Encapsulation of Cardiac Stem Cells to Enhance Cell Retention and Cardiac Repair

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This thesis is submitted to the Faculty of Graduate and Postdoctoral Studies as partial fulfillment of the Master of Science program degree in Cellular and Molecular Medicine.

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This work was previously published as an original research article entitled "*The effect of encapsulation of cardiac stem cells within matrix-enriched hydrogel capsules on cell survival, post-ischemic cell retention and cardiac function*." Biomaterials 2014; 35(1):133-142.

Permission has been granted from the Journal Editor-in-Chief to publish this work as a Master's thesis for completion of my degree. Please see Appendix II page 67.

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Acknowledgements

I would like to recognize and thank the following individuals for their significant contributions to my work:

- Thesis Advisory Committee: Dr. Duncan J Stewart and Dr. David W Courtman
- Davis Laboratory Members:
    - Everad Tilokee: training, experimental design, patient consenting, tissue processing
    -Nicholas Latham: training, experimental design, patient consenting, tissue sample processing
    -Robyn Jackson: patient consenting, tissue sample processing
    -Richard Seymour: animal surgery
    -Dr. Maryam Kamkar: experimental design
    -Dr. Andre Molgat: experimental design
    -Bin Ye: training
- Suuronen Laboratory Members:
    -Dr. Brian McNeill: experimental design, integrin qPCR
- University of Ottawa Heart Institute - Animal Care and Veterinary Services
- Study Collaborators
    -Dr. Buu-Khanh Lam, Dr. Marc Ruel, Dr. Munir Boodhwani, Dr. Vincent Chan, Dr. Fraser Rubens

I would also like to extend my greatest gratitude to my supervisor, Dr. Darryl R Davis, for this project would not have been completed without his guidance and support.

Sources of Funding

This work was supported by the Canadian Institutes of Health Research (Operating Grant 229694). Dr. Davis is funded by Canadian Institutes of Health Research (Clinician Scientist Award).

Abstract

Despite advances in treatment, heart failure remains one of the top killers in Canada. This recognition motivates a new research focus to harness the fundamental repair properties of the human heart, with human cardiac stem cells (CSCs) emerging as a promising cell candidate to regenerate damaged myocardium. The rationale of this approach is simple with *ex vivo* amplification of CSCs from clinical grade biopsies, followed by delivery to areas of injury, where they engraft and regenerate the heart. Currently, outcomes are limited by modest engraftment and poor long-term survival of the injected CSCs due to on-going cell loss during transplantation. As such, we explored the effect of cell encapsulation to increase CSC engraftment and survival after myocardial injection. Transcript and protein profiling of human atrial appendage sourced CSCs revealed strong expression the pro-survival integrin dimers αVβ3 and α5β1- thus rationalizing the integration of fibronectin and fibrinogen into a supportive intra-capsular matrix. Encapsulation maintained CSC viability and expression of pro-survival transcripts when compared to standard suspended CSCs. Media conditioned by encapsulated CSCs demonstrated superior production of pro-angiogenic/ cardioprotective cytokines, angiogenesis and recruitment of circulating angiogenic cells. Intra-myocardial injection of encapsulated CSCs after experimental myocardial infarction favorably affected long-term retention of CSCs, reduced scar burden and improved overall cardiac function. Taken together, cell encapsulation of CSCs prevents detachment induced cell death while boosting the mechanical retention of CSCs to enhance repair of damaged myocardium.

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αSMA	Alpha smooth muscle actin
ABCG2	ATP-binding cassette sub-family G member 2
AKT	Protein kinase B
CAC	Circulating angiogenic cell
CDC	Cardiosphere-derived cell
c-Kit	Tyrosine receptor kinase
CSC	Cardiac stem cell
CSp	Cardiosphere
cTnT	Cardiac troponin T
ECM	Extracellular matrix
EF	Ejection fraction
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FAC	Fractional area change
f/f	Fibrinogen and fibronectin
FG	Fibrin glue
HNA	Human nuclear antigen
HF	Heart failure
HUVEC	Human umbilical vein endothelial cell
IL-6	Interleukin 6
JNK	Jun N-terminal kinase
KDR	Kinase insert domain receptor
LV	Left ventricular
MDR-1	Multidrug resistance protein 1
MEF2C	Myocyte enhancer factor 2C
MI	Myocardial infarction
MSC	Marrow stromal cell
NYHA	New York heart association
SCID	Severe combined immunideficiency
SDF-1	Stromal cell-derived factor 1
SPMG	Superparamagnetic microspheres
SV	Stroke volume
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor

1.0 Introduction

Heart failure is at the top of the list for causes of mortality in Canada, costing the population and government over $20 billion each year (Naylor 1999, Roger 2012). As new pharmaceutical and surgical therapies improve the survival of patients suffering from myocardial infarctions and other acute cardiac diseases, a greater number of patients are left susceptible to the debilitating consequences of irreversible cardiac damage. Currently it is estimated that 500,000 Canadians (10/1000 over the age of 65) are living with heart failure, having a 27% chance of mortality within 1 year of diagnosis and increasing to 50% within 5 years of diagnosis (Roger 2012, Alter 2012, Lee 2010). These statistics illustrate the reality that current therapies cannot prevent disease progression and validate the need for novel ways aiming to reverse and repair an irreversibly failing heart.

1.1 Cardiomyocyte renewal and the search for resident cardiac stem cells

Stem cell therapy has quickly become a promising means of regenerating the heart, with a number of proposed cellular candidates being investigated. An ideal stem cell candidate should be autologous, easy to expand *ex vivo* to reach clinically relevant doses, able to persist in damaged tissue and differentiate into working myocardium after transplantation, improve overall cardiac function and consistently demonstrate product safety. Though preclinical and clinical trials using cell products such as bone marrow mononuclear cells and mesenchymal stem cells have brought hope to the field of stem cell therapy, results are modest at best and often inconsistent amongst trials (Orlic 2001, Strauer 2002, Balsam 2004). Maximal benefits of current stem cell products are limited due to poor acute engraftment, low long-term survival and an inability of these

cells to differentiate into functional cardiomyocytes, thus lacking the ability of direct cardiac repair (Murry 2004, Chavakis 2010, Wang 2011, Jiang 2013, Katritsis 2007). These conclusions have left researchers motivated to find a cardiogenic cell product capable of persistently integrating and differentiating into new working heart tissue with the hopes of truly mending a broken heart.

1.1.1 Confirmation of cardiomyocyte renewal

Traditionally in cardiac biology, the human heart has been considered a terminal post-mitotic organ incapable of self-repair. This dogma was challenged with the discovery that the adult heart exhibits a low level of cardiomyocyte renewal throughout an individual's lifespan (Urbanek 2006, Kajstura 2008). In 2009, Bergmann et al published a landmark study using a carbon dating technique of atmospheric carbon-14 formed during the Cold War nuclear bomb testing. This carbon-14 integrated into genomic DNA, thus allowing for the calculation of cell turnover in affected individuals. Authors estimated that nearly 50% of adult cardiomyocytes are renewed over an individual's lifespan, decreasing with age (averaging 0.5-1.0% per year) (Bergmann, 2009). Though the extent of this turnover remains a controversial topic in the field with other studies estimating annual cardiomyocyte turnover of 22% per year (Kajstura 2010), this data clearly demonstrates that new cardiomyocytes are generated annually. The discovery of cell cycle markers such as Ki67 and incorporation of BrdU in both diseased and normal adult hearts has further confirmed the existence of a replenishing pool of cardiomyocytes (Beltrami 2001, Urbanek 2003). With confirmation of cardiomyocyte turnover being established, groups quickly began searching for the source of these new myocytes; a cell population capable of proliferating and

differentiating into new myocytes, similar to stem cells in the brain and liver (Thorgeirsson 1996, Bjornson 1999).

1.1.2 Discovery and characterization of resident cardiac stem cells

The existence of a resident population of cardiac stem cells (CSCs) was first described by Beltrami et al in 2003, in which they identified a population of cells that satisfied the three key criteria for "stem cell" consideration: multipotency, self-renewing and clonogenic. This cell population was positive for the receptor tyrosine kinase (c-Kit), a well documented stem cell marker, and negative for hematopoietic lineage markers including CD34 and CD45 (He 2011). These c-Kit+ CSCs express transcription factors of early cardiac development including Nkx2.5, MEF2C and GATA4, thus are primed for the adoption of a cardiac fate. Two distinct subpopulations of c-Kit+ CSCs have emerged: the myogenic CSC (c-Kit+/KDR-), found in the interstitial space between cardiomyocytes, and the vasculogenic CSC (c-Kit+/KDR+), found in discreet clusters in the atrial appendage and cardiac apex (D'Amario 2011). In culture, c-Kit+ cells have a doubling time of approximately 40 hours without reaching growth arrest for more than 19 months after their isolation (Beltrami 2003). Clear evidence of biochemical differentiation supported the notion these cells are capable of giving rise to three cardiogenic lineages: endothelial cells, smooth muscle cells, and myocytes (Beltrami 2003).

Additional characterization of CSCs demonstrated the expression of other stem cell surface markers including Sca-1, MDR-1 and ABCG2 (Pfister 2008, Meissner 2006, Martin 2004, Nagai 2013). CSCs expressing these markers were negative for markers of cardiac identity, and demonstrated a strong ability to proliferate and differentiate into

functional cardiomyocytes (Martin 2004, Wang 2006). These cells also had the ability to migrate within the heart to regions of damage in response to ischemic injury, and upon transplantation attenuated left ventricular dysfunction and adverse remodeling (Bailey 2012). Taken together, the studies mentioned demonstrate the presence of a CSC niche within the adult heart that has the potential to repair damaged tisue. The degree to which these cells contribute to cardiomyocyte repopulation and injury remains in question.

1.2 CSCs and their role in cardiomyocyte renewal and injury repair

A recent lineage tracking study has demonstrated that the role of resident CSCs in cardiomyocyte renewal is likely very minimal (Hsieh 2007). In this study, an inducible cardiomyocyte-specific transgenic mouse fate-mapping approach was used in which cardiomyocytes express GFP and cardiac stem cells do not. In normal aging, stem or precursor cells do not refresh uninjured cardiomyocytes at a significant rate, as observed by the little change in percent-GFP expression in the heart over 1 year. After cardiac injury, the role of CSCs in injury repair is more prominent as illustrated by the existence of newly formed GFP- myocytes arising from a non-myocyte (GFP-) source.

The exact source of these cardiomyocytes is still uncertain, but similar studies have demonstrated that endogenous CSCs migrate to towards the infarct and may help reduce cardiac injury (Fransioli 2008). Thus, although CSCs seem to play a role in cardiomyocyte renewal and minor repair, their small population (1 CSC per 10,000 cardiomyocytes) suggests they are incapable of meaningful regeneration in response to significant cardiac injury and cannot prevent ventricular remodeling (Urbanek 2006, Bergmann 2009). These findings left the field requiring culture methods with the

promise of delivering a number of potent cells to repopulate scar tissue and reverse heart failure.

1.3 Differences among CSC-based cell products

1.3.1 Isolation of CSCs from small myocardial biopsies

The isolation and expansion of CSCs from small biopsies of human and murine hearts was first achieved in 2004 by Messina et al. Tissue samples were minced, enzymatically digested and plated on fibronectin-coated dishes. After several days, a monolayer of cells (termed cardiac outgrowth or explant derived cells) spontaneously emigrated from the plated tissue fragments. Though largely unknown, the mechanism behind the formation and migration of these cells is thought to be induced by the role of Notch-1 in epithelial to mesenchymal transition (Zakharova 2012). The resulting outgrowth of cells can be enzymatically harvested and used for downstream applications. The outgrowth is composed of a heterogeneous mixture of cells expressing stem cell markers (c-Kit, Sca-1, and ABCG2), mesenchymal markers (CD90 and CD105), and endothelial markers (CD31 and CD34) (Davis 2010a).

1.3.2 Production of cardiospheres and cardiosphere-derived cells

From this heterogeneous outgrowth population of CSCs, several differing CSC products have emerged. After harvest, CSCs can be grown in suspension on poly-D-lysine plates to form 3-dimensional spherical aggregates termed cardiospheres (CSp), a process initiated by hydrophobic interactions between the cells and the plate surface (Messina 2004). Cardiosphering is thought to increase stimulation of the ERK and VEGF pathways that enhance proliferation of the cardiac progenitor subpopulation, thus enriching the c-Kit+ subpopulation or cell “stemness” (Davis 2009, Davis 2010a, Cho

2012, Cho 2013). Due to the large diameter of CSp (70-100μm), it has been demonstrated that CSp can be re-plated in 2D cultures to generate a population of single cells called cardiosphere-derived cells (CDCs), avoiding the possible risk of thrombosis following intra-coronary infusion (Lee 2011, Smith 2007). A number of preclinical animal trials have illustrated the ability of CDCs to enhance cardiac repair post-myocardial infarction resulting in the first-in-human trial (Malliaras 2012, Li 2012, Johnston 2009, Bonios 2011, Makkar 2012).

1.3.3 Antigenic selection of stem cell markers

In contrast to the strategy of utilizing the cumulative population of CSCs, some groups have opted for a “pure” (i.e. c-Kit+) cardiac progenitor population by using antigenic selection (Bearzi 2007). These cells can be antigenically isolated from the spontaneous outgrowth of plated explant tissue and must be expanded in culture to reach a therapeutically relevant dose as c-Kit+ cells are less than 10% of the total cardiac outgrowth. A small portion of the c-Kit+ progenitor cells also co-express early cardiac commitment transcription factors (Nkx2.5, GATA4 and MEF2C), suggesting these cells are isolated at differing stages of differentiation. Animal studies have demonstrated the ability of human c-Kit+ cells to improve LVEF, attenuate scar formation, electromechanically couple to surrounding myocardium and improve overall ventricular function (Beltrami 2003, Bearzi 2007, Li 2009).

1.3.4 Strategies to simplify CSC culture

Although CSp and c-Kit antigenic selection have a proven ability to regenerate the heart, they require additional complex steps and prolonged culture periods that increase cost and time before obtaining a clinically relevant dose for transplantation. For

these reasons, the therapeutic capacity of the primary heterogeneous CSCs arising from the tissue explants was examined, and it was observed that CSCs have a superior ability to adopt a cardiac phenotype while maintaining an equivalent paracrine profile (Davis 2010b). By using the early aggregate CSCs, it simplifies the culture technique without altering the complimentary cell-to-cell interactions of subpopulations to ultimately reduce culture times and minimize the risk for phenotypic drift or malignant transformation. These CSCs maintain a stem cell character, demonstrating ability for self-renewal, clonogenicity and multipotency (Davis 2010b). In the single head-to-head study performed to date comparing c-Kit+ selected cells and monolayer CDCs, the heterogeneous CDCs demonstrated an enhanced regenerative performance as measured by increased LVEF and lower scar burden. These results support the notion that antigenic sub-selection or sphere expansion may not be necessary to obtain a CSC product with superior therapeutic capabilities (Davis 2010b, Li 2012).

1.3.5 The origin of CSCs

The origin of *ex vivo* proliferated CSCs remains a controversial topic, with the underling thought being CSCs originate from an extra-cardiac region and migrate to the heart prior to tissue collection. This suggestion has been refuted by a recent study of CSCs cultured from transplant recipients. One study examined CSCs obtained from sex-mismatched donor-recipient pairs (male recipient, female donor) demonstrating that CSCs were uniformly XX, whereas peripheral circulating cells were XY (Cho 2013). Furthermore, analysis of the short tandem repeats of *ex vivo* proliferated CSCs were identical to the transplanted donor heart, not the recipients (White 2013). These studies confirm the notion that CSCs possess an intrinsic cardiac origin. Overall, ex vivo

proliferation of CSCs holds the promise of delivering a large number of potent stem cells derived directly from the heart, with the potential of regenerating damaged myocardium into functional heart tissue and reversing HF in the adult heart.

1.4 Mechanism behind CSC mediated repair

1.4.1 Direct trans-differentiation of CSCs

Despite functional improvements observed after the delivery of CSC products, the underlying mechanism driving this cardiac repair remains largely unknown. The initial theory behind stem cell transplantation was to deliver cells to the damaged myocardium, and for these cells to engraft and differentiate into new functional myocardium resulting in improved cardiac function. CSCs display an enhanced capability to adopt endothelial, smooth muscle, and importantly, cardiomyocyte fates making direct differentiation of CSCs into cardiac lineage a unique quality of their regenerative abilities (Beltrami 2003, Bearzi 2007, Davis 2010a, Davis 2010b). Unfortunately, after delivery into the myocardium persistence of cells is very poor, with less than 5% of cells remaining after 3 weeks - suggesting the benefit of first generation CSC products is largely paracrine-mediated (Terrovitis 2009, Terrovitis 2010). Recently the Davis lab demonstrated that human CSCs secrete high amounts of angiopoeitin-1, angiogenin, human growth factor, insulin-like growth factor 1, interleukin 6 and vascular endothelial growth factor (Latham 2013).

