A Collagen Cardiac Patch Incorporating Alginate Microparticles Permits the Controlled Release of HGF and IGF-1 to Enhance Cardiac Stem Cell Migration and Proliferation

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Abstract

Cardiac Stem Cells (CSCs) represent a logical cell type to exploit as a regenerative treatment option for tissue damage accrued as a result of a myocardial infarction (MI). However, the isolation and expansion of CSCs prior to cell transplantation is time-consuming, costly and invasive, and the reliability of cell expansion may also prove to be a major obstacle in the clinical application of CSC based transplantation therapy after a MI. In order to overcome this, we propose the incorporation of growth factor-eluting alginate microparticles (MPs) into collagen-based scaffolds as an implantable biomaterial to promote the recruitment and expansion of CSCs in the myocardium.

In order to obtain scaffolds able to enhance the motogenic and proliferative potential of CSCs, the aim of this work was to achieve a sustained delivery of both Hepatocyte Growth Factor (HGF) and Insulin-Like Growth Factor 1 (IGF1). Both proteins were initially encapsulated in alginate MPs by spray-drying and subsequently incorporated into a collagen scaffold. MPs were seen to homogeneously distribute through the interconnected scaffold pore structure. The resulting scaffolds were capable of extending the release of both proteins up to 15 days, a 3-fold increase over non-encapsulated proteins embedded in the scaffolds. *In vitro* assays with isolated CSCs demonstrated that the sustained release of both bioactive proteins resulted in an increased motogenic and proliferative effect.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/term.2392

As presently practiced, the isolation and expansion of CSCs for autologous cell transplantation is slow, expensive and difficult to attain. Thus, there is need for strategies to specifically activate *in situ* the intrinsic cardiac regenerative potential represented by the CSCs using combinations of growth factors obviating the need for cell transplantation. By favouring the natural regenerative capability of CSCs, it is hypothesised that the cardiac patch presented here will result in positive therapeutic outcomes in myocardial infarction and heart failure patients in the future.

Keywords

Biomaterials

Controlled release

Growth factor delivery

Tissue engineered scaffolds

Cardiac Stem Cells

Microparticles

1. Introduction

A major weakness in existing therapies for myocardial infarction is their inability to regenerate the resulting damaged cardiac muscle. Thus, the goal of current experimental strategies is finding the means for attenuation of the progressive processes leading to tissue destruction, while inducing myocardial tissue regeneration (1, 2).Regenerative cardiology's Holy Grail has been the development of procedures to either replace lost myocytes with transplanted stem cells or to use stem cells to mediate functional repair through paracrine effects (3-6).

The most primitive undifferentiated population of stem cells in the heart, the c-Kit^{POS} Cardiac Stem Cell (CSC) pool which was the first stem cell identified in the rat heart and, up to date, this CSC pool is still the most extensively characterised (7, 8). CSCs are a heterogenic group of cells and they are concentrated in specific areas of the heart, such as the atria or pericardium (9). They represent a logical source to exploit in myocardial regeneration because of their likelihood to be intrinsically programmed to generate viable cardiac tissues *in vitro* and increase its viability *in vivo* (10-13).

The ideal replacement for the lost myocardium after myocardial infarction is functional autologous myocardial tissue, which can bypass issues around immunosuppression associated with allogenic therapies (14). However, as presently practiced, the isolation and expansion of endogenous cardiac stem cells (eCSCs) for autologous cell transplantation is slow and expensive. In addition, endomyocardial biopsies are difficult to attain (12, 15). Thus, there is need for strategies to specifically activate *in situ* the intrinsic cardiac regenerative potential represented by the eCSCs using combinations of growth factors, cytokines and drugs, obviating the need for cell transplantation (15, 16).

Data from mice and dogs show that the regenerative response of eCSCs to an ischemic insult can be enhanced *in situ* by the administration of growth factors (17, 18). These eCSCs possess Insulin like Growth Factor-1 (IGF-1) and Hepatocyte Growth Factor (HGF) receptors that regulate their growth, survival, and migration (17-19). Accumulating evidence supports the notion that the regenerative response of eCSCs can be activated by the presentation of both HGF and IGF-1 in the ischemic myocardium (20-22). The motogenic potential of HGF followed by the proliferative and cytoprotective effect of IGF-1 constitutes a powerful combination in inducing myocardial repair. Since the endogenous levels of these proteins can be too low to induce a pro-angiogenic/motogenic response following an ischemic insult, an extended release of growth factors from implantable biomaterials has the potential to effectively activate eCSCs.

Merging a biocompatible cardiac patch with a growth factor delivery system represents an attractive delivery device that can increase the effect size in terms of the amount of tissue targeted. Of the different materials used for the fabrication of cardiac patches in recent years, collagen, the predominant protein in the extracellular matrix, has been used extensively, both alone and with cells and as a delivery device for biotherapeutics (23-27). In order to ensure the stability of the growth factors during the cardiac patch fabrication process and integration into the damaged tissue site, an appropriate growth factor carrier is required. In addition, the carrier should act as a reservoir, protecting the therapeutics from degradation *in vitro* and *in vivo*. In order to enhance the bioavailability and control the release of growth factors in the cardiac tissue, drug delivery systems have been suggested as a means to protect and dose the protein cargo. Alginate microparticles were utilised, as these biocompatible polymer particles have been shown previously to allow for the controlled delivery of growth factors within collagen scaffolds, while maintaining the existing pore architecture, mechanical properties and proven biological activity of the underlying scaffold (28).

In addition, it is necessary to address the challenge of multiple factor delivery. Proper spatiotemporal presentation of growth factor combinations is imperative in order to increase efficacy. Combined delivery strategies have been shown to produce more favourable outcomes in terms of cardiac tissue regeneration than single agent delivery (20, 29). Herein we describe the development and *in vitro* testing of a biomaterial that combines a collagen scaffold and alginate microparticles loaded with HGF and IGF-1 to enhance cardiac stem cell migration and proliferation.

