 1 x 10^6 cells/mL and 20 x 10^6 cells/mL. (B) Representative image of rMSC within the HyA-RGD hydrogels visualised by live/dead staining. Scale bar=1mm. (C&D) Quantification of rMSC imaged at the centre and the surface of the HyA-RGD hydrogel using confocal microscopy displayed as (C) percentage of live/dead cells and (D) the number of live/dead cells/mm² at day 14. n=1.
2.4 Discussion

While progress has been made in stem cell therapy, significant clinical hurdles remain. One of the greatest challenges in translating cell therapy into routine clinical use is the limited retention and survival of transplanted cells in the host tissue. The delivery of cells in hydrogels that crosslink in situ is a promising strategy which aims to physically retain cells at the target site while acting as a temporary ECM to protect cells and maintain their viability (155). HyA-TA hydrogels formed by the oxidative coupling reaction of HRP and H₂O₂ have been widely used in tissue engineering (250,251). This chapter reports on the suitability of an injectable, RGD-modified HyA-TA hydrogel as a biomaterial carrier for hMSCs. Evaluation of a biological scaffold typically involves two key aspects; whether the mechanical strength of the hydrogel material is suitable for practical applications and if the hydrogel can support cell growth and adhesion (158).

Our results demonstrate that both HyA and HyA-RGD hydrogels formed rapidly. Rapid gelation is a desirable property for in vitro work as it prevents the settling of cells during the encapsulation process (181). For clinical translation, the ability to control gelation is of great importance as a long gelation time could result in leakage of the polymer solution from the injection site and dispersion into non-target organs, while a short gelation time may lead to needle blockage and make delivery via multiple injection sites unachievable (41). The gelation kinetic of HyA-TA hydrogels has been extensively researched and can be easily modified by adjusting the concentration of HRP, with a higher concentration leading to a decreased gelation time (237,240,252).

In this chapter, the HyA-RGD hydrogel was found to have a significantly higher swelling ratio and lower compressive modulus than HyA hydrogel. This may be due to the RGD substitution reducing the crosslinking density. These results indicate the HyA-RGD hydrogel has a larger mesh size, as stiffness is known to be inversely proportional to scaffold porosity and increased swelling is related to a larger average pore size (181,253). A larger average pore size may be favourable for cell
delivery as it allows for greater diffusion of metabolites through the scaffold, which can provide a comfortable environment for cell growth (254). As with gelation time, the mechanical strength of HyA-TA hydrogels can be easily manipulated by adjusting the catalysts or by increasing polymer concentration to alter the crosslinking density of the polymer networks (237,252,255). The ability to control material stiffness is another advantage for clinical translation as the injection of a very stiff material into the myocardium may disrupt cardiac architecture and impair contraction, whereas a soft material may be expelled through the circulatory system by contractile forces (254). Taken together, the compressive modulus of HyA and HyA-RGD hydrogels ranged between 5.5-7kPa, which is lower than normal cardiac muscle (10-15kPa) and that of a myocardial scar (35-70kPa) (256). For bulking purposes, stiff materials are advantageous as they have been demonstrated to provide structural support to the damaged myocardial wall and improve function (179). However, when designing hydrogels for cell delivery, stiff materials may not always be favourable as increased crosslinking and polymer concentration can negatively affect cellular behaviour. This was demonstrated by Lei et al. who reported that mouse MSCs exhibit less spreading, migration and slower proliferation rates in stiffer RGD-modified HyA hydrogels, compared to softer matrices (246).

The ability of a scaffold to facilitate the adequate delivery of nutrients to resident cells is critical for the success of any scaffold-based tissue engineering venture. The transport of solutes including gases, nutrients and waste within scaffolds occurs mainly by diffusion. Both mesh size and environmental factors such as temperature are known to be of important regulators of solute diffusion (257). Limited diffusion may lead to the death of cells at the inner regions of the scaffold, with viable cells only observed along the periphery (258). The optical transparency of our HyA-RGD hydrogel enabled the visualisation of encapsulated cells in a non-invasive manner. As expected, the number of live hMSCs was greatly reduced at the centre of the large-volume HyA-RGD hydrogel scaffold. This effect may be attributed to nutrient deprivation and the presence of oxygen gradients throughout the scaffold (259). This issue was overcome by reducing the volume of the scaffold to 200μl. This volume is comparable with clinical trials whereby stem cell suspensions were
delivered intramyocardially using injections of 100μl-1mL per injection site (112–114). Also delivered within this range was the implantable hydrogel Algisyl-LVR, which was injected into the myocardium using 10-15 injections of 300μl each (178).

hMSC viability was high (>90%) following encapsulation in HyA and HyA-RGD hydrogels, indicating the crosslinking procedure is non-cytotoxic. While viability was maintained throughout the cultivation period, the RGD peptide did not influence the number of viable cells within the hydrogel. The failure of RGD to increase cell proliferation in a 3D microenvironment has been observed in multiple studies using a variety of cell types including MSCs and osteoblasts (160,260). Interestingly, RGD is known to increase cell proliferation in 2D studies, whereby cells are cultured on top of RGD-modified hydrogels (187,261). The effect of RGD on cell morphology has been widely observed (187,192,246,262,263). As expected, the RGD peptide significantly increased hMSC spreading, with hMSCs in HyA-RGD hydrogels exhibiting a typical spindle shape with protruding pseudopods while hMSCs in HyA hydrogel retained a spherical morphology.

hMSCs in RGD-modified HyA hydrogel secreted more VEGF than hMSCs in the unmodified control. VEGF is a potent angiogenic factor frequently investigated for the treatment of ischaemic heart disease (264,265). Treatment with VEGF protein has been shown to improve coronary blood flow and regional myocardial function in an animal model of chronic myocardial ischaemia (266). Although several phase I trials demonstrated the safety of recombinant VEGF protein for therapeutic angiogenesis in patients, the phase II VIVA trial reported IC VEGF administration was ineffective (267,268). This was later attributed to the short half-life of VEGF and low myocardial uptake following coronary infusion (269). In order to overcome these limitations, researchers are increasingly turning to delivery of the VEGF gene rather than the protein (270,271). However, while the IM injection of VEGF plasmid DNA was shown to be safe (272), both the EUROINJET-ONE (273) and NORTHERN (274) trials found no significant clinical benefit. Alternative strategies to enhance the therapeutic effect of VEGF are currently under investigation including delivery of VEGF-transfected MSCs (275), the sustained release of VEGF through PLGA
microparticles (276) and conjugation of VEGF to an injectable hydrogel (277). The success of these approaches in preclinical models provides strong evidence that the controlled and targeted delivery of VEGF is necessary to achieve clinically relevant vascular growth. The HyA-RGD hydrogel described in this study could potentially be used to immobilise MSCs in myocardial tissue to facilitate the sustained release of VEGF to enhance cardiac repair.

The RGD peptide also significantly enhanced MCP-1 secretion from hMSCs in HyA hydrogel. MCP-1 is a chemotactic factor that is released by a variety of cells in response to pro-inflammatory stimuli to attract white blood cells to sites of insult or injury (278). In addition to recruiting monocytes to the ischaemic myocardium, MCP-1 is known to affect many processes involved in MI. Like VEGF, MCP-1 mediates angiogenesis and has been shown to stimulate the formation of new blood vessels in the infarcted heart (279,280). MCP-1 has been shown to inhibit apoptosis of cardiomyocytes under hypoxia to enhance their survival (281,282). Transgenic mouse studies have demonstrated that cardiac overexpression of MCP-1 prevents LV dysfunction and remodelling after MI, providing further evidence of the cardioprotective effects of MCP-1 (283). Lastly, it has been demonstrated that MCP-1 is involved in the homing of stem cells, including MSCs, to the injured myocardium (284). Neural crest stem cells were found to migrate and assemble at the ischaemic border zone area of infarcted hearts in response to MCP-1, where they contributed to cardiac regeneration (285). These findings make MCP-1 a potential target for therapeutic intervention in MI. The underlying reason why RGD enhances VEGF and MCP-1 release from hMSCs is unclear and merits further examination. However, these findings are consistent with a recent study by Ho et al., who reported hMSCs spheroids in RGD-modified alginate hydrogel secreted more VEGF and MCP-1 than spheroids in unmodified gels (286).

The effects of seeding density on cell-biomaterial interactions are often overlooked. We compared cell viability of rMSCs in HyA-RGD and scaffold integrity at low and high seeding densities (1 million and 20 million cells/mL). Following two weeks in culture, both the low and high densities experienced approximately 10% cell death,
indicating that increased cell number did not compromise viability in HyA-RGD hydrogel. Similar results were reported by Erickson et al. using seeding densities of 20 million and 60 million cells/mL in high macromer concentration HyA hydrogels (287). While the high seeding density reduced scaffold integrity, this was likely due to the dilution of the HyA-TA/HRP precursor solution with the large cell pellet. Future work could take this into account by increasing the concentration of HyA or crosslinking density.

**Conclusion**

The development of a biomaterial scaffold capable of maintaining cell viability while promoting attachment and function represents a major research goal in the field of cardiac tissue engineering. This chapter confirms the suitability of RGD-modified HyA-TA hydrogel for this purpose. hMSCs in RGD-modified HyA hydrogels demonstrated significantly greater cell spreading and protein secretion compared to hMSCs in unmodified HyA hydrogel. The development of a hydrogel system with tunable properties such as HyA-RGD, may give researchers the additional level of control needed to enhance the delivery of cells and cardiac tissue engineering.
Chapter 3: Protective Effect of Insulin-Like Growth Factor-1 on Mesenchymal Stem Cells Cultured under Hypoxia and Reduced Nutrient Conditions
3.1 Introduction

Stem cell therapy has shown great promise in regenerating damaged myocardium and restoring its function. However, poor survival in the ischaemic post-MI environment severely limits the therapeutic efficacy of transplanted cells. For instance, Laflamme et al. reported heat-shocked human cardiomyocytes formed stable grafts when injected into uninjured nude rat hearts, with a 90% engraftment success rate. In contrast, engraftment decreased to 18% in infarcted hearts, with much smaller grafts observed (288,289). The development of strategies to promote the survival of stem cells in an ischaemic environment is critical for successful cellular therapy. In chapter 2, the RGD peptide was reported to promote hMSC attachment in HyA hydrogels under standard culture conditions. We hypothesised that the attachment of hMSCs to RGD could inhibit anoikis and improve hMSC survival under the condition of hypoxia (1% O₂) and reduced nutrients, as is present at ischaemic injury sites. To test this hypothesis, a controlled experimental system was designed to assess cell survival using three groups; a control, an experimental group (RGD) and a positive control (IGF-1) (Fig. 3.1). IGF-1 was chosen as the positive control as it is a well-known anti-apoptotic factor which increases the survival of numerous cell types in vitro (202,290,291).

Figure 3.1 Schematic diagram of controlled experimental system whereby HyA is considered as the control, HyA-RGD is the test group and HyA & IGF-1 is the positive control. ↑ denotes increase.
In vivo, MSCs are maintained in a specialised 3D microenvironment or “niche” that regulates their survival and function, as well as their differentiation and proliferation (27). As shown in Fig. 3.2, MSC fate is influenced by both cellular and acellular factors, such as adherence to adjacent cells via gap junctions and soluble factors such as IGF-1 (292). IGF-1 is an anabolic growth hormone (~7.6kDa) that shares close structural homology with IGF-2 and pro-insulin. It is mainly synthesised in the liver but is also produced locally in the heart. Circulating IGF-1 is bound to binding proteins (IGFBPs) which enhance its bioactivity and half-life (196). IGF-1 plays a vital role in cell metabolism, proliferation and growth, through the stimulation of its tyrosine kinase receptor, IGF-1R. IGF-1R is a heterotetrameric complex, consisting of two extracellular α-subunits attached to two membrane-spanning β-subunits, which project into the cytoplasm (293).

**Figure 3.2** The MSC niche in vivo. Within the niche, signals are exchanged between MSCs, stromal cells, progenitor cells and the ECM. IGFs can act via paracrine (produced locally) or endocrine (delivered by blood supply) signalling to stimulate the IGF-1 receptor, IGF-2 receptor or the insulin receptor on MSCs. IGFBPs can adjust IGF actions and affect their stability and half-life (292).
Following ligand binding, receptor dimerisation occurs and the intracellular subunits are immediately phosphorylated, beginning a cascade of intracellular events. Two main pathways have been identified, the phosphatidylinositol-3 kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (200). Stimulation of the MAPK/ERK pathway is considered essential for the proliferative effects observed with IGF-1, while activation of the PI3K/Akt pathway regulates the metabolic and anti-apoptotic effects of IGF-1 (293). IGF-1 has been shown to induce potent mitogenic responses in multiple cell types including CSCs (201), myoblasts (294) and neural progenitor-like cells (291). However, the proliferative effect of IGF-1 on MSCs is poorly understood. As summarised in Table 3.1, treatment of bone marrow MSCs with IGF-1 has yielded variable results.

**Table 3.1 Proliferative effect of IGF-1 on MSCs. BM = bone marrow.**

<table>
<thead>
<tr>
<th>Factor</th>
<th>MSC source</th>
<th>Treatment</th>
<th>Proliferative effect</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>Rat BM</td>
<td>2.5-20g/mL</td>
<td>No effect on proliferation after 2 days</td>
<td>(206)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Rat BM</td>
<td>5-20ng/mL</td>
<td>Significant reduction in MSC mean doubling time over 8 days</td>
<td>(205)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Ovine BM</td>
<td>10ng/mL in serum-deprived medium</td>
<td>↑ in proliferation after 5 days</td>
<td>(295)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Human BM</td>
<td>100ng/mL in reduced serum medium (2% FBS)</td>
<td>No effect on proliferation after 4 days</td>
<td>(204)</td>
</tr>
</tbody>
</table>

Growth factors such as IGF-1, typically induce multifaceted effects, eliciting a different response depending on the concentration and/or exposure time as well as the target cell phenotype (207). The anti-apoptotic effect of IGF-1 on MSCs has been demonstrated during *in vivo* studies with rMSCs (296,297). Pre-incubation of rMSCs with IGF-1 was found to increase the survival of engrafted cells in the infarcted heart (296,297) (Table 3.2). However, the stem cells in these studies were animal-derived. This chapter aims to elucidate the proliferative and pro-survival effect of IGF-1 treatment on hMSCs from multiple human donors.
Table 3.2 Main studies of MSC conditioning with IGF-1. i.v., intravenous infusion; i.m., intramyocardial administration. (298)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Animal species/model</th>
<th>Route of delivery</th>
<th>Main results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>Rat/MI</td>
<td>i.v.</td>
<td>↑CXCR4 expression, ↑MSC survival, ↑capillary density, ↓LV remodelling, ↑LV function</td>
<td>(296)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Rat/MI</td>
<td>i.m.</td>
<td>↑MSC survival, ↓inflammation, ↓apoptosis, ↑LV function</td>
<td>(297)</td>
</tr>
</tbody>
</table>

We propose that IGF-1 may protect hMSCs against apoptosis induced by hypoxia, reduced nutrient conditions. These experiments were conducted with the aim of using IGF-1 as a pro-survival stimulus in future 3D in vitro experiments to compare and contrast the protective effects of biomaterial modifications versus a known protective mitogen. In addition, to confirm that hMSC rescue is possible under our simulated infarct condition, the pro-survival effect of glucose was assessed. Data have shown that glucose depletion, not exposure to continuous hypoxia is responsible for the impairment of MSC survival and function (299). Therefore it was hypothesised that glucose treatment would enhance hMSC survival under adverse conditions.