1.4.2 Paracrine-mediate CSC repair

The exact mechanism underlying paracrine-mediated repair remains unknown. It has been shown that the wide array of cytokines secreted by CSCs stimulates pro-survival pathways in damaged myocardium resulting in myocardial salvage to promote

the rescue of reversibly damaged heart tissue (Chimenti 2010). Recent studies have demonstrated that recruited endogenous stem cell differentiation and cardiomyoctyte proliferation plays an important role in CSC-mediated cardiac repair (Malliaras 2013). Treatment of injured myocardium with CSCs ultimately leads to the attenuation of scar formation by reducing collagen deposition and fibrosis post-MI through increased secretion of endogenous matrix metalloproteinases (Tseliou 2014). These hearts exhibited enhanced microvessel density which may result in part from a reduction in collagen deposition permitting the migration of endothelial progenitor cells. Other explored mechanisms include exosome transfer from transplanted CSCs and immunomodulatory effects on inflammatory responses, though the degree to which these contribute to cardiac repair remains a highly debated (Vrijsen 2010, Barile 2012, Arslan 2013).

1.5 Advancement of first generation CSC products to clinical trial

Clinical trials using bone marrow or blood derived stem cells for the treatment of HF have resulted in overall poor cardiac repair in patients (Lunde 2005, Erbs 2007, Herbots 2009, Davis 2011). With a number of successful small and large-scale preclinical animal studies behind them, and the fact that CSCs can adapt all cardiac fates, this cell product moved quickly to clinical trials.

1.5.1 CADUCEUS

The “CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction (CADUCEUS)” trial was a phase 1 study designed to demonstrate the safety of intra-coronary infusion of autologous CDCs (Makkar 2012). Patients included in this study had a recent MI with an ejection fraction (EF) of less than 45%, with CDCs

cultured for 4-8 weeks from ventricular biopsies. The primary 6-month patient assessment demonstrated product safety, with no significant differences in patient deaths or adverse cardiac events. Secondary 12-month outcomes using magnetic resonance imaging (MRI) demonstrated an overall reduction in scar size and an increase in viable heart mass after CDC treatment (Malliaras 2014). The safety and efficacy demonstrated by the authors warranted transition to a phase 2 trial powered for efficacy endpoints (*ALLSTAR)*.

1.5.2 ALLSTAR

The success of the *CADUCEUS* trial provided the groundwork for the phase 2 clinical trial entitled “Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration” or ALLSTAR. The allogeneic CDCs had been previously shown to be safe in animal models (Malliaras 2012), and allowed for the delivery of cells more quickly after myocardial infarction. This trial is currently underway and aims to enroll 274 patients by December 2015 (EF <45%) to compare the efficacy of allogeneic CDC delivery to placebo delivery in the treatment of heart failure (ClinicalTrials.gov 2014a). A 12-month follow-up will examine MRI infarct size in patients.

1.5.3 SCIPIO

Recently completed, a second phase 1 trial entitled “cardiac Stem Cells In Patients with Ischaemic cardiOmypathy”, or *SCIPIO*, sought to examine the safety and efficacy of a c-Kit+ purified CSC product (Bolli 2011). Atrial appendages were obtained from patients with a LVEF off less than 40%, from which c-Kit+ cells were isolated and expanded for 113±4 days in culture to reach therapeutic doses. Interim results using echocardiography demonstrated improvements in LVEF and NYHA class. The 12-

month follow-up of all enrolled patients using cardiac MRI showed improvements in LV function and viable tissue, and infarct size (Chugh 2012). This study concluded that the infusion of c-Kit+ CSCs is easy, safe and promotes cardiac repair in patients.

1.5.4 ALCADIA

The third phase 1 clinical trial, AutoLogous Human Cardiac-Derived Stem Cell to Treat Ischemic cArdiomyopathy" (ALCADIA), included patients diagnosed with ischemic cardiomyopathy (15%<EF<35%) between the ages of 20-80 (ClinicalTrials.gov 2014b). Unlike previous trials, *ALCADIA* sought to examine the delivery of human cardiospheres in conjunction with the slow release of basic fibroblast growth factor from a gelatin sheet implanted prior to cell delivery thought to increase the survival of transplanted CSCs (Takehara 2008). Results from the 12-month follow-up were presented at the 2012 American Heart Association Scientific Sessions, but have yet to be published. Findings demonstrated safety and an improvement in NYHA class and LV function, with a trend towards a decrease in infarct size.

1.6 Future directions to enhance CSC therapy

Despite the impressive results of preclinical and clinical trials examining the efficacy of CSCs in cardiac regeneration, poor acute retention (5-20% after 1 hour) and low long term survival (<5% after 3 weeks) likely hinder the full potential of these cells to reverse heart damage (Terrovitis 2009, Terrovitis 2010). Low acute retention ensues very early after injection and is mediated by several factors: mechanical extrusion, clearance by the lymphatic system, the reduced ability of infarcted myocardium to support engrafted cells, and on-going programmed cell death (Bonios 2011, Terrovitis 2009, Karoubi 2009). For this reason, research initiatives are focusing on developing

next generation CSC products that will enhance the engraftment, survival and regenerative capabilities of this ideal cell candidate.

Over the past 50 years, biomaterials have already been widely used in the field of cardiology including artificial cardiac pacemakers, artificial valves and stents. More recently, biomaterials have been designed to aid in the delivery of stem cells to damaged organs, specifically the injured heart. Biomaterials used for cardiac regeneration should ideally be biocompatible and degradable without the formation of toxic metabolites. Biomaterials should also have to ability to provide signals for cellular attachment, proliferation and differentiation, lasting long enough for the cells to persist without impeding the coupling of differentiated cells (Segers 2011).

1.6.1 Enhanced retention and repair using a biomaterials approach

To address the current limitations in stem cell transplantation, several studies have examined the capacity of artificial biomaterials to improve the acute retention of CSCs after injection. One such method uses fibrin glue (FG) – a formulation used to create a fibrin clot – to seal the site of injection (Terrovitis 2009). While the needle was still in situ, one to two drops of FG were applied directly over two injection sites to provide a seal and prevent backwash of CSCs. Short-term retention in the FG-group mice was significantly increased as compared to standard injection, however, ongoing cell loss still occurs and after 3 weeks. This study validates the concept that boosting acute retention provides enhanced functional benefits as the fractional area change of the left ventricle demonstrated a strong trend to increase in the fibrin glue treated groups when compared to cells injected in PBS.

Magnetic-targeting has also been studied as a means of boosting the acute engraftment of CSCs as the pull of a magnet may limit the venous/lymphatic washout of injected CSCs (Cheng 2010, Cheng 2012a). In this investigation, CSCs were labeled with superparamagnetic microspheres (SPMS) containing iron. SPMS-labeled CSCs were injected into the injured myocardium of rats with and without an exterior magnet. When the magnet was applied, cells were visibly attracted towards the ischemic zone. Without the magnet, cells were washed away immediately after injection. Visual inspection showed that magnetic-targeting enhanced 24 hour cell retention and cardiac function when cells were acutely retained by the external magnetic field.

1.6.2 Disruption of cell attachment cues in cell transplantation

Although fibrin glue and magnet-targeting studies increase cell retention, these approaches do not address the ongoing programmed cell death that occurs during transplantation, which begins at cell harvest and continues after cell delivery. In culture, CSCs are grown in an adherent environment on fibronectin-coated plates via integrin-mediated attachments with the cell cytoskeleton (Thomas 1999). During the transplantation process, CSCs are harvested and forced into suspension, resulting in lost attachment signals and reduced pro-survival pathway stimulation leading to anoikis (Grossmann 2002). Anoikis, meaning "to be without a home", is used to describe an anchorage-dependant cell's apoptotic response to the absence of cell-matrix interactions. During anoikis, the Jun N-terminal kinase (JNK) pathway is activated, promoting cell death by the activation of caspase proteolytic activity (Cardone 1997).

1.6.3 The use of biomaterials scaffolds to anchor cells

Recently, biomaterial scaffolds have been used in combination with cell therapy to anchor cells to the damaged myocardium while improving cell survival. These scaffolds contain extra-cellular matrix proteins and provide cells with attachment cues to reduce anoikis while improving cell proliferation and paracrine secretion (Zhang 2008, Kuraitis 2011, Kuraitis 2012). To date, several studies have embedded CSCs in polymerizable hyaluronan-gelatin hydrogel, platelet-rich gel and poly(N-isopropylacrylamide) hydrogel, demonstrating improvements in cell retention, cell proliferation and cardiac differentation (Cheng 2012b, Cheng 2012c, Li 2011). While these methods represent a complimentary means of providing CSCs that may more readily contribute to cardiac repair, delivery to the surface of the beating heart demands invasive surgery with inherent morbidity and mortality.

1.6.4 Encapsulation for cell delivery

In a proof of principle study, the Courtman group looked at the capacity of cellular encapsulation to improve the viability and retention of blood-derived marrow stromal cells (MSCs) injected into rat hind limb (Karoubi 2009). The encapsulation process provides the cell with a three-dimensional 'cocoon' made of biodegradable and biocompatible agarose hydrogel. When adhesion molecules including fibronectin and fibrinogen were incorporated into the hydrogel capsule, viability of the MSCs was significantly increased versus MSCs encapsulated in non-supplemented hydrogel. This increase in survival was most likely attributed to restoration of cell-matrix signaling that is lost during suspension. Upon injection into rat hind limb, an increase in retention was observed in the encapsulated MSCs-treated animals versus non-encapsulated MSCs.

1.7 Encapsulation of CSCs

The goal of this project is to apply the cell encapsulation method to our *ex vivo* proliferated CSC product to increase their acute retention after intra-myocardial injection into damaged myocardium. The cell encapsulation method avoids the invasive administration of bulky fragile cell sheets and provides a minimally invasive way to inject CSCs encapsulated within supportive adhesion-rich cocoons. It is anticipated the capsules will boost acute retention and permit the cocooned stem cells to maintain adhesion signals and avoid anoikis prior to mobilizing from the capsule to regenerate the damaged myocardium.

1.7.1 Capsule composition for CSC encapsulation

This project will utilize an agarose-based capsule, previously shown to be biocompatible, biodegradable and permeable to cell nutrients and waste (Karoubi 2009). By providing a three-dimensional microenvironment for the CSCs that is supplemented with key adhesion molecules such as fibronectin and fibrinogen, it will provide the cells with the necessary attachment signals allowing for long-term survival (**Fig. 1**). Fibronectin and fibrinogen are superior choices as they bind to important cell integrins mediating survival signaling pathways, and stimulate cell proliferation (Pereira 2002, Pankov 2002). Fibronectin is capable of binding to dozens of integrins, and plays an important role in cell adhesion, migration and growth (Pankov 2002).

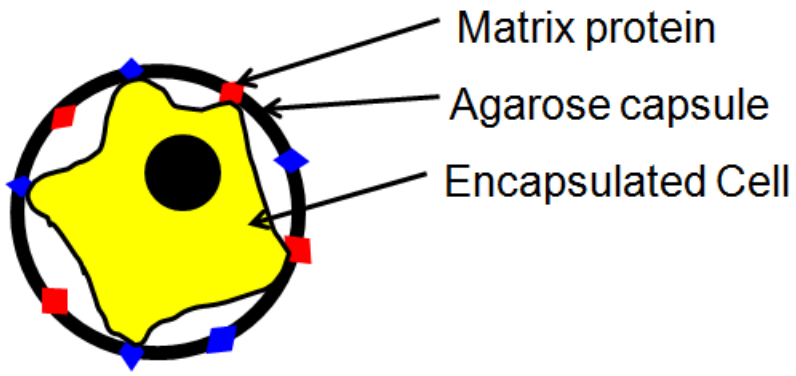


Figure 1: Cartoon depiction of an encapsulated cell within an agarose capsule supplemented with matrix proteins.

Overall, it is predicted that the encapsulation of CSCs will provide an easy and fast way to treat the cells prior to injection. More importantly, it will be non-toxic and will not fundamentally alter the character of these cells thus providing a clinically acceptable means of providing an effective cell product quickly with the assurance that it will be retained in the target organ.

2.0 Study Aim, Hypothesis and Specific Objectives

2.1 Study aim

To explore the impact of cell encapsulation on CSC-mediated cardiac repair using an immunodeficient mouse model of myocardial infarction.

2.2 Study hypothesis

Encapsulation of CSCs will enhance post-infarct cardiac repair by improving cytokine release, angiogenesis, resident stem cell recruitment, and the survival and engraftment of injected CSCs following intra-myocardial transplantation into ischemic mouse myocardium.

2.3 Specific objectives

1. To characterize the size of the capsules and determine ideal capsule components (agarose concentration and proteins).

2. To examine the viability and proliferation of encapsulated CSCs.

3. To evaluate the ability of encapsulated CSCs to secrete cytokines and to promote angiogenesis and stem cell recruitment.

4. To examine the ability of CSC encapsulation to improve cell engraftment after injection into an immunodeficient mouse model of myocardial infarction.

5. To assess the functional effects of encapsulation on CSC-mediated cardiac repair after injection into an immnodeficient mouse model of myocardial infarction: ventricular function, scar size, re-vascularization and apoptosis.

3.0 Methods

3.1 Patients and cell culture

Human CSCs were cultured from the atrial appendages obtained from patients undergoing clinically-indicated surgery using established methods approved by the University of Heart Institute Ethics Board (Messina 2004, Davis 2010b). Briefly, atrial appendage tissue were minced and digested with collagenase IV (Gibco) prior to being explanted on fibronectin-coated plates in cardiac explants media (CEM; Iscove's Modified Dulbecco's Medium (Invitrogen), 20% Fetal Bovine Serum (FBS; Invitrogen), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen), 2mmol/l L-glutamine (Invitrogen) and 0.1 mmol/l 2-mercaptoethanol (Invitrogen)). Samples were cultured at physiological 5% O_2 and 37°C. After 7-10 days in culture, the heterogeneous population of cells that spontaneously emigrated from the plated tissue was harvested using mild trypsinization (0.05% trypsin; Invitrogen) (**Fig. 2A**). After harvesting, plated tissue was cultured and harvested up to 3 more times, resulting in a total of 4 harvests. Following harvest, cells were frozen in freezing media (70% IMDM, 20% FBS, 10% DMSO) and later thawed when used for downstream application.

Circulating angiogenic cells (CACs) were isolated from peripheral blood samples donated by patients undergoing clinically indicated coronary angiography as previously described (Ruel 2005). Briefly, using density-gradient centrifugation (Histopaque 1077; Sigma-Aldrich), mononuclear cells were isolated and cultured in endothelial media (EBM-2, 2% FBS, 50 ng/ml human vascular endothelial growth factor (VEGF), 50 ng/ml human insulin-like growth factor-1 and 50 ng/mL human epidermal growth factor; Clonetics). One week later, CACs were harvested and used for experimentation.

3.2 Cell encapsulation

Human CSCs were harvested and suspended in media and mixed with low melt agarose (Sigma-Aldrich). The agarose was supplemented with human fibronectin (0.25 mg/ml; Sigma-Aldrich) or human fibrinogen (0.15 mg/ml; Sigma-Aldrich), both derived from human plasma. To form capsules, the cell/matrigel mixture was added drop-wise to agitated dimethylpolysiloxane (Sigma-Aldrich) and then rapidly cooled using a combination of cold HBSS and ice (**Fig. 2A**). The mixture was then centrifuged and capsules were filtered from the coalesced hydrogel using a 100µm filter (Fischer Scientific) and were re-suspended in appropriate media for testing. The capacity of immobilized matrix proteins to incorporate into matrigel capsules was verified using Oregon-green conjugated fibrinogen (Invitrogen). Fibrinogen was visualized in empty capsules under fluorescent microscopy at 470nm to demonstrate protein localization.