2. Materials and Methods

2.1. Fabrication of alginate microparticles

Protein loaded and unloaded alginate (Sigma, Ireland, low viscosity alginic acid sodium salt from Macrocystis pyrifera) microparticles were manufactured by a spray-drying method. Fluorescein isothiocyanate labelled BSA (FITC-BSA, Sigma) was initially incorporated as a model drug to determine the effects of the spray-drying process on the loaded protein. A 0.5% w/v alginate feed solution was mixed with either HGF (RhHGF294, CF (R&D systems, UK)) or IGF-1 (RhIGF-1291, CF (R&D systems, UK)) (1 µg protein/mg polymer) and spray-dried using a Buchi Mini Spray-Dryer B290 according to the following drying parameters: compressed air 5-8 bar, air flow rate 400-600 L/h, inlet temperature 140°C, aspirator at 80% of maximum capacity, and pump flow rate at 15%. Unloaded microparticles were prepared as

controls. Briefly, spray-drying involves transformation of a solution into dried particulates by feeding the solution into an atomiser to generate high surface area droplets for subsequent vaporisation of the solvent (water). The obtained microparticles were collected and dispersed in acetonitrile under sonication. The dispersion was then poured into 1.2% (w/v) calcium chloride solution under magnetic stirring for 10 min to allow particle crosslinking. The particles were filtered on 0.45 µm nylon filter paper, washed twice with distilled water and dried at room temperature overnight. The recovered microparticles were stored at 4ºC in a desiccator until further use.

The mass yield of product after spray-drying was determined according to the below equation.

$$\text{Yield (\%)} = \frac{\text{weight of microparticles following spray-drying (g)}}{\text{Weight of polymer loaded (g)}} \times 100$$

The mean particle size and size distribution of the microparticles were determined by dynamic light scattering (DLS) with a Malvern MasterSizer Sirocco 2000 by suspending the particles to be analysed in water.

2.2. Fabrication and characterisation of Collagen-Alginate scaffolds

Collagen-Alginate (Coll-Alg) scaffolds used in this study were fabricated using a technique previously developed in our laboratory (30-32). Briefly, collagen (Collagen Matrix Inc., NJ, USA) slurries were produced by the homogenization of 1.8 g fibrillar collagen within 320 mL of 0.5 M acetic acid solution. Slurries were homogenized in a reaction vessel at 4°C, using an overhead blender. The slurry was degassed under vacuum to remove air bubbles. In order to incorporate both HGF loaded microparticles and IGF loaded microparticles into different parts of the same scaffold, a separator column was used. MPs were suspended in 0.5 M acetic acid and added at 1% w/v (weight of microparticles/volume of slurry), equating to 1 µg protein for each protein. The microparticles were then dispersed in the collagen slurry, which was subsequently placed into stainless steel moulds (10x100mm). The moulds were placed into a freeze-dryer and cooled to -10°C at a constant rate of 0.9°C/min. Once freezing was complete, the ice crystals were removed by sublimation for 17 h at 200 mTorr. All scaffolds were crosslinked under an ultraviolet (subtype C, 365 nm λ) lamp for 15 min and were turned half-way through to enhance the mechanical and enzymatic resistance properties of the materials (33, 34). The scaffolds were subsequently sterilized under a UV lamp (254 nm).

For SEM imaging, scaffolds were cut and mounted onto metallic stubs, with the help of carbon-based glue, and sputtered with gold. Images were captured at 5 kV, using secondary electron mode, taken at a working distance of 12–18 mm. Both the surfaces and the cross-sections of the microparticle-containing scaffolds were examined.

2.3. HGF/IGF-1 release from Coll-Alg scaffold

Coll-Alg scaffolds were added to 6-well plates and 2 mL of phosphate buffer (pH 7.1) was added to the wells. The samples were incubated at 37°C whilst shaking at 75 rpm for the duration of the release study (28 days). The phosphate buffer was completely removed and replaced at 4 h, 24 h, day 3, day 5, day 10, day 15, day 20 and day 28 and frozen until analysis. Control samples contained unloaded alginate microparticles. Growth factor release of both HGF and IGF-1 was assessed by

ELISA (rhHGF Duoset ® and rhIGF-1 Duoset ® Elisa development kit, (R&D systems)). 50 µL aliquots were tested in duplicate and compared to a standard curve.

2.4. Isolation of c-Kit^{POS} CSCs from rat myocardial biopsies

In order to assess *in vitro* the bioactivity of the released growth factors from the Coll-Alg scaffolds, it was necessary to isolate and expand the cells that the patch and released growth factors would be targeting *in vivo*. Isolated myocardial tissue from Sprague Dawley rats was cut into 1-2 mm^3 pieces, from which gross connective tissue was removed, then washed and digested three times for 5 minutes 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% fetal calf serum, 100 U/mL penicillin G, 100 g/mL streptomycin and 2 mmol/L L-glutamine) and were cultured as explants in CEM at 37°C and 5% CO_2. Explants were cultured on fibronectin coated plates and were maintained attached during subsequent harvests using plastic coverslips. 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 2-3x10^4 cells/mL on poly-D-lysine coated dishes in cardiosphere growth medium (CGM) (35% complete IMDM/65% DMEM–Ham F-12 mix containing 2% B27, 10 ng/mL epidermal growth factor (EGF), 20 ng/mL basic fibroblast growth factor (bFGF), 40 nmol/L cardiotrophin-1, 40 nmol/L thrombin, antibiotics, and L-Glu, as in CEM). Several days later, cells that remained adherent to the poly-D-lysine coated dishes were discarded, whereas detached cardiospheres in suspension were plated on fibronectin coated flasks and expanded as monolayers. Rat CSCs (rCSCs) were subsequently passaged by trypsinization and split at a density of 1x10^7 cells/T175 flask.

2.5. Flow Cytometry

CSCs were passaged twice as adherent monolayers and then used for flow cytometry experiments with a FACS (BD Canto, BD Biosciences, San Jose, Calif) flow cytometer with quantitative analysis provided by CellQuest software (BD Biosciences, San Jose, Calif). Cells were trypsinized and resupended in FACS buffer (PBS + 1% FBS) twice and left to incubate for 30 min (4°C). Cells were then washed twice again and were incubated with fluorescein isothiocyanate-conjugated antibodies against CD-117 (c-Kit) for 30 min (1:200 Dilution). Isotype-identical antibodies (IgG2b, k) served as negative controls (BD Biosciences, San Jose, Calif).

2.6. CSC migration

Previous data have shown that HGF induces potent motogenic and invasive activity of CSCs (35-38). In order to assess the bioactivity of the released HGF, a migration assay was carried out. This assay is based on the concept that following serum starvation, cells will migrate towards a serum stimulus. rCSCs were harvested from T175 tissue culture flasks via trypsinisation. Cells were washed twice with serum-free growth media at a density of 5x10^5 cells/mL. 7x10^4 CSCs were added to hanging well cell culture inserts with a pore size of 8 µm, suitable for insertion into a 12-well plate (Millipore, Ireland). Then, 2 mL of serum-free rCSC growth media were then added basolaterally and 0.5 mL was added apically. Cells were allowed to incubate in serum free media and were deprived of serum for a total of 2 h, at which point

basolateral media were replaced with growth media supplemented with HGF (25ng/mL)(Positive Control), No serum (Negative Control) or with pooled HGF sample scaffold release media (25 ng/mL). Samples were harvested at 48 h. Insert membranes were gently rinsed with PBS repeatedly. The inserts were immersed in 4% formalin for 10 min to fix adherent cells and stained with haematoxylin for a further 10 min. Following a final rinse with PBS the membranes were removed with a scalpel blade. Membranes were mounted on a glass slide, bottom side down. Cell migration was quantified by counting the cells from five random fields on the underside of the membrane at 20x magnification.