**Objectives**

- The first objective was to assess the proliferative effect of IGF-1 on hMSCs under standard culture conditions.

- The second objective was to determine if IGF-1 confers a protective effect on hMSCs under hypoxia, reduced nutrient conditions.

- The third objective was to identify the IGF-1R on hMSCs.

- The fourth objective was to assess the ability of glucose to enhance hMSC survival under hypoxia, reduced nutrient conditions.
3.2 Materials and Methods

3.2.1 Materials
Lyophilised synthetic IGF-1 was sourced from Pepscan, Lelystad, Netherlands (supplied by our collaborator Celyad, Mont-Saint-Guibert, Belgium).

3.2.2 Human mesenchymal stem cell culture
hMSCs were cultured in T175 tissue culture flasks using low glucose DMEM (D6046) supplemented with 10% FBS, 100U/mL Penicillin and 100µg/mL Streptomycin at 37°C in a 5% carbon dioxide (CO₂) environment. Cells were passaged at 80-90% confluency and were not used beyond passage 5.

3.2.3 Quantification of double stranded DNA (dsDNA)
For each experiment, cell-containing wells were washed using PBS before hMSCs were detached using 0.2 M NaHCO₃, 1% Triton-X lysis buffer. Lysates were stored at -80°C. dsDNA was quantified using Quant-iT™ PicoGreen® dsDNA assay kit (Life Technologies) as per the manufacture’s protocol. PicoGreen is a fluorescent nucleic acid stain which selectively binds to dsDNA, resulting in a 1000-fold increase in its fluorescence. A dsDNA standard curve was prepared in order to create a graph of fluorescence versus dsDNA concentration. Sample dsDNA concentration was determined with respect to the standard curve generated.

3.2.4 Bioactivity of IGF-1 (low dose) on hMSCs under standard culture conditions

3.2.4.1 hMSC proliferation over 3 days
hMSCs were seeded at a density of 50,000 cells per well in 6-well plates and allowed to adhere overnight. Medium was removed and replaced with IGF-1 supplemented medium (5-20ng/mL), which was replenished daily to ensure consistent growth factor levels. Cell proliferation was monitored over 3 days by collection and quantification of dsDNA.
3.2.4.2 hMSC proliferation over 7 days

hMSCs were seeded in 6-well plates at a density of 40,000 cells per well and allowed to adhere overnight. Medium was removed and replaced with IGF-1 supplemented medium (5-20ng/mL). IGF-1 supplemented medium was replenished every 2-3 days. Proliferation was monitored over 7 days by collection and quantification of dsDNA.

3.2.5 Bioactivity of IGF-1 (high-dose) on hMSCs under standard culture conditions

hMSCs were seeded at a density of 50,000 cells per well in 6-well plates and allowed to adhere overnight. Medium was removed and replaced with IGF-1 supplemented medium at increasing concentrations (50-250ng/mL). IGF-1 supplemented medium was replenished daily for three days. Proliferation was determined by collection and quantification of cellular dsDNA.

3.2.6 Bioactivity of IGF-1 (low dose) on hMSCs under serum-deprived conditions

Generally when examining the effect of growth factors, cells are cultured in serum-free or serum-reduced medium to exclude or reduce the activity of FBS on cells (295). Therefore, the ability of IGF-1 to induce hMSC proliferation under serum-free conditions was examined. hMSCs were seeded at a density of 1,000 cells per well in 48-well plates in serum-reduced medium (5% FBS) and allowed to adhere overnight. Serum was gradually reduced from 5% to 0% over 2 days to reduce cell death due to shock. Medium was then removed and replaced with IGF-1 (5-20ng/mL) supplemented serum–free medium, which was replenished every 2-3 days. Cell proliferation was monitored over 7 days by collection and quantification of dsDNA.

3.2.7 Efficacy of IGF-1

Previous data has shown that IGF-1 stimulates proliferation of rat cardiac stem cells (rCSCs) (201). In order to test the efficacy of the IGF-1 used in these experiments, rCSCs proliferation was measured in response to IGF-1 treatment.
3.2.7.1 Cardiac stem cell culture

Isolated myocardial tissue from Sprague Dawley rats was cut into 1-2mm³ pieces, from which gross connective tissue was removed, then washed and digested three times for 5 min at 37°C with 0.2% trypsin (Invitrogen), 0.1% collagenase I (Sigma) and 0.1% Dispase (Sigma). The tissue fragments were washed with complete explant medium (CEM) (Iscove’s Modified Dulbecco’s Medium [IMDM] supplemented with 10% FBS, 100U/mL Penicillin, 100µg/mL Streptomycin and 2mmol/L L-Glutamine) and cultured as explants in CEM at 37°C and 5% CO₂. Explants were cultured on fibronectin-coated plates and carefully covered using coverslips to create slight pressure in order to encourage adherence. After several days, a layer of stromal-like cells arose from adherent explants over which small, round, phase-bright cells migrated. Once confluent, the phase bright cells surrounding the explants were harvested by gentle enzymatic digestion. These cardiosphere-forming cells were seeded at 2-3 x 10⁴ cells/mL on poly-D-lysine coated dishes in complete growth medium (CGM) (35% IMDM/65% DMEM-Ham F12 mix supplemented with 3.5% FBS, 100U/mL Penicillin, 100µg/mL Streptomycin, 2mmol/L L-Glutamine, 2% B27, 10ng/mL epidermal growth factor (EGF), 60ng/mL basic fibroblast growth factor (bFGF), 40nmol/L cardiotrophin-1 and 40nmol/L thrombin). Several days later, cells that remained adherent to the poly-D-lysine coated plates were discarded and detached cardiospheres in suspension were plated on fibronectin coated flasks and expanded as monolayers. rCSCs were subsequently passaged by trypsinisation at a density of 1 x 10⁶ cells/T175 flask (300).

3.2.7.2 rCSC proliferation

rCSCs cells were seeded at a density of 30,000 cells per well in 24-well plates in CGM. CGM was removed and replaced with basal medium (35% IMDM/65% DMEM-Ham F12 mix supplemented with 3.5% FBS, 100U/mL Penicillin, 100µg/mL Streptomycin and 2mmol/L L-Glutamine) for 24 hours. rCSC were then treated with IGF-1 (25-100ng/mL) in basal medium for 3 days. Cell proliferation was monitored by collection and quantification of dsDNA.
3.2.8 Protective effect of IGF-1 on hMSCs cultured under hypoxia, reduced nutrient conditions

3.2.8.1 hMSC survival over 3 days

hMSCs were seeded at a density of 5,000 cells per well in 48-well plates and allowed to adhere overnight. Medium was discarded and wells were washed with PBS. Cells were treated with increasing concentrations of IGF-1 (5-20ng/mL) in DMEM, no-glucose medium (D11966, Thermo Fisher) supplemented with 1% FBS, 100U/mL Penicillin and 100µg/mL Streptomycin. Samples were incubated at 1% O₂ (5% CO₂) using a hypoxia chamber (Model #856-HYPO, Hypoxia Chamber Glove Box, Plas-Labs, Inc™. USA). Cell proliferation was monitored over 3 days by collection and quantification of dsDNA.

3.2.8.2 hMSC survival over 7 days

hMSCs were prepared as in section 3.2.8.1. Medium was replenished every 2-3 days and cell proliferation was monitored over 7 days by collection and quantification of dsDNA.

3.2.9 Flow cytometry

3.2.9.1 hMSC IGF-1R expression under standard culture conditions

hMSC were passaged 5 times as adherent monolayers before being used for flow cytometry experiments with a FACS (BD Canto, BD Biosciences, San Jose, California) flow cytometer with quantitative analysis provided by CellQuest software (BD Biosciences, San Jose, California). Cells were trypsinised and resuspended in FACs buffer (PBS + 1% FBS). Cells were then washed x3 before they were incubated with R-phycoerythrin (PE) conjugated antibodies against CD221 (IGF-1R) for 30mins (1:200 dilution). Isotype-identical antibody (IgG1 k) served as a negative control (BD Biosciences, San Jose, California).

3.2.9.2 hMSC IGF-1R expression under hypoxia, reduced nutrient conditions

To assess the effect of hypoxia and reduced nutrient conditions on IGF-1R expression, hMSCs were cultured as adherent monolayers in T75 flasks under standard or hypoxia, reduced nutrient conditions. After 72 hours, cells were
trypsinned and resuspended in FACs buffer (PBS + 1% FBS). Cells were then washed x3 before they were incubated with R-phycoerythrin (PE) conjugated antibodies against CD221 (IGF-1) for 30mins (1:200 dilution). Isotype-identical antibody (IgG1k) served as a negative control (BD Biosciences, San Jose, California). Flow cytometry experiments were conducted with a FACS (BD Canto, BD Biosciences, San Jose, California) flow cytometer with quantitative analysis provided by CellQuest software (BD Biosciences, San Jose, California).

### 3.2.10 Protective effect of glucose on hMSCs under hypoxia, reduced nutrient conditions

hMSCs were seeded in 24-well plates at a density of 10,000 cell per well and allowed to adhere overnight. Medium was removed and wells were rinsed with PBS before medium was replaced with increasing concentrations of glucose (0.15–0.2g/L) in DMEM, no glucose medium (D11966, Thermo Fisher) supplemented with 1% FBS, 100U/mL Penicillin and 100µg/mL Streptomycin. Samples were incubated at 1% O₂ (5% CO₂) for 3 days using a hypoxia chamber (Model #856-HYPO, Hypoxia Chamber Glove Box, Plas-Labs, Inc™. USA).

### 3.2.11 Statistical analysis

Statistical analysis of results was carried out using GraphPad Prism software, version 5.01. Two-way ANOVA analysis was performed followed by Bonferroni post-test comparing all columns to the control. Results are expressed as mean ± standard deviation (SD) and significance was determined using a probability value of P<0.05. To account for donor variability, results were averaged from three independent experiments using three different hMSC donors where specified. A minimum of n=3 replicates were performed for all experiments.
### 3.3 Results

#### 3.3.1 Bioactivity of IGF-1 on hMSCs under standard culture conditions

Stimulation of hMSCs with IGF-1 (5-20ng/mL) revealed no effect on proliferation compared to the untreated control (Fig. 3.3, A & B). Treatment with IGF-1 significantly decreased hMSC dsDNA at day 5 at a concentration of 10ng/mL and day 7 at concentrations of 5-20ng/mL (Fig. 3.3, B).

![Figure 3.3](image)

**Figure 3.3** Effect of IGF-1 treatment on hMSC proliferation under standard culture conditions. Quantification of dsDNA from hMSCs treated with increasing doses of IGF-1 (5-20ng/mL), untreated cells were used as the control. (A) Stimulation of hMSCs with IGF-1 over 3 days. Data shown as mean + SD of three independent experiments using three different hMSC donors (n=3). (B) Stimulation of hMSCs with IGF-1 over 7 days. Data shown as mean + SD (n=3). *p<0.05. **p<0.01. ***p<0.001.
3.3.2 Toxicity induced by high doses of IGF-1

To further evaluate the effect of IGF-1 on hMSCs, cells were treated with IGF-1 at a higher concentration ranging from 50-250ng/mL. Concentrations ≥50ng/mL were found to significantly decrease the dsDNA of hMSCs after 1, 2 and 3 days, compared to the untreated control (Fig. 3.4), indicating cytotoxicity.

![Graph showing dsDNA levels over time](image)

**Figure 3.4 Effect of high-dose IGF-1 treatment on hMSCs.** Quantification of dsDNA from hMSCs treated with 50-250ng/mL for 1, 2 and 3 days. Results are expressed as mean ± SD (n=3). ***p<0.001.
3.3.3 Response to IGF-1 treatment following exposure to low serum medium

Under serum-deprived conditions, IGF-1 (5-20ng/mL) did not increase hMSC proliferation over 7 days (Fig. 3.5).

![dsDNA vs. days for different IGF-1 concentrations](image)

Figure 3.5 hMSC response to IGF-1 treatment under reduced serum conditions. Quantification of dsDNA following the treatment of cells with IGF-1 for 7 days in the absence of FBS. IGF-1 treatment had no effect on hMSC proliferation under these conditions. Data shown as mean ± SD of three independent experiments using three different hMSC donors.
3.3.4 Efficacy of IGF-1

To assess the efficacy of the IGF-1 used in these experiments, rCSCs were treated with increasing concentrations of IGF-1 (25-100ng/mL) for 3 days in the absence of growth factors, such as EGF and bFGF, to exclude the proliferative effects of these proteins (Fig. 3.6). IGF-1 significantly increased rCSC proliferation after 2 and 3 days, with a dose-dependent response observed at day 3 between 25-50ng/mL. This result confirmed that the IGF-1 used in this chapter was bioactive.

Figure 3.6 Effect of IGF-1 on rCSC proliferation. Quantification of dsDNA demonstrated that stimulation with IGF-1 ≥25ng/mL significantly increased rCSC proliferation after 2 and 3 days. IGF-1 was observed to have a dose-dependent response between 25-50ng/mL at 72h. Results are expressed as mean + SD (n=3). **p<0.01. ***p<0.001.
3.3.5 IGF-1 does not confer a protective effect on hMSCs under hypoxia, reduced nutrient conditions

To determine the effect of IGF-1 on hMSC survival under hypoxia, reduced nutrient conditions, hMSCs were treated with increasing concentrations of IGF-1 (5-20ng/mL) and cultivated for up to 7 days. As shown in Fig. 3.7, A & B, IGF-1 did not enhance hMSC survival.

![Graph A](image1.png)

Figure 3.7 Effect of IGF-1 on hMSCs survival under hypoxia, reduced nutrient conditions. (A) Quantification of dsDNA revealed that IGF-1 treatment has no effect on hMSC dsDNA over 3 days. Data shown as mean ± SD of three independent experiments using three different hMSC donors. (B) IGF-1 treatment had no effect on hMSC dsDNA over 7 days. Results are expressed as mean ± SD (n=3).
3.3.6 Fluorescence-activated cell sorting analysis

3.3.6.1 IGF-1R expression under standard culture conditions

Analysis of IGF-1R expression revealed that hMSCs do not normally express this receptor. 0% of hMSCs expressed IGF-1R (Fig. 3.8).

Figure 3.8 Fluorescence-activated cell sorting analysis of hMSCs. IGF-1 receptor expression was analysed within the density plots and shown as a percentage of positive events. 0% of hMSCs expressed the IGF-1 receptor. Data shown as mean ± SD of three independent experiments using three different hMSC donors.
3.3.6.2 *IGF-1R expression under hypoxia, reduced nutrient conditions*

To investigate the effect of hypoxia, reduced nutrient conditions on the activation of IGF-1R, we examined the receptor expression following culture under standard and hypoxia, reduced nutrient conditions. As shown in Fig. 3.9, IGF-1R was not detected, indicating IGF-1R expression was not upregulated by exposure to hypoxia, reduced nutrient conditions.