3.3 Integrin profile expression

The heterogenous population of first harvest CSCs harvested from the plated tissue was sorted using a FACSAria flow cytometer (BD Biosciences) with isotype matched immunoglobulin antibodies used as controls (c-kit, 9816-11, Southern Biotech; CD90, BD Biosciences). Total RNA was extracted using PARIS^{TM} RNA extraction kit (Invitrogen) and treated with 2U DNase I (Invitrogen) for 15 min at room temperature to eliminate genomic DNA. From 0.5 µg total RNA, first strand cDNA synthesis was performed using GoScript^{TM} reverse transcriptase (Promega) and 0.5 µg random hexamer primers (Integrated DNA Technologies) for 1h at 40 °C. With gene specific primers designed using DNAMAN software (Lynnon Biosoft) and primer3 (v.0.4.0; Rozen and Skaletsky, 2000), target gene mRNA levels were assessed by RT-qPCR

using BRYT Green GoTaq qPCR Master Mix (Promega) and a LightCycler 480 Real-Time PCR system (Roche). Relative changes in mRNA expression of target genes were determined using the ∆-∆Ct method normalized against *18S* and *Gapdh* (Pfaffl 2001). Western blot analysis was used to look at protein expression of integrins in c-Kit+/CD90-, c-Kit-/CD90- and c-Kit-/CD90- subpopulations (in some instances from different cells than those used for mRNA level assessment). Briefly, 20 µg of denatured protein was run on an 8% SDS-PAGE gel with loading buffer (Laemmli Sample Buffer; Biod-RAD). Gels were run at 80V for 0.5hr, followed by 110V for 1.5hrs. Gel transfers were completed at 110V for 1hr in cold transfer buffer. Membranes were blocked for 1hr in 5% milk solution. Primary antibodies were applied as per manufacturer guidelines, and a horseradish peroxidise detection reagent was used to visualized the bands on X-ray film (HRP; Bio-RAD). Representative bands shown were traced from different blots and combined to form one figure; densitometry was normalized to the corresponding *Gapdh* from each blot.

3.4 Cell viability and proliferation

The colorimetric WST-8 assay (Cell counting kit 8, Dojindo Molecular Technologies, Inc.) was used to assess CSC proliferation on first harvest CSCs. Normal culture conditions (adherent cells) were compared to cells in suspension (using Poly-HEMA-coated dishes), encapsulated cells (capsules supplemented with fibronectin and fibrinogen), encapsulated cells (capsules unsupplemented) and cells that were exposed to blocking antibodies for α3, αV, β1 and β3 prior to encapsulation. A baseline CCK-8 assay was performed at 16 hours, followed by a CCK-8 assay performed at 48 hours.

Fold-change was calculated as the 48 hr absorbance reading over the 16 hr absorbance reading, and expressed as the difference from 1.

3.5 Pro-survival and pro-anoikis gene expression

RNA was extracted from first or second harvest CSCs after 48 hours of culture in adherent, encapsulated or suspended conditions using the TRIzol (Invitrogen) protocol. Activation of attachment mediated pro-survival pathways (AKT, ERK) was quantified by RT-qPCR expression of downstream targets (*Bcl-2, Fos*) using a LightCycler 480 Real-Time PCR system (Roche). Levels of the cell anoikis markers caspase-3 and JNK (*Casp3 p17subunit and Jun*) were quantified in the same manner. Transcript-specific hydrolysis primer probes were designed and ordered from IDT. Relative changes in mRNA expression of target genes were determined using the Δ-ΔCt method normalized to (Pfaffl 2001). Mouse RNA was used as a negative control.

3.6 Conditioned media for paracrine profiling, angiogenesis, and CAC recruitment

Conditioned media was obtained from adherent, encapsulated and suspended third harvest CSCs after 48 hours of culture in hypoxic conditions (1% oxygen). Cells were seeded at 90% confluency in low serum basal media (Iscove's Modified Dulbecco's Medium, 1% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 ug/ml streptomycin, 2 mmol/l L-glutamine and 0.1 mmol/l 2-mercaptoethanol) on 6-well plates (Corning). The influence of cell encapsulation upon the paracrine signature of CSC was verified by comparing conditioned media using commercially available enzyme-linked immunosorbent assays (ELISA; R&D Systems, USA; angiogenin (DAN00), interleukin-6 (IL-6; D6050), stromal cell-derived factor-1α (SDF-1α; DSA00) and vascular endothelial growth factor (VEGF; DVE00)).

The capacity of encapsulated CSCs to promote angiogenesis was assessed using a growth factor-depleted matrigel assay (ECM625, Millipore) as directed by the manufacturer's instructions. Human umbilical vein endothelial cells (HUVECs, 5.0 x 10^4) were seeded on matrigel with stem cell conditioned media (adherent, encapsulated or suspended), serum free DMEM supplemented with 100 µM VEGF (positive media control) or DMEM alone (negative media control). After 18 hours of incubation, random fields (10x magnification, 5 random fields) were sampled using phase contrast microscopy. Cumulative capillary-network growth was determined using Image J software plug-in, NeuronJ (National Institutes of Health (NIH); http://rsb.info.nih.gov/ij).

The effects of cell encapsulation upon stem cell recruitment was assessed using fibronectin coated trans-well plates (24 wells, 3.0 µm pores; Corning) with 5.0 x 10^4 CACs plated in the upper well in serum-free DMEM while conditioned media (adherent, encapsulated or suspended) was placed in the bottom well. Serum free DMEM containing 100 ng VEGF was used as a positive control to normalize individual variations in CAC migration, and DMEM alone (negative media control). After 24 hours of normoxic incubation, the inserts and the remaining upper compartment CACs were removed. CACs that had successfully migrated through the polycarbonate membrane were fixed (4% paraformaldehyde) and stained with DAPI (Sigma-Aldrich). Fluorescent microscopy (10x magnification, 6 random fields) was used to determine the average number of cells per random field (ImageJ, ICTN plug-in, Center for Bio-Image).

3.7 Myocardial infarction, cell injection, and functional evaluation

The ability of encapsulated human CSCs to promote myocardial retention and influence cardiac repair was assessed using male NOD-SCID mice (8-9 weeks old;

Charles River, Wilmington, MA, USA) after left anterior descending artery (LAD) ligation (**Fig. 2B**). One week after LAD ligation, mice were injected with 1 x 10^5 encapsulated second harvest CSCs (3% agarose), 1 x 10^5 suspended (non-encapsulated) second harvest CSCs, or the negative vehicle control (PBS) using a 23G needle with injections split between the cardiac apex and lateral infarct border zone. Twenty-one and 28 days after LAD ligation, the effect of cell therapy was evaluated from the left ventricular ejection fraction, fractional area change and stroke volume (LVEF, FAC, SV; VisualSonics V1.3.8, VisualSonics, Toronto, Canada). The myocardial retention of transplanted cells was assessed 1 hour and 28 days after LAD ligation in a subset of mice using qPCR for non-coding human ALU repeats (mouse DNA used as negative control and to assess specificity). Genomic DNA was extracted (DNeasy kit; Qiagen) from the excised heart and qPCR was performed using transcript specific hydrolysis primer probes (IDT). Cell number was derived from standard curves normalized to the mass sampled and was expressed as a percentage of the cell count delivered.

3.8 Histology

After the final assessment of myocardial function, the hearts were excised, fixed with 4% paraformaldehyde, embedded in optimal cutting temperature compound and sectioned. Tissue viability within the infarct zone was calculated from Masson's trichrome (Invitrogen) stained sections by tracing the infarct borders manually and then using ImageJ software to calculate the percent of viable myocardium within the overall infarcted area. CSC engraftment was confirmed by staining frozen sections for human nuclear antigen (HNA; SAB4500768, Sigma-Aldrich) and differentiation to a cardiac lineage was identified by staining with unconjugated α-SMA (ab125266; Abcam), cTnT

(ab66133; Abcam) and vWF (11778-1-AP; Proteintech Group) – refer to **Table 1** for secondary antibodies used. Apoptosis within the infarct region was achieved using an *In Situ* cell death detection kit (TUNEL technology, Roche). The number of TUNEL+ cells counted was normalized to the number of DAPI+ cells per random field image. Re-vascularization was examined through staining with isolectin B4 (B-1205, Vector Laboratories). Arteriole density was measured using ImageJ (ICTN plug-in, Center for Bio-Image) and normalized to the number of DAPI+ cells per random field image.

Table 1: List of secondary antibodies used for histology.

Name	*Company*	*Catalogue number*
Alexa 488	Life Technologies	A11008
Alexa 594	Abcam	Ab96873
Streptavidin, Texas Red	Life Technologies	1028680

3.9 Statistical analysis

All data is presented as mean ± standard error of the mean (SEM). To determine if differences existed within groups, data was analyzed by a one-way ANOVA; if such differences existed, a two-tailed t-test was used to determine the group(s) with the difference(s) (Microsoft Office Excel 2007). Differences in categorical measures were analyzed using a Chi Square test. A final value of $P \leq 0.05$ was considered significant for all analyses. All probability values reported are 2-sided.

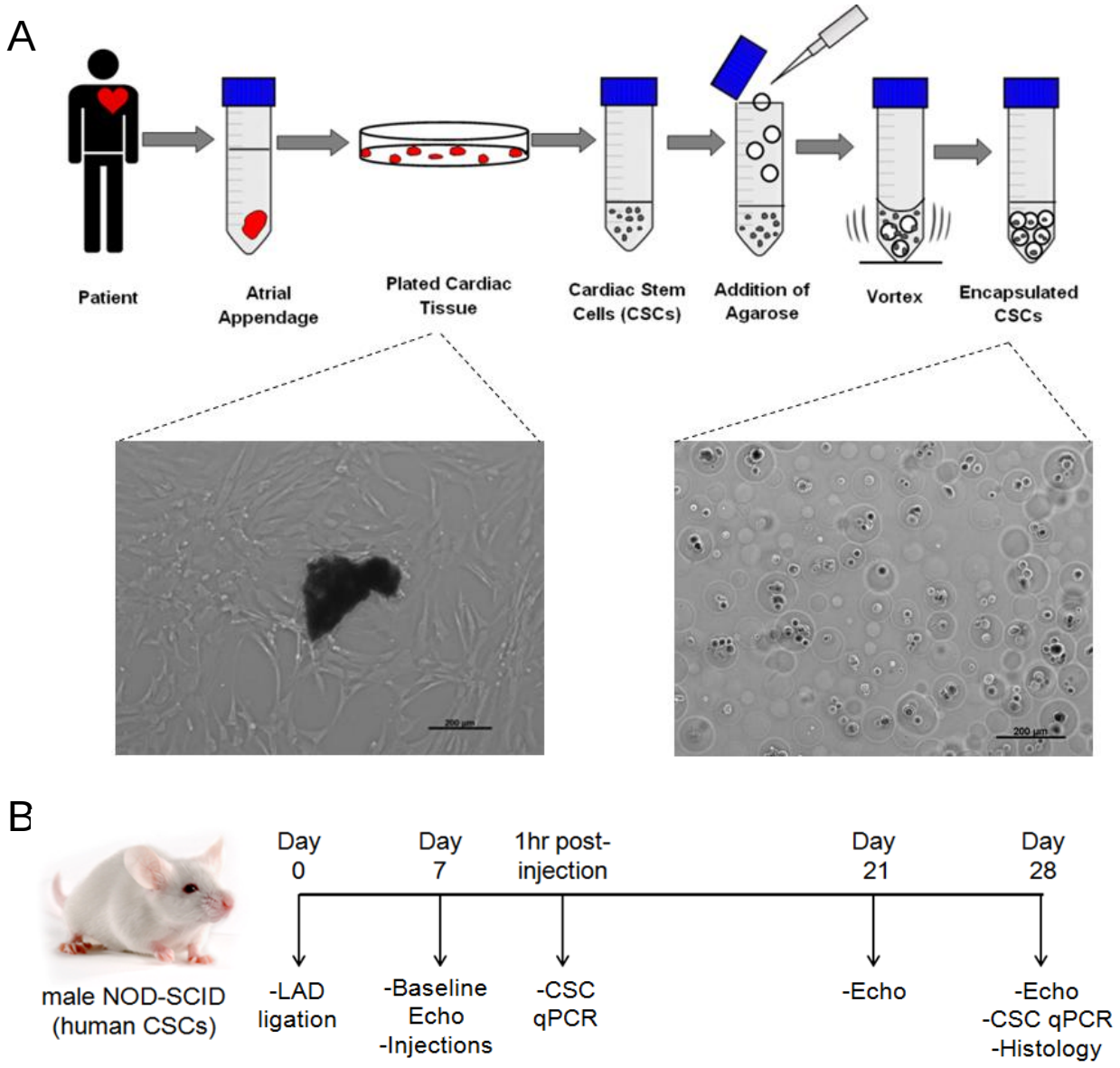


Figure 2. Experimental design. **(A)** Method of encapsulation of CSCs (top). Atrial appendages were obtained from patients undergoing clinically indicated cardiac surgery. After 7-10 days in culture, spontaneous outgrowth surrounding the plated cardiac tissue appeared (bottom left); CSCs were harvested and encapsulated in an agarose capsule (bottom right). Scale bar = 200 µm. **(B)** Overview of *in vivo* experiments injecting human CSCs into SCID mice.

4.0 Results

4.1 Baseline patient demographics

Twenty-one patients (81% male; age 65±10 years; BMI 28±5 kg/m^2, (**Table 2**) consented to donating atrial appendages and were enrolled in the study. All patients had a history of stable cardiac disease with several cardiovascular risk factors, including diabetes (35%; HbA1c 6.0±0.01%), hypertension (68%), dyslipidemia (76%) and ongoing smoking (50%). The patients who consented to donating atrial appendages for the *in vivo* study tended to have fewer co-morbidities (less ongoing smoking and peripheral vascular disease) while requiring less cardiac medications. Though variances were observed between the samples used for animals studies compared to those used for *in vitro* experiments, there were no differences observed within groups. The majority of patients underwent elective cardiac surgery for coronary bypass alone (68%) with the remainder undergoing valve repair/replacement alone (11%) or coronary bypass with valve repair/replacement (21%). All patients were on stable cardiac medications for at least six months prior to surgery. Atrial appendage specimens were collected at the time of cardiac surgery and were processed within one hour of harvest. After one week, CSCs were harvested from the spontaneous outgrowth of plated tissue and used directly for experimentation.

There still remains some controversy as to whether CSCs isolated from more healthy donors are superior to those isolated from "sicker" patients. A recent study demonstrated that CDCs from heart failure donors had superior regenerative capabilities than those isolated from patients soon after MI or who were healthy (Cheng 2014). In contrast, studies from the Davis lab have suggested that diabetes and LTS

score plays a role in reducing the efficacy of CSCs (unpublished data). To control for individual variation, all cell lines used in this study served as their own control (i.e., encapsulated vs. non-encapsulated).

Table 2: Patient Demographics. Differences between patients used for *in vivo* and *in vitro* indicated with an asterisk. Cells obtained from one of the patients were used for both *in vitro* and *in vivo* testing.

	All patients (N=21)	*In Vitro* patients (N=19)	*In Vivo* patients (N=3)
Age (yrs)	65±10	65±11	66±2
BMI	28±5	28±5	30±1
Gender (%male)	81%	84%	33% *
Hypertension	68%	70%	66%
Dyslipidemia	76%	79%	66%
Ongoing Smoking	50%	63%	0% *
Diabetes (avg HbA1C)	35% (0.06±0.01)	32% (0.06±0.002)	33% (0.06±0.004)
Thyroid or other endocrine disease	10%	5%	33% *
Peripheral vascular disease	16%	18%	0% *
History of MI	42%	41%	33%
Valvular Heart Disease	42%	41%	66% *
Coronary Artery Disease	84%	88%	66% *
Congestive Heart Failure	0%	0%	0%
NYHA class	2±1	2±1	1±1
LV ejection fraction	51±10	49±8	59±12
CCS class	3±1	3±1	2±1
Creatinine (umol/L)	77±20	78±21	72±16
Medications:			
Anti-platelet/Anti-Coagulant	79%	82%	33% *
Beta-Blocker	84%	88%	33% *
Statins	74%	76%	33% *
Diuretics	17%	19%	33%
ACEI or ARB	37%	41%	66% *
Calcium channel blocker	21%	24%	0% *
Antiarrhythmic drug	0%	0%	0%
Insulin	16%	18%	0% *
Oral hypoglycemics	26%	24%	33%

4.2 CSC expression of adhesion molecules

To identify the optimal extracellular matrix (ECM) environment for CSC proliferation and survival, the integrin expression profile of CSCs was profiled using quantitative PCR (n=3). Flow cytometry separation of the various sub-populations within CSCs permitted the direct comparison of cardiac progenitor cells (c-Kit+) with mesenchymal progenitor cells (CD90+) and the subpopulation negative for both markers (c-Kit-/CD90-). Profiling demonstrated that 13 integrin subunits α2-7, α11, αD, αE, αL, αV, αX and β1-8 were variably expressed within CSCs in culture (**Fig. 3A**). Amongst the 25 integrins profiled, none were differentially expressed by c-Kit+ cells alone while 5 subunits (αM, β2, β3, β7 and β8) were below qPCR detection. The CD90+ sub-fraction demonstrated strong expression of the α2, β1and β6 subunits ($p \leq .0.05$ vs. c-Kit+ or c-Kit-/CD90- cells) while β3, β4 and β7 were not detected. The c-Kit-/CD90- subpopulation was distinguished by strong expression of αE and α5 ($p \leq .0.05$ vs. c-Kit+ or CD90+ cells) while the β7 and β8 sub-units were not detected. This data demonstrates that while CSCs are spontaneously migrating in culture from the plated tissue biopsy, pro-survival integrins are selectively up-regulated within the subpopulations that comprise CSCs, with discreet integrins that define the c-Kit- subpopulations but not the c-Kit+.