2.7. CSC proliferation

Previous data have shown that IGF-1 induces proliferative and anti-apoptotic effects on CSCs (35, 39, 40). In order to assess the bioactivity of the released IGF-1, CSC proliferation was measured in response to the release media. rCSCs were cultured as described above**.** rCSCs were passaged and $3x10^4$ cells were seeded in 24-well plates in CSC medium for 24 h. Culture medium was then replaced with basal medium and starved for 24 h and then treated with either pooled release media containing 25 ng/mL IGF or no IGF for 72 h. The cells were then removed and homogenised through the addition of 500 µL 0.2 M $NaHCO_3$, 1% Triton-X lysis buffer. Lysates were stored at −80°C and underwent three freeze–thaw cycles prior to analysis. Double-stranded DNA (dsDNA) levels within the cell lysates were measured using the QuantiT PicoGreen dsDNA assay (Molecular Probes, Invitrogen, Ireland) according to the manufacturer's protocol.

Statistical analysis

One way analysis of variance (ANOVA) or Two Way ANOVA were performed followed by pairwise multiple comparison procedures (Tukey 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 replicates were performed for all experiments, unless otherwise stated.

3. Results

3.1. Schematic of cardiac patch manufacturing process

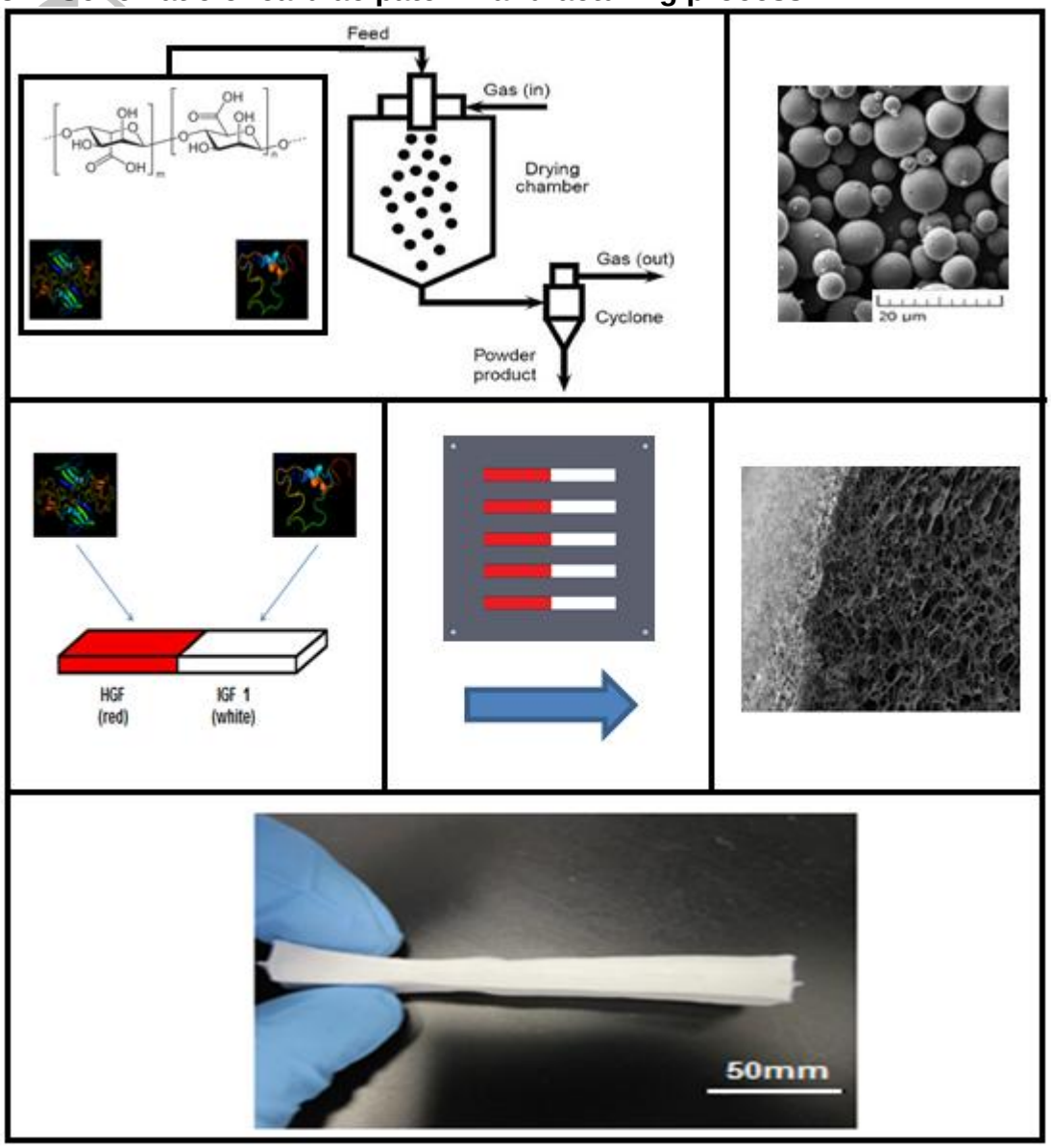

Figure 1 Step 1 (A) Schematic showing the production of IGF-1 and HGF loaded alginate microparticles by spray drying (41) and **(B)** resulting microparticles. **Step 2 (C)** Microparticles are then combined with collagen slurry with the HGF microparticles on the left (red portion of patch) and the IGF-1 microparticles on the right (White portion of patch) **(D)** and placed in a 10X200mm stainless steel mould and subsequently freeze dried at -10°C for 26hrs. **(E)** Transverse SEM image showing the interconnected pore structure of the scaffold. **Step 3 (F)** Image of final patch design.

3.2. Growth factor loaded microparticle characterisation

A process yield of 49.77±3.38% was determined for blank alginate microparticles. The content of encapsulated protein was found to be 48%±5.67% as determined by ELISA. Scanning electron microscopy (SEM) images of the protein loaded microparticles showed generally smooth and spherical particles with heterogeneous size **(Figure 2A)**, as previously described for alginate microparticles fabricated by spray-drying (28, 42). Laser diffraction measurements confirmed a size distribution range (0.1-20µm), **(Figure 2B)**, with an average size of 2.2 µm.