Figure 3.9 Fluorescence-activated cell sorting analysis of hMSCs following standard and hypoxia, reduced nutrient conditions. IGF-1 receptor expression was analysed within the density plots. Three different hMSC donors were investigated.
3.3.7 Glucose protects hMSCs under hypoxia, reduced nutrient conditions

Treatment with 0.15-0.2g/L of glucose significantly increased hMSC survival under hypoxia and reduced nutrient conditions after 3 days (Fig. 3.10).

![Graph showing effect of glucose treatment on hMSC survival under hypoxia, reduced nutrient conditions.](image)

Figure 3.10 Effect of glucose treatment on hMSC survival under hypoxia, reduced nutrient conditions. Quantification of dsDNA demonstrated that glucose is capable of significantly increasing hMSC survival under hypoxia, reduced nutrient (H/RN) conditions. Results are expressed as mean ± SD (n=3) with significance displayed compared to the control (H/RN). ***p<0.001.
3.4 Discussion

According to Karpov et al., there are two main strategies to increase the therapeutic effects of MSCs: (1) to prevent MSC death in ischaemic tissue and (2) to increase the production of growth factors and cytokines for cardiac repair with transplanted MSCs (298). While multiple strategies have been shown to enhance MSC viability including genetic modification, preconditioning and tissue engineering, (298) we aimed to elucidate the effect of a chemical agent, IGF-1 on hMSC survival. Pre-treatment with IGF-1 has been shown to enhance the viability of transplanted rMSC in animal models of MI (296,297); however, its effect on hMSC survival has not been documented. Our results show that IGF-1 does not enhance hMSC proliferation or survival in vitro. In addition, FACs analysis failed to identify IGF-1R on hMSCs from multiple donors. Interestingly, glucose treatment was found to significantly increase hMSC survival under ischaemic culture conditions. Taken together, our results demonstrate that it is possible to rescue hMSC from adverse conditions, but IGF-1 is not suitable for this purpose.

Initial studies in this chapter examined the effect of IGF-1 on hMSC proliferation. Our results demonstrate that low-dose IGF-1 had no effect on hMSC growth under standard or serum-deprived culture conditions. These findings are in line with data published by Collins et al., who found stimulation of hMSCs with IGF-1 did not enhance proliferation over 4 days (204). While their study utilised hMSC from just one donor, our study examined multiple donors to account for donor-to-donor variability. Following this result, we tested the efficacy of our IGF-1 using c-kit+ rCSCs. CSCs are self-renewing, clonogenic and multipotent, giving rise to endothelial cells, smooth muscle cells and cardiomyocytes (77). IGF-1 has previously been reported to promote the proliferation of mouse CSCs through the activation of the PI3/Akt-1 pathway, which in turn phosphorylates FoxO3a leading to its nuclear export. In the absence of IGF-1, PI3/Akt-1 is not stimulated, leaving FoxO3a localised in the nucleus where it upregulates the expression of cell-cycle inhibitors such as p27kip1 and p57kip2 to inhibit CSC proliferation (201). As expected,
IGF-1 treatment significantly increased the proliferation of rCSCs over 3 days, confirming the bioactivity of the growth factor.

Next, we assessed the effect of IGF-1 on hMSC survival. The survival of MSCs in the ischaemic environment relies on the balance between hypoxia-induced pro-apoptotic signalling mainly mediated via Toll-like receptor 4 (TLR4) and G protein-coupled receptors, and pro-survival pathways such as the PI3K/Akt and ERK pathways (298). IGF-1 is a well-known activator of the pro-survival Akt pathway. Therefore, it was expected that IGF-1 would activate its receptor to decrease cell death by stimulating the pro-survival Akt pathway. Approaches which target the Akt pathway of transplanted cells have demonstrated success in preclinical models of MI. For example, bone marrow-derived MSCs transduced with Akt exhibit enhanced cardiac repair when transplanted into both ischaemic rat (141) and porcine myocardium (301). In the present study, we show that IGF-1 does not enhance hMSC survival under ischaemic culture conditions. In addition, IGF-1R was not detected on hMSC following culture under adverse culture conditions. As the anti-apoptotic effects of IGF-1 are mediated by IGF-1R, this may explain why IGF-1 has no effect on our hMSCs. However, this result is surprising as IGF-1R expression and concentration is known to be upregulated when cells such as human hepatocytes (302) and neuronal growth cones (303) are exposed to hypoxic conditions. Hypoxia has also been reported to activate expression of IGF-1R in a population of gefitinib-resistant lung cancer stem cells (304).

Having ruled IGF-1 out as a possible pro-survival stimulus and positive control under ischaemic conditions, we next sought to identify an alternative soluble factor capable of eliciting the desired response. To this end, the effect of glucose on hMSC survival was evaluated. In the ischaemic environment, MSCs encounter both low oxygen tension and nutrient deprivation (305). Most studies have focused on the role of oxygen in cell survival because it is known to regulate a number of critical cellular processes such as adhesion (306), metabolism, proliferation (307) and differentiation (308). In addition, oxygen is a poorly diffusive molecule whose limited diffusivity poses a challenge in the design of tissue engineering scaffolds.
(309). However, while oxygen deprivation may significantly affect cell viability, glucose deprivation is the more significant limiting factor in cell survival (299,310). In this study, the ability of glucose to significantly enhance hMSC survival under ischaemic conditions was demonstrated. This increase in cell viability is in line with the work reported in other studies which successfully employed glucose as a survival stimulus under adverse conditions in vitro (310,311). Deschepper et al. provided the first in vivo evidence of the pro-survival function of glucose on hMSCs. hMSCs in ectopically implanted hydrogels supplemented with glucose exhibited four- to five-fold higher viability and the gels were more vascularised compared with scaffolds without glucose (311). These observations suggest that glucose may be an essential component of the ideal scaffold for cell delivery. However, the encapsulation of bioactive molecules within scaffolds presents specific challenges such as possible structural damage to proteins during encapsulation and rapid release and degradation in vivo. In this respect, HyA-RGD hydrogels are advantageous as the RGD ligand is already covalently cross-linked to the HyA backbone.

### Conclusion

In this chapter, we developed an in vitro model to investigate the deleterious effects of ischaemia on hMSC survival. Treatment with IGF-1 did not influence viability under these conditions. In addition, the IGF-1R was not identified on hMSCs following exposure to normal or simulated pathological conditions. In comparison, glucose treatment was found to increase hMSC survival under oxygen and reduced nutrient conditions. Our results demonstrate that while it is possible to rescue hMSC from adverse conditions, IGF-1 is not suitable for this purpose.
Chapter 4: Functionalisation of HyA Hydrogel to Enhance the Delivery of Mesenchymal Stem Cells into the Ischaemic Myocardium
4.1 Introduction

The cell microenvironment has emerged as a key regulator of cell behaviour and function in development, physiology and pathophysiology. The ECM serves not only as a structural support for cells but also as a source of biophysical and biochemical cues that influence cell behaviours such as spreading, proliferation, migration, differentiation and apoptosis. Therefore, it is desirable to develop materials that mimic the structures, properties and functions of native ECM and enable the study of cells in vitro in a realistic and adaptable cell microenvironment (312). Owing to their similarity with the ECM, natural polymers such as HyA are often employed as cell carriers for tissue regeneration. However, the use of single component scaffolds over-simplifies the complex microenvironment found within biological tissues. Although HyA is an essential component of the ECM, it is a natural barrier to cell proliferation, adhesion and migration. Thus it is often necessary to engineer HyA scaffolds to influence encapsulated cell behaviour. Typically, hydrogels are engineered with motifs found in the ECM of the target tissue (313). Principal components of the ECM are listed in Table 4.1 (314).

Table 4.1 Principal components of the ECM. Adapted from (314).

<table>
<thead>
<tr>
<th>ECM Components</th>
<th>Structural components</th>
<th>Non-structural components</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collagen (types I, III, IV, V, VI, XI, XIII, XV, XVIII), laminins, elastin, emilin/multimerins, fibronectin, fibronectin EDA, fibuline, fibrillin, nidogen/enactin</td>
<td>Hyaluronic acid, syndecan, veriscan, perlecan, biglycan, lumucan, decorin, agrin, glypican, haplns</td>
</tr>
</tbody>
</table>

Biologically active molecules can be incorporated into the hydrogel in suspension or covalently bound to the polymer to stimulate biochemical and biophysical cues (242). As illustrated in Fig. 4.1, combinatorial strategies may improve the encapsulated stem cell survival and function (315). Because HyA acts as a barrier to cell adhesion and proliferation in vitro, it can be used as a blank slate to investigate the influence of different ligands on cell behaviour (313). In this chapter, the effect of RGD and nidogen-1 on hMSC survival and function in HyA hydrogels is...
systematically quantified under the condition of hypoxia and reduced nutrients, as is present in ischaemic injury sites. Few studies have examined the effect of multi-component scaffolds on stem cells under the condition of hypoxia and reduced nutrients (211). This dearth in knowledge undermines the therapeutic potential of MSC therapy for ischaemic heart disease. We aim to provide a detailed analysis of hMSC behaviour in these dual-factor hydrogel scaffolds in order to further progress towards identifying the ideal scaffold for cardiac tissue engineering.

**Figure 4.1** Schematic diagram of injectable hydrogel delivery system for improved stem cell-delivered therapeutics. (a) The encapsulation of stem cells within injectable hydrogels designed to provide cells with mechanical support and biochemical cues along with co-encapsulation of bioactive factors. (b) The use of injectable hydrogels combined with bioactive factors may protect cells during injection and subsequently in vivo. The hydrogel should eventually degrade and be gradually replaced by cell-secreted ECM (315).

### 4.1.1 RGD
In chapter 2, RGD-modified HyA hydrogel was shown to significantly increase hMSC adhesion and spreading compared to unmodified HyA under standard culture conditions. As anchorage-dependent cells such as hMSCs rely on cell adhesion for survival, strategies that enhance cellular attachment may have considerable beneficial effects on the efficacy of MSC based therapies. In this chapter, the ability
of RGD-modified HyA hydrogel to influence hMSC viability, morphology and function under oxygen and nutrient deprived conditions was assessed. Unmodified HyA hydrogel which was used as the control, provides an ideal milieu for studying the effects of specific cell-matrix interactions due to the notable lack of cell adhesion as reported in chapter 2.

The ability of cells to attach to the ECM is a key determinant of cytoskeleton organisation and cellular morphology. In addition to influencing cell shape, cell-matrix interactions also control a cell’s ability to proliferate, migrate and differentiate. Cell adhesion happens in three stages: attachment, spreading and formation of focal adhesions and stress fibres (Fig. 4.2) (316). Focal adhesions are protein complexes which act as transmembrane links between the ECM and the actin cytoskeleton (317). Following initial cell-ligand attachment, cells increase their surface area contact through the formation of actin microfilaments and spreading. If the appropriate signals are provided, cells continue to organise their cytoskeleton as indicated by the formation of focal adhesions and actin-containing stress fibres (316). The initial attachment and early spreading of cells is a passive process which does not require the cell to expend metabolic activity, while the later stage is an active process (318).

![Image: Figure 4.2 Cell spreading and attachment. Adapted from (316).](image_url)
4.1.2 Nidogen-1

Nidogen-1, also known as entactin, is a basement membrane (BM) glycoprotein that is responsible for maintaining the integrity of cardiac tissue (314). Nidogen-1 is primarily synthesised by mesenchymal cells and deposited in the BM during development (319). The basement membrane (BM) is a thin layer of specialised ECM tissue, which separates cell monolayers from underlying connective tissue. It consists of four major components; type IV collagen, laminin, nidogen and perlecan. As shown in Fig. 4.3, the general structure of the BM is a mat-like structure whereby laminin and collagen connect to form networks which are noncovalently interconnected by nidogen and perlecan (320). A recent study by Kappler et al. reported that human cardiac ECM microparticles containing fibronectin, collagen and nidogen-1 exerted a cytoprotective effect on cardiomyocytes subjected to simulated ischaemia-like conditions, i.e., hypoxia and glucose/serum starvation (210). However, the direct role of nidogen-1 under pathological conditions is largely unknown (314). The aim of this chapter is to determine the ability of nidogen-1 to confer a cytoprotective effect under ischaemic culture conditions.

**Figure 4.3** Schematic diagram of the basement membrane (BM). (a) The interconnections of the four major BM elements lead to a mat-like texture and (b) the interactions of BM proteins based on *in vitro* binding data (320).
Objectives
Transplanting stem cells in instructive biomaterial scaffolds may increase cell survival and improve their therapeutic efficiency.

- The first objective was to assess the ability of RGD to enhance hMSC viability and function under hypoxia, reduced nutrient conditions.
- The second objective was to successfully encapsulate nidogen-1 in HyA hydrogel.
- The third objective was to determine the effect of nidogen-1 on hMSCs in HyA hydrogel under standard culture conditions.
- The fourth objective was to assess the ability of nidogen-1 to enhance hMSC viability and function under hypoxia, reduced nutrient conditions.
4.2 Materials and Methods

4.2.1 Effect of RGD on hMSC survival, morphology and function under hypoxia, reduced nutrient conditions

HyA hydrogel was prepared as described in Section 2.2.3. FGF-treated hMSCs were trypsinsised and encapsulated in HyA and HyA-RGD hydrogels at a density of 1 x 10^6 cells/mL. Hydrogel samples were cultured under reduced nutrient conditions (DMEM 11966 no glucose medium supplemented with 1% FBS, 100U/mL Penicillin and 100µg/mL Streptomycin, Sigma Aldrich, Ireland) in a hypoxia chamber (1% O_2, 5% CO_2) for 11 days. Cell viability was monitored by CCK8 as described in Section 2.2.8.1. Live/dead staining was performed and confocal microscopy was used to capture an image of the entire cross section at the surface and centre of the hydrogel and as described in Section 2.2.8.3. VEGF and MCP-1 detection was carried out as described in Section 2.2.8.6.

4.2.2 Effect of cell adhesion to RGD on hMSC survival, morphology and function under hypoxia, reduced nutrient conditions

In Chapter 2, significant cell spreading was reported in HyA-RGD hydrogels under normoxic culture conditions after 4 days (Section 2.3.5). In this experiment, FGF-treated hMSCs were encapsulated in HyA and HyA-RGD hydrogels (prepared as described in Section 2.2.3) and cultured under standard culture conditions (21% O_2, 10% FBS) for 4 days in order to allow adhesion to the RGD ligand. Following this, samples were rinsed with PBS and transferred to oxygen and nutrient deprived conditions as described in Section 4.2.1 for 11 days. Cell viability was monitored by CCK8 as described in Section 2.2.8.1. Live/dead staining was performed and confocal microscopy was used to capture an image of the entire cross section at the surface and centre of the hydrogel and as described in Section 2.2.8.3. VEGF and MCP-1 detection was carried out as described in Section 2.2.8.6. The experimental timeline is summarised in Fig. 4.4.
Figure 4.4 Experimental timeline. hMSCs were encapsulated in HyA-RGD hydrogel and cultured under normoxic culture conditions for 4 days in order to allow cellular adhesion prior to exposure to harsh conditions. At T0, cell-laden hydrogels were transferred to adverse culture conditions and monitored over 11 days.