For an integrin protein to be functional and bind to the ECM, it is essential that dimerization between an alpha and beta subunit occurs (Grossmann 2002, Karoubi 2009). Thus, we profiled the expression of operative integrin proteins that would maintain CSC survival signalling: αVβ3 and α5β1, binding partners to fibronectin and fibrinogen. It has been shown that these ECM proteins play a pivotal role by mediating

cell growth and cell migration (Knowlton 1992, Pankov 2002, Takagi 2003). Western blot analysis confirmed these four subunits are present on the surface of the CSCs and rationalize the use of fibronectin and fibrinogen as ideal candidates to customize the agarose capsule to support CSCs (n=3, **Fig. 3B**).

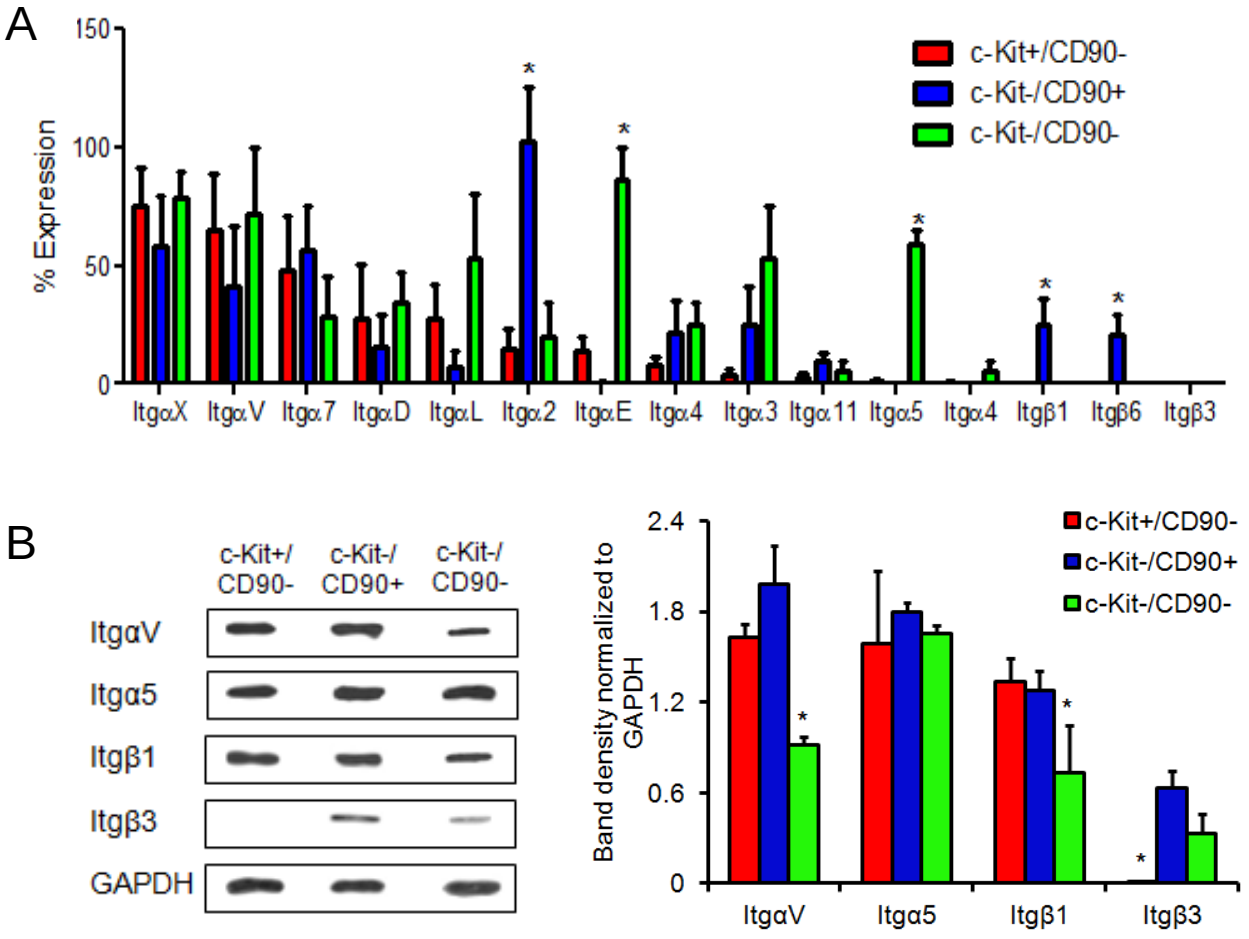
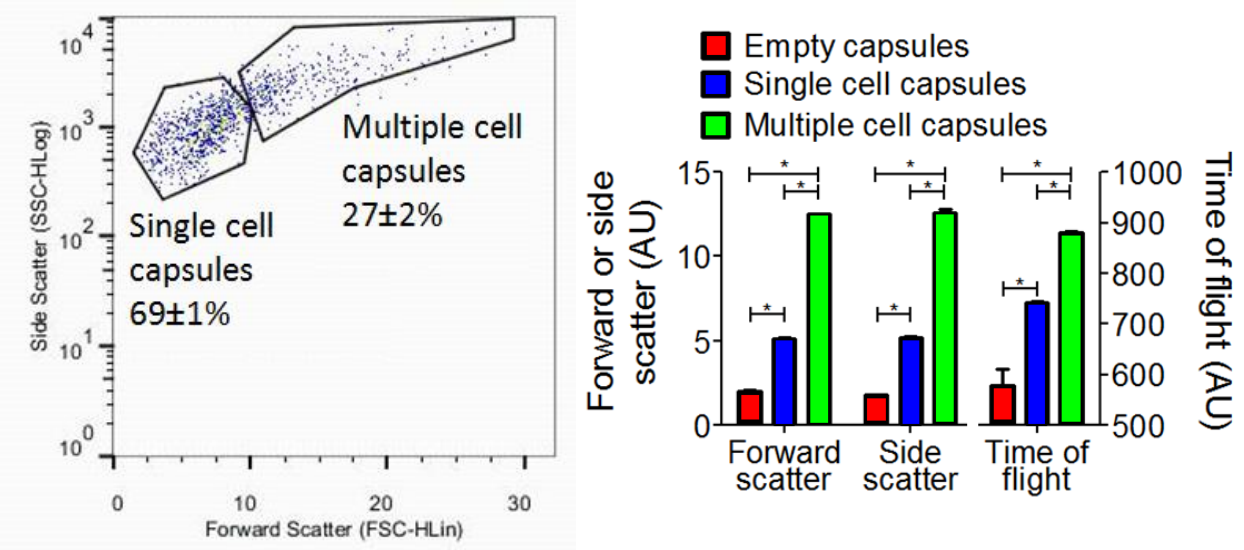


Figure 3: Surface expression of CSC integrins. **(A):** qPCR analysis of integrin expression on relevant sub-populations within the CSC admixture. Data is presented as the percent of total gene expression of CSC integrins normalized to *Gapdh*; n=3. **(B):** Western Blot analysis with corresponding densitometry graph; n=3.

4.3 Assessment of capsule characteristics

CSCs were harvested at day 7-10 and encapsulated within low-melt agarose through drop-wise addition to agitated dimethylpolysiloxane prior to rapid cooling and filtration through a 100μm filter. The capsules average 50-60μm in diameter and contain between zero and approximately 6 cells per capsule, with an encapsulation efficiency of >95%. Flow cytometry analysis confirmed that single cell capsules were smaller in size (decreased time of flight and decreased forward scatter) compared to capsules containing multiple cells (**Fig. 4A**).The mixture of Oregon-green fibrinogen with agarose alone prior to capsule formation demonstrated that ECM proteins efficiently incorporate into the capsule (**Fig. 4B**).

A



B

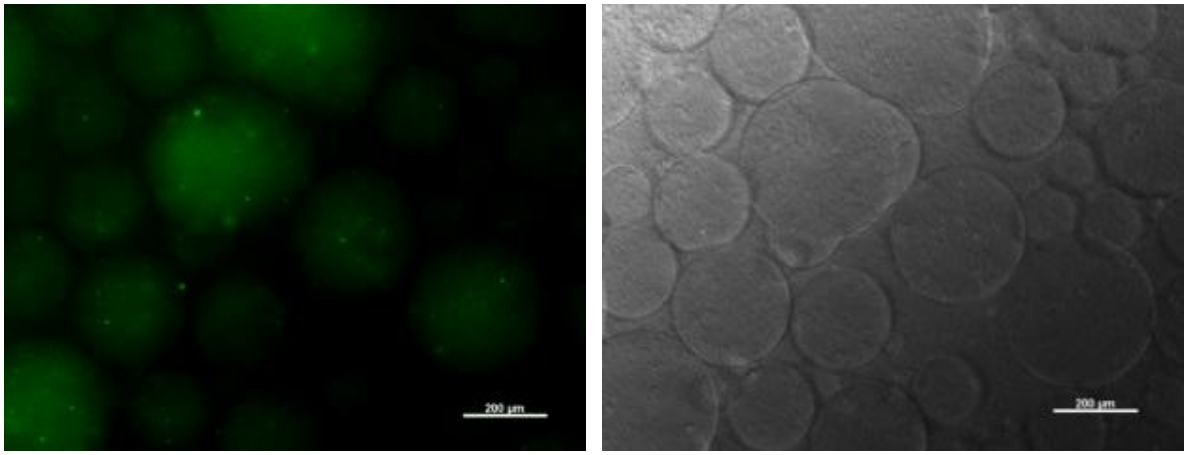


Figure 4: Characterisation of the agarose capsules. **(A)** Flow cytometry analysis of capsule size (n=3). **(B)** Fluorescent microscopy of empty matrigel capsules supplemented with Oregon-green fibrinogen. Scale bar = 200 µm

4.4 CSC migration from the capsule

Given that the biophysical properties of the capsule may alter the capacity of CSCs to emerge from injected capsules, we examined the influence of variable capsular ECM protein and agarose concentrations on the capacity of CSCs to spontaneously migrate from the capsule and the ability of the capsule to remain intact after 24 hours in culture (**Fig. 5**). At a constant 3% agarose content, alterations in the capsule ECM protein concentrations did not affect the ability of CSCs to emerge from the capsules ($p=0.6$). Under fixed protein conditions (0.15mg/ml fibrinogen and 0.25mg/ml fibronectin), reducing the agarose concentration promoted the spontaneous migration of CSCs from the capsule to cover the culture area ($p<0.01$ between all groups), likely due to the reduced stability of the capsule. This *in vitro* data confirms the notion that increasing the rigidity of the capsule limits the spontaneous migration of CSCs and suggests that altering capsule rigidity may influence the kinetics of CSC delivery to the myocardium after intra-myocardial injection. Based on these results, subsequent experiments were performed at a constant agarose concentration (3%) and fixed protein content (0.15mg/ml fibrinogen and 0.25mg/ml fibronectin).

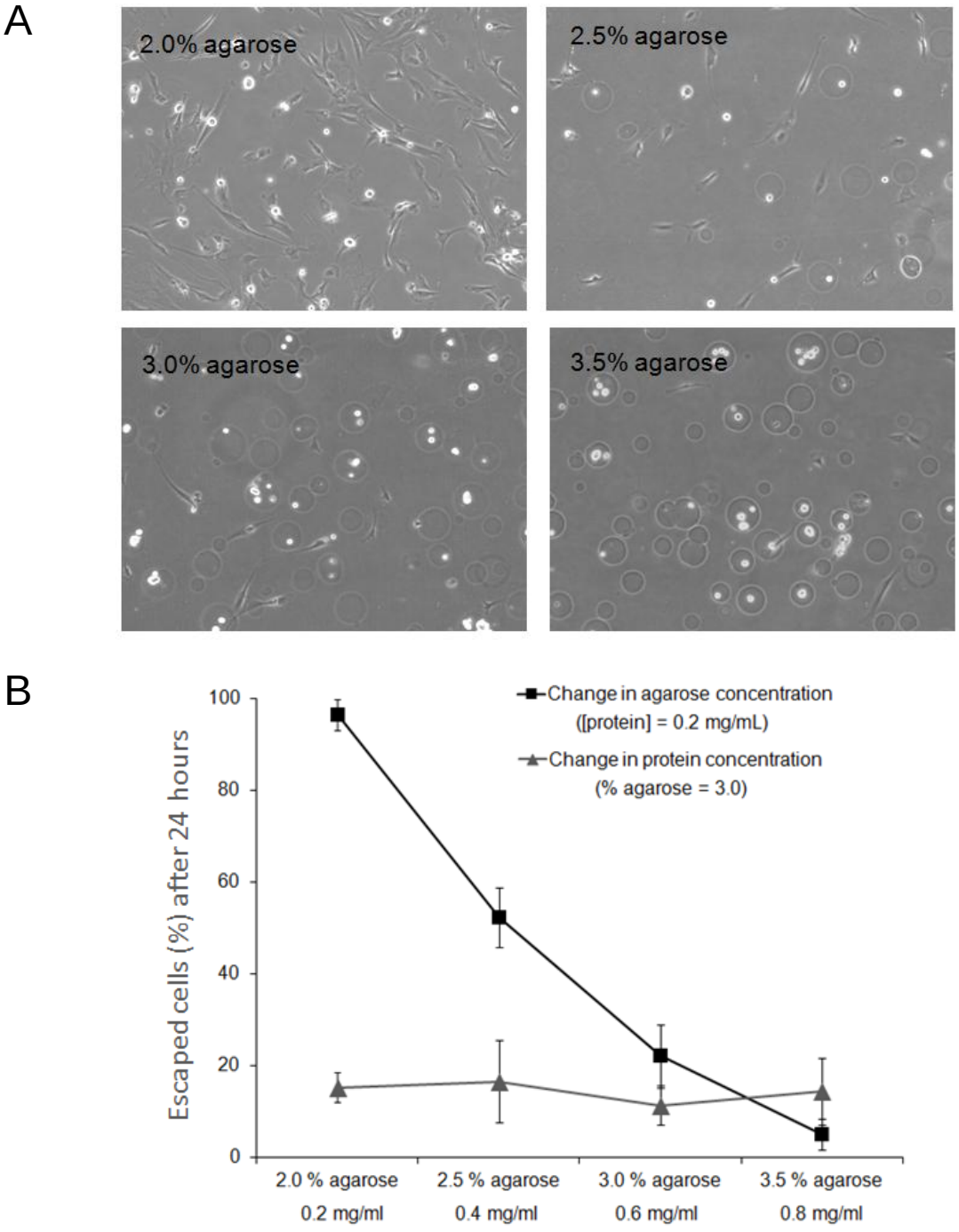


Figure 5: Spontaneous migration of CSCs out of the capsule. **(A)** Representative images of encapsulated CSCs at increasing agarose concentrations after 24 hours of culture. **(B)** Random field analysis demonstrating variable effects of capsule agarose and protein content on extra-capsular CSC migration after 24 hours of culture (n=3).

4.5 Assessment of CSC viability in capsules

The effects of cell encapsulation on CSC viability were assessed by examining the survival and fold-change of CSCs in culture after encapsulation within fibronectin and fibrinogen supplemented capsules compared with standard adherent and suspended (Poly-HEMA) culture conditions. Using a colorimetric dehydrogenase activity assay, baseline cell metabolic activity was assessed 16 hours after plating to allow cells to settle and recover, and compared to readings performed at 48 hours (**Fig. 6A**). When compared to cells in suspension, encapsulation significantly improved the viability of CSCs at baseline (1.3±0.1 vs. 0.5±0.02, respectively; $p=0.002$) and after 48 hours (2.2±0.2 vs. 0.4±0.04; $p=0.001$). No differences were observed when the encapsulated CSCs were compared to the normal adherent culture condition (1.3±0.1 vs. 1.3±0.08 at baseline and 2.2±0.2 vs. 2.1±0.1 at 48 hours, respectively; $p>0.5$). To verify cell rescue was due to the addition of ECM proteins to the capsule, two additional groups were investigated: CSCs encapsulated in unsupplemented capsules and CSCs treated with αV, α5, β1 and β3 blocking antibodies prior to encapsulation to inhibit cell binding to the capsule matrix proteins in fibronectin and fibrinogen supplemented capsules. Cells in suspension had a significant decline in metabolic activity at baseline (0.5±0.04) and 48 hours (0.5±0.06) when compared to cells encapsulated in supplemented capsules ($p<0.05$). Blocking antibodies used to prevent cells from forming attachments with supplemented capsules decreased cell metabolic activity at baseline (0.9±0.08, $p=0.07$) and 48 hours (1.0±0.01, $p=0.009$) when compared to cells encapsulated in supplemented capsules.