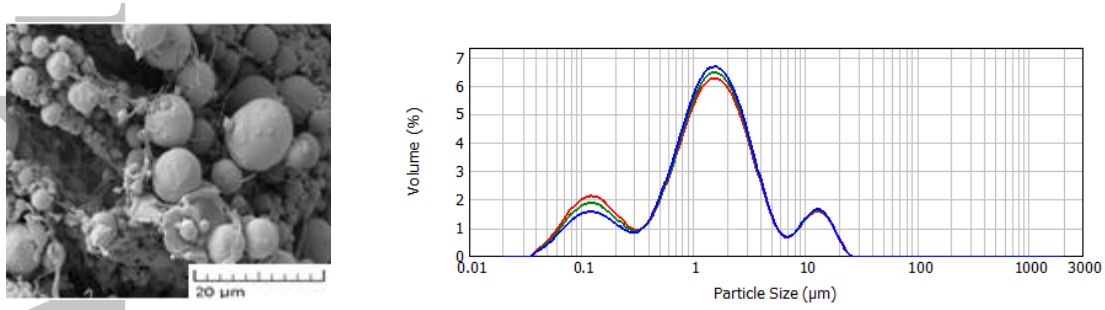

Figure 2 Microparticle characterization. **(A)** SEM image of alginate MPs prepared by spray-drying showing heterogeneous particle sizes **(B)** Microparticle size distribution, indicating particle diameters in the range 0.1-20 µm. Average particle size was 2.2 µm, with a process yield of 49.77±3.38% and an encapsulation efficiency of 48%±5.67%.

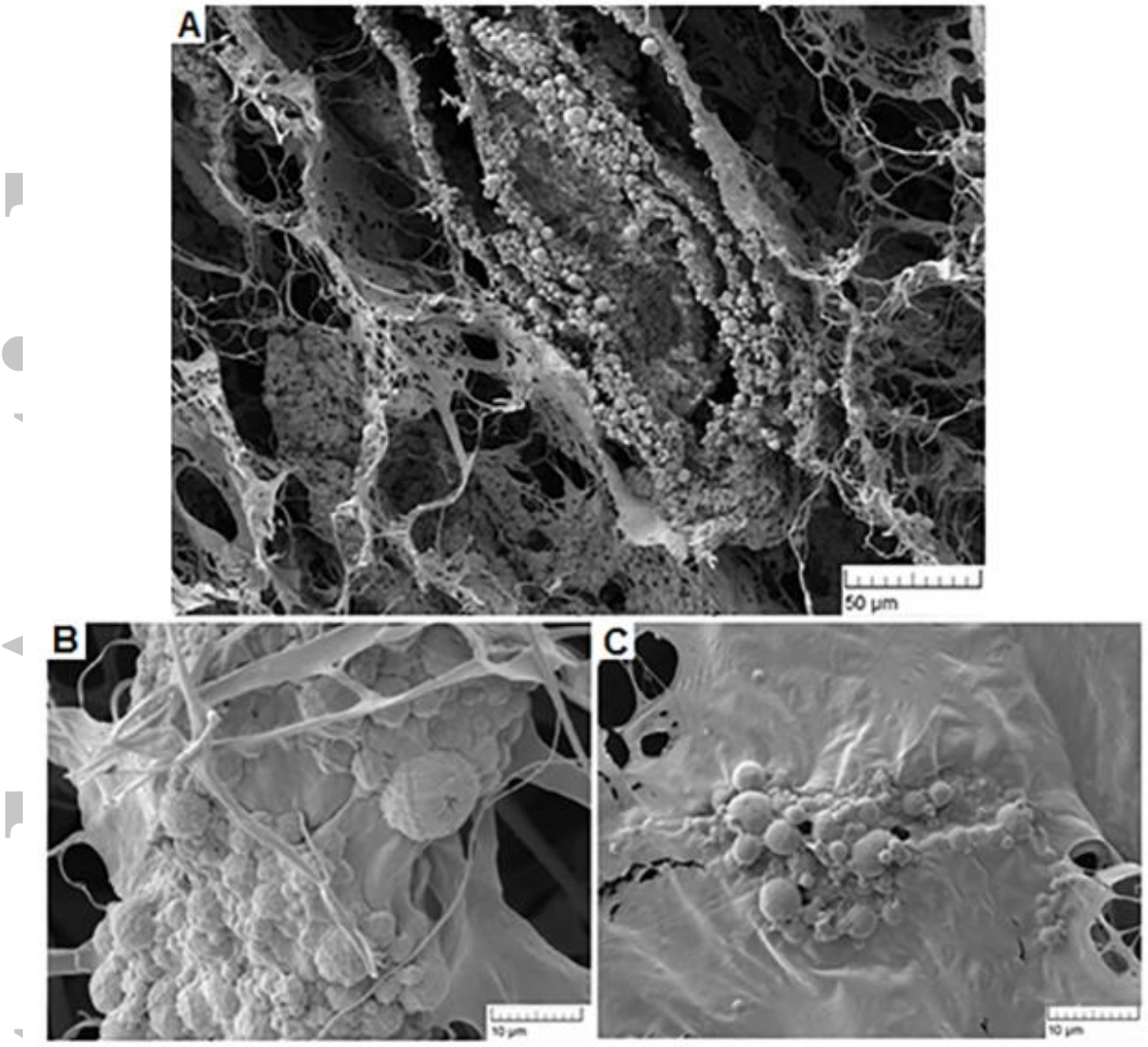

Figure 3 (A/B/C) SEM images of cross-sections of scaffolds incorporating 1% w/v alginate microparticles. **(C)** Representative SEM image of the surface of the scaffolds showing the incorporated alginate microparticles. Scale bar = 50 µm **(A)**, 10 µm **(B/C)**.

SEM examination of the alginate microparticles in the collagen scaffolds **(Figure 3A)** revealed that the particles kept their spherical morphology and a smooth surface after incorporation into the scaffolds. The microparticles appear as clusters embedded within the collagen fibres **(Figure 3B)**. Alginate microparticles were also visible on the surface of the collagen scaffold. Similar to the transverse images, the microparticles lay within close proximity to each other **(Figure 3C)**.