4.2.3 Physical entrapment of nidogen-1 in HyA hydrogel

HyA hydrogel was prepared as described in Section 2.2.3. Nidogen-1 was added to the HRP-HyA solution at 12μg/mL, to give a final concentration of 6μg/mL once hydrogels were formed. Hydrogel disks of 200μl (total protein 1.2μg) were immersed in 20mL release medium composed of PBS with or without 2.5U/mL or 100U/mL hyaluronidase. The samples were incubated at 37°C in a shaking waterbath at 100rpm. At selected timepoints, 1mL of the release medium was removed and stored in siliconised centrifuge tubes to prevent non-specific adsorption of the protein to the plastic surface. 1mL of solution with or without hyaluronidase was added to maintain the total release medium at 20mL. The collected samples were stored at -20°C. Nidogen-1 concentration was determined by ELISA assay as per the manufacturer’s instructions (R&D Systems).

4.2.4 Effect of nidogen-1 on hMSC viability, morphology and function under standard culture conditions

HyA hydrogel was prepared as described in Section 2.2.3. Nidogen-1 was added to the HRP-HyA solution at 12μg/mL to give a final concentration of 6μg/mL once hydrogels were formed. FGF-treated hMSCs were trypsinised and encapsulated in HyA and HyA-nidogen-1 hydrogel at a density of 1 x 10⁶ cells/mL and cultured in 2mL of DMEM (D6046, Sigma) supplemented with 10% FBS, 100U/mL Penicillin,
100µg/mL Streptomycin (Sigma Aldrich, Ireland) and 5ng/mL FGF at 37°C in a 21% oxygen, 5% CO₂ environment for 11 days. Cell viability was monitored by CCK8 as described in Section 2.2.8.1. Live/dead staining was performed and confocal microscopy was used to capture an image of the entire cross section at the surface and centre of the hydrogel and as described in Section 2.2.8.3. VEGF and MCP-1 detection was carried out as described in Section 2.2.8.6.

4.2.5 Effect of nidogen-1 on hMSC survival, morphology and function under hypoxia, nutrient deprived conditions

HyA hydrogel was prepared as described in Section 2.2.3. Nidogen-1 was added to the HRP-HyA solution at 12µg/mL to give a final concentration of 6µg/mL once hydrogels were formed. FGF-treated hMSCs were trypsinised and encapsulated in HyA and HyA-nidogen-1 hydrogels at a density of 1 x 10⁶ cells/mL. Hydrogel samples were cultured under oxygen and nutrient deprived conditions as described in Section 4.2.1 for 11 days. Cell viability was monitored by CCK8 as described in Section 2.2.8.1, live/dead staining was performed and confocal microscopy was used to capture an image of the entire cross section at the surface and centre of the hydrogel and as described in Section 2.2.8.3. VEGF and MCP-1 detection was carried out as described in Section 2.2.8.6.

4.2.6 Effect of cell interaction with nidogen-1 on hMSC survival, morphology and function under hypoxia, nutrient deprived conditions

HyA hydrogel was prepared as described in Section 2.2.3. Nidogen-1 was added to the HRP-HyA solution at 12µg/mL to give a final concentration of 6µg/mL once hydrogels were formed. FGF-treated hMSCs were trypsinised and encapsulated in HyA and HyA-nidogen-1 hydrogels at a density of 1 x 10⁶ cells/mL. Following encapsulation, hMSCs were allowed to adapt to the 3D environment for 4 days under standard culture conditions in order to allow interaction with nidogen-1. At day 4, samples were transferred to oxygen and nutrient deprived conditions as described in Section 4.2.1 to mimic the harsh environment of the infarcted tissue.
4.2.7 Statistical Analysis

Statistical analysis of results was carried out using GraphPad Prism software, version 5.01. Two-way ANOVA followed by Bonferroni post-test analysis was performed unless otherwise stated. Error is reported as standard deviation (SD) and significance was determined using a probability value of $P<0.05$. A minimum of $n=3$ replicates were performed for all experiments.
4.3 Results

4.3.1 Effect of weak adhesion to RGD on hMSC survival, morphology and function under hypoxia, reduced nutrient conditions

To determine the effect of RGD on hMSCs under hypoxia with reduced nutrient conditions, hMSCs were encapsulated in HyA and HyA-RGD hydrogels and cultured under 1% O₂ and 1% FBS conditions over a period of 11 days. The CCK8 assay was used to assess hMSC viability. As shown in Fig. 4.5, A, hMSC viability was significantly lower in HyA-RGD hydrogels after 4 days, with no significant difference observed thereafter. With the live/dead assay, no differences in the number of live or dead cells were observed between unmodified and RGD-modified hydrogels (Fig. 4.5, B & C). Taken together, these results indicate that RGD does not improve hMSC survival under adverse culture conditions.

Figure 4.5 RGD does not increase on hMSC viability under hypoxia, reduced nutrient conditions. (A) Viability of hMSCs encapsulated in HyA and HyA-RGD hydrogels after 4, 7 and 11 days determined by CCK8 assay. ***p<0.001. (B&C) Quantification of live/dead hMSCs in HyA and HyA-RGD hydrogel. Data shown as (B) the percentage of total live/dead cells and (C) the number of live/dead cells/mm². Mean ± SD (n≥3).
The degree of cell spreading under hypoxia, reduced nutrient conditions was classified by cell circularity. As shown in **Fig. 4.6, A**, hMSC spreading was significantly enhanced in RGD-modified HyA hydrogel from day 4 onwards, compared to unmodified HyA hydrogel. Representative images of dapi/phalloidin staining show hMSCs cultured in HyA and HyA-RGD hydrogel following 11 days in culture (**Fig. 4.6, B**). hMSC spreading is evident in HyA-RGD hydrogels.

**A**

![Bar chart showing cell circularity](image)

**B**

![Images showing cell spreading](image)

**Figure 4.6 Effect of RGD on encapsulated hMSC spreading. (A)** Cell circularity was quantified. A value of 1 indicates a perfect circle and as the value approaches 0, the shape is increasingly elongate. hMSC spreading was significantly increased in HyA-RGD hydrogel, compared to HyA hydrogel. Results are expressed as the mean ± SD (n≥3). ***p<0.001. (B) DAPI (blue) and phalloidin (red) stained images of hMSCs seeded in HyA hydrogel (with or without RGD) following 11 days in culture. Magnification 20x. Scale bar =20µm.
After 4, 7 and 11 days in hypoxia, reduced nutrient conditions, conditioned medium was collected and analysed for VEGF and MCP-1 content using ELISA, to determine if the RGD modification enhanced protein release from encapsulated hMSCs. As shown in Fig. 4.7, A & B, neither VEGF nor MCP-1 secretion was increased in response to RGD.

![Graph A: VEGF secretion over time](image_url)

![Graph B: MCP-1 secretion over time](image_url)

**Figure 4.7 Paracrine factor release.** Release of (A) VEGF and (B) MCP-1 from hMSCs encapsulated in HyA and HyA-RGD hydrogels at various time points determined by ELISA. Results are expressed as the mean + SD (n=4).

**In summary, the functionalisation of HyA hydrogels with RGD promotes cell spreading following direct transfer to adverse culture conditions but does not increase cell survival or function.**
4.3.2 Effect of strong adhesion to RGD on hMSC survival, morphology and function following transfer to hypoxia, reduced nutrient conditions

Having determined that RGD does not enhance hMSC survival when immediately exposed to adverse culture conditions, we next sought to determine if adhesion to RGD before exposure to adverse culture conditions would enhance hMSC survival and function. We hypothesised that during the pre-culture period under standard condition, hMSCs would form strong adhesions to the matrix and subsequently promote the survival and function following transfer to adverse culture conditions. Our results show that the adhesion of hMSCs to the RGD peptide prior to exposure to adverse culture conditions had no effect on the metabolic activity of hMSC under hypoxia, reduced nutrient conditions over a period of 11 days, compared to HyA hydrogel alone (Fig. 4.8, A). However the adhesion of hMSCs to RGD was found to significantly increase the percentage of live cells within the hydrogel, as well as significantly reduce the number of dead cells, compared to unmodified HyA hydrogel (Fig. 4.8, B & C).
Figure 4.8 Effect of cell adhesion to RGD on hMSC viability following transfer to hypoxia, reduced nutrient conditions. (A) Viability of hMSCs encapsulated in HyA and HyA-RGD hydrogels after 3, 7 and 11 days determined by CCK8 assay. (B&C) Quantification of live/dead hMSCs in HyA and HyA-RGD hydrogel. Data shown as (B) the percentage of total live/dead cells and (C) the number of live/dead cells/mm². Results are expressed as the mean ± SD (n≥3). **p<0.01. ‘bbb’ refers to a significant difference between the number of dead cells, p<0.001.
Cell spreading was significantly enhanced in HyA-RGD hydrogels following transfer from normoxia to hypoxia, reduced nutrient culture conditions (Fig. 4.9, A). Representative images of dapi/phalloidin staining show hMSCs spreading in HyA-RGD hydrogel (Fig. 4.9, B).

![Graph showing cell circularity over time](image)

**Figure 4.9** Effect of cell adhesion to RGD on hMSC morphology following transfer to hypoxia, reduced nutrient conditions. (A) Cell circularity was quantified. A value of 1 indicates a perfect circle and as the value approaches 0, the shape is increasingly elongate. hMSC spreading was significantly increased in HyA-RGD hydrogel, compared to HyA hydrogel. Results are expressed as the mean + SD (n≥3). ***p<0.001. (B) DAPI (blue) and phalloidin (red) stained images of hMSCs seeded in HyA hydrogel (with or without RGD) following 11 days in culture. Magnification 20x. Scale bar =20µm.
hMSC adhesion to RGD did not enhance VEGF release (Fig. 4.10, A). MCP-1 release was significantly increased from hMSCs in HyA-RGD (T-4) hydrogel at T0 only (Fig. 4.10, B).

In summary, adhesion of hMSCs to RGD before exposure to adverse culture conditions increased hMSC survival after 11 days. Early release of MCP-1 was significantly enhanced. Cell spreading remained significantly increased for the duration of the study.
4.3.3 Physical entrapment of nidogen-1 in HyA hydrogel

In order to examine the effect of nidogen-1 on hMSCs in HyA hydrogel, it was first necessary to demonstrate successful entrapment of the nidogen-1 protein in HyA hydrogels. Fig. 4.11 shows the release of nidogen-1 from HyA hydrogels in the presence of PBS, 2.5U/mL and 100U/mL hyase. Nidogen-1 was retained in hydrogels suspended in PBS over a period of 11 days, demonstrating successful physical entrapment within the HyA hydrogel. In the presence of 2.5U/mL and 100U/mL hyase, nidogen-1 was released gradually.

![Graph showing cumulative nidogen-1 release from HyA hydrogels](image)

**Figure 4.11** Physical entrapment of nidogen-1 in HyA hydrogel. Cumulative release of nidogen-1 from HyA-Tyr hydrogels in the presence of PBS, 2.5U/mL and 100U/mL hyase. Results expressed as mean ± SD (n=3).
4.3.4 Effect of nidogen-1 on encapsulated hMSC viability, morphology and function under standard culture conditions

To examine the effect of the nidogen-1 on hMSC viability, hMSCs were encapsulated in HyA and HyA-nidogen-1 hydrogel and cultured under standard conditions for a period of 11 days. Nidogen-1 had no affect the metabolic activity of hMSCs (Fig. 4.12, A). As shown in Fig. 4.12, B, cell death was less than 20% at each timepoint, indicating nidogen-1 did not have a cytotoxic effect on hMSCs. The percentage of live cells in HyA-nidogen-1 hydrogels was found to be significantly lower at day 4 and significantly higher after 11 days in culture (Fig. 4.12, C). This data is also displayed as the number of live and dead cells/mm² (Fig. 4.12, D). The number of dead cells was found to be significantly increased at day 4 and subsequently significantly decreased at day 11 in HyA-nidogen-1 hydrogel, compared to unmodified HyA. Taken together, these results demonstrate that HyA-nidogen-1 hydrogels can support long-term hMSC culture and may play a role in cell viability.
Figure 4.12 Viability of hMSCs in HyA and HyA-nidogen-1 hydrogel under standard culture conditions. (A) Metabolic activity of hMSCs determined by CCK8 assay (B) LDH release from hMSCs determined by LDH assay. Values are normalised to the control; cells treated with lysis buffer, taken as 100% dead. (C&D) hMSCs in hydrogels were stained with calcein AM (live-green) and EthD-1 (dead-red). Cell viability is depicted as (C) the percentage of live/dead cells and (D) the number of live/dead cells/mm². Results are expressed as mean ± SD (n≥3). *p<0.05. ‘b’ refers to a significant difference between the number of dead cells, p<0.05. ‘bb’ refers to a significant difference between the number of dead cells, p<0.01.
As shown in Fig. 4.13, A, the incorporation of nidogen-1 did not increase hMSC spreading in HyA hydrogel. Quantification of spreading indicated hMSCs in HyA-nidogen-1 hydrogels exhibited a significantly rounder morphology than those in HyA hydrogel. Live/dead staining shows hMSCs remained ‘balled-up’ with a spherical morphology in both HyA and HyA-nidogen-1 hydrogels over a period of 11 days (Fig. 4.13, B). Dapi/phalloidin staining revealed circular morphology of hMSCs in both HyA and HyA-nidogen-1 hydrogel (Fig. 4.13, C).

**Figure 4.13 Effect of nidogen-1 on hMSC circularity in HyA hydrogel under standard culture conditions.** (A) Quantification of cell spreading. Results are expressed as the mean + SD (n≥3). ***p<0.001. (B&C) Representative images of hMSCs cultured for 11 days in HyA hydrogel with or without nidogen-1. (B) Live (green)/dead (red) staining. Magnification 10x. Scale bar=100µm. (C) DAPI (blue) and phalloidin (red) staining. Magnification 20x. Scale bar=20µm.
Nidogen-1 significantly enhanced encapsulated hMSC protein release. A significant increase in both VEGF and MCP-1 release was observed after 11 days (Fig. 4.14, A & B).

**Figure 4.14 Effect of nidogen-1 on hMSC protein release from HyA hydrogel.** Release of (A) VEGF and (B) MCP-1 from hMSCs encapsulated in HyA and HyA-nidogen-1 hydrogels at various time points determined by ELISA. Results expressed as mean ± SD (n=4). *p<0.05. **p<0.01.

*In summary, nidogen-1 significantly increased the percentage of live cells within HyA hydrogel under standard culture conditions after 11 days. Nidogen-1 did not increase hMSC spreading. Nidogen-1 significantly increased VEGF and MCP-1 release from hMSCs in HyA hydrogel.*
4.3.5 Effect of nidogen-1 on encapsulated hMSC viability, morphology and function under hypoxia, reduced nutrient conditions

Next, the effect of nidogen-1 on hMSCs under adverse culture conditions was investigated. No significant differences in metabolic activity were observed between hMSCs cultured in HyA and HyA-nidogen-1 hydrogels under hypoxia, reduced nutrient conditions (Fig. 4.15, A). While nidogen-1 did not increase the percentage of live/dead cells throughout the construct, a significant increase in the number of dead cells was observed after 7 days in culture (Fig. 4.15, B & C).