When examining the change in metabolic activity over time (indirectly corresponding to cell proliferation) the fold-change in absorbance between 16 and 48 hours was calculated. When comparing CSCs cultured in adherent conditions with matrix supplemented capsules no obvious difference was detected (1.6±0.02 vs. 1.8±0.1 fold increase over 32 hours; p=0.2). CSCs cultured in suspended conditions or unsupplemented capsules declined 0.9±0.01 and 0.7±0.1 fold over 32 hours ($p<0.05$ vs. adherent or matrix supplemented capsules) (**Fig. 6B**). Encapsulated CSCs that had been treated with blocking antibodies experienced a significant decline in cell proliferation when compared to untreated encapsulated CSCs (1.1±0.09 vs. 1.8±0.1 fold increase over 32 hours; p=0.004). A trypan blue stain indicated a 19.9% increase in cell death in the suspended culture vs. the normal adherent after 48 hours (29.2±3% vs. 9.4±2% cell death; p=0.001).

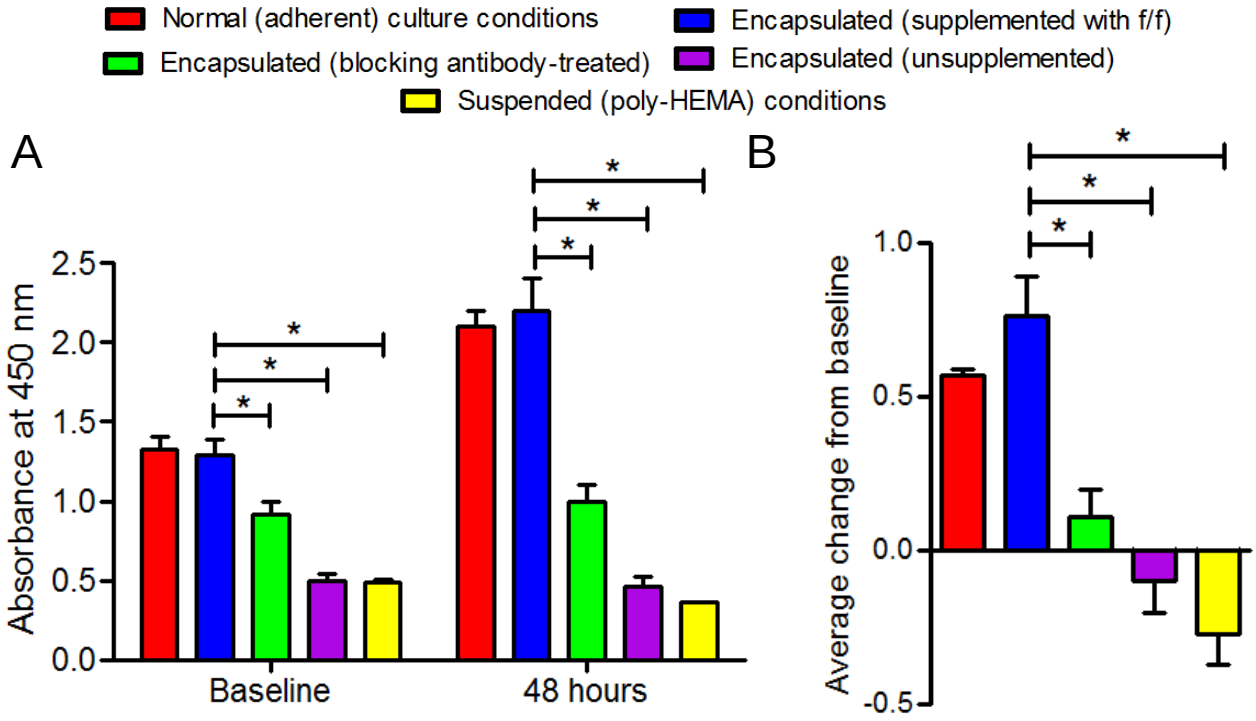


Figure 6: CCK-8 viability assay; f/f= fibronectin and fibrinogen. **(A)** CSC viability using CCK-8 at baseline (16 hours) and after 48 hours in culture, n=3. **(B)** Metabolic activity of CSCs represented by the absorbance fold change, n=3.

4.6 Expression of pro-survival and pro-anoikis genes

The attachment of ECM proteins to cell integrins is critical in maintaining CSC survival and proliferation. The ability of cell encapsulation to imitate normal cell culture on plates was examined by comparing the expression of downstream transcripts within the AKT and ERK survival pathways in CSCs cultured under adherent, encapsulated (supplemented with fibrinogen and fibronectin) and suspended conditions. There were no observed differences in the expression of *Fos* and *Bcl-2* in CSCs cultured for 48 hours under adherent and encapsulated conditions ($p>0.2$; **Fig. 7**). CSCs cultured in suspension consistently increased the expression of *Fos* and *Bcl-2* reflecting the apoptotic stresses applied to the cells with loss of vital integrin-dependent attachments to the ECM ($p=0.04$, $p=0.03$ vs. adherent, respectively; and $p=0.05$, $p=0.09$ vs. encapsulated culture conditions, respectively).

The expression pro-anoikis markers JUN and caspase-3 (*Jun*, *casp3-p17 subunit)* was also examined. Results demonstrated a considerable up-regulation of gene expression in the suspended cell culture compared to the adherent and encapsulated cultures ($p=0.01$, $p= 0.04$ and $p=0.09$, $p=0.03$, respectively; **Fig. 7**).

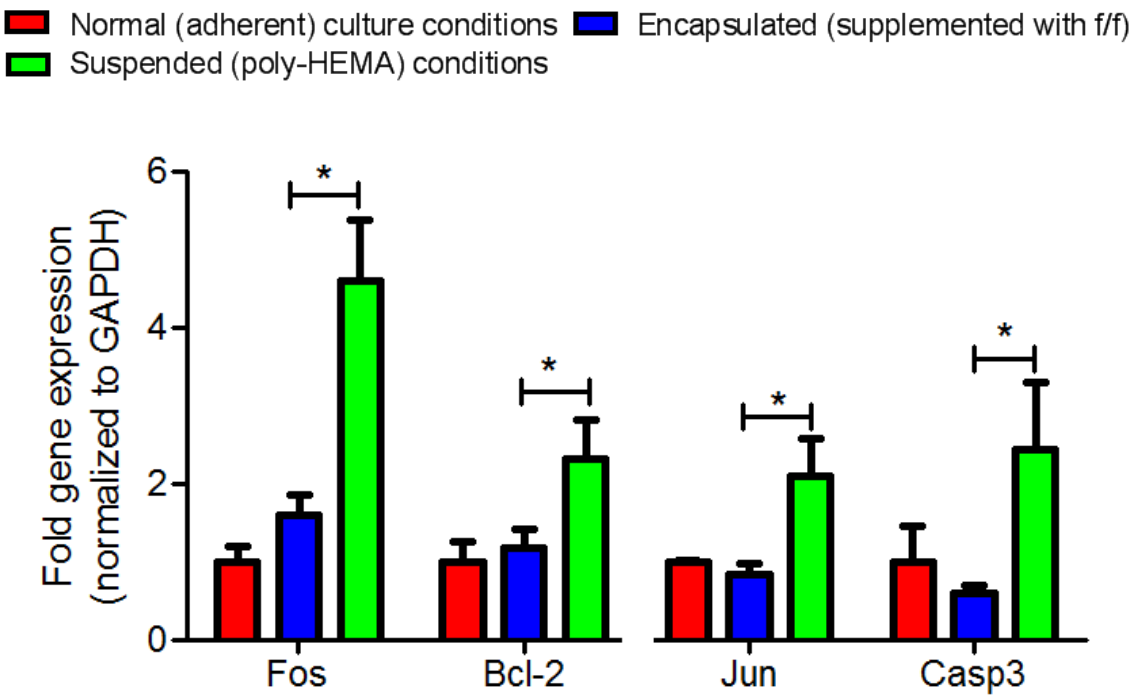


Figure 7: Expression patterns of pro-survival (*Fos, Bcl-2*) and pro-anoikis (*Jun, Casp3*) transcripts; f/f= fibronectin and fibrinogen. RT-qPCR data is expressed as the relative gene expression normalized to *Gapdh* after 48 hours in culture, with adherent culture values set to 1 to represent all genes on the same graph (n=3/transcript).

4.7 Paracrine profile analysis of encapsulated CSCs

Although direct trans-differentiation into functional cardiomyocytes provides a portion of CSC-mediated benefits, several studies have suggested that the secretion of cardio-protective and pro-angiogenic cytokines plays a pivotal role in the positive outcomes observed from cell transplantation (Chimenti 2010, Davis 2010a). To ensure that the production of cytokines was not inhibited by encapsulating CSCs, a representative panel of cytokines that are important in angiogenesis and cell recruitment was profiled with the use of ELISAs (**Fig 8**). Conditioned media was collected under stress conditions (1% oxygen tension and low serum media) from adherent, encapsulated and suspended CSCs. There was no observed difference in angiogenin, SDF-1, VEGF and IL-6 secretion from cells into the conditioned media in adherent and encapsulated culture conditions ($p=0.3$, $p=0.7$, $p=0.4$, $p=0.6$, respectively). Suspension culture resulted in a uniform reduction in the cytokine content of conditioned media ($p=7E-04$, $p=6E-04$, $p=1E-04$, $p=8E-07$ vs. adherent and $p=5E-07$, $p=0.02$, $p=0.04$, $p=0.004$ vs. encapsulated culture conditions, respectively).

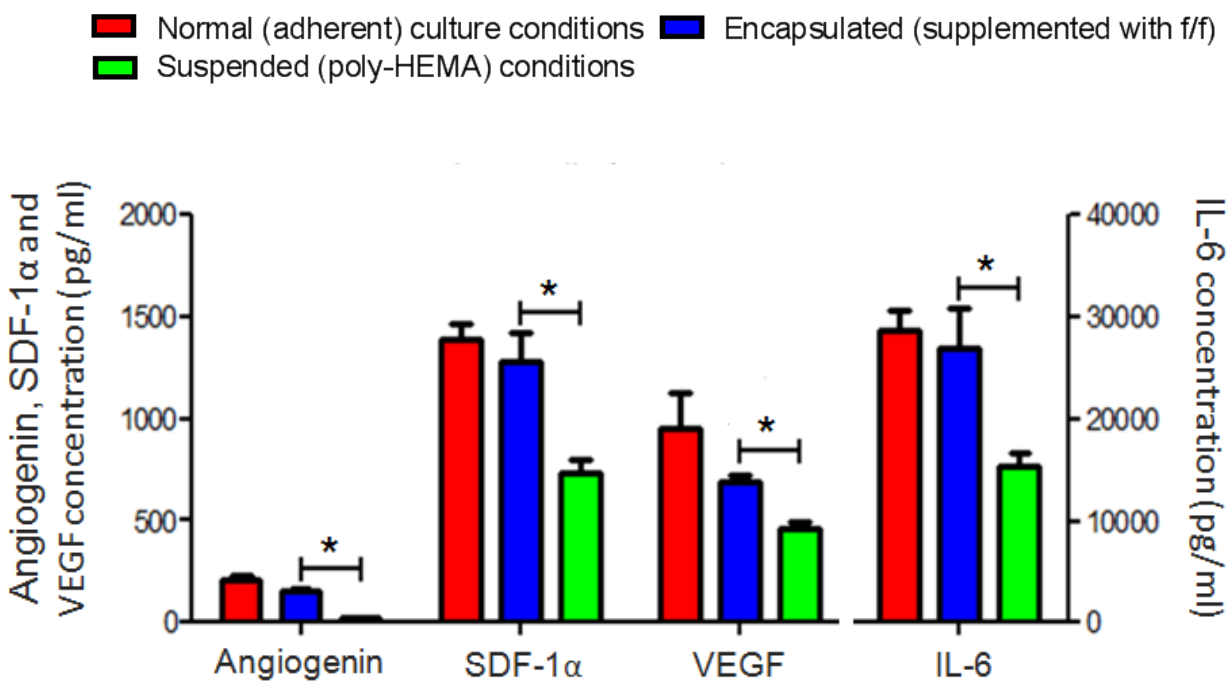


Figure 8: ELISA quantification of angiogenin, SDF-1α, VEGF and IL-6 release; f/f= fibronectin and fibrinogen. Conditioned medium was collected after 48 hours of hypoxic (1% O_2) and low serum (1% FBS) conditions (n=3/cytokine).

4.8 Effect of encapsulation on angiogenesis and CAC recruitment

The effect of CSC encapsulation upon vascular repair was assessed by comparing the ability of conditioned media from adherent, encapsulated and suspended CSCs to promote the formation of capillary-like networks in culture. To ensure results were represented on a cell-per-cell basis, all results using the CM from the suspended cell culture were normalized by 19.9% to account for cell death over 48 hours as indicated previously by trypan blue staining. No difference was observed between the network lengths formed by HUVECs exposed to conditioned media from encapsulated CSCs compared to conditioned media from standard adherent culture conditions (286±26% vs. 220±31% of VEGF control tubule formation, p=0.2; **Fig. 9**). In contrast, the HUVEC networks resulting from conditioned media of suspended cells were significantly shorter than those from both adherent and encapsulated conditioned media (112±12%, p=0.005 and p=2E-05 respectively; **Fig. 9**).

The recruitment of endogenous stem cells is a key driver of tissue regeneration after cell transplantation, thus the ability to attract circulating angiogenic cells (CACs) was compared in the conditioned media from adherent, encapsulated and suspended CSCs. The number of CACs attracted was maintained by the adherent and encapsulated conditioned media (100±11% vs. 108±17% of positive control; p=0.7; **Fig. 10**). Fewer CACs were attracted by the conditioned media from suspended cells (53±6%, p=0.002 and p=0.009, respectively; **Fig. 10**). Collectively, this data suggests that cells in suspension have an inferior production of cytokines, resulting in a reduced ability to promote angiogenesis and to recruit endogenous stem cells.

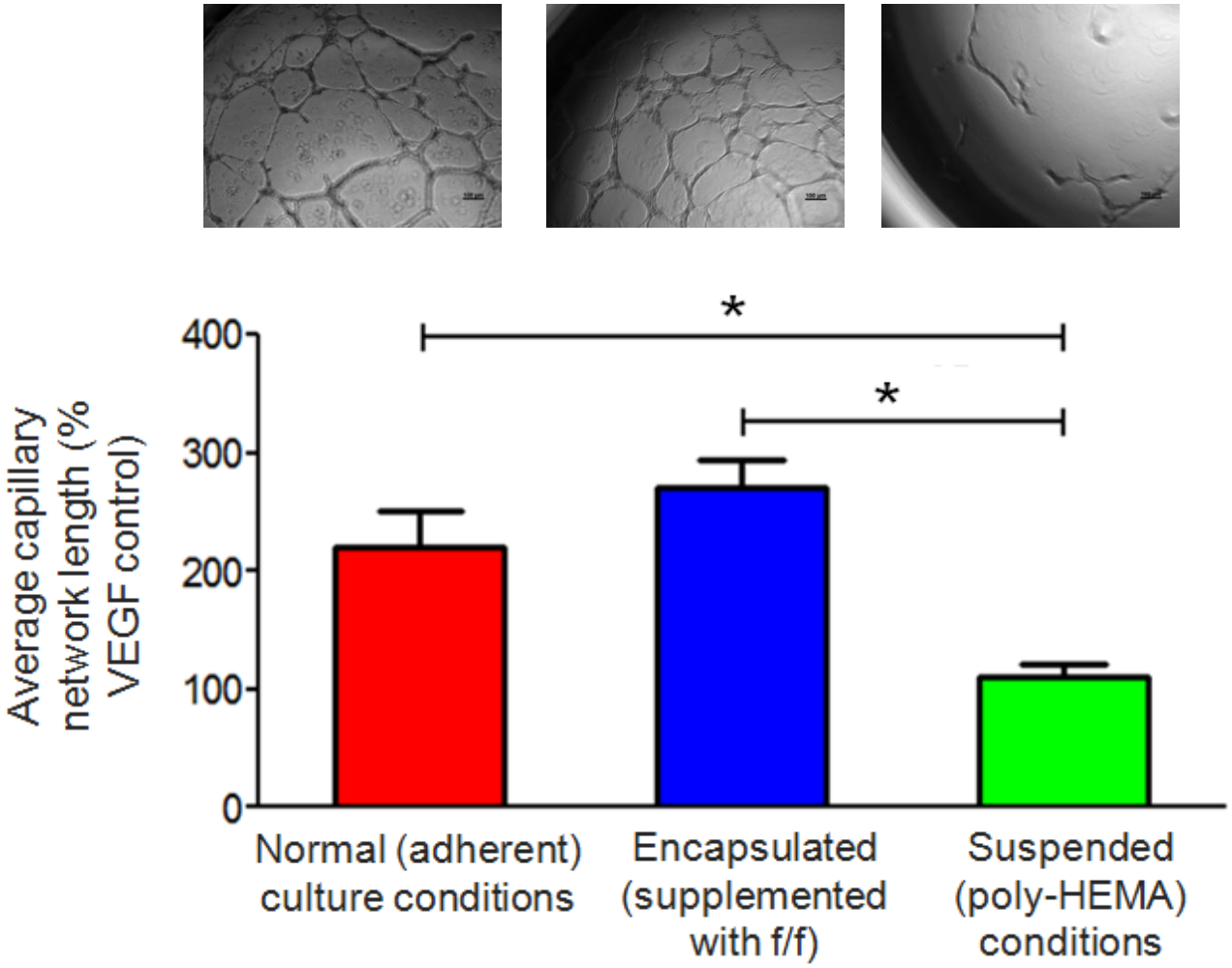


Figure 9: Angiogenesis assay performed using CSC conditioned media; f/f= fibronectin and fibrinogen. Representative images of matrigel-plated HUVECs after 18 hours of culture in conditioned media from adherent, encapsulated and suspended CSCs (top, scale bar = 100 μm) and quantified network lengths (bottom; n=3).