3.3. Analysis of free growth factor release from cardiac patches

Release kinetics of free growth factors incorporated into the collagen scaffolds was first assessed, prior to the addition of alginate MPs. The total amount of HGF released from the scaffolds over 5 days was 37% (367 ng) of the initial amount incorporated. The total amount of IGF-1 released from the scaffolds was 18% (178 ng) of the initial amount. Both growth factors exhibited an initial burst release (43% and 35% total release of HGF and IGF-1 detected, respectively) in the first 24 h. No

further release of growth factors was detected following day 5 and up to 28 days **(Figure 4)**. The release of HGF (1 µg) from microparticle loaded cardiac patches was assessed using quantitative ELISA over a period of 28 days. The samples showed a slow initial release with a large burst at day 3. Cumulatively over 50% of the HGF is released in the first 3 days. Release drops off until day 10 where another burst is observed. HGF release is negligible after day 15. The release of IGF-1 (1 µg) from microparticle loaded cardiac patches was also assessed using quantitative ELISA over a period of 28 days. Release of IGF-1 showed a more sustained continuous release profile compared to the release of HGF from the scaffold over the same period. Similar to HGF release, IGF-1 release dropped off by day 15 with very little release observed after this timepoint **(Figure 4)**.

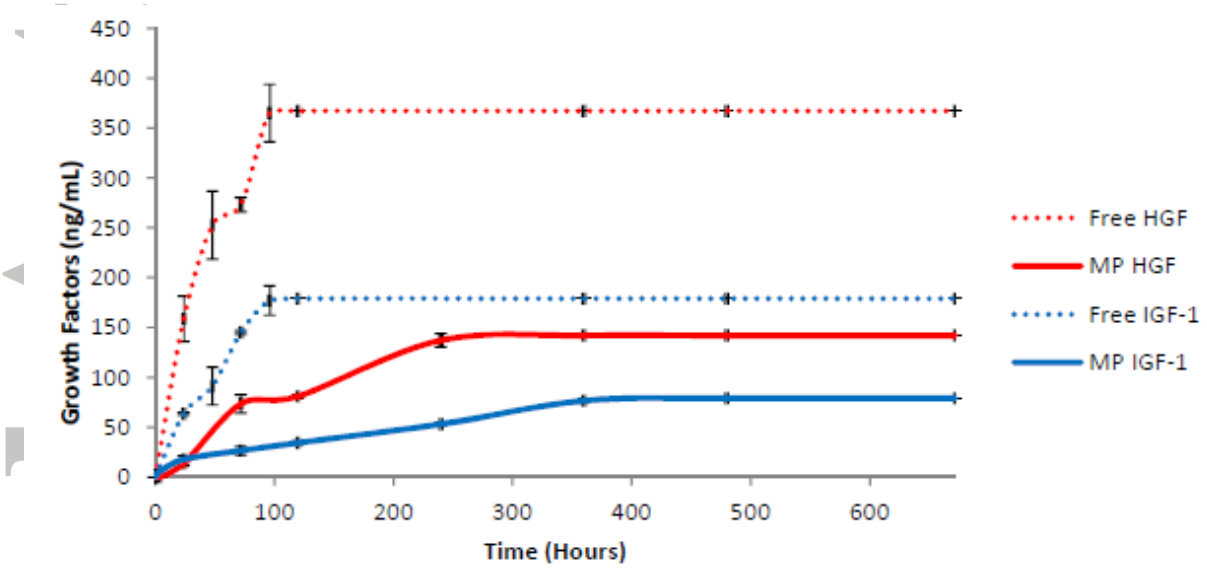

Figure 4 Cumulative release of free loaded HGF and IGF-1 from collagen scaffolds over 28 days (mean±SD) and cumulative HGF (1 µg) and IGF-1 (1 µg) release profiles from microparticle loaded scaffolds over 28 days. 141 ng of HGF is released in total over the course of the study while 79 ng of IGF-1 is released over the same period exhibiting a 50% lower amount released. HGF release was faster and intermittent while IGF-1 showed a slower but continuous release profile. HGF and IGF-1 free loaded scaffolds released larger amounts of both growth factors (367 & 178ng respectively) but at 3X faster rate compared to microparticle loaded scaffolds (mean ± SD).

3.4. Isolation and expansion of rat cardiac stem cells

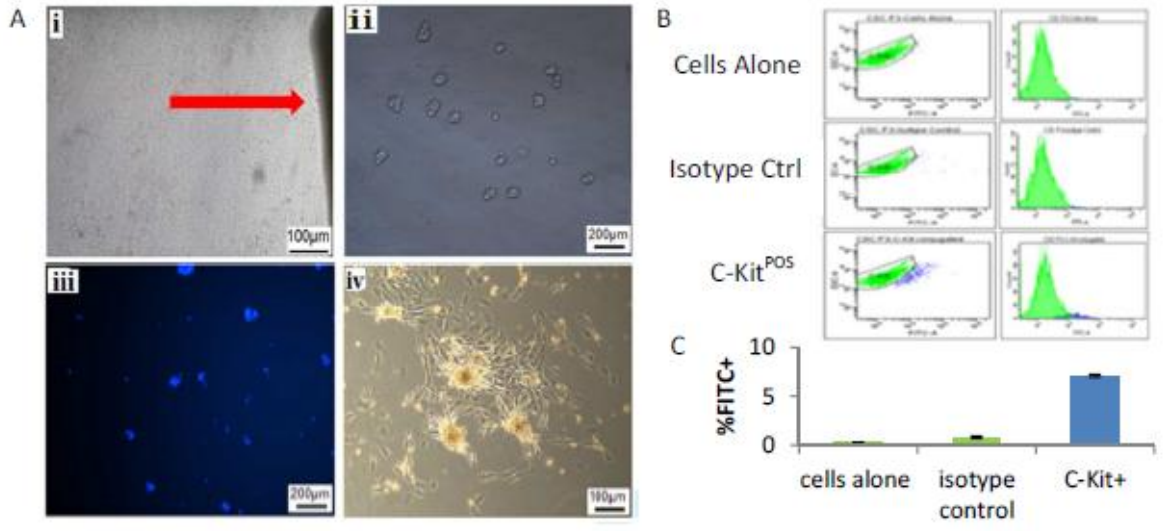

Figure 5 (A) Microscopy images depicting the steps involved in specimen processing for cardiosphere growth and CDC expansion: **(i)** Endomyocardial biopsy fragment on day 7 after plating showing stromal-like and phase-bright cells. **(ii)** Cardiosphere-forming cells collected from the explant after 12 days and plated on poly-D-lysine for 10 days. **(iii)** DAPI staining on day 7 after plating to show Cardiosphere viability. **(iv)** CDCs during passage 2, plated on fibronectin for expansion. CSCs can be seen to grow out of the spheres after plating on fibronectin. **(B)** Fluorescence activated cell sorting analysis of rat CSCs. The phenotype profile for c-Kit expression was analysed within the density plots and shown as a percentage of positive events. 6.1% of the CSC population expressed the stem cell marker c-Kit (mean±SD).