Figure 4.15 Effect of nidogen-1 on hMSC viability in HyA hydrogel under hypoxia, nutrient reduced conditions. (A) Metabolic activity of hMSCs determined by CCK8 assay (B&C) Cell viability is depicted as (C) the percentage of live/dead cells and (D) the number of live/dead cells/mm². Results are expressed as mean ± SD (n≥3). ‘b’ refers to a significant difference between the number of dead cells, p<0.05.
Nidogen-1 did not increase hMSC spreading in HyA hydrogel under hypoxia, nutrient reduced conditions (Fig. 4.16, A & B).

Figure 4.16 Effect of nidogen-1 on hMSC circularity in HyA hydrogel under hypoxia, reduced nutrient conditions. (A) Quantification of cell spreading. Results expressed as the mean + SD (n≥3). (B) Representative DAPI (blue) and phalloidin (red) images of hMSCs cultured for 11 days in HyA hydrogel with or without nidogen-1. Magnification 20x. Scale bar=20µm.
The release of VEGF from hMSCs was significantly reduced following 11 days in HyA-nidogen-1 hydrogel, compared to unmodified HyA hydrogel (Fig. 4.17, A). In contrast, the release of MCP-1 from hMSCs in HyA-nidogen-1 hydrogel was significantly increased after 4 days (Fig. 4.17, B).

**Figure 4.17** Effect of nidogen-1 on hMSC protein release from HyA hydrogel under hypoxia, reduced nutrient conditions. Release of (A) VEGF and (B) MCP-1 from hMSCs encapsulated in HyA and HyA-nidogen-1 hydrogels at various time points determined by ELISA. Results are expressed as mean ± SD (n=4). *p<0.05. ***p<0.001.

**In summary, the incorporation of nidogen-1 in HyA hydrogels was not found to increase hMSC viability, spreading or VEGF release under hypoxia and reduced nutrient conditions. Early release of MCP-1 was increased from hMSCs in HyA–nidogen-1 hydrogel.**
4.3.6 Effect of cell interaction with nidogen-1 on encapsulated hMSC viability, morphology and function under hypoxia, reduced nutrient conditions

The adhesion of hMSCs to RGD before exposure to adverse culture conditions was shown to significantly enhance hMSC survival. Therefore, we investigated if preconditioning of hMSCs in HyA-nidogen-1 hydrogel could induce any beneficial effects on hMSCs when transferred to hypoxia and reduce nutrient conditions. No significant differences in metabolic activity were observed between hMSCs cultured in HyA and HyA-nidogen-1 HyA following transferral to hypoxia, reduced nutrient conditions (Fig. 4.18, A). A significant decrease in the percentage of live was observed in HyA-nidogen-1 hydrogel after 3 days in adverse culture conditions. No differences were observed in the number of live/dead cells in HyA and HyA-nidogen-1 hydrogels (Fig. 4.18, B & C).
Figure 4.18 Effect of cell interaction with nidogen-1 on hMSC viability in HyA hydrogel under hypoxia, nutrient reduced conditions. (A) Metabolic activity of hMSCs determined by CCK8 assay (B&C) Cell viability is depicted as (B) the percentage of live/dead cells and (C) the number of live/dead cells/mm². Results are expressed as mean ± SD (n≥3). *p<0.05.
Despite a brief culture period with nidogen-1 in normoxia, nidogen-1 did not increase hMSC spreading in HyA hydrogel following transferral to hypoxia, nutrient reduced conditions (Fig. 4.19, A & B).

![Figure 4.19](image)

**Figure 4.19** Effect of cell interaction with nidogen-1 on hMSC circularity in HyA hydrogel under hypoxia, reduced nutrient conditions. **(A)** Quantification of cell spreading. Results are expressed as the mean + SD (n≥3). **(B)** Representative DAPI (blue) and phalloidin (red) images of hMSCs cultured for 11 days in HyA hydrogel with or without nidogen-1. Magnification 20x. Scale bar =20µm.
No significant increase in the release of VEGF or MCP-1 was observed from hMSCs encapsulated in HyA-nidogen-1 hydrogel (Fig. 4.20, A & B).

Figure 4.20 Effect of cell interaction with nidogen-1 on hMSC protein release from HyA hydrogel under hypoxia, reduced nutrient conditions. Release of (A) VEGF and (B) MCP-1 from hMSCs encapsulated in HyA and HyA-nidogen-1 hydrogel at various time points determined by ELISA. Results expressed as the mean ± SD (n=4).

*In summary, pre-conditioning of hMSCs with nidogen-1 in HyA hydrogel does not enhance hMSC survival, spreading or protein secretion when transferred to hypoxia and reduced nutrient conditions.*
4.4 Discussion

Injectable, in situ forming hydrogels are often employed as delivery vehicles to immobilise cells at the injection site and enhance their therapeutic potential by allowing the continuous release of paracrine signalling at the site of injury. The ideal hydrogel should not only improve cellular retention, but also provide cells with pro-survival cues (313). Although HyA has frequently been used for tissue engineering purposes, it does not support cell adhesion or spreading and requires functionalisation in order to enhance cell attachment (321,322). This study presents novel findings in relation to the effects of RGD and nidogen-1 functionalisation of HyA hydrogels on hMSC survival, spreading and function under ischaemic culture conditions in vitro. RGD is the principal integrin-binding domain present within ECM proteins such as fibronectin, which is a critical component of both the cardiac ECM and stem cell niche (210,323,324). Nidogen-1 is a component of the cardiac ECM that is responsible for maintaining the integrity of cardiac tissue (314).

In chapter 2, RGD was shown to promote the spreading of hMSCs under standard culture conditions, with hMSCs exhibiting an elongated, spindle-like morphology indicative of strong adhesion. As adhesion is known influence cell survival, we sought to examine the effect of hMSC adhesion to RGD on survival and function under adverse culture conditions. Under oxygen and nutrient deprived conditions, RGD was found to increase hMSCs spreading compared to unmodified hydrogel; however, visualisation of hMSCs in HyA-RGD hydrogel revealed a spherical morphology indicative of weak adhesion to the ligand. The initial attachment of cell integrins to ECM components is a passive process. However after attachment, the transition from early spreading to late spreading is an active process that requires energy expenditure (316). The inability of hMSCs to progress to late spreading and generate strong adhesions to RGD under adverse conditions is likely due to the absence of glucose, which is the primary source of energy for hMSCs. Further analysis of cell viability and protein release found the weak attachment of hMSCs to RGD did not enhance cell survival or function under adverse conditions, compared to unmodified HyA hydrogel.
In light of these findings, a new experimental plan was devised whereby hMSCs were given time to adhere strongly to RGD in HyA hydrogel before exposure to adverse culture conditions. In chapter 2, we identified hMSC spreading in HyA-RGD hydrogels after 4 days in standard culture conditions. This is in line with multiple other reports that have identified cell spreading in RGD modified hydrogels after 3-5 days in culture (192,218,286). Therefore hMSCs were given 4 days under standard culture conditions before they were transferred to adverse culture conditions. Adhesion is a reversible process, and such reversal may occur under both normal and pathological conditions (316). Following transfer to adverse culture conditions, hMSCs in HyA-RGD were found to maintain their elongated, spindle-like morphology after 11 days in adverse culture conditions, demonstrating de-adhesion did not occur. Strong adhesion of hMSCs was found to increase the percentage of live cells within the hydrogel construct. A significant reduction in the number of dead cells was observed in HyA-RGD hydrogel after 11 days, possibly due to inhibition of anoikis. Early release of the chemoattractant protein MCP-1 was also increased compared to unmodified HyA. MCP-1 is typically released by cells in response to pro-inflammatory stimuli, growth factors or cytokines in order to attract and activate white blood cells to sites of insult or injury (278). These observations provide evidence that the adhesion of cells to RGD prior to transplantation may subsequently enhance their longevity and function in the ischaemic myocardium.

The delivery of stem cells in RGD-modified hydrogels to the infarcted heart has been extensively studied in animal models of MI (187,230,325). Typically, cells are suspended in the polymer solution immediately before injection into the ischaemic tissue. The results in our study argue that RGD-modification of hydrogel may not enhance the survival or therapeutic effect of encapsulated cells in ischaemic conditions compared to unmodified hydrogel. In order to harness the pro-survival effect of RGD, it may be necessary to allow stem cells to adhere to the RGD peptide in hydrogel ex vivo, prior to transplantation. The delivery of preformed hydrogel would require the development of a novel catheter. Given the importance of glucose in cell metabolism, it is possible that the inclusion of glucose in HyA-RGD
hydrogels scaffolds could provide cells with the energy required to progress from weak attachment to strong adhesion under ischaemic condition. This has the potential to overcome the need for ex vivo expansion in preformed hydrogels.

To our knowledge, this is the first report characterising the effect of nidogen-1 on hMSCs entrapped in HyA hydrogels. Following the successful encapsulation of nidogen-1 in HyA hydrogel, we assessed the effect of the nidogen-1 protein on hMSCs under standard culture conditions. The percentage of live cells was significantly increased in HyA-nidogen-1 hydrogel after 11 days, indicating it may play a role in hMSC viability. Cells in HyA-nidogen-1 hydrogel did not spread and exhibited a spherical morphology. Both VEGF and MCP-1 secretion was increased in HyA-nidogen-1 hydrogel compared to unmodified HyA hydrogel. Taken together, these results demonstrate that while nidogen-1 does not influence hMSC spreading, it plays a role in hMSC viability and function.

Next the effect of nidogen-1 was assessed under hypoxia and reduced nutrient conditions. Under adverse culture conditions nidogen-1 had no effect on hMSC survival, spreading or VEGF release. Nidogen-1 did however increase the secretion of MCP-1 after 4 days. From these results, we can conclude that nidogen-1 is not a promising pro-survival stimulus for hMSCs under ischaemic culture conditions. Though it appears to have a role in the immunomodulatory behaviour of hMSC. In contrast the aforementioned RGD results, the cultivation of hMSCs with nidogen-1 under standard conditions for 4 days before transfer to adverse culture conditions did not improve hMSC survival, spreading or function. This observation suggests that a pre-exposure cultivation period is only necessary when enhancing cell survival with adhesion ligands. When developing biomaterials for cellular attachment, the RGD sequence has multiple advantages over large proteins. First, short peptides are simple and cheap to synthesise. Second, peptides can preserve their bioactivity in harsh chemical conjugating conditions that can easily cause protein denaturation. The use of RGD also minimises the risk of immune reactivity or pathogen transfer particularly where xenograft proteins are used (323,326).
A limitation to these studies is the low levels of cell death observed under the harsh ischaemic in vitro conditions. This may be due to the inherent pro-survival effect of the HyA hydrogel through the cell-surface glycoprotein receptor CD44 (327). Future work will examine the modification of HyA hydrogel with other cardiac ECM proteins such as decorin and collagen IV on hMSCs behaviour. Following the investigation of individual proteins and peptides, combinatorial scaffolds can be designed in order to control encapsulated stem cell fate and promote survival under ischaemic conditions.

**Conclusion**

The results in the chapter demonstrate the suitability of HyA hydrogel as a blank slate biomaterial to investigate the effect of single factor ECM proteins and peptides under normoxic and hypoxia, reduced nutrient conditions in vitro. Strong adhesion of hMSCs to the RGD peptide was shown to improve cell survival under adverse culture conditions. This finding may have a significant effect on the use of adhesive ligands to enhance stem cell engraftment in ischaemic injury sites. The effect of nidogen-1 on hMSCs in HyA hydrogel was reported for the first time. Although nidogen-1 does not influence hMSC spreading, it appears to play a role in cell viability and function under standard culture conditions. Nidogen-1 may play a role in the immunomodulatory effects of hMSCs at ischaemic injury sites.
Chapter 5: Encapsulation of Cardiac Stem Cells in an RGD-Functionalised Hyaluronic Acid Hydrogel to Enhance Cell Survival under Hypoxia and Reduced Nutrient Conditions
5.1 Introduction
CSCs were first identified in 2003 (77). Since then, several types of CSCs have been isolated and characterised, including cardiosphere-derived cells (CDCs), c-kit⁺ cells, stem cell antigen-1 (Sca-1⁺) cells and Isl-1⁺ cells (77,328–330). In the mammalian heart, clusters of CSCs are found predominantly in the atrial and apical myocardium where they reside in a dynamic microenvironment, or niche, which maintains stemness. The niche can be defined as a randomly oriented ellipsoid structure made up of both cellular and extracellular components (324). As shown in Fig. 5.1, fibronectin is distributed throughout the niche and accumulates around c-kit⁺ CSCs (324).

![Figure 5.1 Cardiac stem cell niche. Schematic image demonstrating the cellular and extracellular components of the cardiac stem cell microenvironment (331).](image)

The pool of cells found within the niche is heterogeneous, consisting of both quiescent and actively proliferating cells, and uncommitted and early committed cells (331). Aside from their possible involvement in cardiomyocyte turnover, their exact physiological function of CSCs has not yet been elucidated. Their role in
pathological situations is also ambiguous since in the case of myocardial injury following MI, their potential regenerative capacity is clearly overwhelmed (332). Nevertheless, as a resident cell type with the innate ability to generate de novo cardiomyocytes, CSCs represent an ideal candidate for cardiac cell therapy (333). Animal studies have documented the ability of ex vivo expanded CSCs to improve heart function following transplantation in animal models of MI. A meta-analysis of 80 preclinical studies including 1970 animals (1176 treated, 794 controls) reported CSC treatment resulted in a 10.7% improvement in ejection fraction compared with control animals (334). The success of these studies has paved the way for clinical trials. While bone marrow-derived stem cells are the most common cell source used in clinical studies, there is a clear and growing interest in the use of second-generation cardiac-derived stem cells/pluripotent stem cells, stemming from the desire to closely match the target organ (Fig. 5.2) (79).

![Figure 5.2](image)

**Figure 5.2** Clinical trials using first- and second-generation cells. # indicates follow-up study (79).

The SCIPIO (cardiac Stem Cells In Patients with Ischemic Cardiomyopathy) trial was the first study of CSCs in humans. Autologous c-kit+ CSCs were delivered by IC infusion into patients with ischaemic heart failure who had undergone coronary artery bypass grafting (CABG). This phase I trial confirmed the safety of CSC delivery as well as the feasibility of isolating and expanding CSCs from heart tissue obtained during CABG surgery. Functional benefits in response to CSC therapy included a
reduction in infarct size, an increase in viable myocardium and a marked improvement in LVEF and NYHA functional class at 4 and 12 months post treatment (215,335). The second trial, CADUCEUS (CArdiosphere-Derived aUtolo-gous stem CELls to reverse ventricUlar dySfunction), confirmed the safety of IC infusion of autologous CDCs following MI. This study documented a reduction in scar size alongside an increase in viable myocardium; however, no differences in LVEF were observed between groups 12 months after treatment (336,337). These trials are summarised in Table 5.1.