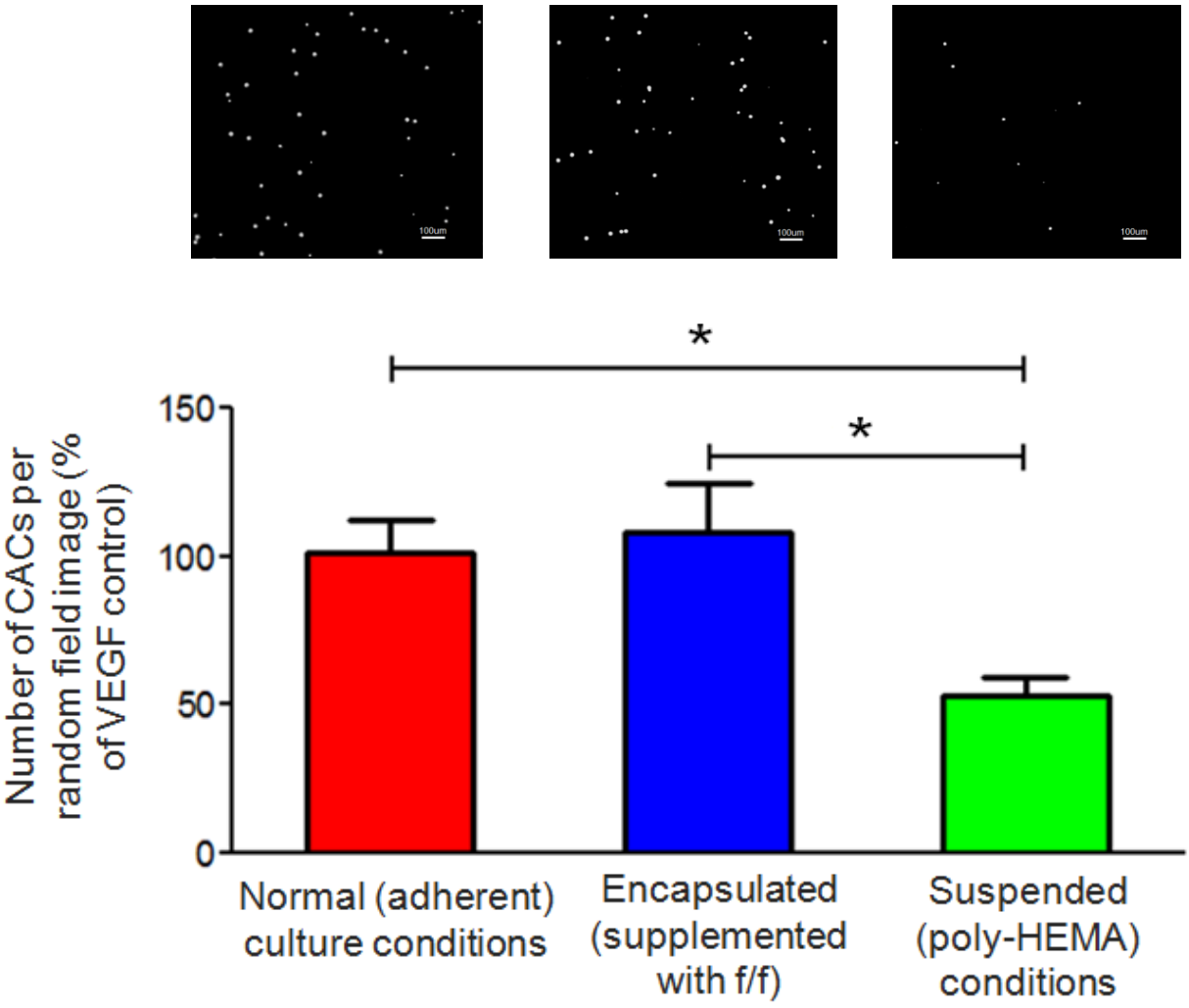


Figure 10: Migration assay performed using CSC conditioned media; f/f= fibronectin and fibrinogen. Representative images of migrated CACs through the transwell membrane (top, scale bar = 100 μm) and quantified number of migrated cells (bottom, n=3).

4.9 Effect of encapsulation on cell engraftment

To examine the effect of encapsulation on cell engraftment, encapsulated CSC retention was compared to non-encapsulated CSCs. A 23G needle was used to deliver encapsulated and non-encapsulated CSCs as the added encapsulation bulk reduced cell release from the previously used 25G needle (**Fig 11A**). Echocardiography-guided intra-myocardial injection of encapsulated CSCs into immunodeficient mice 1 week after experimental myocardial infarction provided a 3±1 fold enhanced acute engraftment when compared to injection of non-encapsulated CSCs (20±4% vs. 6±1% CSCs retained 1 hour after delivery from the 23G needle, respectively; p=0.006, **Fig. 11B**). Although encapsulation did not completely eliminate ongoing cell loss, long-term cell persistence was greatest in mice that were treated with encapsulated CSCs compared to the non-encapsulated CSCs (10±1% vs. 4±1% of the engrafted CSCs remaining after 3 weeks, respectively; p=0.03).

Eventually, the remodeled ischemic heart of heart failure patients will be the ideal venue for encapsulation-based retention as durable long-term engraftment and differentiation of injected cells will be needed to reverse the progressive remodeling that ensues long after cardiac damage. As proof of feasibility, we injected encapsulated human CSCs into SCID mice 4 weeks post MI. In a manner that mirrors injection of CSCs 1 week post MI, encapsulation boosted acute (1 hour) engraftment by 3±1 fold over standard suspension injection (10.6±3 non-encapsulated CSCs vs. 24.5±5% encapsulated CSCs retained one hour post injection, $p<0.05$; **Fig 11C**).

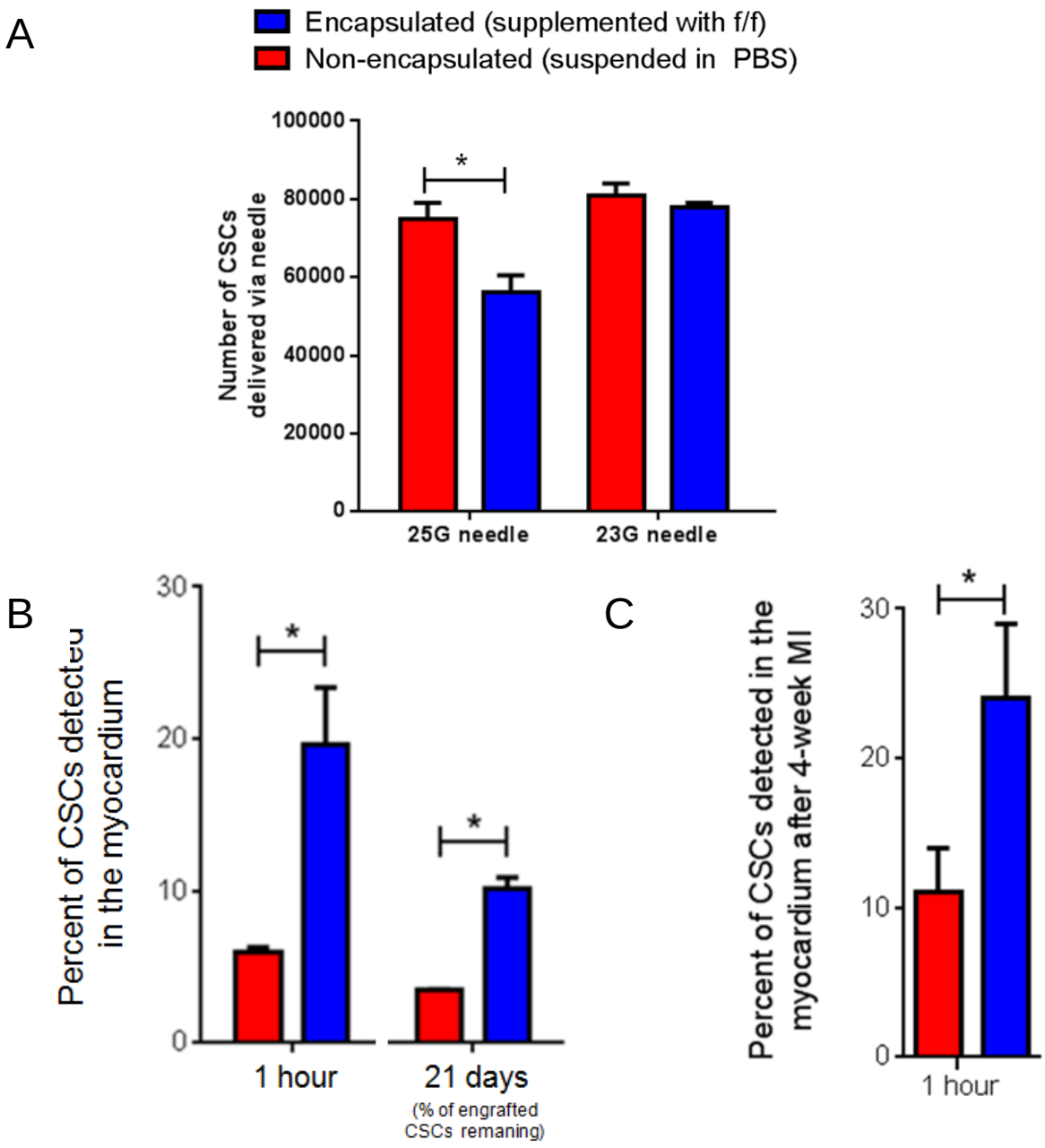


Figure 11: Post-ischemic cell engraftment; f/f= fibronectin and fibrinogen. **(A)** Number of CSCs delivered from a 25G or 23G needle. **(B)** RT-qPCR analysis of the percent of non-encapsulated and encapsulated CSCs delivered 1 week post-MI detected 1 hour (n=3/group) or 21 days (n=4/group) post injection or **(C)** delivered 4 weeks post-MI detected 1 hr post injection (n=3/group).

Co-localization of HNA+ cells with markers of cardiac identity (alpha smooth muscle actin, cardiac troponin-T and Von Willebrand factor) illustrated the ability of CSCs to differentiate into a cardiac phenotype (**Fig. 12**). Analysis of histological sections demonstrated that encapsulation did not influence the fate of injected CSCs, with fewer engrafted CSCs taking on a smooth muscle phenotype compared to an endothelial or cardiomyocyte phenotype (8.7±2.6% αSMA+ vs. 27.6±11.6% cTnT+ and 29.1±9.3% vWF+ in non-encapsulated CSCs-treated mice compared to 6.2±2.2% αSMA+ vs. 29.9±6.0% cTnT+ and 36.4±7.7% vWF+ with encapsulated CSCs-treated mice; **Fig. 13A**).

Long-term engraftment was confirmed by the staining of histological sections for human nuclear antigen (HNA) (**Fig 13B**). HNA positive cells were predominantly found in the infarct border zone and within the infarct region. Treatment of mice with encapsulated CSCs provided a two-fold increase in the number of engrafted CSCs observed as compared to the transplantation of non-encapsulated CSCs (17.4±3 vs. 7.8±2 HNA+ cells per $1mm^2$ random field, respectively; $p<0.05$).

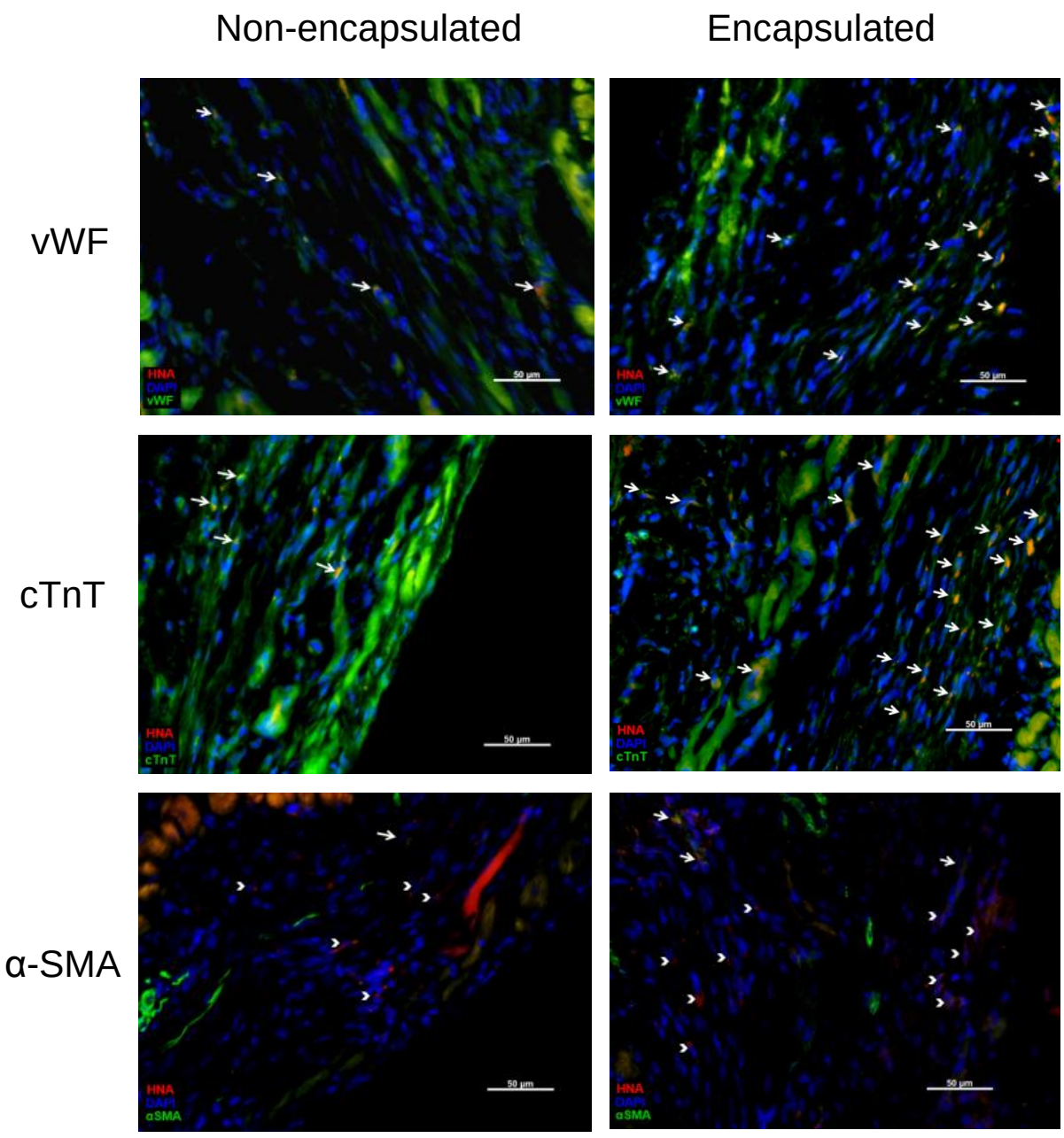


Figure 12: Immunohistochemistry for markers of cardiac phenotype. Random field images of staining of frozen sections day 21 post-delivery for HNA+ cells (red), cardiac lineage markers (green) vWF, cTnT and α-SMA and DAPI (blue). Scale bar = 50 µm.