Hearts from donors (single whole) were stored on ice in complete explant media (CEM) and processed within 2 hours. A typical biopsy fragment, or explant, is shown after mincing and partial enzymatic digestion (red arrow), on day 7 **(Figure 5Ai)**. Harvesting of cardiosphere (CS)-forming cells, the loosely adherent cells that spontaneously shed from the explants, was initially performed 12 or more days after a specimen was obtained. Typical cardiospheres are shown **(Figure 5Aii)** 10 days after harvest, when growth was robust. Cardiospheres appeared to be viable during this period as assessed by DAPI staining **(Figure 5Aiii)**. Cardiospheres were plated for expansion 4 to 28 days after harvest and the resultant Cardiosphere Derived Cells (CDCs) which are termed Cardiac Stem Cells (CSCs) are passaged at 7 to 14 day intervals thereafter. The typical morphology of CSCs is evident during expansion at passage 2 **(Figure 5Aiv)**. Phenotypic analysis of newly developing rCSCs revealed expression of the stem cell marker c-Kit. **Figure 5B** illustrates that 6.1% of the CSC population expressed the c-Kit marker.

3.5. Analysis of the bioactivity of HGF/IGF-1 microparticle loaded cardiac patches on CSCs

In order to assess the bioactivity of HGF released from the cardiac patches, rCSCs were added to hanging well cell culture inserts with a pore size of 8 µm at a density of $3x10^4$ cells/insert **(Figure 6A)**. Cells were allowed to incubate in serum free media for 2 h and were then treated with free HGF and HGF scaffold release media (25 ng/mL), or supplemented with fresh HGF (positive control), or left in serum free media (negative control). After 48 h incubation, cells were scraped off the apical side, and the basolateral side of the inserts was stained with haematoxylin and cell

migration was quantified by counting the cells in 5 random fields. Cells that had been exposed to a fresh HGF exhibited a high level of migration through the membrane, while cells exposed to no serum stimulus exhibited minimal migration**.** Cells that were treated with the HGF release media from the scaffold showed an ability to migrate through the membrane although to a lesser extent compared to the fresh HGF **(Figure 6B/C).**

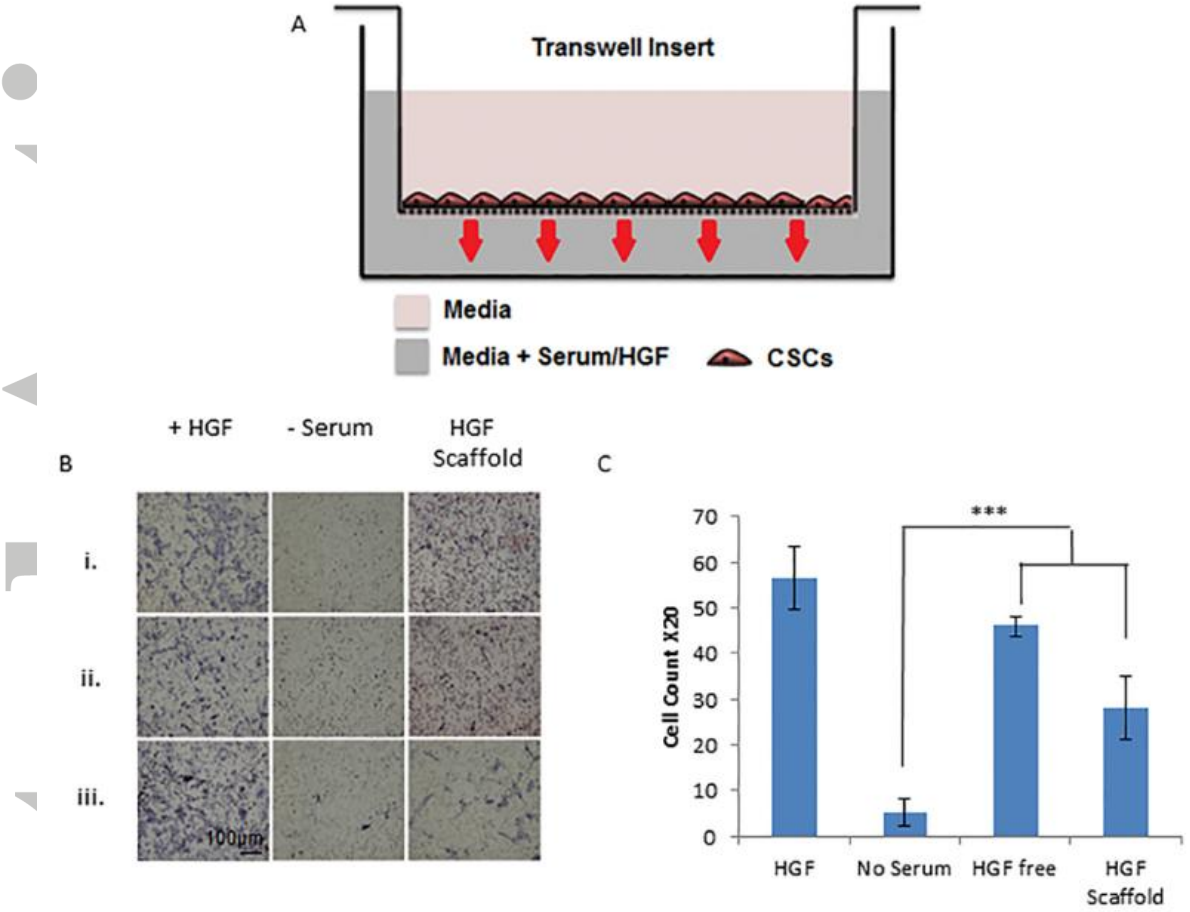

Figure 6 Schematic depicting the migration assay of CSCs through a transwell insert in response to a media stimulus. Cellular migration of CSCs was measured with cells cultured in fresh HGF, No serum media or release media from HGF-containing cardiac patches (25 ng/mL) and free HGF (25 ng/mL). Cells were serum starved and then permitted to migrate through the membrane in response to a serum/growth factor stimulus. Cells were collected on the membrane of a 12-well transwell cell culture insert which was stained with haematoxylin. Cells were counted at 5 random fields of view at 20x magnification per membrane **(B/C)**. The significant increase in migration for the HGF treated media as compared to no serum media demonstrates the functionality of the protein after release from the scaffold on native CSCs (mean ± SD, n=3). Note: *** denotes $p<0.0001$.