**Table 5.1** Clinical trials using cardiac stem cells (79).

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of patients</th>
<th>Type of patients</th>
<th>Duration (months)</th>
<th>Imaging modality</th>
<th>Changes in left ventricular ejection fraction (LVEF)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCIPO (2011)</td>
<td>23</td>
<td>HF</td>
<td>4</td>
<td>Echocardiography</td>
<td>30.1 ± 2.4-30.2 ± 2.5% to 30.3 ± 1.9-38.5 ± 2.8%</td>
<td>+8.2% (335)</td>
</tr>
<tr>
<td>SCIPO+ (2012)</td>
<td>33</td>
<td>HF and MI</td>
<td>4 and 12</td>
<td>Echocardiography</td>
<td>N/A to 27.5 ± 1.0-39.1 ± 2.4% (4th month) and 41.2 ± 4.5% (12th month)</td>
<td>+7.6% (4th month) + 13.7 (12th month) (215)</td>
</tr>
<tr>
<td>CADUCEUS</td>
<td>25</td>
<td>MI</td>
<td>6</td>
<td>MRI</td>
<td>38-44.8% to 38-43.4%</td>
<td>+5.4 (336)</td>
</tr>
<tr>
<td>CADUCEUS+</td>
<td>25</td>
<td>MI</td>
<td>12</td>
<td>MRI</td>
<td>42.5 ± 11.3-48.2 ± 11.4% to 42.4 ± 8.9-48.2 ± 10.3%</td>
<td>+5.4 (337)</td>
</tr>
</tbody>
</table>

N/A, not applicable (placebo group was not included in trial); HF, heart failure; MI, myocardial infarction; LV, left ventricular; MRI, magnetic resonance imaging.

*Follow-up studies.

**Table 5.1** Clinical trials using cardiac stem cells (79).

While transplantation of *ex vivo* proliferated CSCs is an emerging therapy for ischaemic heart disease, their therapeutic effects are limited by poor engraftment and long-term survival (102,338,339). Biomaterials possess multiple beneficial features such as biocompatibility, biodegradability, low-immunogenicity and low cytotoxicity to overcome these hurdles (156). The delivery of CDCs in hydrogel has been shown to enhance cellular engraftment and therapeutic efficacy in a mouse model of MI, compared to cells in saline (340). This chapter examines the survival of rat c-kit+ CSCs encapsulated in an *in situ* crosslinking HyA-RGD hydrogel under hypoxic/reduced serum conditions, as present at ischaemic injury sites. C-kit+ CSC are able to differentiate into myocytes, smooth muscle cells and endothelial cells *in vitro*, and therefore may contribute to myocardial regeneration following transplantation *in vivo* (77). RGD is the principle integrin-binding domain within the
niche protein fibronectin, which is known to accumulate around c-kit+ CSCs (323). We hypothesise that RGD will enhance c-kit+ CSC viability in an ischaemic environment through the inhibition of cell death by anoikis (151,341).

**Objectives**

The primary objective of this study was to investigate whether HyA-RGD hydrogel could be used to enhance the survival of CSCs under ischaemic conditions. For these experiments, the well-known cardioprotective agent IGF-1 was first tested as a pro-survival stimulus for rCSCs. Previously in chapter 3, we set out to establish IGF-1 as a positive control (Fig. 3.1) for our hMSC studies. While hMSCs did not respond to IGF-1 treatment, CSC proliferation was shown to be enhanced following IGF-1 stimulation. Therefore this chapter aimed to determine the ability of IGF-1 to increase CSCs survival under ischaemic conditions so it could be included as a positive control in hydrogel experiments. The delivery of IGF-1 in microparticles incorporated in HyA hydrogel was also assessed.

- The first objective was to determine the level of c-kit marker on rCSCs.

- The second objective was to assess the effect of RGD on rCSC viability in HyA hydrogel under standard culture conditions.

- The third objective was to establish IGF-1 as a positive control under ischaemic culture conditions.

- The fourth objective was to analyse the ability of RGD to enhance rCSC survival in HyA hydrogel under ischaemic culture conditions.

- The fifth objective was to assess the bioactivity of IGF-1 released from PLGA microparticles incorporated into HyA hydrogels.
5.2 Materials and Methods

5.2.1 Cardiac stem cell isolation and culture
CSCs were previously isolated in our laboratory from Sprague Dawley rats and cultured as described previously in Section 3.2.7.1 (300). Cells were not used above passage 9.

5.2.2 Flow Cytometry
Our laboratory group recently reported that after two passages, 6.1% of the rCSC population express the stem cell marker c-kit (300). The experiments in this thesis used rCSCs up to passage 9. In order to characterise the expression of c-kit on late-passage rCSCs, cells were passaged 9 times as adherent monolayers and then used for flow cytometry experiments with a FACS (BD Canto, BD Biosciences, San Jose, California) flow cytometer with quantitative analysis provided by CellQuest software (BD Biosciences, San Jose, California). Cells were trypsinised and resuspended in FACs buffer (PBS + 1% FBS). Cells were then washed three more times before they were incubated with fluorescein isothiocyanate (FITC) 7 conjugated antibodies against CD-117 (c-kit) for 30mins (1:200 dilution). Isotype-identical antibodies (IgG2b, k) served as negative controls (BD Biosciences, San Jose, California).

5.2.3 Effect of RGD on rCSC viability in HyA hydrogels under standard culture conditions
The interaction between cells and their ECM is known to play a role in maintaining their viability and proliferation. The effect of the adhesion peptide RGD on rCSC viability in HyA hydrogels was examined. rCSCs were encapsulated in HyA and HyA-RGD hydrogels at a density of $1 \times 10^6$ cells/mL and culture in CGM (Section 3.2.7.1) at 21% oxygen. The viability of encapsulated rCSCs was determined by CCK8 assay and LIVE/DEAD assay as described in Section 2.2.8.1 and Section 2.2.8.3 respectively, over a period of 11 days.
5.2.4 Effect of IGF-1 on cell survival under hypoxia, low-serum conditions

The pro-survival effect of IGF-1 on rCSCs in stress conditions was explored. rCSCs were passaged and seeded in 24-well plates at a density of 30,000 cells per well in CGM (Section 3.2.7.1) for 24h. Wells were rinsed with PBS before rCSCs were cultured under one of three conditions for 6 days (i) CGM in normoxia (21% oxygen) (ii) reduced-serum (1% FBS) basal medium in hypoxia (1% oxygen) (iii) reduced-serum (1% FBS) basal medium supplemented with IGF-1 25-250ng/mL in hypoxia (1% oxygen). Basal medium was prepared as described previously in Section 3.2.7.2. Cell survival was monitored by collection and quantification of dsDNA by PicoGreen assay as described in Section 3.2.3.

5.2.5 Effect of RGD on rCSC survival in HyA hydrogels under hypoxia/low serum conditions

rCSCs were encapsulated in HyA and HyA-RGD hydrogels at a density of 1 x 10^6 cells/mL and cultured in low-serum (1% FBS) basal medium in hypoxia (1% oxygen) for 11 days. rCSCs encapsulated in HyA hydrogel and cultured in 150ng/mL IGF-1 supplemented medium were used as a positive control for these experiments. The viability of cells within the hydrogels was determined by CCK8 and live/dead assay as described in Section 2.2.8.1 and Section 2.2.8.3. Live/dead data shown as the percentage live/dead cells, the number of live cells/mm^2 and the number of dead cells/mm^2.

5.2.6 Release of IGF-1 from PLGA microparticles in HyA

IGF-1-loaded PLGA microparticles (MPs) were developed in the laboratory at RCSI by double emulsion solvent evaporation method (unpublished data). To measure IGF-1 release from MPs in HyA hydrogel, IGF-1-loaded MPs were encapsulated in HyA hydrogels (1µg/200µl hydrogel) and incubated in glass vials at 37°C in a shaking water bath (Grant Instruments, Cambridge, UK) at 50rpm. PBS was removed at given timepoints (1, 4 hours, 1, 2, 4, 8 and 11 days) and replaced with fresh PBS. Collected supernatants were stored at -20°C. IGF-1 concentration was determined by ELISA assay as per the manufacturer’s instructions (R&D Systems). Release from free-loaded IGF-1 (1µg/200µl hydrogel) was used as the control.
5.2.7 Bioactivity of IGF-1 released from PLGA microparticles in HyA on rCSCs

In order to assess the bioactivity of IGF-1 released from MPs in HyA hydrogel, IGF-1-loaded MPs were encapsulated in HyA hydrogel as in Section 5.2.6 and suspended in 400µl of PBS. Samples were incubated in glass vials in a shaking water bath at 37°C at 50rpm. A single timepoint was collected after 48 hours. rCSCs were seeded in 24-well plates at a density of 30,000 cells/well in CGM (Section 3.2.7.1) and allowed to adhere overnight. Culture medium was removed, wells were washed with PBS and medium was replaced with BM (Section 3.2.7.2) for 24hrs. rCSCs were then treated with BM supplemented with 10ng/mL IGF-1 release medium from IGF-1-loaded MPs in HyA hydrogel. CGM and free IGF-1 in BM were used as controls. After 72 hours, dsDNA was collected and quantified by PicoGreen assay as in Section 3.2.3.

5.2.8 Statistical analysis

Statistical analysis of results was carried out using GraphPad Prism software, version 5.01. Two-Way ANOVA was performed followed by pairwise multiple comparison procedures (Bonferroni test). Error is reported as standard deviation (SD) and significance was determined using a probability value of P<0.05. A minimum of n=3 technical replicates were performed for all experiments, unless otherwise stated.
5.3 Results

5.3.1 Fluorescence-Activated Cell Sorting Analysis

Phenotypic analysis of late passage rCSCs revealed expression of the stem cell marker c-kit. 1.8% of the CSC population expressed the c-kit marker (Fig. 5.3).

![Fluorescence-activated cell sorting analysis of rCSCs](image)

Figure 5.3 Fluorescence-activated cell sorting analysis of rCSCs. The phenotype profile for c-kit expression was analysed within the density plots and shown as a percentage of positive events. 1.8% of the rCSC population expressed the stem cell marker c-kit (n=1).
5.3.2 Effect of RGD on rCSC viability in HyA hydrogel under standard culture conditions

The effect of the RGD peptide on encapsulated rCSC viability was assessed over 11 days. RGD was found to significantly increase rCSC metabolic activity after 7 days in culture (Fig. 5.4, A). RGD also significantly increased the percentage of live cells/decreased the percentage of dead cells after 4 days (Fig. 5.4, B). RGD was found to significantly decrease the number of dead cells after 4 days, and significantly increased the number of live cells after 7 days (Fig. 5.4, C). Taken together, these results demonstrate that RGD significantly enhances encapsulated rCSC viability compared to unmodified HyA hydrogel.

Figure 5.4, D demonstrates rCSC morphology in HyA and HyA-RGD hydrogels following 11 days in culture. As observed during 2D rCSC expansion, rCSCs are small, rounded cells with short cellular processes which fall below the limit of detection. Therefore, it was not possible to quantify and compare rCSC spreading for these experiments.
**Figure 5.4** Effect of RGD on rCSC viability under standard culture conditions. (A) Metabolic activity of rCSCs encapsulated in HyA and HyA-RGD hydrogels determined by CCK8 assay. **p<0.01. (B & C)** Comparison of live.dead cells in HyA and HyA-RGD hydrogels. Encapsulated rCSCs imaged using confocal microscopy following live.dead staining at times indicated. (B) Statistical difference observed between the percentage of live.dead cells when exposed to RGD at day 4 compared to the control. ***p<0.001. (C) Statistical difference observed between the number of dead cells at day 4 and the number of live cells at day 7 in HyA-RGD hydrogels compared to the control. ‘a’ refers to a significant difference between the number of live cells. ‘b’ refers to a significant difference between the number of dead cells. p<0.05. Mean ± SD (n≥3). (D) Representative confocal images showing rCSC morphology in HyA and HyA-RGD hydrogels following 11 days in culture. Magnification 10x. Scale bar=200µm.
5.3.3 Effect of IGF-1 on rCSC survival under hypoxia, reduced serum conditions (2D)

To evaluate the protective effect of IGF-1 on rCSC survival in stress conditions, rCSCs were treated with 25-250ng/mL of IGF-1 and exposed to hypoxia (1% O₂), low serum conditions (342–344). After 6 days, IGF-1≥25ng/mL was found to significantly increase rCSC proliferation compared to the untreated control (Fig. 5.5).

![Figure 5.5 Effect of IGF-1 on rCSC survival under hypoxia, reduced serum conditions.](image)

*Figure 5.5 Effect of IGF-1 on rCSC survival under hypoxia, reduced serum conditions.* The effect of IGF-1 on cell proliferation was determined by PicoGreen assay after stimulation of cells with increasing doses of IGF-1 under hypoxic, serum reduced conditions. IGF-1 increases the survival of rCSCs at day 6. H/RM, hypoxia/regular medium. H/LS, hypoxia/low serum. Results are expressed as the mean ± SD (n=3). ***p<0.001.
5.3.4 Effect of RGD on rCSC survival in HyA hydrogel under hypoxia, reduced serum conditions

Having established IGF-1 as a suitable pro-survival stimulus for rCSCs in 2D, we next sought to include IGF-1 as a positive control in our 3D hydrogel study. rCSCs were encapsulated in (i) HyA hydrogel (control), (ii) HyA-RGD hydrogel or (iii) HyA hydrogel cultured with IGF-1 (positive control) and cultured under 1% O₂ and 1% FBS conditions for 11 days. When interpreting these results, rCSC survival in HyA and HyA-RGD hydrogels is first compared, then rCSCs in HyA-RGD and HyA-IGF-1, before finally commenting on the differences between HyA and HyA-IGF-1.

While the RGD peptide was not found to enhance the metabolic activity of encapsulated rCSCs, compared to unmodified HyA hydrogel (Fig. 5.6, A), a significant increase in the percentage of live cells along with a corresponding decrease in the percentage of dead cells was observed at day 11 (Fig. 5.6, B). The number of viable rCSCs is also displayed as live/dead cell per mm². In the HyA RGD hydrogels, a significant difference was observed in both live and dead cells at day 4, compared to HyA hydrogels, indicating an overall increase in the total number of cells within the hydrogel construct at this timepoint (Fig. 5.6, C & D). Although no differences in the number of live cells were observed for the remainder of the study, the RGD peptide was found to significantly decrease the number of dead cells at day 11 compared to HyA hydrogel. Taken together, these results indicate RGD promotes rCSC survival under adverse culture conditions.

Compared to rCSCs cultured in HyA-IGF-1, the metabolic activity of rCSCs in HyA-RGD was significantly lower at day 7 (Fig. 5.6, A). No significant difference was observed in the percentage of live and dead cells or the number of live cell/mm² (Fig. 5.6, B & C). Lastly, a significant increase in dead cells was observed in HyA-RGD hydrogels at day 4 compared to HyA-IGF-1, however, no significant differences were observed after this timepoint (Fig. 5.6, D). Overall, the effect of RGD was found to be comparable to our positive control, IGF-1.
Compared to HyA alone, IGF-1 treatment was found to significantly increase the percentage of live cells with a corresponding decrease in the percentage of dead cells (Fig. 5.6, B). In terms of dead cell/mm², IGF-1 treatment significantly decreased the number of dead cells at day 11 compared to HyA hydrogel alone (Fig. 5.6, D). Overall, these results demonstrate the suitability of IGF-1 as a pro-survival stimulus for rCSC in 3D hydrogel experiments.