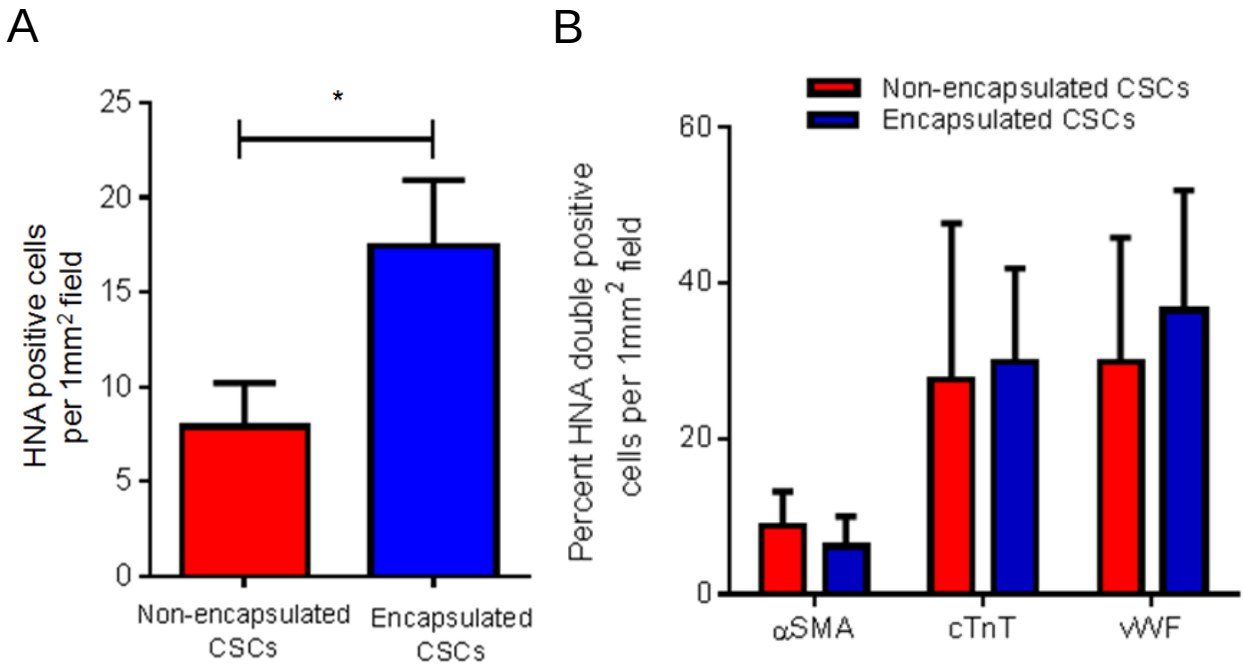


Figure 13: Quantification of HNA staining and markers of cardiac lineage. **(A)** Number of HNA+ cells per field image in animals injected with non-encapsulated (n=3) or encapsulated CSCs (n=3). **(B)** Percent of HNA+ cells co-localized with markers of cardiac lineage vWF, cTnT and α-SMA in animals injected with non-encapsulated (n=3) vs. encapsulated CSCs (n=3).

4.10 Effect of encapsulation on myocardial repair

To examine the effects of encapsulation on CSC-mediated cardiac repair, echocardiography at 2 and 3 weeks post-transplantation was used to examine LVEF, FAC and SV (**Fig. 14A-C**). Baseline LVEF at the time of injection (one week after experimental myocardial infarction) were similar in the vehicle, non-encapsulated and encapsulated treated animals (36±3 vs. 35±1 vs. 33±2%, respectively; p=0.9); suggesting similar infarct sizes in all mice. Progressive post-infarct remodeling resulted in a gradual decline in LVEF within the group of control animals treated with vehicle (PBS) only (final LVEF 28±3%; p=0.05 vs. baseline). Three weeks after transplantation, mice treated with non-encapsulated CSCs demonstrated a 6±1% (p=0.02) and 11±3% (p=0.005) increase in LVEF when compared to baseline or vehicle treatment, respectively. Intra-myocardial injection of encapsulated CSCs enhanced the LVEF three weeks after transplant by 15±2% compared to baseline (p=7E-05), 10±2% compared to transplant of non-encapsulated CSCs (p=1E-04), and 22±3% compared to PBS control (p=6E-04).

When examining FAC, similar results were observed. Mice injected with encapsulated CSCs had a significantly greater improvements in FAC three weeks after transplant compared to mice treated with non-encapsulated CSCs or PBS only (+11.4±1.1% vs. +5.5±1.6% or -1.7±1.5%, p=0.01 and 7E-05; respectively). While treatment with non-encapsulated CSCs yielded modest improvements in SV (+2.0±1.9 μL from baseline), mice treated with encapsulated CSCs had a marked improvement (+14.4±2.0 μL from baseline, p=7E-04). Isolectin B4 staining 3 weeks post-treatment demonstrated a 2±1 increase in arteriole density within the infarct and infarct border

zone in hearts of mice injected with encapsulated CSCs compared to non-encapsulated CSCs and PBS only groups (465±90 µm vs. 278±60 µm and 51±22 µm, p=0.002 and p=1E-04, respectively; **Fig 15**).

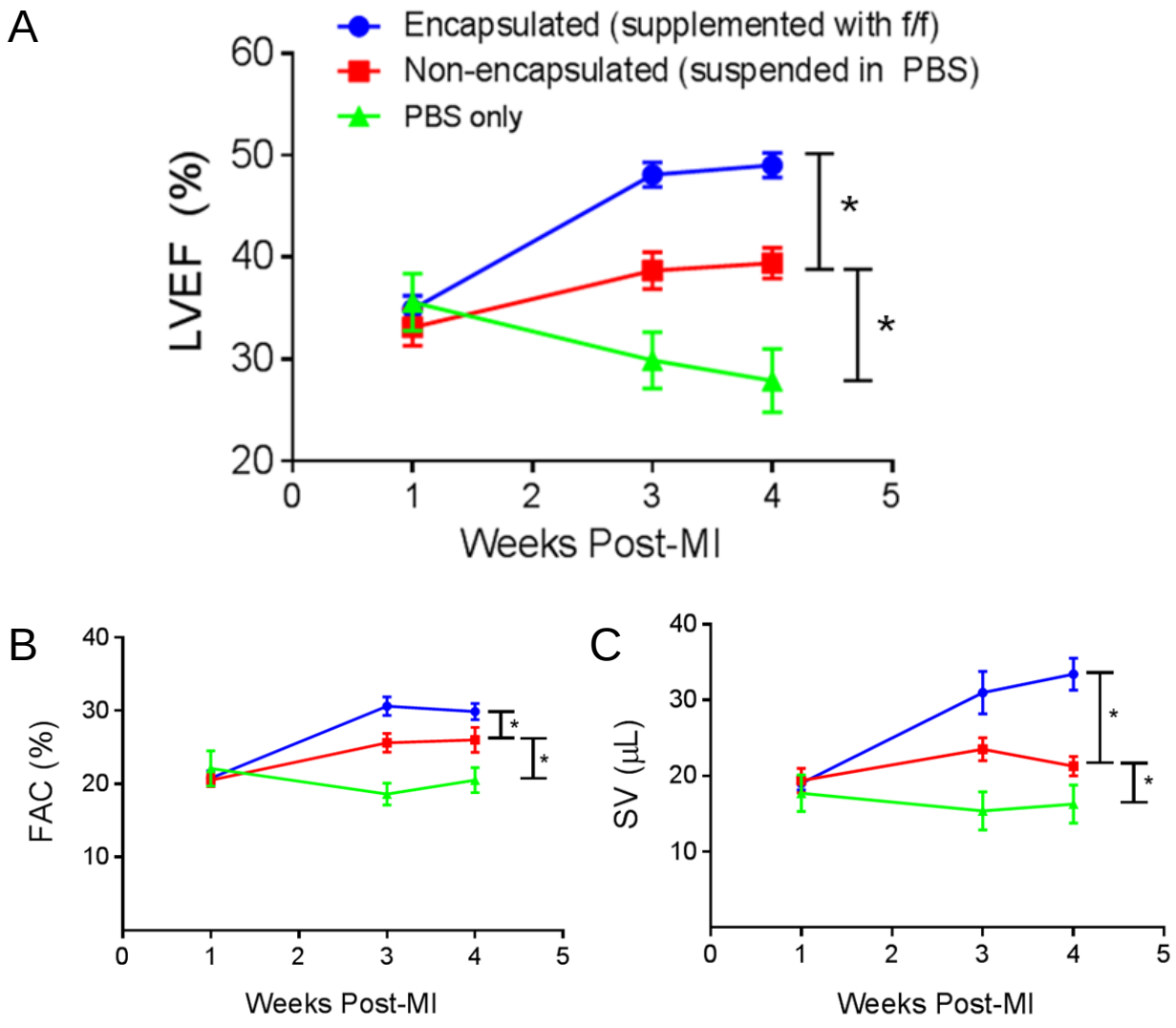


Figure 14: Post-ischemic cardiac function; f/f= fibronectin and fibrinogen. Measurements of **(A)** LVEF, **(B)** FAC, and **(C)** SV of mice injected with non-encapsulated CSCs in PBS (n=7), encapsulated CSCs in PBS (n=7) or standard PBS control (n=4) at week 1, 3 and 4 post-MI.

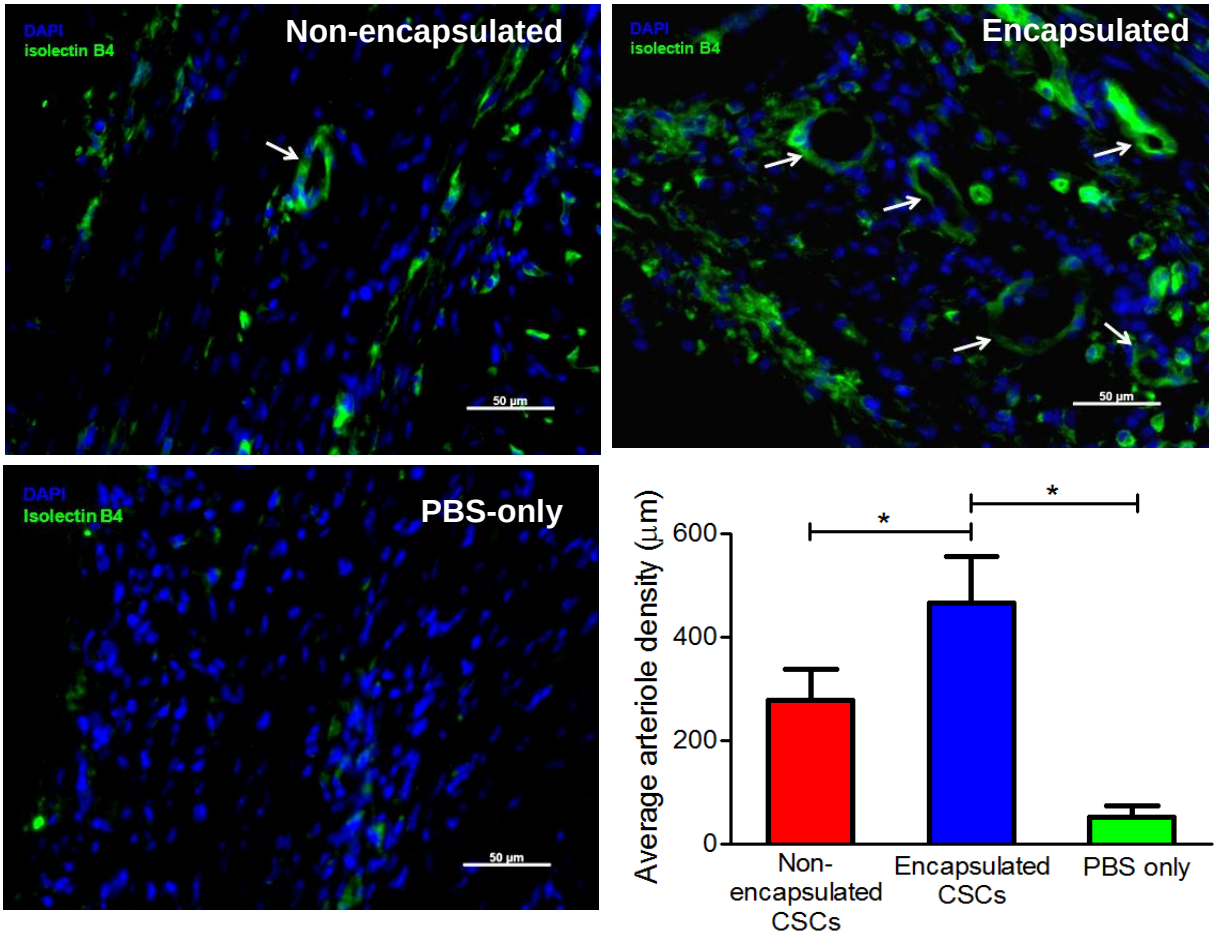


Figure 15: Immunohistochemistry for arteriole density *in vivo*. Histological sections stained for Isolectin B4 (green, depicted by arrows) and DAPI (blue). Representative images and quantification of staining 3 weeks post-treatment (n=3/group).

TUNEL staining of histological sections indicated a reduced amount of apoptosis within the infarct region of mice injected with encapsulated CSCs compared to those treated with non-encapsulated CSCs and the vehicle control (1.5±0.7 vs. 4.0±0.8 and 7.6±1.0 TUNEL+ cells per 100 DAPI+; p=0.02 and p=0.001, respectively; **Fig. 16**). Infarct size measurements from Masson's trichrome sections demonstrated that

treatment with encapsulated CSCs significantly reduced the final scar burden as compared to animals treated with non-encapsulated CSCs or PBS only (9.0±0.8% vs. 15.5±1.6% or 19.4±0.2% scarring of the left ventricle 4 weeks LAD ligation; p=2E-04 and 4E-06, respectfully; **Fig. 17**).

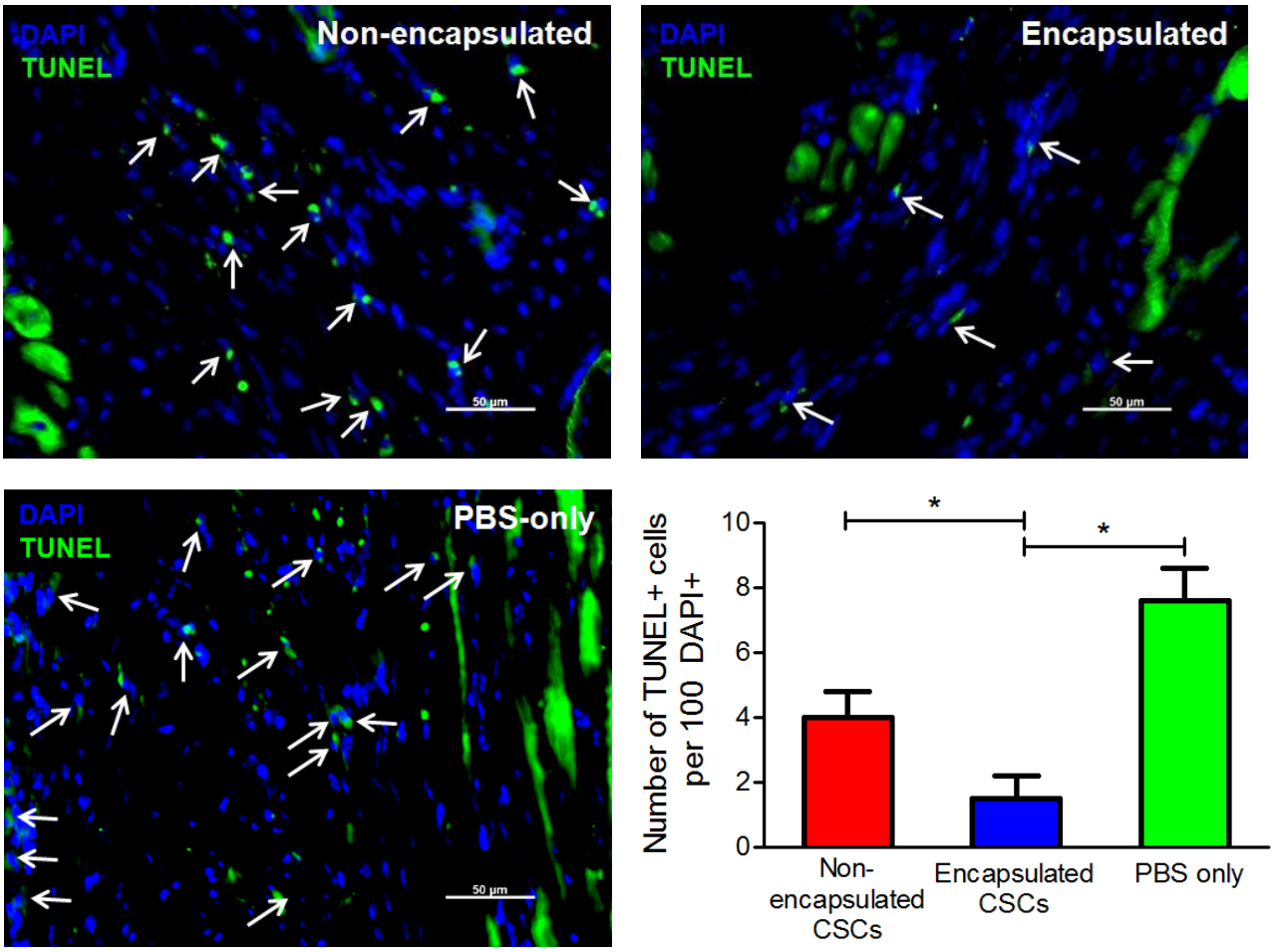


Figure 16: Immunohistochemistry for apoptosis *in vivo***.** Histological sections stained for TUNEL (green) and DAPI (blue). Representative images and quantification of staining 3 weeks post-treatment (n=3).