In order to assess the bioactivity of IGF-1 released from the cardiac patches, rCSCs were incubated with free IGF-1 and IGF-1 (25 ng/ml) scaffold release media and without release media (control). Cells were seeded and serum starved for 24 h prior to the addition of IGF release media. After 72 h incubation, the growth of cells was assessed quantitatively via a picogreen double-stranded (ds) DNA assay **(Figure 7)**. IGF-1 bioactivity was confirmed by the increased expression in cellular growth compared to the untreated control.

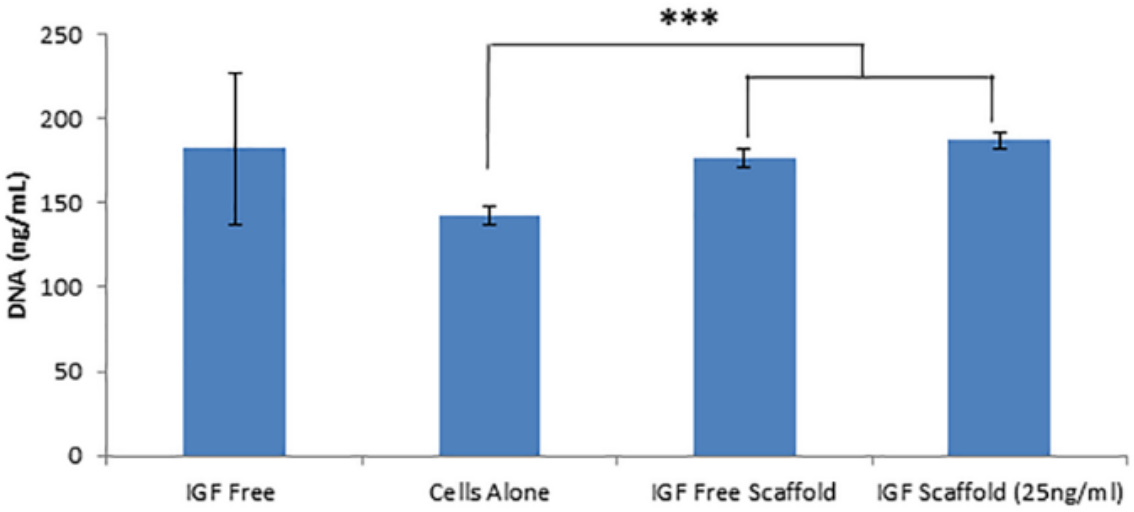

Figure 7 ds DNA levels of free IGF-1 and IGF-1 (25 ng/ml) scaffold release media treated rCSCs compared against untre ated rCSCs cultured over a period of 72 h as assessed by picogreen assay. IGF-1 treated cultures show a higher cell number compared to the untreated control group indicating the functionality of the protein to induce cellular proliferation. This indicates the IGF-1 remains bioactive after release from the scaffold (mean ±SD, n=3). Note: *** denotes p<0.0001.

4. Discussion

Cardiac-specific stem cells offer promise of enhanced cardiogenesis compared to other cell sources in transplantation therapy for ischemic heart disease (7, 17, 43, 44). The observation that the adult heart possesses a pool of resident CSCs allows myocardial regeneration by inducing endogenous cardiac cells to migrate and proliferate *in situ* replacing lost cardiomyocytes and inducing their beneficial effects through paracrine mechanisms (7, 10, 13). The ability to activate CSCs *in situ* represents an attractive treatment option that could negate costly cell expansion and transplantation procedures. The aim of this study was to develop a scaffold that could be implanted on the epicardial surface of the heart and induce local activation of CSCs by controlling the release of encapsulated growth factors. This study has developed and characterised a cardiac patch that exploits the sustained release of growth factors to promote the recruitment and expansion of CSCs.

CSCs are able to reconstitute dead myocardial tissue and recover cardiac function (7). Linke *et al* demonstrated that resident CSCs within the heart possess HGF and IGF-1 receptor systems that can be activated to induce their migration, proliferation and survival (18). When these growth factors are injected into the heart, they have been shown to regulate resident CSCs to promote significant restoration of dead tissue in a dose-dependent manner (17, 20, 21). Previous work on the therapeutic efficacy of these growth factors has relied on transepicardial injections given as a one off bolus or necessitating repeated administrations during open chest surgery as the route of delivery (17, 18, 21). In addition, the therapeutic use of these growth factors is limited by their short half-life *in vivo* (45, 46). A scaffold based depot that can release these growth factors in a sustained manner would obviate the need for repeated administrations and could provide a spatiotemporal co-release to favour myocardial repair.

Both HGF and IGF-1 were encapsulated in alginate using a spray-drying technique in order to protect them from degradation and extend their release period **(Figure 1)**. The spray-drying process was chosen since it is widely used at an industrial level for the manufacture of particulate systems, as it enables production of small-diameter microparticles (MPs) with high yields and drug encapsulation efficiencies (47). In accordance with previous reports using the same technique (28, 42), the alginate MPs had an average size of 2.2µm **(Figure 2)**. Loaded alginate MPs were incorporated into scaffolds composed of type 1 collagen. This protein has been previously shown to improve recovery of the heart after MI through several processes independent of exogenous cells, including mechanical support, facilitation of cell migration and angiogenesis (23, 48). In addition, the collagen patch will provide a physical support for the MPs to locally release their payload. As observed in **Figure 3,** the MPs preserved their morphological features after incorporation into the collagen scaffolds. Previous work combining alginate MPs with collagen scaffolds loaded with VEGF for bone regeneration demonstrated similar results in terms of the MPs integrity (49). Using a separator column we successfully generated a scaffold that contained two different growth factors on opposing sides with a mean pore size >100 µm. This pore size is ideal in order to create a microenvironment in which recruited CSCs can promote myocardial regeneration. HGF MPs are loaded on the patch away from the infarct site and once released will instruct cells to migrate towards the patch. IGF-1 MPs are loaded on the patch at the infarct site which will instruct migrating CSCs to proliferate. The microenvironment and the growth factors can act synergistically to induce recruited cells to regenerate the damaged cardiac tissue (7, 50).