Figure 5.6 Effect of RGD on rCSC survival under hypoxic, reduced serum conditions. (A) Metabolic activity of rCSCs encapsulated in HyA, HyA-RGD and HyA &IGF-1 hydrogels determined by CCK8 assay. (B) Comparison of the percentage of live/dead cells in HyA, HyA-RGD and HyA &IGF hydrogels. Encapsulated rCSCs imaged using confocal microscopy following live/dead staining at times indicated. Graph comparing the number of (C) live cells/mm² and (D) dead cells/mm² in HyA, HyA-RGD and HyA & IGF-1 hydrogels. Note: * indicates a significant difference between HyA and HyA-RGD. # indicates a significant difference between HyA-RGD and HyA & IGF. x indicates a significant difference between HyA and HyA & IGF. */#/## denotes p<0.05, **/## denotes p<0.01. *** denotes p<0.001. Mean ± SD (n≥3).
5.3.5 IGF-1 release from PLGA microparticles in HyA hydrogel

In order to assess the ability of MPs to enhance the release of IGF-1 from HyA hydrogel, the release of IGF-1 from PLGA MPs encapsulated in HyA hydrogel was assessed over the course of 11 days (Fig. 5.7, A & B). Release of free-loaded IGF-1 from HyA hydrogel was used as the control for this study. Results indicate IGF-1 was more rapidly released from MP-loaded hydrogels. MPs significantly increased the fraction of IGF-1 released compared to the free-loaded IGF-1, indicating a certain degree of retention of the growth factor in the HyA hydrogel when PLGA MP are not used.

**Figure 5.7 Release IGF-1 from HyA hydrogel** Cumulative release of IGF-1 from HyA hydrogel, expressed as (A) μg/mL release and (B) percentage release of the total IGF-1 loaded in each hydrogel. Results are expressed as the mean ± SD (n=3).
5.3.6 Bioactivity of IGF-1 release from microparticles in HyA hydrogel.

In order to assess the bioactivity of IGF-1 released from PLGA MPs in HyA hydrogel, rCSCs were incubated with BM supplemented with 10ng/mL IGF-1 release medium. IGF-1 bioactivity was confirmed by the increase in cellular growth compared to the untreated control (BM) (Fig. 5.8). This indicates the IGF-1 remains bioactive after release from MPs loaded into HyA hydrogels.

Figure 5.8 Bioactivity of IGF-1 release from MPs loaded into HyA Hydrogel. dsDNA of rCSCs cultured for 72h with either (i) CGM, (ii) BM, (iii) BM supplemented with free IGF-1 or (iv) BM supplemented with IGF-1 released from MPs encapsulated in HyA hydrogel. IGF-1 at 10ng/mL. Results are expressed as the mean + SD (n=3). CGM denotes complete growth medium. BM denotes basal medium. MP denotes microparticles. All columns compared to the control, BM. **p<0.01. ***p<0.001.
5.4 Discussion

The issue of poor cell retention and survival in the infarcted heart is a major factor which has been shown to affect the efficacy of cellular therapy to date (81). The combination of cells with hydrogels has been widely explored as a means to protect cells from mechanical washout and the harmful inflammatory environment, to support their long-term survival and function. The ideal hydrogel should undergo gelation under mild conditions without harming cells, providing cells with a suitable 3D environment which facilitates diffusion of biomolecules and preferably possess peptide sequences easily recognisable by cell-surface receptors (164). Our results show that HyA-RGD hydrogel meets these design requirements and has good biocompatibility with rCSCs. Furthermore, when the cells were exposed to an ischaemic environment, RGD scaffolds protected rCSCs against cell death. The RGD peptide has been widely used to functionalise a variety of hydrogels for a multitude of applications (160,187,192,218,326). It is known to bind to integrins on the cell surface, functioning as a bio-physical cue for the attachment of cells to the hydrogel (323). RGD may act to prevent anoikis (341).

In chapter 3, the ability of IGF-1 to enhance rCSC proliferation under normoxic conditions was reported. IGF-1 is a potent proliferative factor and a known cardioprotective agent which has been shown to enhance cardiomyocyte survival while also stimulating cardiac regeneration through the activation and migration of endogenous c-kit+ CSCs in vivo (345). In this chapter, IGF-1 treatment was included as a positive control in the 3D hydrogel study and was shown to significantly increase rCSC survival under hypoxic, reduced serum conditions as planned. Further analysis of this study would include quantification of proteins secreted in response to IGF-1 and RGD in order to conduct a more detailed comparison.

The hematopoietic stem cell marker c-kit has been used to isolate and characterise adult cardiac stem cells in numerous studies (78,201,300,330,346). C-kit+ CSCs are resident cardiac stem cells that exhibit stem cell properties of clonogenicity, self-renewal and the ability to differentiate into adult cardiac lineages (77). While the
functional contribution of c-kit+ CSCs to in vivo cardiomyocyte turnover is one of the highly debated concerns regarding their regenerative capacity (347), preclinical and clinical studies have provided strong evidence to show their in vitro abilities of cardiac lineage differentiation as well as in vivo ability to enhance cardiac structure and function after implantation (77,81,215). The rCSCs used in this chapter were previously isolated in our laboratory from rat heart explants. From the explants, approximately 6% of the cell population were found to express the c-kit marker (300). This falls in line with c-kit levels from other publications, where expression levels are reported to vary between 4-12% (78,346). Our results show that at a late passage, less than 2% of the cell population express the c-kit marker, demonstrating levels of c-kit expression declines with time in culture. This finding is consistent with data published by Davis et al. who reported a reduction in c-kit expression following extended culture of CPCs (346).

The oxygen-serum deprivation model used in our studies aims to replicate the in vivo pathological environment faced by cells upon transplantation. These conditions have been commonly described and utilised by other research groups working with cardiac-derived stem cells (342,343). A limitation of this system is the inability to account for all of the complexities of the in vivo infarct. While this set up provides a more specific study of the role of hypoxia and serum reduction on cell viability, it neglects other negative influences on cell viability such as inflammatory cytokines and free radicals which could be present in the injured myocardium (129).

To date, clinical trials evaluating CSCs for cardiac repair following MI have utilised IC cell infusion (215,336), which is unsurprising given the familiarity of most cardiologists with this procedure (98). However, preclinical comparative studies of IC and IM CSC delivery have demonstrated retention is lower with IC than IM (at 24 h, 12.7% of the cells present after 5 minutes were found in the heart after IC infusion vs. 22.5% after IM injection; at 7 days, the corresponding percentages were 3.5% and 7.6%, respectively) (81). In addition, IM delivery has demonstrated improved CSC engraftment, compared with IC infusion (111). Recent studies have focused on the combination of cells with biomaterial carriers to further enhance
the efficacy of CSCs following IM injection to the infarcted heart. One such study, reported that encapsulation of CSCs in hydrogel microcapsules promoted engraftment and long-term survival after injection, leading to enhanced cardiac function compared to non-encapsulated CSCs (344). However, for meaningful myocardial tissue replacement, biomaterials should not only improve retention of stem cells but also provide mechanical support to the compromised ventricular wall (348). The injectable, in situ gelling HyA-RGD hydrogel described in this chapter has the potential to not only improve CSC survival in the ischaemic heart, but also confer passive mechanical support to improve wall mechanics.

In addition to biomaterial strategies, genetic modification of c-kit+ CSCs has been shown to enhance their reparative capacity. C-kit+ CSCs genetically modified to overexpress the proto-oncogenic serine-threonine kinase Pim1 have been shown to reduce scar size, increase viable tissue and restore cardiac function in animal models of MI (229,349). Genetic enhancement of IGF-1 in human CSCs has also been shown to enhance cardiac repair in an animal model of MI by improving long-term survival of transplanted cells and surrounding myocardium (342). Although gene therapy has shown promising results, clinical progression faces several problems including transfection efficiency, limiting the mutagenic potential, reducing cytotoxicity and controlling gene targeting. The delivery of proteins and small molecules may circumvent the safety concerns of genetic manipulation; however, this approach has limited success due to uncontrolled protein release and the short half-life of proteins in vivo. In this chapter, we demonstrated the bioactivity of IGF-1 released from MP incorporated into HyA hydrogel. The delivery IGF-1-loaded MPs in HyA hydrogel in combination CSCs may act as a multi-modal therapy to promote both endogenous and transplanted CSC viability.
Conclusion

In this chapter, we established a controlled experimental system where IGF-1 treatment was used as a positive control to compare and contrast different pro-survival stimuli under ischaemic culture conditions. In summary, this chapter demonstrates that both biophysical (in the case of RGD) and bio-chemical (in the case of IGF-1) strategies can be employed to enhance rCSC survival under adverse culture conditions.
Chapter 6: Discussion
6.1: Overview

Heart failure is a progressive, debilitating disease commonly caused by the irreversible loss of functional cardiac tissue after MI. Aside from organ transplantation, current treatment options are considered palliative as they improve quality of life and possibly prolong survival but fail to address the underlying damage to cardiac tissue. Therefore there have been significant efforts to develop new therapies that could repair and regenerate the myocardium. To date, clinical efforts towards cardiac regeneration have focused on cell-based therapies. Although the safety and feasibility has been demonstrated using multiple cell types, only a minimal recovery of LV function has been achieved. The curative potential of stem cells is hindered by poor retention and survival of transplanted cells, in part due to the disrupted ECM in the infarcted myocardium that can lead to apoptosis of transplanted cells by anoikis. Although preconditioning, genetic manipulation and co-transplantation of cells may extend the longevity of transplanted cells, these approaches fail to address the physical factors affecting cellular retention including dispersion from the injection site and anoikis. Tissue engineering strategies such as injectable carriers and patches are currently under investigation to overcome this issue. As patch implantation requires an invasive surgical procedure, it is believed that injectable biomaterials, which can be delivered in a minimally invasive manner, will facilitate more rapid clinical translation. In addition to their use as cell delivery vehicles, hydrogels may also be used to stabilise the heart wall and prevent adverse LV remodelling.

Naturally in the body, stem cells reside in highly specialised tissue specific microenvironments known as “niches”. Within the niche, cells are exposed to biophysical properties and biochemical cues which have major regulatory functions on the cells. The stem cell niche is an important consideration for the encapsulation of cells within a surrogate 3D matrix as it functions to physically maintain cells and govern their fate. The ideal hydrogel would not only improve cellular retention, but also provide instructive cues to help control the fate of engrafted cells. Natural biomaterials such as HyA are commonly used for cell delivery due to their intrinsic
ability to generate scaffolds that are analogous to native tissue. However, the use of unmodified HyA hydrogel for cell delivery is not ideal, as single-factor ECM scaffolds over-simplify the multicomponent ECM of the stem cell niche and do not provide bioactive cues to promote the long-term survival and function of encapsulated cells.

The overall aim of this research was to develop a functionalised HyA hydrogel capable of improving stem cell survival and function under the condition of hypoxia with reduced nutrients, as is present in ischaemic injury sites. Our HyA was modified with tyramine to enable gel formation via enzyme mediated crosslinking of tyramine moieties using HRP and H$_2$O$_2$. As the optimal cell source is unknown, we applied this research to both CSCs and MSCs, two of the leading candidates for cardiac regeneration.

Evaluation of a biological scaffold typically involves two key aspects: whether the mechanical strength of the hydrogel is suitable for practical applications and if the hydrogel can support cell growth and adhesion. HyA is known to resist cellular attachment, therefore RGD, the most widely studied adhesive peptide in the biomaterial field, was incorporated to facilitate cell adhesion. RGD, the tripeptide sequence Arg-Gly-Asp, can be found in many ECM proteins such as fibronectin. In Chapter 2, both HyA and HyA-RGD hydrogels were found to gelate rapidly to form porous scaffolds which exhibited minimal swelling and appropriate mechanical properties. While hMSC viability was maintained in both RGD-modified and unmodified HyA hydrogels under standard culture conditions, RGD was found to facilitate the strong adhesion of hMSCs to the 3D matrix and significantly enhance VEGF and MCP-1 secretion from encapsulated cells compared to unmodified HyA. These observations demonstrated the ability of RGD functionalisation to promote strong cell-matrix interactions which subsequently lead to enhanced secretion of paracrine factors from hMSCs.

In order to accurately determine the ability of RGD to promote hMSC survival and function under adverse culture conditions, we sought to establish a controlled
experimental system using the well-known anti-apoptotic factor IGF-1 in Chapter 3. IGF-1 is known to enhance the growth and survival of multiple cell types; however, its effect on hMSCs is poorly understood. IGF-1 treatment was found to have no effect on hMSC proliferation or survival. In addition, expression of IGF-1R was not detected following exposure to normoxic or hypoxic, reduced nutrient conditions. The bioactivity of our IGF-1 protein was confirmed using rCSCs, which demonstrated significant proliferation in response to IGF-1 stimulation.

Having determined the ability of hMSCs to adhere to RGD under standard culture conditions in chapter 2, Chapter 4 investigated the ability of RGD to promote the survival of hMSCs under hypoxic and reduced nutrient conditions. In addition, the effect of the ECM protein nidogen-1 on hMSC survival in HyA hydrogel was investigated. To our knowledge, this is the first report characterising the effect of nidogen-1 on hMSCs in hydrogel.

The optimal source of cells for regenerating damaged myocardium is a topic of intensive research. CSCs represent an ideal candidate for cardiac cell therapy given their innate ability to generate de novo cardiomyocytes. CSCs are reported to reside within a stem cell niche in the heart surrounded by fibronectin (350). RGD is the principal integrin-binding domain within fibronectin; therefore in chapter 5 the interaction between RGD and rCSCs was investigated and its effect on cell survival under hypoxia, reduced nutrient conditions was analysed. RGD was found to decrease cell death compared to unmodified HyA hydrogel under adverse culture conditions, indicating the cell-RGD interaction inhibits cell death due to anoikis. IGF-1 was confirmed as a suitable pro-survival stimulus and positive control for these studies.

The following sections will summarise the main findings of each chapter and their implications.
6.2 Chapter 2: Mesenchymal Stem Cell Cultivation in an Injectable \textit{in situ} Forming Hyaluronic Acid Hydrogel Modified with RGD

A growing body of preclinical evidence demonstrates that the combination of cells and hydrogel can achieve enhanced cardiac repair, compared to injection of cells alone (230–232). This chapter investigates the potential of an RGD-modified HyA hydrogel to act as surrogate matrix to maintain hMSC viability, attachment and function under standard culture conditions. HyA-RGD hydrogel was compared in these studies with unmodified HyA hydrogel.

The mechanical properties of enzymatically crosslinked tyramine substituted HyA (HyA-TA) hydrogels have been extensively researched and can be easily modified by adjusting the ratio of crosslinking agents or by increasing polymer concentration (237,252,255). Our results demonstrate that crosslinking of HyA and HyA-RGD precursors resulted in rapid gelation. This property is desirable for clinical translation to reduce leakage of liquid scaffold and to effectively retain cells at the site of injection, thereby maximising the therapeutic effect. HyA-RGD hydrogel was shown to have a higher swelling ratio than HyA hydrogel which may be due to the RGD substitution reducing crosslinking density. The compressive moduli of HyA and HyA-RGD hydrogels ranged between 5.5-7kPa, which is similar to that of Algisyl-LVR™ (3-5kPa) but lower than both normal and pathological cardiac tissue (178,256). For bulking purposes, stiff materials are advantageous as they may provide structural support to the damaged myocardial wall and improve function (179). However, as reported by Lei \textit{et al.}, stiff materials may not be favourable for cell delivery as increased crosslinking and polymer concentration can negatively affect hMSC spreading, migration and proliferation, compared to softer matrices (246).