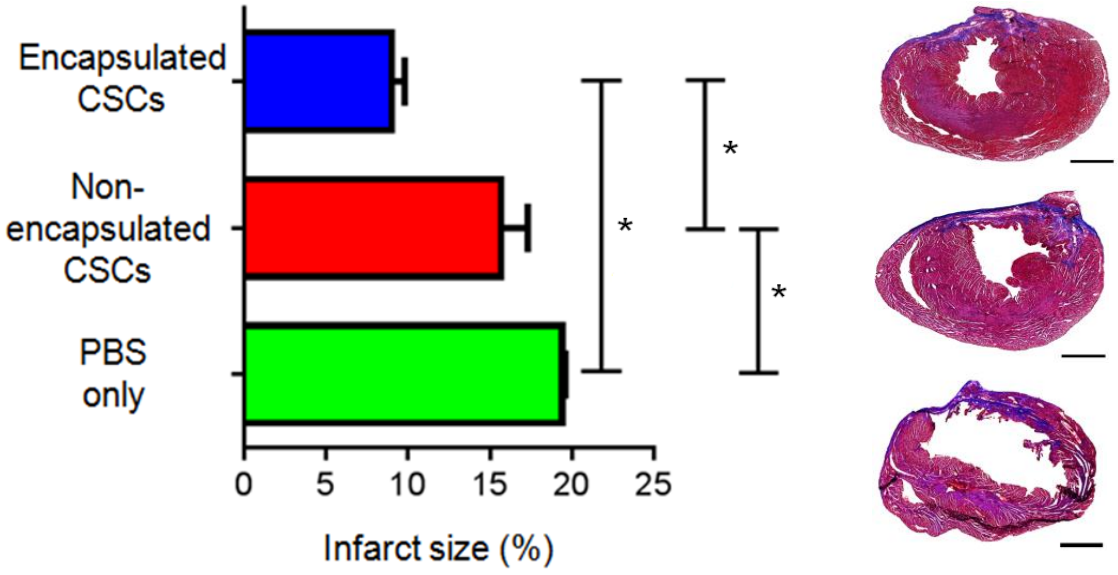


Figure 17: Infarct size measurements *in vivo***.** Masson's trichrome staining and infarct size quantification in mice injected with standard PBS vehicle (n=3), non-encapsulated CSCs in PBS (n=3) or encapsulated CSCs in PBS (n=3) at 3 weeks post-injection. Scale bar = 1 mm.

Taken together, the *in vivo* data suggests that encapsulation boosts the acute retention of CSCs by improving the survival of the cells throughout the transplantation process, with a greater number of engrafted cells surviving long-term. Preventing post-transplant anoikis and improving retention translated to enhanced CSC-mediated post-infarct cardiac repair and contractility (demonstrated through improved LVEF, FAC and SV), attenuation of scar formation and improvements in revascularization in treated mice.

5.0 Discussion

With the discovery that the human heart is not static and the identification of superior cell types for heart regeneration, stem cell therapy using CSCs has moved quickly to clinical trials. Current first generation CSCs appear to function in a paracrine-based fashion by recruiting endogenous stem cells and salvaging damaged cardiomyocytes. In this sense, CSCs are not fully being utilized as a true cardiac progenitor cell capable of directly replacing injured myocytes. Promising clinical results with CSCs have left the field hopeful and motivated to find novel ways of improving the current limitations to CSC therapy.

In recent years, remarkable progress has been made towards the design of biomaterials that promote tissue repair and regeneration in the damaged myocardium. The combination of a biomaterials approach with stem cell therapy represents a natural extension given substantial data demonstrating the modest persistence of injected cells alone. As such, a variety of biomaterials have entered active development with the intent of providing a supportive scaffold for injected cells that aids improving cell survival and engraftment.

In this study, we investigated the effect of encapsulating CSCs in a three-dimensional agarose capsule supplemented with integrin-binding proteins. As compared to similar strategies developed to boost retention of CSCs, cell encapsulation is an attractive candidate for clinical translation given the use of simple general public licensed-compatible materials (inert hydrogel and human-derived ECM proteins). The novelty of this study includes 1) being the first to characterize the expression of pro-survival adhesion molecules on CSCs, 2) provides a clinically translatable means of

enhancing CSC retention, 3) documents sustained cardiac repair through long-term follow-up, and 4) cultures stem cells directly from patients who will require this therapy in the future.

5.1 Effect of encapsulation on CSC viability and survival

In order for the capsules to be effective in preventing cell anoikis, they must be able to provide attachments for the cocooned CSCs. Fibronectin and fibrinogen are capable of binding to multiple integrin dimers, namely αVβ3 and α5β1. With confirmation that CSCs express all of these integrin subunits on their cell surface, a cell viability assay was performed and we demonstrated that encapsulation improves cell viability and metabolic activity compared with cells in suspension. When fibronectin and fibrinogen were removed from the capsule, or CSCs were treated with integrin blocking antibodies, the effects were reversed suggesting the ECM binding proteins within the capsule are re-establishing vital cell-matrix attachments lost during cell suspension.

The examination of pro-survival (*Fos, Bcl-2*) and pro-anoikis (*Jun, Casp3*) gene expression was also used to determine the condition of the encapsulated CSCs. Overall, no difference in gene expression levels were observed when comparing the encapsulated CSCs to the CSCs grown in an adherent culture. This suggests that encapsulation is providing similar attachments signals to the CSCs, allowing them to maintain "normal" levels of gene expression. In contrast, marked differences in gene expression were observed in the suspended cell cultures. During anoikis, it has been reported that the activity of the JNK pathway (regulator of *Jun*) in required, and activation of JNK requires caspase activity (Frisch 1996, Cardone 1999). In the suspended cell culture, high levels of *Jun* and *Casp3* expression were detected,

suggesting the anoikis pathway has been activated in these cells. Interestingly, increased levels of pro-survival genes *Fos* and *Bcl-2* were also observed in the suspended cells. Given regulation of these pathways is not restricted to integrin-cell interactions, it is believed that a compensatory mechanism is in effect as the cell attempts to survive despite being in suspension, possibly through the suppression of anoikis by *Bcl-2* (Frisch 1996).

5.2 Effect of encapsulation on CSC cytokine release

Emerging evidence has suggested that the local production of cardioprotective and pro-angiogenic cytokines plays an important role in CSC-mediated cardiac repair (Chimenti 2010, Davis 2010a). Results demonstrate that encapsulation does not inhibit the release of cytokines by CSCs, and no differences in secretion levels were observed when compared to adherent CSCs. The comparable paracrine profile between the two groups translated to similar abilities to promote angiogenesis and recruit stem cells *in vitro*. In contrast, conditioned media collected from cells in suspension demonstrated an inferior paracrine profile with a decline in cytokine release – despite normalization on a cell-to-cell basis – translating to a marked reduction in angiogenic and recruitment capabilities. Overall, this data suggests that encapsulated CSCs have an enhanced ability to promote indirect cardiac repair compared to cells in suspension.

5.3 Effect of encapsulation on CSC-mediated cardiac repair

This study demonstrated that CSC encapsulation improves acute retention, long-term survival, and overall cardiac function in a direct comparison to non-encapsulated CSCs. Although encapsulation significantly preserved LV function when administered soon after myocardial infarction, progressive loss of transplanted cells still occurs and

likely reflects: 1) ongoing clearance via lymphatic or mechanical extrusion, 2) reduced intra-capsular oxygen tension after transplantation into the ischemic border zone, 3) progressive loss of CSCs damaged during or prior to transplant, and 4) the harsh conditions CSCs encounter after migration out of the capsule. Mechanistically, this improvement is in part mediated by an increase of pro-angiogenic and cardioprotective cytokines being released in the post-transplant period due to improved acute retention. Results demonstrated this led to improvements in scar attenuation, revascularization of the infarct, reduction in apoptosis, and possibly superior recruitment of endogenous stem cells in encapsulated CSC-treated mice. However, direct differentiation of engrafted CSCs also plays an important role as demonstrated by the greater number HNA+ CSCs colocalized with cardiac muscle marker cTnT in animals treated with encapsulated CSCs, resulting in marked enhancements in the LVEF, FAC and SV.

5.4 Study limitations and future directions

Overall, this encapsulation study has brought great promise to improving CSC retention, though the method has some important limitations. Although the process is rapid and uncomplicated, ongoing cell loss occurs during encapsulation as one attempts to regulate capsule size. As a result, the final cell product yield approximates 20% of the initial starting product. Using a microfluidics approach would be beneficial as single cells are successively surrounded by the desired hydrogel/protein mixture, leading to a reproducible final cell product yield near 100%. In addition, the encapsulation approach may benefit from further capsule engineering to improve the acute retention of injected cells. Possible enhancements include the addition of extra-capsular proteins (such as

proteoglycans) to aid in capsular binding to the host ECM, or cross-linking proteins (such as factor 13) to covalently fix capsules to the host ECM proteins.

The obvious on-going cell loss provides support for the notion that additional buffers against apoptosis or necrosis will be needed to fully exploit the advantages conferred by enhanced acute retention of CSCs. Future methods to be explored include: 1) enhanced customization of capsule ECM constituents to promote CSC proliferation, and 2) priming the cells to improve survival upon injection into the harsh environment (ex: IGF-1 pre-treatment or genetic modification).

6.0 Conclusions

In this study, we demonstrate that encapsulation within matrix-supplemented agarose capsules improve CSC viability and proliferation, and maintains pro-survival pathways through the re-introduction of important cell-matrix attachments. When compared to cells in suspension, encapsulated CSCs have superior secretion of cardio-protective and pro-angiogenic cytokines, which translated to enhanced blood vessel formation and circulating progenitor cell attraction *in vitro*. In an *in vivo* model of 1-week MI, we demonstrate that CSC encapsulation boosts the acute engraftment and long-term survival after injection, translating to enhanced cardiac function compared to non-encapsulated CSCs. Engraftment data in a 4-week myocardial infarction model demonstrated the potential for encapsulation to be effective in the treatment of established ischemic cardiomyopathy. In conclusion, CSC encapsulation provides an attractive way to treat the cells prior to injection through a fast, non-toxic and clinically acceptable means.

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8.0 Appendix I

Table 3: CP values from qPCR pathway expression. **(A)** Fos, **(B)** Jun, **(C)** Bcl-2, and corresponding **(D)** Gapdh. **(E)** Casp3 with **(F)** corresponding Gapdh.

A	Fos		
	Adherent	Encapsulated	Suspended
Patient 1	29.1	29.48	27.54
	26.52	28.9	31.06
	25.96	29.89	24.53
Patient 2	33.91	30.47	28.52
	33.59	30.89	32.46
	32.24	30.42	30.86
Patient 3	25.26	28.63	25.77
	26.77	27.82	24.45
	23.62	27.49	24.2

B	Jun		
	Adherent	Encapsulated	Suspended
Patient 1	27.11	28.19	24.96
	24.55	27.14	27.62
	23.98	26.9	22.92
Patient 2	30.59	26.09	25.79
	29.66	26.48	27.79
	28.07	25.58	25.62
Patient 3	22.95	25.81	23.25
	23.44	25.04	22.8
	21.64	24.58	22.19

C	Bcl-2		
	Adherent	Encapsulated	Suspended
Patient 1	37.4	35.5	33.54
	35.2	34.78	37.35
	33.8	36.98	34.52
Patient 2	37.7	34.4	33.83
	37.64	34.51	36.22
	37.01	35.11	34.65
Patient 3	32.15	33.49	32.51
	34.92	32.65	31.43
	31.09	32.16	31.86

D	Gapdh		
	Adherent	Encapsulated	Suspended
Patient 1	22.6	24.64	22.05
	20.98	22.74	25.58
	20.56	23.34	21.88
Patient 2	25.68	21.72	21.92
	24.88	21.89	23.46
	23.73	21.53	21.41
Patient 3	18.56	22.51	20.86
	18.4	21.12	20.55
	18.96	21.11	20.23

E	Casp3		
	Adherent	Encapsulated	Suspended
Patient 1	29.1	27.7	25.76
	29.06	27.9	25.81
Patient 2	?	28.6	27.01
	27.33	?	?
Patient 3	26.65	27.21	26.13
	26.92	27.92	26.06

F	Gapdh		
	Adherent	Encapsulated	Suspended
Patient 1	25.94	22.86	23.83
	25.94	22.86	23.71
Patient 2	22.99	24.31	22.89
	22.76	24.16	22.92
Patient 3	21.22	22.67	22.73
	20.84	22.62	22.71

Table 4: CP values for ALU sequence qPCR and ALU concentration from standard curve. **(A)** 1 hour post-injection and **(B)** 21 days post-injection in a 1 week MI model.

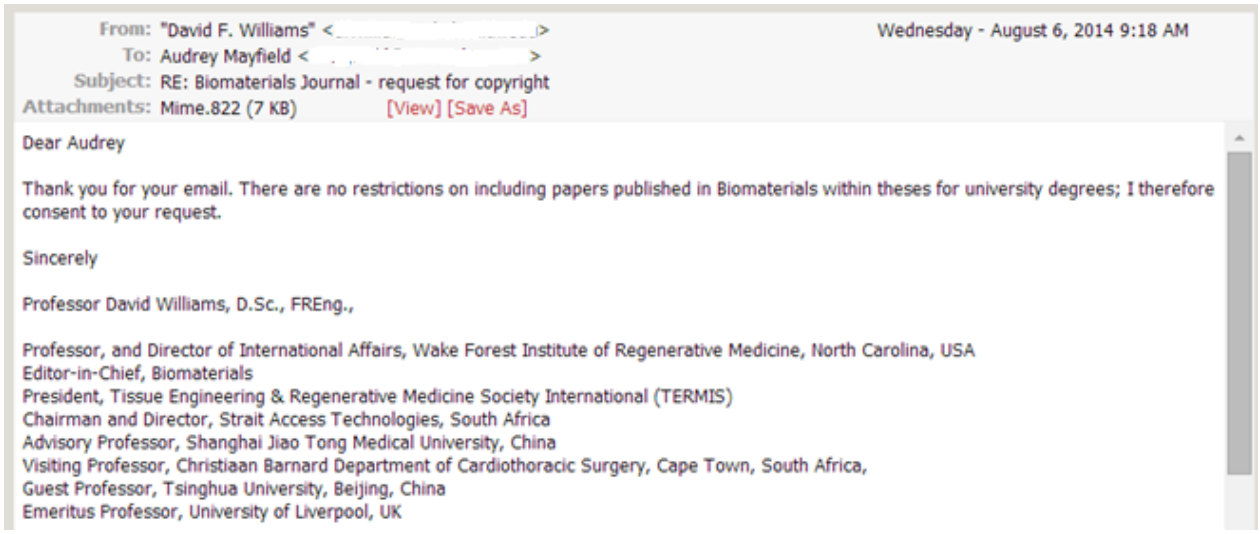
A

1 hr	Non-encapsulated		Encapsulated		Negative Control	
	CP value	ALU conc	CP value	ALU conc	CP value	ALU conc
Mouse 1	20.47	1.41E-02	19.86	1.94E-02	26.63	4.42E-07*
	20.55	1.20E-02	20.43	1.33E-02	26.69	4.20E-07*
Mouse 2	20.6	1.28E-02	19.12	3.24E-02		*extrapolated
	20.57	1.17E-02	18.93	3.69E-02	Standard Curve Point	
Mouse 3	21.41	6.67E-03	19.86	1.95E-02	8.83	5.15E+01
	21.35	6.98E-03	19.9	1.90E-01	8.91	4.86E+01

B

21 days	Non-encapsulated		Encapsulated		Negative Control	
	CP value	ALU conc	CP value	ALU conc	CP value	ALU conc
Mouse 1	24.72	5.77E-04	20.18	1.70E-02	27.2	9.14E-09*
	24.71	5.77E-04	20.23	1.59E-02	27.3	7.74E-09*
Mouse 2	25.94	7.60E-05	22.04	4.70E-03		*extrapolated
	25.98	7.47E-05	21.99	4.64E-03	Standard Curve Point	
Mouse 3	24.76	5.47E-04	23.57	1.64E-03	8.48	4.93E+01
	24.8	5.33E-04	23.58	1.62E-03	8.44	5.07E+01
Mouse 4	23.8	1.40E-03	23.59	1.62E-03		
	23.76	1.47E-03	23.64	1.52E-03		

8.1 Appendix II



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