In addition to maintaining protein stability and bioactivity, a cardiac patch designed for protein delivery needs to ensure a homogeneous distribution that allows adequate protein release (51). The release profile of both free loaded HGF and IGF-1 from the collagen scaffolds showed an initial burst release with no further release being detected by day 5 **(Figure 4)**. Similar IGF-1 release kinetics in which the majority of IGF is released in the first 4 days was previously shown for collagen glycosaminoglycan scaffolds (GAG) (52). After 5 days, the cumulative release of IGF-1 was 18%. HGF release followed the same rapid release profile as IGF-1 but cumulative release was considerably higher (37%). Comparable release behaviour for HGF from collagen scaffolds (32% release after 168 hours) has been reported before (53). Free IGF-1 has a very short half-life which would account for the variation in concentrations detected of each growth factor. Overall, the patch demonstrated dual release kinetics but release of the growth factors was rapid.

Studies using CSCs transplanted directly into the infarcted myocardium have demonstrated left ventricular restoration as have studies that have carried out direct intramyocardial injection of HGF and IGF-1 (54). Paracrine factors released from CSCs (HGF, IGF-1 and VEGF) could only be detected in the infarcted heart up to day 7 (54). In terms of direct injection of HGF and IGF-1 only one injection was performed 20 days post infarction (55). Both studies identified extending and enhancing paracrine potency as prime candidates to boost overall efficacy. In terms of inducing migration of CSCs, it has also been suggested that long term release of HGF is required in order to exert a functional benefit (56, 57). It is therefore necessary to extend release past what is achievable with free loaded growth factors. Release from the scaffold could be extended up to 15 days with both growth factors encapsulated in MPs and by doing so could potentially extend efficacy.

In addition to the extended release profile that the patch provides for both HGF and IGF-1, the release kinetics of both growth factors is particularly advantageous. Similarly to the behaviour of free loaded growth factors, HGF showed a higher release in the first 72 hours compared to IGF-1 **(Figure 4)**. 50% of the total amount of HGF detected was released during that period. The initial burst release is likely due to the protein being released from the MPs in contact with the acidic environment of the collagen slurry prior to the freeze drying process, this could also account for the differences in growth factor levels observed in the microparticle loaded scaffolds. Proteins typically accumulate on the outside surface of spray-dried MPs and as such the aggregated protein on the MP surface may release in an uncontrolled manner (58). This release behaviour has been observed before in alginate MP loaded scaffolds (49). However this fast released HGF from the patch may be beneficial to provide an immediate strong migratory signal to eCSC resident in viable portions of the heart. Both growth factors then show a sustained release for the next 12 days. HGF release also showed another spike in release at day 10. HGF can also mediate processes required at later phases of infarct repair, such as angiogenesis induction, more favourable extracellular matrix ECM remodelling and fibrosis reduction (59, 60). Slower, yet continuous, release of IGF-1 can mediate cellular proliferation and processes required at later phases of infarct repair such as rescuing the remaining functional myocardium and reduction of cell apoptosis and loss after the initial ischemic event (35).

In order to assess the bioactivity of the growth factors released from the Coll-Alg patch it was first necessary to isolate CSCs from rat heart explants. The c-Kit^{POS} eCSCs are characterized by expression of c-Kit (CD117), the receptor for stem cell factor (61). We isolated c-Kit^{POS} cells no fewer than 6-10 rats at a time. Given the relatively small number of c-Kit^{POS} CSCs that can be isolated from a single heart, pooling explants circumvents the issue of very low eCSCs density (62). From the explants we have cultivated, over 6% of the cell population express the stem cell marker c-Kit **(Figure 5)**. This falls in line with c-Kit levels from other publications that have been isolated from rat cardiac explants. Although levels vary between publications, they are uniformly low and between 4-10% c-Kit^{POS} as CSCs derived from explants include subpopulations of other cell types such as mesenchymal stem cells, in addition to cardiac progenitor cells (62-64).

Although there is little conclusive information regarding the specific HGF concentration required to induce an *in vitro* biological response in CSCs, data on HGF concentration has been demonstrated in mesenchymal stem cells (MSCs) (36) to induce a migratory response. Activation of these growth factor signals has been confirmed by phosphorylation of c-Met and their downstream targets in both CSCs (35) and MSCs (65). We elected to use 25 ng/mL of released HGF, which was sufficient to induce a migratory response in CSCs cultured on a transwell insert. As shown in **Figure 6**, HGF released from the Coll-Alg scaffold presented an increased CSC migration relative to no stimulus control although not as effective as the high serum positive control.

The longer sustained release of IGF-1 is expected to instruct the proliferation of CSCs. Migration studies have shown that HGF promotes motogenic and invasive activity of CSCs, whereas IGF-1 had little effect. Conversely, IGF-1 showed more antiapoptotic and proliferative effects on CSCs compared with HGF (35). Previous work has shown that IGF-1 can promote cell proliferation in mouse CSCs even in the

absence of other growth factors such as EGF and bFGF. This proliferative effect was seen to be associated with the phosphorylation of Akt-1 and FoxO3a. C-kit^{POS} CSCs that express IGF-1R display enhanced proliferation, differentiation and survival in response to the IGF-1/IGF-1R interaction (66). We elected to use 25 ng/mL of released IGF to induce a proliferative effect on treated CSCs. Cells exposed to IGF-1 showed an increase in dsDNA compared to untreated controls **(Figure 7)**. This illustrates that the MP-encapsulated IGF maintained its bioactivity after release from the patch. This increase in cell proliferation observed has also previously been shown to be dose respondent (40) and that this dose-dependent increase on CSCs occurs after growth factor deprivation.

The cardiac patch described herein represents a promising approach for overcoming the associated problems of cell transplantation for cardiac tissue engineering. Both the spray-drying and freeze-drying techniques, used for the fabrication of MPs and scaffolds, respectively, are reproducible processes that can easily be scaled up to industrial production (67, 68). Embedding the growth factors in the MPs and their position on the cardiac patch allowed for the spatiotemporal release of multiple agents. This cardiac patch formulation described within, requires further application and investigation at a pre-clinical level to advance the fields of cardiac regeneration, and potentially help alleviate the suffering caused by ischemic heart disease.

5. Conclusions

Stem cell therapies are under development as treatments for cardiovascular disease with the premise of providing a competent cell source to replace and replenish the cardiomyocytes lost after MI. However, a caveat to this treatment option is that cells have to be isolated and expanded to considerable numbers before preparation for delivery back to the damaged heart. This patch design would be available as an off-the-shelf option, and would not require lengthy culture periods or patient specific culture preparations and methods. The scaffold would take advantage of the resident stem cell population in the heart through the dual spatiotemporal release of growth factors and enhance healing through the activation of the endogenous stem cell compartment. It could be a more feasible and economical approach to the treatment of cardiovascular disease compared to stem cell transplant therapies and could be used in conjunction with pharmaceutical management strategies but as a higher order therapy that targets the underlining degenerative loss in the failing heart.

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