The effect of scaffold size on encapsulated cell viability was assessed using live/dead staining to visualise hMSCs throughout large-volume (500μl) and small-volume (125μl) hydrogels. In small-volume hydrogels, live hMSCs were visible throughout the scaffold, indicating the adequate diffusion of nutrients. In large-volume
hydrogels, live cells were restricted to the periphery of the scaffold, with few viable cells observed at the centre of the construct. Following these observations, new moulds were designed to generate cylindrical hydrogel constructs of 200µl. Visualisation of hMSCs within 200µl hydrogel constructs revealed cells were homogenously distributed throughout the entire hydrogel construct and cells remained viable at the surface and centre over a period of 11 days.

The effect of RGD on encapsulated hMSC behaviour was assessed. Analysis by CCK8 assay, live/dead staining and LDH release revealed that cell viability post-encapsulation in HyA and HyA-RGD hydrogel was high, indicating the gelation process was non-cytotoxic, as expected. Several small animal studies have demonstrated that HyA-TA is safe and does not evoke an immune response when injected subcutaneously (237–240). Subsequently, both HyA and HyA-RGD hydrogels were found to maintain cell viability over an 11 day period. RGD was found to increase hMSC spreading after 4 days in culture, as visualised by live/dead and Dapi/phalloidin staining. RGD functionalisation also promoted the secretion of the angiogenic factor VEGF and the chemoattractant factor MCP-1. These results corroborate with a recent study by Ho et al., who found hMSC spheroids in RGD-modified alginate hydrogel exhibited an elongated morphology after 5 days and secreted more VEGF and MCP-1 than unmodified hydrogel (286). The effect of cell seeding density on cell viability was evaluated. Both low and high seeding density (1 million and 20 million cells/mL) experienced approximately 10% cell death after 14 days, indicating that increased cell number does not compromise viability with the scaffold.

In summary, these results demonstrate the suitability of RGD-modified HyA hydrogel to maintain hMSC viability and to promote cellular attachment and function under standard culture conditions.
6.3 Chapter 3: Protective Effect of Insulin-Like Growth Factor-1 on Mesenchymal Stem Cells Cultured under Hypoxia and Reduced Nutrient Conditions

In Chapter 2, we demonstrated that RGD-modified HyA hydrogel could support hMSC viability, attachment and function under standard culture conditions. We hypothesised that the adhesion of anchorage-dependent hMSCs to RGD would enhance their survival and therapeutic effect under adverse culture conditions (1% oxygen, reduced nutrients) representative of the infarcted heart. In order to determine the effect of RGD under simulated infarct conditions in vitro, a controlled experimental system was designed, whereby the well-known anti-apoptotic agent IGF-1 was proposed as a positive control. IGF-1 is known to stimulate both proliferative and anti-apoptotic pathways in multiple cell types (201,291,294,296,297). However, its effect on hMSCs is not fully understood. The results from our study demonstrate that IGF-1 does not increase hMSC proliferation or survival. However, treatment with glucose was shown to have a significant effect on hMSC survival under adverse culture conditions.

Stimulation of hMSCs with IGF-1 (5-20ng/mL) revealed no effect on proliferation compared to the untreated control over a 7 day period. Treatment with high doses of IGF-1 resulted in cell death, possibly due to protein toxicity. Typically, growth factors are evaluated in the reduced serum or serum-free medium in order to exclude the possible proliferative effect of growth factors in FBS. hMSCs treated with IGF-1 under serum-deprived conditions did not proliferate over a period of 7 days. To test the efficacy of our synthetic IGF-1, rCSCs were treated with increasing doses of IGF-1 (25-100ng/mL) for 3 days in the absence of growth factors, to exclude the proliferative effects of these mitogens as reported by Johnson et al. (201). rCSC proliferation was significantly enhanced after 2 days, confirming the efficacy of our IGF-1 protein, with a dose dependent response observed between 25ng-50ng/mL after 3 days.
We next sought to assess the anti-apoptotic effects of IGF-1 on hMSCs under hypoxia and reduced nutrient conditions. Little is currently known about the mechanisms involved in the response of MSCs to an ischaemic environment. Ischaemia includes both oxygen and glucose deprivation, and these have varied effects depending on the cell source (351). It was hypothesised that IGF-1 treatment under hypoxia, reduced nutrient conditions, would activate IGF-1R, to decrease cell death by stimulating the pro-survival Akt pathway. Our results show that stimulation of hMSCs (5-20ng/mL) did not enhance hMSC survival over a period of 7 days. Using hMSCs from three different donors, FACs analysis revealed that IGF-1R was not expressed on the hMSC surface following culture under standard or stress conditions.

Glucose deprivation, not severe hypoxia, is responsible for the impairment of hMSC survival and function (299). Glucose treatment of hMSCs under oxygen and nutrient deprived conditions was shown to significantly enhance their survival. Our results suggest that it is possible to rescue hMSCs from adverse conditions, but IGF-1 is not suitable for this purpose.

In summary, this chapter we developed an in vitro model to investigate the deleterious effects of ischaemia on hMSC survival. Stimulation of hMSCs with IGF-1 did not increase their proliferation or survival. In addition, IGF-1R was not identified on hMSCs following exposure to normal or adverse culture conditions. In comparison, glucose treatment was shown to improve hMSC survival under oxygen and reduced nutrient conditions.

6.4 Chapter 4: Functionalisation of HyA Hydrogel to Enhance the Delivery of Mesenchymal Stem Cells into the Ischaemic Myocardium

When designing hydrogels for cell encapsulation, it is important to consider that stem cells depend upon both biophysical and biochemical environmental signals for their cellular activities. Transplanting stem cells in instructive biomaterial scaffolds may increase cell survival and improve their therapeutic efficiency. In Chapter 2,
HyA-RGD hydrogel was found to support hMSC attachment and enhance the secretion of VEGF and MCP-1 under standard culture conditions. In this chapter, the effect of RGD functionalisation on hMSC survival under ischaemic culture conditions was assessed. In addition, HyA hydrogel was modified with the basement membrane protein nidogen-1, which is a constituent of the cardiac ECM (210). The effect of nidogen-1 on hMSCs under standard and adverse culture conditions was investigated.

When transferred directly to hypoxia, reduced nutrient conditions, hMSCs in HyA-RGD hydrogels attached to the RGD ligands but failed to form strong adhesions. In the absence of strong adhesion, hMSCs viability and protein release in HyA-RGD hydrogel was comparable to unmodified HyA hydrogel over an 11 day period. Following these observations, hMSCs were encapsulated in HyA and HyA-RGD hydrogels and cultured under standard culture conditions for 4 days, in order to allow hMSCs to adhere strongly to the RGD ligand before hMSC-laden hydrogels were transferred to hypoxia, reduced nutrient conditions. Once hMSCs had adhered to RGD, they exhibited enhanced survival under adverse culture conditions compared to unmodified HyA hydrogel. Significantly less dead cells were observed in HyA-RGD hydrogels after 11 days in adverse culture conditions, indicating RGD protected cells from anoikis. Dapi/phalloidin staining revealed hMSCs in HyA-RGD hydrogels retained an elongated morphology under hypoxia, reduced nutrient conditions after 11 days, indicating de-adhesion did not occur under ischaemic conditions. Early release of MCP-1 was also increased from hMSCs in HyA-RGD hydrogel.

The cardiac ECM protein nidogen-1 was successfully entrapped in HyA hydrogel during crosslinking. Under standard culture conditions, the percentage of live cells was significantly increased after 11 days, with a corresponding decrease in the number of dead cells/mm² compared to unmodified HyA. Although hMSC spreading was unaffected, both VEGF and MCP-1 release was significantly increased after 11 days. Under hypoxia, reduced nutrient conditions, nidogen-1 was not found to enhance hMSC survival, spreading or VEGF release over a period of 11 days. The
secretion of MCP-1 from hMSCs in HyA-nidogen-1 hydrogel was found to be enhanced after 4 days only.

As described previously using RGD, hMSCs were encapsulated in HyA and HyA-nidogen-1 hydrogels and cultured under standard conditions for 4 days to allow cell interaction with nidogen-1 prior to exposing to adverse culture conditions. Despite the inclusion of this step, hMSCs in HyA-nidogen-1 did not exhibit enhanced viability, spreading or protein release compared to unmodified HyA. This result suggests a normoxic precondition period is only advantageous when using adhesive peptides and proteins.

In summary, while nidogen-1 was found to confer some beneficial effects under standard culture conditions, it was unable to enhance hMSCs survival under adverse culture conditions. To our knowledge, this is the first report characterising the effect of nidogen-1 on hMSCs entrapped in HyA hydrogels. Multiple in vivo studies have utilised RGD-modified HyA for cell delivery in animal models of MI (151,325). Our results suggest RGD-modified hydrogels may not enhance hMSC survival and function compared to unmodified hydrogel unless cells are given time to adhere to the adhesive ligand prior to transplantation. This finding may have major implications for the use of adhesion peptides and proteins for stem cell delivery to ischaemic tissues.

6.5 Chapter 5: Encapsulation of Cardiac Stem Cells in an RGD-Functionalised Hyaluronic Acid Hydrogel to Enhance Cell Survival under Hypoxia and Reduced Nutrient Conditions

While the optimal cell source for cardiac regeneration is currently unknown, CSCs are an ideal candidate given their innate ability to generate de novo cardiomyocytes. In Chapter 3, rCSCs were found to proliferate in response to IGF-1. In this chapter, we investigated the effect of IGF-1 on rCSCs under hypoxia, reduced serum conditions with an aim of including it as a positive control in hydrogel studies with rCSCs.
We first confirmed the expression of c-kit+ on rCSCs at a late passage. Our results demonstrate that receptor expression was reduced with time in culture. Next we assessed biocompatibility of rCSCs with our HyA hydrogels and investigated the effect of RGD on hMSC viability over a period of 11 days under standard culture conditions. rCSC in HyA and HyA-RGD hydrogels maintained high viability throughout the study, rCSCs in HyA-RGD exhibited increased viability at multiple timepoints throughout the study.

rCSC survival under hypoxia and reduced serum conditions was increased in response to IGF-1 (25-250μg/mL) in a 2D study in vitro. No toxicity was observed with high-dose treatment. Following this result, the effect of RGD on rCSC survival in HyA and HyA-RGD hydrogel was the assessed under hypoxia, reduced serum using IGF-1 as a positive control as planned. Compared to unmodified HyA, rCSC viability was significantly enhanced in both HyA-RGD hydrogel and HyA hydrogel treated with IGF-1.

IGF-1 was encapsulated in PLGA MPs previously developed in the RCSI laboratory. IGF-1-loaded MPs were encapsulated in HyA and the release medium was collected. rCSCs were treated with IGF-1 released from MPs in HyA hydrogel in order to assess if the protein was still bioactive. Proliferation of rCSCs was shown to significantly increase in response to both free IGF-1 and released IGF-1, indicating IGF-1 MPs can be successfully loaded in HyA scaffolds to enhance both endogenous and transplanted CSC survival.

In summary, RGD significantly enhanced the viability of rCSCs under hypoxia, reduced serum conditions. IGF-1 was identified as a pro-survival stimulus for rCSCs under adverse culture conditions. The encapsulation of IGF-1 in MPs allows for the controlled release of IGF-1 from HyA hydrogel.
6.6 Future work

- In Chapters 2, 4 and 5, HyA hydrogel was used as a blank slate material to investigate the roles of different ECM ligands and proteins on encapsulated cell behaviour under standard and adverse culture conditions. Future work will incorporate other ECM-derived proteins into HyA hydrogels in order to further study cell-matrix interaction and the effect of biological cues on encapsulated cell behaviour. Cardiac specific ECM proteins which may be investigated include collagen IV, decorin, laminin and elastin.

- As reported in chapter 4, different peptides and proteins elicit different cellular responses. Future work will aim to engineer a biomimetic scaffold using a combination of ECM materials to enhance cardiac tissue engineering.

- In chapter 4, the strong adhesion of hMSCs to RGD was found to decrease cell death under ischaemic culture conditions. Future work will consider the clinical translation of this strategy. It is envisaged that this will require ex vivo cultivation of hMSCs in preformed HyA-RGD hydrogel. The main disadvantage of preformed material is that it must be implanted. This issue may be circumvented by making the preformed gel into micro- or nanoparticles. Alternatively, novel catheters can be developed to allow to delivery of preformed hydrogels as a “bullet”. Work is currently ongoing with industry partners to address the delivery of complex and viscous cell-laden materials.

- In chapter 3, Glucose was shown to enhance hMSC survival under hypoxia, reduced nutrient conditions. As glucose is the primary energy source of MSCs, future work will determine if the glucose-loaded HyA hydrogel can improve the longevity of encapsulated hMSCs under adverse culture conditions. Future work will determine if glucose can facilitate the spreading of hMSCs (which is known to be an active process that requires energy) in
HyA-RGD hydrogel under ischaemic culture conditions to reduce cell death by anoikis.

- The strength of our HyA hydrogels ranged between 5.5-7kPa which is in line with the acellular hydrogel Algisyl-LVR™. Delivery of Algisyl-LVR™ to the heart enhanced cardiac function but implantation required open chest surgery and full anaesthesia. Future work will assess if the minimally invasive delivery of our HyA hydrogel can provide mechanical support to the compromised ventricular wall and enhance cardiac function.
6.7 Thesis conclusions

- The research presented in this PhD thesis has demonstrated the suitability of an injectable *in situ* crosslinking HyA hydrogel +/- RGD to act as a surrogate matrix to maintain the viability of stem cells for cardiac cell therapy. The functionalisation of HyA hydrogel with RGD was found to enhance hMSC spreading and function under standard culture conditions.

- IGF-1 is not a suitable pro-proliferative or pro-survival factor for *in vitro* studies using hMSCs. hMSCs do not express IGF-1R under normal or pathological conditions.

- RGD may improve encapsulated hMSC survival under the condition that cells adhere to the ligand prior to exposure to ischaemic culture conditions. Nidogen-1 enhanced hMSC viability and function under standard culture conditions but did not improve encapsulated hMSC survival or spreading under ischaemic culture conditions.

- HyA-RGD hydrogel was found to promote rCSC viability under both standard and ischaemic culture conditions, compared to unmodified HyA. IGF-1 was shown to enhance rCSC viability under adverse culture conditions and may be used as a pro-survival stimulus in experiments assessing rCSC survival.
Overall Conclusion

Collectively, the research presented in this thesis demonstrates the suitability of HyA hydrogel as a blank slate biomaterial to investigate the effect of single factor ECM proteins and peptides under normoxic and hypoxia, reduced nutrient conditions \textit{in vitro}. Functionalisation of HyA with the RGD peptide was shown to enhance both hMSC and CSC survival under adverse culture conditions, compared with non-functionalised HyA hydrogel. In addition, the effect of nidogen-1 on hMSCs in HyA hydrogel was reported for the first time. Our results demonstrate that nidogen-1 may play an important role in hMSC viability and paracrine factor release.
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